# In Search for Potent and Selective NPY $Y_4$ Receptor Ligands: Acylguanidines, Argininamides and Peptide Analogs

**Dissertation**

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.) der

Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie –

der Universität Regensburg


vorgelegt von

**Melanie Kaske**

aus Memmingen

2012

Die vorliegende Arbeit entstand in der Zeit von Mai 2008 bis Mai 2012 unter der Anleitung von Herrn Prof. Dr. Armin Buschauer am Institut für Pharmazie der Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie – der Universität Regensburg.

Das Promotionsgesuch wurde eingereicht im Mai 2012.

Tag der mündlichen Prüfung: 01. Juni 2012

Prüfungsausschuss: Prof. Dr. F.-M. Matysik (Vorsitzender)
Prof. Dr. A. Buschauer (Erstgutachter)
Prof. Dr. G. Bernhardt (Zweitgutachter)
Prof. Dr. J. Wegener (Drittprüfer)

*„Zwei Dinge sind zu unserer Arbeit nötig: Unermüdliche Ausdauer und die Bereitschaft, etwas, in das man viel Zeit und Arbeit investiert hat, wieder zu verwerfen.“*

Albert Einstein

# Danksagungen

An dieser Stelle möchte ich mich bedanken bei:

Herrn Prof. Dr. Armin Buschauer für die Möglichkeit an diesem vielseitigen und herausfordernden Projekt arbeiten zu dürfen, seine wissenschaftlichen Anregungen, seine Förderung und seine konstruktive Kritik bei der Durchsicht der Arbeit,

Herrn Prof. Dr. Günther Bernhardt für seine stete Hilfsbereitschaft und Unterstützung in allen fachlichen Fragen, sein Interesse am Fortgang der Experimente, seine konstruktive Kritik bei der Durchsicht dieser Arbeit sowie die Erstellung des Zweitgutachtens,

Herrn Dr. Max Keller für die tolle Zusammenarbeit im Rahmen des NPY-Projekts, seine Hilfsbereitschaft, seine Begeisterungsfähigkeit, die Beantwortung aller möglichen wie auch unmöglichen Fragen sowie die Bereitstellung von Synthesevorstufen und Argininamid-Derivaten,

Herrn Dr. Stefan Weiß für die Bereitstellung der Argininamid-Derivate,

Frau Dr. Anja Kraus und Herrn Dr. Patrick Igel für die Bereitstellung der Acylguanidine,

Herrn Prof. Dr. Oliver Reiser, Herrn Dr. Lukasz Berlicki, Frau Raquel Gutiérrez Abad und Herrn Ludwig Pilsl für die Bereitstellung von Peptid-Analoga und die gute Zusammenarbeit im Rahmen des Peptid-Projekts,

Frau Prof. Dr. Chiara Cabrele und ihrem Mitarbeiter Fabrizio Zanta für die Einführung in die Peptidsynthese, die anregenden Diskussionen und die Bereitstellung einiger Peptide,

Frau Dr. Nathalie Pop und Herrn Dr. Miroslaw Lopuch für die Unterstützung bei der Durchführung von Konfokalmikroskopie-Experimenten,

Herrn Prof. Dr. Sterner und seinem Mitarbeiter Bernd Reisinger für die Hilfe bei der Aufnahme von CD-Spektren,

allen Mitarbeitern der Zentralen Analytik für die Aufnahme und die Hilfestellung bei der Interpretation von NMR- und Massen-Spektren,

Frau Elvira Schreiber und Frau Brigitte Wenzl für die Durchführung unzähliger funktioneller Assays und Bindungsstudien,

Frau Maria Beer-Krön für die Durchführung der GTPase Assays und Kristallviolettassays sowie für die vielen netten und aufbauenden Gespräche,

Herrn Peter Richthammer für die netten Gespräche, seine stete Hilfsbereitschaft und Unterstützung bei allen technischen Herausforderungen sowie die tolle Zusammenarbeit im Rahmen der verschiedenen Praktika,

Frau Martina Wechler, Frau Siliva Heinrich, Frau Karin Reindl und Frau Uta Hasselmann für die Unterstützung bei allen organisatorischen Fragen und Problemen,

meinen Laborkollegen Herrn Dr. Roland Geyer, Frau Dr. Miriam Ertel und Frau Carolin Meyer für eine unvergesslich schöne Zeit im Labor, die tolle Zusammenarbeit sowie die netten Gespräche, die so manchen grauen Laboralltag aufheiterten,

allen aktuellen und ehemaligen Mitgliedern des Lehrstuhls für eine tolle Zusammenarbeit und die stets gute Kollegialität sowie die schöne Zeit, auch außerhalb der Uni,

Frau Nicole Kagermeier und Frau Stefanie Bauer für den sportlichen Ausgleich verbunden mit vielen netten Gesprächen nach langen Labortagen,

der Deutschen Forschungsgemeinschaft für die finanzielle Förderung im Rahmen des Graduiertenkollegs GRK 760,

und ganz besonders meinen Eltern, die mir immer zur Seite standen, mir jegliche Unterstützung und Hilfe zukommen ließen und immer an mich geglaubt haben sowie meinen Großeltern, die meinen Weg immer gespannt verfolgt und mich stets unterstützt haben.

# Poster Presentations and Professional Training

**Poster Presentations:**

Kaske, M., Keller, M., Weiß, S., König, B., Bernhardt, G., Buschauer, A.: Argininamides as neuropeptide Y (NPY) $Y_4$ receptor antagonists; 5th Summer School Medicinal Chemistry, Regensburg, 13.09. – 15.09.2010

Kaske, M., Keller, M., Weiß, S., König, B., Bernhardt, G., Buschauer, A.: Monovalent and bivalent argininamide-type antagonists of the human NPY $Y_4$ receptor. EFMC-ISMC, 21st International Symposium on Medicinal Chemistry, Brüssel, 05.09. – 09.09.2010

Kaske, M., Keller, M., Buschauer, A.: Bivalent Argininamides as Ligands of the Human NPY $Y_4$ Receptor. Frontiers in Medicinal Chemistry; Münster, 14.03. – 17.03.2010

Kaske, M., Pop, N., Igel, P., Bernhardt, G., Buschauer, A.: In search for non-peptidic ligands of the human NPY $Y_4$ receptor starting from acylguanidine-type histamine $H_2$ receptor agonists. Frontiers in Medicinal Chemistry; Heidelberg, 15.03. – 18.03.2009

**Professional Training:**

Since October 2007 associated member of the Research Training Group (Graduiertenkolleg 760) “Medicinal Chemistry: Molecular Recognition – Ligand Receptor Interactions“

# Contents

# CHAPTER 1

# General Introduction

## 1.1 The Neuropeptide Y (NPY) Family

Neuropeptide Y (NPY) belongs together with peptide YY (PYY) and pancreatic polypeptide (PP) to a peptide family of neuroendocrine hormones. The three members of the so called neuropeptide Y family all consist of 36-amino acids with C-terminal amidation, which is essential for biological activity,[1] and share considerable amino acid homology (**Table 1.1**).

**Table 1.1**: Amino acid sequences of the peptides of the human neuropeptide Y family.

| | |
|---|---|
| **hNPY** | YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-$NH_2$ |
| **hPYY** | YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-$NH_2$ |
| **hPP** | APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-$NH_2$ |

NPY, one of the most abundant neuropeptides in the central and peripheral nervous system,[2] was first isolated by Tatemoto and coworkers from porcine brain in 1982,[3] and was proven to be highly conserved across species.[4] PYY, discovered while searching for C-terminally amidated peptides in the extracts of porcine intestine,[5] shows greater variability, whereas PP, isolated as a contaminant in chicken insulin (1968),[6, 7] only reveals 50 % identity within mammals.[8]

In the central nervous systems, NPY was discovered in the basal ganglia, hypothalamus, amygdala and the hippocampus, where it acts as cotransmitter, e.g. with noradrenaline or GABA.[9-12] In the periphery, NPY is present as a cotransmitter in sympathetic neurons, where it is co-stored and co-released with noradrenaline.[13, 14] Additionally, NPY was found in the parasympathetic nervous system. The gut hormones PYY and PP are released in the gastrointestinal tract, where they regulate pancreatic and gastric secretion.[3, 15] Furthermore, the hormones of the neuropeptide Y family are associated with a broad range of biological effects such as stimulation of food intake,[16] memory processes,[17] anxiolysis,[18] hypothermia,[19] etc. Moreover, they are considered to be implicated in various diseases such as dysregulation of food intake and obesity,[20-24] mood disorders,[25-29] alcoholism,[27, 30-32] bone physiology,[33-35] pain[36-38] and cancer.[39-43]

The crystal structure of the avian pancreatic polypeptide (aPP) was resolved by X-ray analysis.[44, 45] The 3D structure comprises a polyproline-like helix at the N-terminus (1-8), followed by a β-turn (9-13) and an anti-parallel α-helical residue (14-31). Thereby, the C-terminal part (32-36), revealing the most crucial residues for receptor binding, was considered rather flexible. The hairpin-like conformation, the so called “PP-fold”, which leads to a close proximity of N- and C-terminus, stabilized by hydrophobic interactions, was also proposed for porcine NPY (**Figure 1.1**) due to its high sequence homology (50 %).[46] A comparable tertiary structure could later be confirmed for PYY.[47]

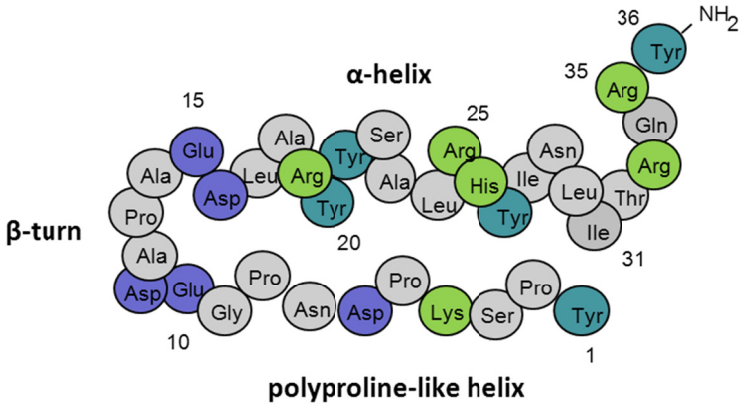



**Figure 1.1:** Tertiary structure of porcine NPY (according to Allen et al.[46]); ● basic residues; ● acid residues; ● tyrosine residues.

The three-dimensional structure of NPY and PP in solution was intensely investigated by several groups over the last decades using NMR techniques and CD spectroscopy as well as FRET-based approaches. While some of these studies confirmed the PP-fold structure (cf. **Figure 1.2**: A),[48-51] others reported contradictory findings, e.g. dimeric structures through α-helical contacts and conformations with non-helical and flexible N-termini (cf. **Figure 1.2**: B).[52-56]

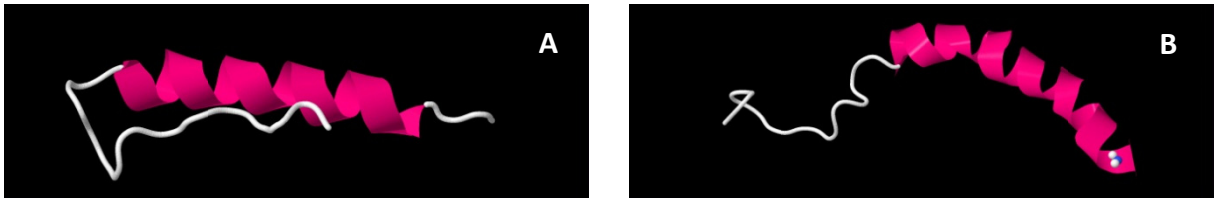



**Figure 1.2**: Structure of (**A**) bPP in solution according to Li et al.[51] supporting a hairpin-like conformation determined by X ray crystallography; (**B**) pNPY in solution according to Monks et al.[54] contradicting the PP-fold theory and proposing a flexible N-termini. Structural data are obtained from the Protein Data Bank (PDB access code 1BBA and 1RON, http://www.pdb.org/pdb/home/home.do)

However, conformational NMR studies are predominantly performed under unphysiological conditions with high concentrations of NPY in the millimolar range or pH values considerably lower than 7.4 in order to increase the solubility of the investigated peptides. Therefore, the relevance of these data with respect to the biologically active conformation of NPY under physiological conditions must be put into question. Interestingly, CD spectroscopic analyses revealed different conformations of NPY, co-existing in a dynamic equilibrium depending on the peptide concentration and the pH value. Probably, a hairpin-like conformation is favored under physiological conditions.[57] Additionally, interactions of NPY and PP with the surface of the cell membrane were studied suggesting a conformation of NPY and PP which differ from the PP-fold structure(cf. **Figure 1.3**).[56, 58-60]

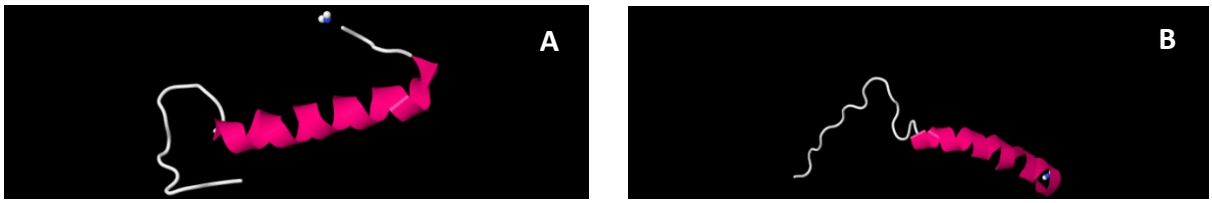



**Figure 1.3**: Structure of (**A**) bPP bound to micelles according to Lerch et. al.;[56] (**B**) pNPY bound to micelles according to Bader et al.[58]: The N-terminal part is flexible, while the C-terminal α-helix interacts with the membrane surface. In case of bPP a part of the N-terminus is also associated to the membrane; no PP-fold conformation could be observed. Structural data are obtained from the Protein Data Bank (PDB access code 1LJV and 1F8P, http://www.pdb.org/pdb/home/home.do).

## 1.2 Mammalian NPY Receptor Subtypes

The neuropeptide Y, PYY and PP interact with a family of seven transmembrane G-protein coupled receptors (GPCRs) belonging to the rhodopsin like superfamily (class 1) of receptors. To date, five mammalian NPY receptor subtypes, termed $Y_1$, $Y_2$, $Y_4$, $Y_5$ and $y_6$ receptor, have been cloned.[24, 61-67] The $y_6$ receptor was found to be functional in mice, but non-functional in most mammalian species; while being a pseudogene in many mammals, it is missing at all in the rat genome.[68] All NPY receptor subtypes were shown to activate pertussis toxin sensitive $G_{i/o}$ proteins, mediating the inhibition of forskolin-stimulated cAMP accumulation.[69, 70] In addition, NPY receptors ($Y_1$, $Y_2$, $Y_4$, $Y_5$) are reported to couple to phospholipase C, resulting in an increase of the intracellular calcium concentration.[61, 71-77] Thereby, the extent of the calcium response depends on the cell type.[69]

In **Table 1.2** the binding profiles, the localization and the physiological role of the mammalian NPY receptor subtypes (except for $y_6$) are summarized.

**Table 1.2**: Binding profile, localization and physiological role of the mammalian NPY receptor subtypes.[78-83]

| | **Binding Profile** | **Localization** | **Involvement** |
|---|---|---|---|
| **$Y_1$** | NPY ≈ PYY ≈ [Leu$^{31}$, Pro$^{34}$]NPY > NPY2-36 > NPY3-36 ≥ PP > NPY13-36 | smooth vascular muscles (postjunctionally), cerebral cortex, hypothalamus, colon, human adipocytes | regulation of blood pressure, seizures and food intake, anxiety, pain sensitivity, depression, angiogenesis, alcohol consumption |
| **$Y_2$** | NPY ≥ NPY2-36 ≈ NPY3-36 ≈ NPY13-36 >> [Leu$^{31}$, Pro$^{34}$]NPY | hippocampus, hypothalamus, nerve endings, (pre- and post-synaptic), renal tubules | regulation of blood pressure, seizures and food intake, anxiety, pain sensitivity, depression, angiogenesis, hypothalamic regulation of bone formation, regulation of GI motility |
| **$Y_4$** | PP > PYY ≥ NPY > NPY2-36 | brain, coronary arteries, ileum | food intake, regulation of GI motility |
| **$Y_5$** | NPY ≈ PYY ≈ NPY2-36 > hPP > NPY13-36 > rPP | Hypothalamus | food intake, seizures, anxiety |

## 1.2.1 NPY $Y_1$, $Y_2$ and $Y_5$ Receptors and their Ligands

### 1.2.1.1 The NPY $Y_1$ Receptor

The $Y_1R$ was the first NPY receptor to be cloned in 1990 as a rat orphan receptor by Eva et al.[84] The identification of the human gene followed two years later.[63, 85] The $Y_1R$ consists of 384 amino acids and is highly conserved, showing 90 – 96 % overall identity across orders of mammals.[68] Additionally, a high tendency to agonist induced internalization was observed for the $Y_1R$ as described for radioligand binding studies[86], confocal microscopy with fluorescent ligands[87] or GFP tagged $Y_1R$[88].

The most characteristic property of the $Y_1R$ is the enormously decreased affinity of N-terminally truncated analogs of the endogenous peptides NPY or PYY.[89] NPY(2-36) reveals a 75-fold decrease in affinity in comparison to NPY. Further truncated sequences such as NPY(3-36), NPY(13-36) and NPY(18-36) are almost devoid of $Y_1R$ affinity with $K_i$ values in the micromolar range.[90] By contrast, modifications in the C-terminal part were successful with respect to selectivity for the $Y_1R$ over the $Y_2R$, for instance, replacement of single amino acids e.g. [Leu$^{31}$,Pro$^{34}$]NPY.[91] In 2001 Mullins et al.[92] reported on selective $Y_1R$ agonists obtained by the introduction of D-amino acids, e.g. [D-Arg$^{25}$]pNPY and [D-His]pNPY. A further increase in selectivity was obtained by central truncation and cyclization of the endogenous ligands (e.g. Des-AA11-18[Cys$^{7,21}$, D-Lys$^{9}$(Ac), D-His$^{26}$, Pro$^{34}$]NPY). Surprisingly, even compounds lacking the N-terminal part of NPY could be developed within the last decades. Takebayashi et al. for example published a cyclized peptide c[D-Cys29-L-Cys34]NPY Ac$^{29-36}$ (YM-42454).[93, 94] Additionally, high $Y_1R$ affinity and selectivity was observed for the linear peptide pNPY(25-36) containing β-ACC (β-aminocyclopropanecarboxylic acid) in position 34 and/or 32.[95] As this high level of $Y_1R$ affinity and selectivity was not obtained with truncated analogs comprising the natural sequence, one may speculate that the unnatural β-amino acid stabilizes a secondary structure which is preferred upon $Y_1R$ binding. Introduction of non-proteinogenic amino acids such as norleucine (Nle) or 4-benzoylphenylalanine (Bpa) into the C-terminal nonapeptide of NPY, recently reported by Zwanziger et al.,[96] led to the identification of the first $Y_1R$ selective peptide with reduced size and affinity in the low nanomolar range.

The first non-peptidic $Y_1R$ antagonist described in literature was the arpromidine-type compound BU-E-76 (HE 90481), originally designed as $H_2R$ agonist,[97] with a $pA_2$ value of 4.4 in human erythroleukemia cells.[98] To date, a wide variety of highly potent and selective non-peptidic $Y_1R$ antagonists with affinities in the nanomolar and subnanomolar range have been developed, including BIBP 3226, the first compound published as highly potent and selective for the $Y_1R$ subtype. A selection of non-peptidic $Y_1R$ antagonists is shown in **Figure 1.4**.

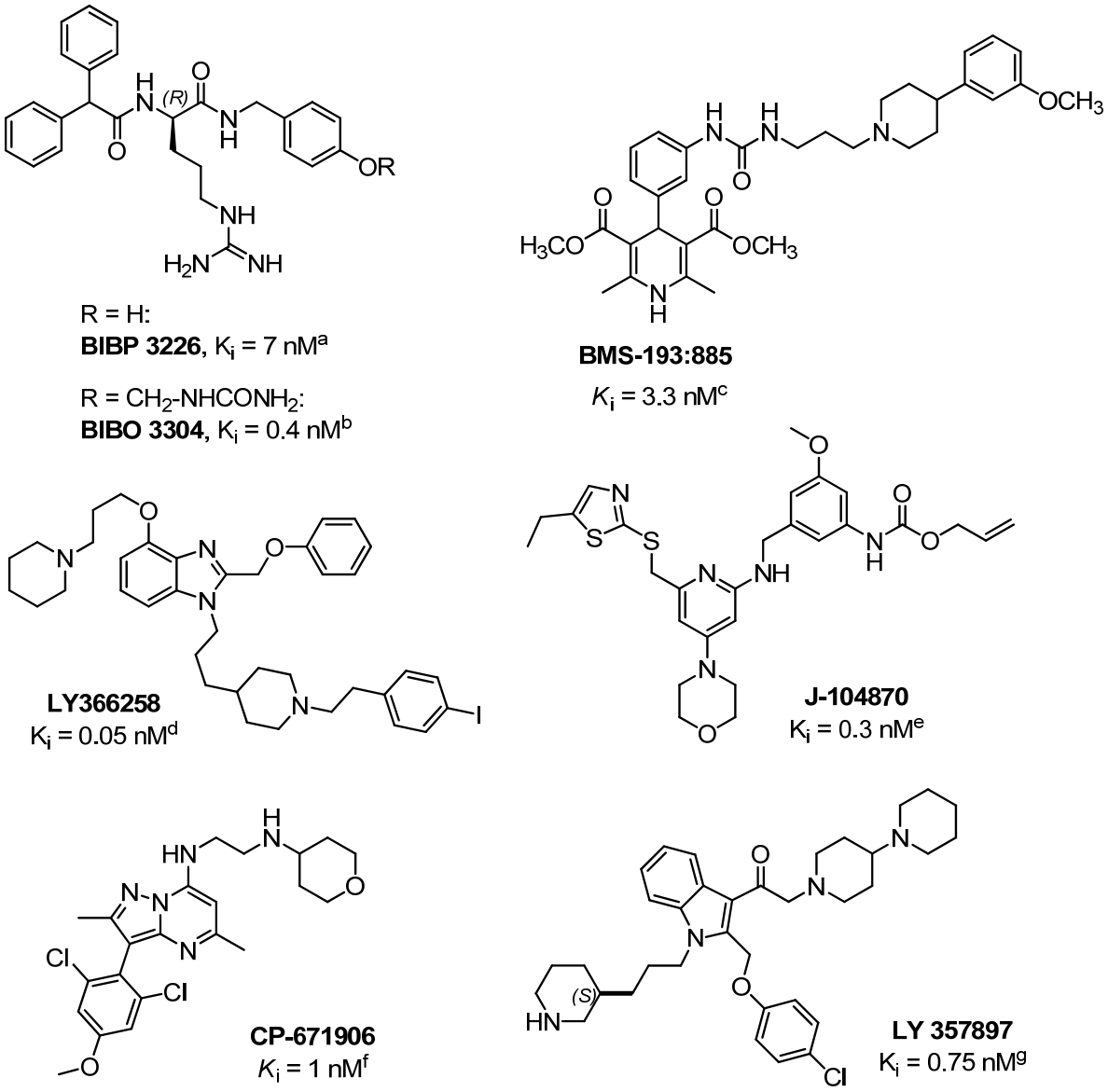



**Figure 1.4**: Examples of non-peptidic and selective $Y_1R$ antagonists. a: Rudolf at al.[99], b: Wieland et al.[100], c: Sit et al.[101], d: Zarrinmayeh et al.[102], e: Kanatani et al.[103], f:Griffith et al. [104], g: Hipskind et al.[105]

### 1.2.1.2 The NPY $Y_2$ Receptor

In 1986 the $Y_2$ receptor was identified by pharmacological studies with N-terminally truncated analogs of NPY and PYY using vascular preparations (e.g. NPY(3-36) and NPY(13-36)).[1] In 1995, the human $Y_2R$, which consist of 381 amino acids, was cloned from SMS-KAN cells and subsequently from human brain cDNA libraries.[106, 107] Although this receptor subtype turned out to be highly conserved across species with a sequence homology of 90 – 96 %, it shares only 30 % overall identity with the $Y_1R$ and the $Y_4R$, respectively.[86, 108] Reports in terms of $Y_2R$ internalization are contradictory. Thus, desensitization of the receptor after agonist stimulation could be detected in LN319 cells,[73] but in contrast to the $Y_1R$, the internalization process was observed only after prolonged agonist excitation[86, 88] and was described to be quite slow.[109]

In contrast to the $Y_1R$, N-terminally truncated analogs such as NPY(13-36), PYY(3-36), [Leu$^{31}$]PYY(24-36) and AcPYY(22-36)still revealed full agonistic activities with comparable potencies compared to the endogenous ligands, and have been intensively studied in the past.[90, 110-113] On the other hand, the $Y_2R$ seems to be more sensitive to substitutions in the C-terminus. Thus, replacement of the amino acids in positions 31 and 34 of NPY with the corresponding amino acids of the PP sequence yielding [Pro$^{34}$]NPY and [Leu$^{31}$,Pro$^{34}$]NPY show only low $Y_2R$ affinity, indicating that the C-terminal part is more important for receptor binding.[91, 114] However, centrally truncated analogs connecting, e.g., NPY(1-4) and NPY(25-36) via a 6-aminohexanoic acid linker affording [Ahx$^{5-24}$]NPY, showed $Y_2R$ affinity comparable to that of the native ligand NPY.[115] Therefore, one may speculate that the C-terminal part of NPY is crucial for key interactions with the $Y_2R$, whereas the N-terminus seems to be less important. This is supported by the results obtained from an alanine scan. The amino acids Arg$^{35}$ and Tyr$^{36}$ were identified to play a key role in binding.[116] Currently, the development of $Y_2R$ agonists is focused on modified and truncated PYY analogs such as PYY(22-36) (BT-48), branched PYY(3-36) derived ligands or replacements of the phenolic OH group of the C-terminal tyrosinamide in PYY(3-36) with a halogen or an amine function.[117-119]

In 1999, BIIE 0246 was reported as the first highly selective non-peptide $Y_2R$ antagonist with a one-digit nanomolar affinity.[120] The design and development of BIIE 0246 was based on the truncated NPY analogs.[121] The potential of $Y_2R$ ligands as therapeutics for the treatment of obesity led to an intensified search and identification of low molecular weight compounds mainly with the help of high throughput screening. A selection of such small $Y_2R$ antagonists is depicted in **Figure 1.5**.

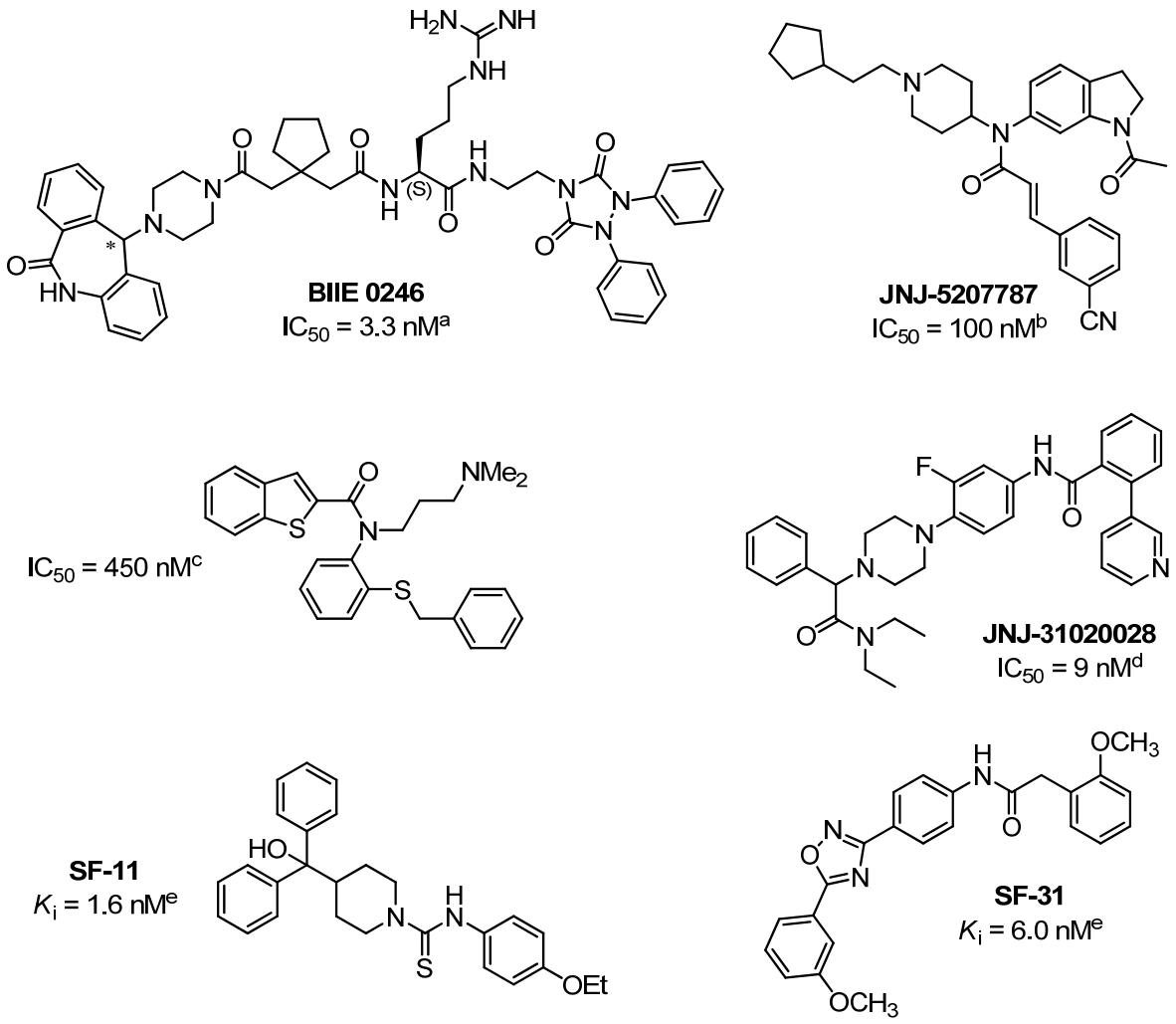



**Figure 1.5:** Examples of non-peptidic and selective $Y_2R$ antagonists: a: Doods et al.[120], b: Bonaventure et al.[122], c: Andres et al.[123], d: Shoblock et al.[124], e: Brothers et al.[125]

### 1.2.1.3 The NPY $Y_5$ Receptor

The existence of the $Y_5$ receptor was suggested based on the observation that both, NPY and NPY(2-36), produced a large increase in feeding after intracerebroventricular administration. Due to the fact that NPY(2-36), is less potent at the $Y_1R$ than the native sequence, this observed orexigenic effect was supposed to be mediated by another NPY receptor subtype which was firstly referred to as "feeding receptor".[126, 127] Cloning of the NPY $Y_5R$ from rat hypothalamus was published in 1996.[24, 64] Within the NPY receptor family, this subtype represents the largest protein. It consists of 445 amino acids with an extended third intracellular loop comprising about 100 amino acids more than the other NPY receptor subtypes and a quite short C-terminus. Furthermore, the $Y_5R$ shows a high homology within species with an 88 – 90 % overall identity.[128, 129] Similar to the $Y_1R$ a high tendency of receptor internalization was observed for the $Y_5R$.[130]

The first $Y_5R$ selective NPY analog was [D-Trp$^{32}$]NPY, an antagonist published by Balsubramaniam et al.[131] A $Y_5R$ selective agonist was designed based on NPY analogs and NPY/PP-chimeras containing Ala and Aib (aminoisobutyric acid) substitutions, which were identified as a key motif for $Y_5R$ selective compounds. Thereby, [Ala$^{31}$,Aib$^{32}$]NPY turned out to be a highly potent and selective $Y_5R$ agonist which was able to induce feeding in rats.[90, 132]

In 1997, the first selective non-peptidic antagonist, CPG 71683A was published. However, due to unfavorable properties (poor solubility) and, in particular, off-target effects, this compound turned out to be an imprecise tool for investigations on the role of the $Y_5R$. Nevertheless, numerous $Y_5R$ ligands covering a broad spectrum of chemical structures were designed and synthesized within the last decade (examples cf. **Figure 1.6**), and several compounds, e.g. MK-0557 (Merck & Co., Inc.), even entered clinical trials.[22]

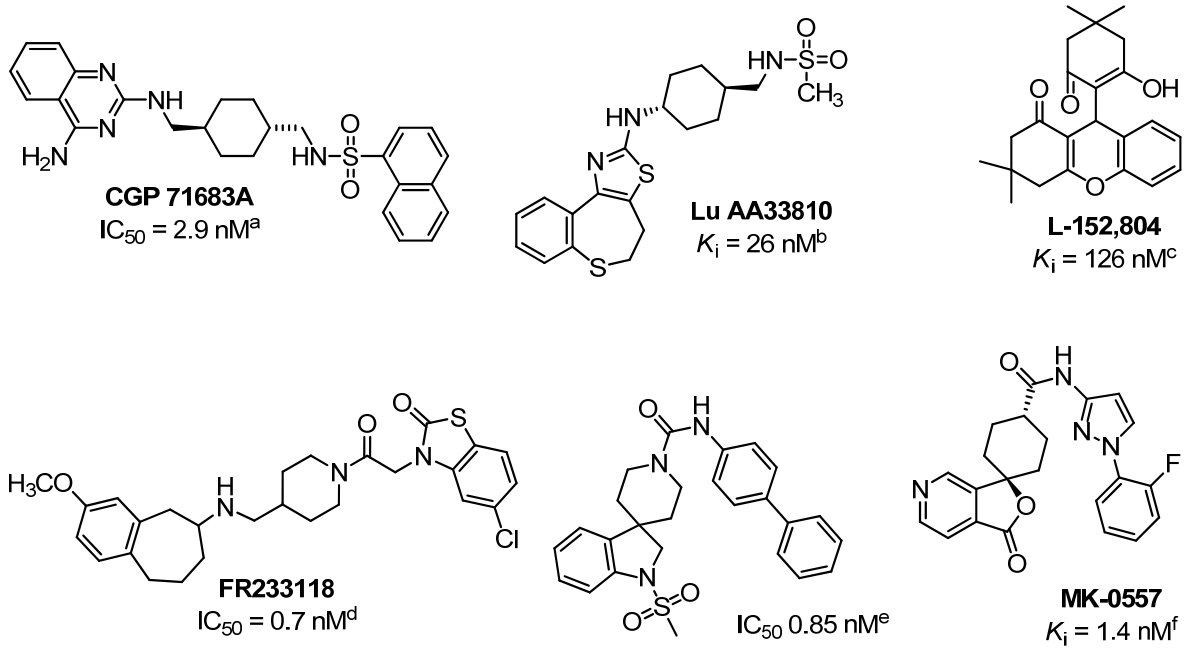



**Figure 1.6:** Examples of selective, non-peptidic $Y_5R$ antagonists. a: Criscione et al.[72], b: Walker et al.[133], c Kanatani et al.[134], d: Itani et al.[135], e: Sakamoto et al.[136], f: Erondu et al.[137]

## 1.2.2 The NPY $Y_4$ Receptor and its Ligands

The NPY $Y_4R$, previously designated as PP1 receptor because of its preference of PP over NPY and PYY, was identified as a novel receptor by an $Y_1R$ homology-based approach.[66, 138] The overall sequence identity of $Y_4R$ and $Y_1R$ amounts to 42 %. A $Y_4R$ clone was first isolated in 1995 by Lundell et al., followed by Bard et al., by sequence homology screening of $Y_1R$ probes.[66, 71] Meanwhile, orthologs such as the rat[24, 138] and the murine[62] $Y_4R$ have been cloned. In terms of receptor internalization contradictory results have been reported: while $Y_4R$ transfected CHO cells did show neither receptor internalization nor desensitization even after 24 hours of incubation with PP,[139] a rapid sequestration

of the guinea pig $Y_4R$ expressed in CHO same cells was observed, when [$^{125}$I]PP was used as an agonist.[130]

Interestingly, the NPY $Y_4R$ shows a low degree of sequence identity across species. The rat $Y_4R$, for example, only shares 75 % sequence homology with the human ortholog.[138] Hence, the $Y_4R$ is considered one of the most rapidly evolving GPCRs known to date.[78] This does not seem surprising as the preferred ligand, PP, only shares a low homology across species, either. Thus, PPs from other species often have much lower affinity to the human $Y_4$ receptor. For example, the affinity of rPP for the hY4 receptor is more than 50 to 100- fold lower than that of hPP.[140] The most characteristic property of the $Y_4R$ is its preferential and high affinity binding of hPP ($K_i$ = 13.8 pM)[66] and its significantly lower affinity for the endogenous ligand NPY. Whereas a $K_i$ value of > 1 µM was reported by Voisin et al.,[139] other workgroups reported quite different data for NPY at the $Y_4R$, with $K_i$ values of 9.9 nM[66] or 2 nM[71]. Probably, $Y_4R$ affinity is highly dependent on the cell type or the radioligand used in the individual experiments. PYY ($pK_i$ = 9.06) and its [Pro$^{34}$]-substituted analog ($pK_i$ = 9.93) showed high affinity for the $Y_4R$ subtype. Surprisingly, some high affinity $Y_1R$ ligands, initially supposed to be selective for this receptor subtype, turned out to be rather potent $Y_4R$ ligands, as well: [Leu$^{31}$, Pro$^{34}$]NPY as well as the homodimeric peptide GW1229, depicted in **Figure 1.7**, were identified as potent $Y_4R$ agonists.[141-144]

In search for $Y_4R$ selective ligands, several PP/NPY-chimeras have been developed revealing high potency and affinity for the $Y_4R$ in the picomolar range such as [cPP$^{1-7}$,pNPY$^{19-23}$,His$^{34}$]-hPP ($IC_{50}$ = 60 pM)[145] or [K$^4$,RYYSY$^{19-23}$]PP(2-36)[146] ($K_i$ = 4 pM). Unfortunately, these ligands bind to the $Y_5R$ with affinities of 40 and 29 pM, either. A $Y_4R$ selective PP-derived analog with improved properties – the exact structure is not described – was developed by 7TM Pharma (Lyngby, Denmark) even entering clinical trials. Again a very successful approach by Balasubramaniam et al. was the synthesis of homodimeric compounds connecting the C-terminal pentapeptide of NPY – with single amino acid substitutions of position 32 and 34 – by suberic acid, as depicted in **Figure 1.8**, resulting in a $Y_4R$ selective homodimer with picomolar affinity ($K_i$ = 0.05 nM).

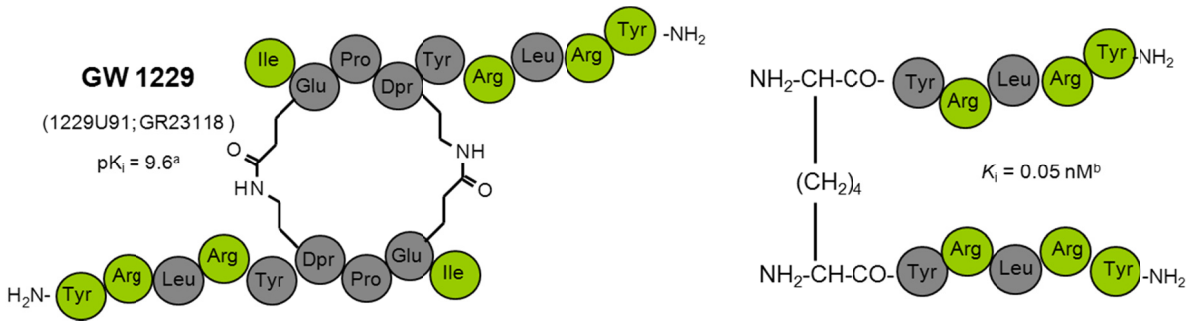



**Figure 1.7**: Structures of highly affine $Y_4R$ peptide dimers: a: GW1229, developed as $Y_1R$ antagonist also revealed high affinity for the $Y_4R$.[143]; b: $Y_4R$ selective agonist synthesized by Balasubramaniam et al.[147]

Recently, the peptide VD-11 (C-terminal oxymethylation product of GW1229) was reported to act as competitive antagonist at the $Y_4R$, as it did not promote the internalization of [$^{125}$I]hPP as observed for other $Y_4$ receptor agonists.[148] However, VD-11 did neither inhibit forskolin-stimulated cAMP formation nor stimulate [$^{35}$S]GTP-γ-S binding.[148] Additionally, in a cAMP assay presented in a former study, the peptide (100 nM) was not able to cause a rightward-shift of the concentration-response of PP.[149] Thus, the hypothesis of $Y_4R$ antagonism of VD-11 is not supported by these functional studies.

Highly affine non-peptide $Y_4R$ ligands have not been described in literature to date. Nevertheless, several acylguanidine-type compounds (cf. **Figure 1.8**), which were initially designed and synthesized as histamine receptor ligands in our workgroup, were identified as weak $Y_4R$ antagonists. In search for non-peptidic $Y_4R$ ligands, acylguanidines were further explored as discussed in this thesis.

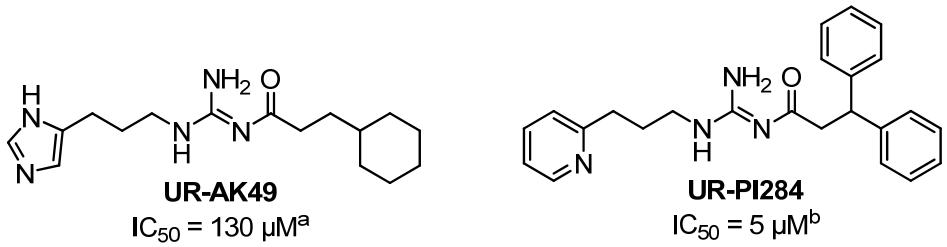



**Figure 1.8**: Acylguandine-type ligands originally designed as histamine receptor ligands and found to be weak $Y_4R$ antagonists; a: Ziemek et al.[150], b: Pop et al.[151]

# 1.3 (Non)Peptide Ligands for NPY Receptors – Development and Therapeutic Potential

## 1.3.1 NPY Receptors in Health and Disease

The NPY receptors and their endogenous ligands are involved in a broad variety of physiological processes and considered to be (potentially) implicated in many human diseases. Over the last decades, a lot of effort has been put on exploring the role of NPY receptors in the regulation of food intake and energy homeostasis, as obesity has become a severe and increasing health concern in industrialized countries. Additionally, the NPY system as a complex network of receptors and peptides has been discovered to play an important role in alcoholism, pain, depression and anxiety, cancer, cardiovascular regulation, bone physiology as well as in the gastrointestinal tract. Some of these topics will be discussed in more detail in the following paragraphs.[79]

**NPY and obesity[20, 22, 79, 152]**

First studies in 1950 with ob/ob mice (obese mice) revealed that these animals are leptin-deficient.[153] Leptin is connected to NPY pathways, and an overexpression of NPY in the hypothalamus of these animals could be detected in later studies.[154] The implication of NPY in increased food intake and

appetite regulation was already stated in the early 1980s.[155, 156] From then on, NPY became a popular target as it was considered the key for the treatment of obesity. In fact, several studies on NPY receptor knockout mice revealed that $Y_1R$, $Y_2R$ and $Y_5R$ deficient mice have higher body weights.[157-159] Additionally, $Y_2R$ and $Y_5R$ knockout mice show increased food intake.[158, 159] By contrast, the $Y_4R$ seems to play an opposite role, as $Y_4R$ knockout mice showed reduced body weight.[160] Recent studies revealed that additional factors such as anxiety and stress influence the NPY mediated effects in metabolic diseases. Chronic stress combined with a poor diet led to abdominal obesity via NPY activation.[21, 161] Obviously, the role of NPY receptors in the regulation of food intake and energy homeostasis and the correlation to obesity is highly complex. Antagonism at $Y_1R$ and $Y_5R$ and agonism at $Y_2R$ and $Y_4R$ result in anti-obesity effects, according to investigations using subtype selective NPY receptor ligands.[162] Nevertheless, selective $Y_1R$ and $Y_5R$ ligands failed in preclinical models of food intake showing no meaningful effects on weight-loss.[22] Maybe future investigations may also consider targeting both receptors by designing dual antagonists, because $Y_1/Y_5R$ heterodimers have been suggested. Additionally, the anorectic effects of peripheral agonism at $Y_2R$ and $Y_4R$ will attract more attention in particular with the recent development of a $Y_2/Y_4$-dual agonist of 7TM Pharma with potential for the treatment of obesity.[20, 163]

**NPY and cancer[39, 164]**

The neuropeptide Y family has been suggested to be implicated in numerous processes of tumor progression, including cell proliferation, angiogenesis and metastasis. Thereby, neuroendocrine tumors, prostate and breast cancers seem to be mainly effected and often show an overexpression of NPY receptors and their respective hormone family.[39] Studies on smooth muscle cells and neural crest tumors, for example, revealed NPY induced promotion of cell proliferation via the $Y_1R$ or the $Y_1R$ and the $Y_2R$, respectively. Furthermore, an NPY-mediated effect in case of angiogenesis was reported by Zukowska-Grojec et al.[165] Interestingly, a reduced angiogenic effect of NPY was observed in $Y_2R$ knockout mice.[166] $Y_2R$ antagonists might be useful to control tumor growth. Apart from targets in anticancer treatment, NPY receptors could be diagnostic markers for *in vivo* imaging of tumors. Thus, PET-, radiolabeled and receptor-targeted cytotoxic NPY derivatives might be useful in cancer patients.[164]

**NPY in depression, stress and anxiety[27, 167]**

Numerous studies on the role of NPY in psychiatric disorders have been reported in literature.[25-28] An influence of NPY on the pathophysiology of depression is supposed. In several studies, reduced NPY levels found in the cerebrospinal fluid were discussed to be correlated to anxiety-like behavior.[168, 169] The $Y_2R$, which is presynaptically located in NPY-ergic neurons and controls NPY-release, is supposed

to play a major role in emotionality. Hence, blocking of this receptor subtype should lead to increased NPY levels which might have a positive effect in the treatment of psychiatric disorders.[28] This hypothesis is corroborated by experiments with $Y_2R$ knockout mice.[170] Additionally, anxiolytic-like effects were observed upon injection of the selective $Y_2R$ agonist NPY(13-36).[171] By contrast, administration of an $Y_2R$ antagonist, BIIE 0246, led to antidepressant-like effects in mice.[172]
Moreover, the $Y_1R$ seems also to be involved in anxiety-like behavior and to mediate antidepressant-like properties of NPY.[40, 173]

**NPY and alcoholism[30, 32, 125]**

A first hint to a correlation of NPY signaling and regulation of alcohol consumption came from animal studies. NPY overexpressing mice consumed less alcohol, whereas mice with inactivation of the NPY encoding gene showed strongly increased alcohol consumption.[174] First data from human studies were reported by Mayfield et al. in 2002.[175] Accordingly, reduced NPY levels were found in the brains of alcoholics compared to the control group. However, it could not be proven, whether the reduced NPY levels are a consequence of chronic alcohol abuse or if they were already present before the manifestation of alcoholism. Although $Y_1$, $Y_2$ and $Y_5$ receptor seem to be implicated in the modulation of alcohol intake, the $Y_2R$ is considered to play a major role. Thus, $Y_2R$ knockout mice showed a reduced alcohol preference compared to wild type mice.[32] Nevertheless, additional studies are necessary to get an idea of the importance of the NPY receptor system in terms of drug and alcohol abuse.

### 1.3.2 NPY Receptor Ligands and their Therapeutic Potential

Regarding the influence of the NPY receptor and peptide family on metabolic regulations and its role in various diseases, the search for potent NPY receptor ligands seems quite important. During the last decades many efforts have been made to identify potent and selective NPY receptor ligands. Unfortunately, only few compounds were suitable to enter clinical trials. Thereby, the NPY receptor research mainly focused on the development of anti-obesity drugs. The therapeutic potential of a selection of NPY receptor ligands – peptides as well as non-peptidic ligands – will be presented in this section.

**BIBP 3326 and BIIE0246**

BIBP 3326 and BIIE0246 were the first non-peptidic antagonists which showed selectivity for $Y_1R$ and $Y_2R$, respectively.[99, 120] They have been proven to be important pharmacological tools to study the functions of the respective receptors. Moreover, inhibition of NPY-induced food intake in rats could be successfully proven for BIBP 3226.[176-178] Nevertheless, the use of these compounds as therapeutics

is limited due to lack of oral bioavailability and inability to cross the blood brain barrier. Additionally, relatively high molecular weight as well as a submicromolar affinity for other receptors, e.g. the α1A adrenergic receptor disqualify the $Y_2R$ ligand BIIE 0246 as an appropriate drug candidate.[125] To date novel $Y_1$ and $Y_2$ receptor selective ligands with lower molecular weight have been synthesized (see section 1.2.1.1 and 1.2.1.2), showing the desired *in vitro* and *in vivo* activities, but still lacking optimal pharmacokinetic properties.[20]

**CGP 71683A and MK-0557**

CPG 71683A was published in 1997 as a highly potent and selective $Y_5R$ ligand.[72] Although it was an adequate pharmacological tool for studying $Y_5R$ functions, it revealed the same problems already described for $Y_1$ and $Y_2R$ non-peptidic ligands, lacking sufficient oral bioavailability and access to the CNS. Nevertheless, numerous high affinity $Y_5R$ selective antagonists with improved pharmacokinetic properties have been disclosed. For example, MK-0557, developed by Merck & Co., described as a highly potent and selective $Y_5R$ ligand with good pharmacokinetic properties (e.g. oral availability) entered into clinical trials and was tested for the treatment of obesity, but was withdrawn due to lack of clinically relevant effects.[22] Obviously, the weight loss in patients treated with MK-0557was statistically significant, but less than observed upon treatment with other weight-reducing drugs. Additionally, weight-regain after diet-induced weight-loss could not be reduced.[137, 179] Hence, it was speculated that the $Y_5R$ alone does not play the major role in appetite regulation.

**PP and PYY-derived ligands**

The two hormones PP and PYY are produced in the pancreatic islet cells (PP) or the intestinal L-cells (PYY) and are secreted after meals depending on the calories consumed.[180-184] Thereby, PYY exists in two different forms: PYY(1-36), which shows agonistic activity at the $Y_1$, $Y_2$ and $Y_5$ receptor and the N-terminally truncated analog PYY(3-36), which seems to be only active at the $Y_2R$ subtype.[185-187] After the first report on a weight-loss inducing effect of the selective $Y_2R$ agonist PYY(3-36), the anorectic properties could not be confirmed in animal models.[188-190] Hence, PYY(3-36) was included in clinical trials. After infusion of this peptide, a decrease in food intake in healthy volunteers could be observed.[191] Furthermore, administration of an intranasal formulation of PYY(3-36) confirmed a reduction of energy intake.[192] However, due to significant side effects (nausea and vomiting) observed in both studies, especially at the highest administered doses, which were necessary to obtain significant effects, the development of PYY(3-36) as a drug candidate was encountered.[193] As PYY(3-36) is supposed to act via the $Y_2R$, Bayer developed N-terminally truncated PEG-conjugated PYY derivatives with improved stability. One ligand, PEG20-PYY(24-36)-L31 was even identified to exert anti-obesity effects in mice.[22]

Administration of the high affinity endogenous $Y_4R$ ligand PP revealed a reduction in food intake and an increase in energy expenditure in obese mice. Additionally, the effect of PP was studied in children with Prader-Willi syndrome resulting in a moderate reduction (12 %) in food intake.[194] Recent studies in lean human volunteers showed PP-induced reduction of food intake and a delay in gastric emptying.[195, 196] Nevertheless, the applicability of PP as a drug is severely compromised because of rapid degradation. Therefore, long-acting $Y_4R$ agonists are urgently needed.[193] As already described for PYY derivatives, Bayer also tried to stabilize PP derived ligands by *N*-PEGylation. To date no pharmacological data of these selective $Y_4R$ agonists were provided.[22]

**TM30338 (Obinepitide) and TM30339**

TM30338 (Obinepitide) and TM30339, a $Y_2/Y_4$ dual agonist and a $Y_4$-agonist, respectively, are peptide ligands derived from PP and PYY. As communicated by 7TM Pharma, the company that developed these peptides, both compounds showed reduction in food intake and body weight in clinical trials (Phase I/II for obinepitide and Phase I for TM30339).[20, 22] TM30339 was also observed to reduce ghrelin levels in healthy obese volunteers;[22] and to date no serious side effects were reported.[197]

### 1.3.3 Strategies for the Development of (Non)Peptide NPY Receptor Ligands

There is a need for the development of selective (non)peptide NPY receptor ligands, on the one hand to clarify the pathophysiological role of NPY and on the other hand to assess the therapeutic potential of NPY receptor ligands and pathways.[79]

Besides the identification and optimization of non-peptidic hits and leads with the aid of high throughput screening (HTS), peptide-based drug design will become more important. With regard to the high molecular weight of the endogenous ligands and the lack of drug-like properties, the identification of small, low-molecular weight peptide-derived ligands is challenging. However, it should be noticed that the development of the first potent non-peptidic $Y_1$ and $Y_2$ receptor antagonists, BIBP 3226[99] and BIIE 0246[120], may be considered a first peptide mimicking strategy. Based on an alanine scan of NPY, especially the amino acids $Arg^{35}$ and $Tyr^{36}$ in the C-terminus of NPY were identified as highly important for receptor binding and activity and were therefore integrated into the design and synthesis of potential NPY receptor ligands.[116]

Peptidic structures could be advantageous due to high specificity and affinity as well as due to their low toxic potential.[198] However, peptides as therapeutic targets lack drug-like properties such as oral bioavailability and stability against biodegradation by serum and tissue proteases, resulting in short half-lives.[199] In addition, the large-scale production of peptides has been very complex and costly.[200] However, during the last years, problems of technical production of peptides could be overcome by introduction and optimization of automated solid phase peptide synthesis techniques.[198, 201]

Furthermore, progress in stabilization of peptides and elongation of half-lives has been made. N-acetylation and C-amidation turned out to increase peptide stability. Additionally, the introduction of PEG-chains to N- or C-terminus or the attachment of peptides to human serum proteins such as albumins are widely accepted methods for increasing stability, which could be successfully applied to peptide therapeutics, e.g. Interferon-α-2a and 2b (Roche and Bayer-Schering), to extend the half-life from 5 to 50 h.[199, 200]

The design of peptide-mimicking compounds also referred to as peptidomimetics, will attract increased attention in the synthesis of specific ligands for peptidergic receptors. Peptidomimetics are defined as compounds that mimic natural peptides or proteins by retaining the ability to interact with the biological target and produce the same biological effects. The major advantage of such compounds compared to native peptides is their improved drug-like properties, in particular, regarding stability and bioavailability.[202] The development of such peptidomimetic structures starts with the identification of the minimum active sequence of the natural peptide and the determination of key residues for receptor binding using e.g. an alanine or D-amino acids scan. The introduction of unnatural amino acids, β-amino acids or D-amino acids often results in conformationally constrained peptide structures with improved affinity or selectivity. Such modifications can induce structural stabilization or hairpin-like structures which are preferred in receptor binding. Furthermore the incorporation of β- and D-amino acids can enhance the peptide stability and bioavailability.[202-205] First steps of a peptidomimetic approach to potent and selective NPY $Y_4$R ligands starting from truncated derivatives of the endogenous ligands pNPY and hPP will be presented within this work.

## 1.4 References


1. Wahlestedt, C.; Yanaihara, N.; Hakanson, R. Evidence for different pre-and post-junctional receptors for neuropeptide Y and related peptides. *Regul. Pept.* **1986,** 13, 307-318.
2. Gray, T. S.; Morley, J. E. Neuropeptide Y: anatomical distribution and possible function in mammalian nervous system. *Life Sci.* **1986,** 38, 389-401.
3. Tatemoto, K.; Carlquist, M.; Mutt, V. Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* **1982,** 296, 659-660.
4. Larhammar, D. Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. *Regul. Pept.* **1996,** 62, 1-11.
5. Tatemoto, K. Isolation and characterization of peptide YY (PYY), a candidate gut hormone that inhibits pancreatic exocrine secretion. *Proc. Natl. Acad. Sci. USA* **1982,** 79, 2514-2518.
6. Kimmel, J. R.; Hayden, L. J.; Pollock, H. G. Isolation and characterization of a new pancreatic polypeptide hormone. *J. Biol. Chem.* **1975,** 250, 9369-9376.

7. Kimmel, J. R.; Pollock, H. G.; Hazelwood, R. L. Isolation and characterization of chicken insulin. *Endocrinology* **1968,** 83, 1323-1330.
8. Conlon, J. M. The origin and evolution of peptide YY (PYY) and pancreatic polypeptide (PP). *Peptides* **2002,** 23, 269-278.
9. Chronwall, B. M. Anatomy and physiology of the neuroendocrine arcuate nucleus. *Peptides* **1985,** 6 Suppl 2, 1-11.
10. Fetissov, S. O.; Kopp, J.; Hokfelt, T. Distribution of NPY receptors in the hypothalamus. *Neuropeptides* **2004,** 38, 175-188.
11. Heilig, M.; Widerlov, E. Neurobiology and clinical aspects of neuropeptide Y. *Crit. Rev. Neurobiol.* **1995,** 9, 115-136.
12. Stanic, D.; Mulder, J.; Watanabe, M.; Hokfelt, T. Characterization of NPY Y2 receptor protein expression in the mouse brain. II. Coexistence with NPY, the Y1 receptor, and other neurotransmitter-related molecules. *J.Comp. Neurol.* **2011,** 519, 1219-1257.
13. Ekblad, E.; Edvinsson, L.; Wahlestedt, C.; Uddman, R.; Hakanson, R.; Sundler, F. Neuropeptide Y co-exists and co-operates with noradrenaline in perivascular nerve fibers. *Regul. Pept.* **1984,** 8, 225-235.
14. Sundler, F.; Böttcher, G.; Ekblad, E.; Hakanson, R. *PP, PYY, and NPY - Occurence and distribution in the periphery. In Handbook of Experimental Pharmacology. Colmers, W.F. and Wahlestedt, C. (eds)*. New Jersey: Humana Press: Totowa, **1993**; p 157-196.
15. Hazelwood, R. L. The pancreatic polypeptide (PP-fold) family: gastrointestinal, vascular, and feeding behavioral implications. *Proc. Soc. Exp. Biol. Med.* **1993,** 202, 44-63.
16. Clark, J. T.; Kalra, P. S.; Kalra, S. P. Neuropeptide Y stimulates feeding but inhibits sexual behavior in rats. *Endocrinology* **1985,** 117, 2435-2442.
17. Flood, J. F.; Hernandez, E. N.; Morley, J. E. Modulation of memory processing by neuropeptide Y. *Brain Res.* **1987,** 421, 280-290.
18. Bacchi, F.; Mathe, A. A.; Jimenez, P.; Stasi, L.; Arban, R.; Gerrard, P.; Caberlotto, L. Anxiolytic-like effect of the selective neuropeptide Y Y2 receptor antagonist BIIE0246 in the elevated plus-maze. *Peptides* **2006,** 27, 3202-3207.
19. Jolicoeur, F. B.; Bouali, S. M.; Fournier, A.; St-Pierre, S. Mapping of hypothalamic sites involved in the effects of NPY on body temperature and food intake. *Brain Res. Bull.* **1995,** 36, 125-129.
20. Kamiji, M. M.; Inui, A. Neuropeptide Y Receptor Selective Ligands in the Treatment of Obesity. *Endocr. Rev.* **2007,** 28, 664-684.
21. Kuo, L. E.; Kitlinska, J. B.; Tilan, J. U.; Li, L.; Baker, S. B.; Johnson, M. D.; Lee, E. W.; Burnett, M. S.; Fricke, S. T.; Kvetnansky, R.; Herzog, H.; Zukowska, Z. Neuropeptide Y acts directly in the

periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. *Nat. Med.* **2007,** 13, 803-811.

22. Sato, N.; Ogino, Y.; Mashiko, S.; Ando, M. Modulation of neuropeptide Y receptors for the treatment of obesity. *Expert Opin Ther Pat* **2009,** 19, 1401-1415.
23. Kamiji, M. M.; Inui, A. NPY Y2 and Y4 receptors selective ligands: promising anti-obesity drugs? *Curr. Top. Med. Chem.* **2007,** 7, 1734-1742.
24. Gerald, C.; Walker, M. W.; Criscione, L.; Gustafson, E. L.; Batzl-Hartmann, C.; Smith, K. E.; Vaysse, P.; Durkin, M. M.; Laz, T. M.; Linemeyer, D. L.; Schaffhauser, A. O.; Whitebread, S.; Hofbauer, K. G.; Taber, R. I.; Branchek, T. A.; Weinshank, R. L. A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature* **1996,** 382, 168-171.
25. Eaton, K.; Sallee, F. R.; Sah, R. Relevance of neuropeptide Y (NPY) in psychiatry. *Curr. Top. Med. Chem.* **2007,** 7, 1645-1659.
26. Wahlestedt, C.; Ekman, R.; Widerlov, E. Neuropeptide Y (NPY) and the central nervous system: distribution effects and possible relationship to neurological and psychiatric disorders. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **1989,** 13, 31-54.
27. Thorsell, A.; Karlsson, R. M.; Heilig, M. NPY in alcoholism and psychiatric disorders. *EXS* **2006**, 183-192.
28. Thorsell, A.; Slawecki, C. J.; El Khoury, A.; Mathe, A. A.; Ehlers, C. L. The effects of social isolation on neuropeptide Y levels, exploratory and anxiety-related behaviors in rats. *Pharmacol. Biochem. Behav.* **2006,** 83, 28-34.
29. Morales-Medina, J. C.; Dumont, Y.; Quirion, R. A possible role of neuropeptide Y in depression and stress. *Brain Res.* **2010,** 1314, 194-205.
30. Thorsell, A. Neuropeptide Y (NPY) in alcohol intake and dependence. *Peptides* **2007,** 28, 480-483.
31. Thorsell, A.; Rimondini, R.; Heilig, M. Blockade of central neuropeptide Y (NPY) Y2 receptors reduces ethanol self-administration in rats. *Neurosci. Lett.* **2002,** 332, 1-4.
32. Thiele, T. E.; Sparta, D. R.; Hayes, D. M.; Fee, J. R. A role for neuropeptide Y in neurobiological responses to ethanol and drugs of abuse. *Neuropeptides* **2004,** 38, 235-243.
33. Baldock, P. A.; Lee, N. J.; Driessler, F.; Lin, S.; Allison, S.; Stehrer, B.; Lin, E. J.; Zhang, L.; Enriquez, R. F.; Wong, I. P.; McDonald, M. M.; During, M.; Pierroz, D. D.; Slack, K.; Shi, Y. C.; Yulyaningsih, E.; Aljanova, A.; Little, D. G.; Ferrari, S. L.; Sainsbury, A.; Eisman, J. A.; Herzog, H. Neuropeptide Y knockout mice reveal a central role of NPY in the coordination of bone mass to body weight. *PLoS One* **2009,** 4, e8415.

34. Lee, N.; Allison, S.; Enriquez, R.; Sainsbury, A.; Herzog, H.; Baldock, P. Y2 and Y4 Receptor Signalling Attenuates the Skeletal Response of Central NPY. *J. Mol. Neurosci.* **2011,** 43, 123-131.

35. Ducy, P.; Amling, M.; Takeda, S.; Priemel, M.; Schilling, A. F.; Beil, F. T.; Shen, J.; Vinson, C.; Rueger, J. M.; Karsenty, G. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* **2000,** 100, 197-207.

36. Seybold, V. S.; McCarson, K. E.; Mermelstein, P. G.; Groth, R. D.; Abrahams, L. G. Calcitonin gene-related peptide regulates expression of neurokinin1 receptors by rat spinal neurons. *J. Neurosci.* **2003,** 23, 1816-1824.

37. Moran, T. D.; Colmers, W. F.; Smith, P. A. Opioid-like actions of neuropeptide Y in rat substantia gelatinosa: Y1 suppression of inhibition and Y2 suppression of excitation. *J Neurophysiol* **2004,** 92, 3266-3275.

38. Brumovsky, P.; Shi, T. S.; Landry, M.; Villar, M. J.; Hökfelt, T. Neuropeptide tyrosine and pain. **2007,** 28, 93-102.

39. Ruscica, M.; Dozio, E.; Motta, M.; Magni, P. Role of neuropeptide Y and its receptors in the progression of endocrine-related cancer. *Peptides* **2007,** 28, 426-434.

40. Hansel, D. E.; Eipper, B. A.; Ronnett, G. V. Neuropeptide Y functions as a neuroproliferative factor. *Nature* **2001,** 410, 940-944.

41. Kitlinska, J. Neuropeptide Y (NPY) in neuroblastoma: effect on growth and vascularization. *Peptides* **2007,** 28, 405-412.

42. Kitlinska, J. Neuropeptide Y in neural crest-derived tumors: effect on growth and vascularization. *Cancer Lett.* **2007,** 245, 293-302.

43. Kitlinska, J.; Abe, K.; Kuo, L.; Pons, J.; Yu, M.; Li, L.; Tilan, J.; Everhart, L.; Lee, E. W.; Zukowska, Z.; Toretsky, J. A. Differential effects of neuropeptide Y on the growth and vascularization of neural crest-derived tumors. *Cancer Res.* **2005,** 65, 1719-1728.

44. Blundell, T. L.; Pitts, J. E.; Tickle, I. J.; Wood, S. P.; Wu, C. W. X-ray analysis (1. 4-A resolution) of avian pancreatic polypeptide: Small globular protein hormone. *Proc. Natl. Acad. Sci. USA* **1981,** 78, 4175-4179.

45. Glover, I.; Haneef, I.; Pitts, J.; Wood, S.; Moss, D.; Tickle, I.; Blundell, T. Conformational flexibility in a small globular hormone: x-ray analysis of avian pancreatic polypeptide at 0.98-A resolution. *Biopolymers* **1983,** 22, 293-304.

46. Allen, J.; Novotny, J.; Martin, J.; Heinrich, G. Molecular structure of mammalian neuropeptide Y: analysis by molecular cloning and computer-aided comparison with crystal structure of avian homologue. *Proc. Natl. Acad. Sci. USA* **1987,** 84, 2532-2536.

47. Keire, D. A.; Kobayashi, M.; Solomon, T. E.; Reeve, J. R., Jr. Solution structure of monomeric peptide YY supports the functional significance of the PP-fold. *Biochemistry* **2000,** 39, 9935-9942.

48. Boulanger, Y.; Chen, Y.; Commodari, F.; Senecal, L.; Laberge, A. M.; Fournier, A.; St-Pierre, S. Structural characterizations of neuropeptide tyrosine (NPY) and its agonist analog [Ahx5-17]NPY by NMR and molecular modeling. *Int. J. Pept. Prot. Res.* **1995,** 45, 86-95.

49. Darbon, H.; Bernassau, J. M.; Deleuze, C.; Chenu, J.; Roussel, A.; Cambillau, C. Solution conformation of human neuropeptide Y by 1H nuclear magnetic resonance and restrained molecular dynamics. *Eur. J. Biochem.* **1992,** 209, 765-771.

50. Haack, M.; Beck-Sickinger, A. G. Towards understanding the free and receptor bound conformation of neuropeptide Y by fluorescence resonance energy transfer studies. *Chem. Biol. Drug Des.* **2009,** 73, 573-583.

51. Li, X.; Sutcliffe, M. J.; Schwartz, T. W.; Dobson, C. M. Sequence-specific proton NMR assignments and solution structure of bovine pancreatic polypeptide. *Biochemistry* **1992,** 31, 1245-1253.

52. Bettio, A.; Dinger, M. C.; Beck-Sickinger, A. G. The neuropeptide Y monomer in solution is not folded in the pancreatic-polypeptide fold. *Protein Sci.* **2002,** 11, 1834-1844.

53. Cowley, D. J.; Hoflack, J. M.; Pelton, J. T.; Saudek, V. Structure of neuropeptide Y dimer in solution. *Eur. J. Biochem.* **1992,** 205, 1099-1106.

54. Monks, S. A.; Karagianis, G.; Howlett, G. J.; Norton, R. S. Solution structure of human neuropeptide Y. *J. Biomol. NMR* **1996,** 8, 379-390.

55. Saudek, V.; Pelton, J. T. Sequence-specific 1H NMR assignment and secondary structure of neuropeptide Y in aqueous solution. *Biochemistry* **1990,** 29, 4509-4515.

56. Lerch, M.; Gafner, V.; Bader, R.; Christen, B.; Folkers, G.; Zerbe, O. Bovine Pancreatic Polypeptide (bPP) Undergoes Significant Changes in Conformation and Dynamics upon Binding to DPC Micelles. *J. Mol. Biol.* **2002,** 322, 1117-1133.

57. Nordmann, A.; Blommers, M. J.; Fretz, H.; Arvinte, T.; Drake, A. F. Aspects of the molecular structure and dynamics of neuropeptide Y. *Eur. J. Biochem.* **1999,** 261, 216-226.

58. Bader, R.; Bettio, A.; Beck-Sickinger, A. G.; Zerbe, O. Structure and dynamics of micelle-bound neuropeptide Y: comparison with unligated NPY and implications for receptor selection. *J. Mol. Biol.* **2001,** 305, 307-329.

59. Lerch, M.; Mayrhofer, M.; Zerbe, O. Structural similarities of micelle-bound peptide YY (PYY) and neuropeptide Y (NPY) are related to their affinity profiles at the Y receptors. *J. Mol. Biol.* **2004,** 339, 1153-1168.

60. Thomas, L.; Scheidt, H. A.; Bettio, A.; Huster, D.; Beck-Sickinger, A. G.; Arnold, K.; Zschornig, O. Membrane interaction of neuropeptide Y detected by EPR and NMR spectroscopy. *Biochim. Biophys. Acta.* **2005,** 1714, 103-113.

61. Gerald, C.; Walker, M. W.; Vaysse, P. J.; He, C.; Branchek, T. A.; Weinshank, R. L. Expression cloning and pharmacological characterization of a human hippocampal neuropeptide Y/peptide YY $Y_2$ receptor subtype. *J. Biol. Chem.* **1995,** 270, 26758-26761.

62. Gregor, P.; Millham, M. L.; Feng, Y.; DeCarr, L. B.; McCaleb, M. L.; Cornfield, L. J. Cloning and characterization of a novel receptor to pancreatic polypeptide, a member of the neuropeptide Y receptor family. *FEBS Lett.* **1996,** 381, 58-62.

63. Herzog, H.; Hort, Y. J.; Ball, H. J.; Hayes, G.; Shine, J.; Selbie, L. A. Cloned human neuropeptide Y receptor couples to two different second messenger systems. *Proc. Natl. Acad. Sci. USA* **1992,** 89, 5794-5798.

64. Hu, Y.; Bloomquist, B. T.; Cornfield, L. J.; DeCarr, L. B.; Flores-Riveros, J. R.; Friedman, L.; Jiang, P.; Lewis-Higgins, L.; Sadlowski, Y.; Schaefer, J.; Velazquez, N.; McCaleb, M. L. Identification of a novel hypothalamic neuropeptide Y receptor associated with feeding behavior. *J. Biol. Chem.* **1996,** 271, 26315-26319.

65. Larhammar, D.; Blomqvist, A. G.; Yee, F.; Jazin, E.; Yoo, H.; Wahlested, C. Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. *J. Biol. Chem.* **1992,** 267, 10935-10938.

66. Lundell, I.; Blomqvist, A. G.; Berglund, M. M.; Schober, D. A.; Johnson, D.; Statnick, M. A.; Gadski, R. A.; Gehlert, D. R.; Larhammar, D. Cloning of a human receptor of the NPY receptor family with high affinity for pancreatic polypeptide and peptide YY. *J. Biol. Chem.* **1995,** 270, 29123-29128.

67. Rose, P. M.; Fernandes, P.; Lynch, J. S.; Frazier, S. T.; Fisher, S. M.; Kodukula, K.; Kienzle, B.; Seethala, R. Cloning and functional expression of a cDNA encoding a human type 2 neuropeptide Y receptor. *J. Biol. Chem.* **1995,** 270, 22661-22664.

68. Larhammar, D.; Wraith, A.; Berglund, M. M.; Holmberg, S. K.; Lundell, I. Origins of the many NPY-family receptors in mammals. *Peptides* **2001,** 22, 295-307.

69. Holliday, N. D.; Michel, M. C.; Cox, H. M. NPY receptor subtypes and their signal transduction. *Handb. Exp. Pharmacol.* **2004,** 162, 45-73.

70. Michel, M. C.; Beck-Sickinger, A.; Cox, H.; Doods, H. N.; Herzog, H.; Larhammar, D.; Quirion, R.; Schwartz, T.; Westfall, T. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev.* **1998,** 50, 143-150.

71. Bard, J. A.; Walker, M. W.; Branchek, T. A.; Weinshank, R. L. Cloning and functional expression of a human $Y_4$ subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. *J. Biol. Chem.* **1995,** 270, 26762-26765.

72. Criscione, L.; Rigollier, P.; Batzl-Hartmann, C.; Rueger, H.; Stricker-Krongrad, A.; Wyss, P.; Brunner, L.; Whitebread, S.; Yamaguchi, Y.; Gerald, C.; Heurich, R. O.; Walker, M. W.; Chiesi, M.; Schilling, W.; Hofbauer, K. G.; Levens, N. Food intake in free-feeding and energy-deprived lean rats is mediated by the neuropeptide $Y_5$ receptor. *J. Clin. Invest.* **1998,** 102, 2136-2145.

73. Grouzmann, E.; Meyer, C.; Burki, E.; Brunner, H. Neuropeptide Y Y2 receptor signalling mechanisms in the human glioblastoma cell line LN319. *Peptides* **2001,** 22, 379-386.

74. Michel, M. C. Concomitant regulation of Ca2+ mobilization and G13 expression in human erythroleukemia cells. *Eur. J. Pharmacol.* **1998,** 348, 135-141.

75. Selbie, L. A.; Darby, K.; Schmitz-Peiffer, C.; Browne, C. L.; Herzog, H.; Shine, J.; Biden, T. J. Synergistic interaction of Y1-neuropeptide Y and alpha 1b-adrenergic receptors in the regulation of phospholipase C, protein kinase C, and arachidonic acid production. *J. Biol. Chem.* **1995,** 270, 11789-11796.

76. Motulsky, H. J.; Michel, M. C. Neuropeptide Y mobilizes Ca2+ and inhibits adenylate cyclase in human erythroleukemia cells. *Am. J. Physiol.* **1988,** 255, E880-885.

77. Aakerlund, L.; Gether, U.; Fuhlendorff, J.; Schwartz, T. W.; Thastrup, O. Y1 receptors for neuropeptide Y are coupled to mobilization of intracellular calcium and inhibition of adenylate cyclase. *FEBS Lett.* **1990,** 260, 73-78.

78. Berglund, M. M.; Hipskind, P. A.; Gehlert, D. R. Recent developments in our understanding of the physiological role of PP-fold peptide receptor subtypes. *Exp. Biol. Med. (Maywood)* **2003,** 228, 217-244.

79. Brothers, S. P.; Wahlestedt, C. Therapeutic potential of neuropeptide Y (NPY) receptor ligands. *EMBO Mol. Med.* **2010,** 2, 429-439.

80. Gehlert, D. R. Introduction to the reviews on neuropeptide Y. *Neuropeptides* **2004,** 38, 135-140.

81. Gehlert, D. R. Neuropeptide Y (NP Y) and its Receptors. Encylop. Neurosci., **2009**; 837-842.

82. Pluym, N. Application of the Guanidine–Acylguanidine Bioisosteric Approach to NPY Y2 Receptor Antagonists: Bivalent, Radiolabeled and Fluorescent Pharmacological Tools. Doctoral Thesis, **2011**.

83. Schneider, E. Development of Fluorescence-Based Methods for the Determination of Ligand Affinity, Selectivity and Activity at G-Protein Coupled Receptors. Doctoral Thesis, **2005**.

84. Eva, C.; Keinanen, K.; Monyer, H.; Seeburg, P.; Sprengel, R. Molecular cloning of a novel G protein-coupled receptor that may belong to the neuropeptide receptor family. *FEBS Lett.* **1990,** 271, 81-84.

85. Larhammar, D.; Blomqvist, A. G.; Yee, F.; Jazin, E.; Yoo, H.; Wahlested, C. Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. *J. Biol. Chem.* **1992,** 267, 10935-10938.

86. Parker, S. L.; Kane, J. K.; Parker, M. S.; Berglund, M. M.; Lundell, I. A.; Li, M. D. Cloned neuropeptide Y (NPY) Y1 and pancreatic polypeptide Y4 receptors expressed in Chinese hamster ovary cells show considerable agonist-driven internalization, in contrast to the NPY Y2 receptor. *Eur. J. Biochem.* **2001,** 268, 877-886.

87. Fabry, M.; Langer, M.; Rothen-Rutishauser, B.; Wunderli-Allenspach, H.; Hocker, H.; Beck-Sickinger, A. G. Monitoring of the internalization of neuropeptide Y on neuroblastoma cell line SK-N-MC. *Eur. J. Biochem.* **2000,** 267, 5631-5637.

88. Gicquiaux, H.; Lecat, S.; Gaire, M.; Dieterlen, A.; Mely, Y.; Takeda, K.; Bucher, B.; Galzi, J.-L. Rapid internalization and recycling of the human neuropeptide Y Y1 receptor. *J. Biol. Chem.* **2002,** 277, 6645-6655.

89. Beck-Sickinger, A. G.; Jung, G. Structure-activity relationships of neuropeptide Y analogues with respect to Y1 and Y2 receptors. *Biopolymers* **1995,** 37, 123-142.

90. Cabrele, C.; Beck-Sickinger, A. G. Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J. Pept. Sci.* **2000,** 6, 97-122.

91. Fuhlendorff, J.; Gether, U.; Aakerlund, L.; Langeland-Johansen, N.; Thogersen, H.; Melberg, S. G.; Olsen, U. B.; Thastrup, O.; Schwartz, T. W. [Leu31, Pro34]neuropeptide Y: a specific Y1 receptor agonist. *Proc. Natl. Acad. Sci. USA* **1990,** 87, 182-186.

92. Mullins, D.; Kirby, D.; Hwa, J.; Guzzi, M.; Rivier, J.; Parker, E. Identification of potent and selective neuropeptide Y $Y_1$ receptor agonists with orexigenic activity in vivo. *Mol. Pharmacol.* **2001,** 60, 534-540.

93. Soll, R. M.; Dinger, M. C.; Lundell, I.; Larhammer, D.; Beck-Sickinger, A. G. Novel analogues of neuropeptide Y with a preference for the $Y_1$-receptor. *Eur. J. Biochem.* **2001,** 268, 2828-2837.

94. Takebayashi, Y.; Koga, H.; Togami, J.; Inui, A.; Kurihara, H.; Koshiya, K.; Furuya, T.; Tanaka, A.; Murase, K. Design of the $Y_1$-receptor-selective cyclic peptide based on the C-terminal sequence of neuropeptide Y. *J. Pept. Res.* **2000,** 56, 409-415.

95. Koglin, N.; Zorn, C.; Beumer, R.; Cabrele, C.; Bubert, C.; Sewald, N.; Reiser, O.; Beck-Sickinger, A. G. Analogues of neuropeptide Y containing beta-aminocyclopropane carboxylic acids are the shortest linear peptides that are selective for the Y1 receptor. *Angew. Chem. Int. Ed. Engl.* **2003,** 42, 202-205.

96. Zwanziger, D.; Bohme, I.; Lindner, D.; Beck-Sickinger, A. G. First selective agonist of the neuropeptide $Y_1$-receptor with reduced size. *J. Pept. Sci.* **2009,** 15, 856-866.

97. Buschauer, A. Synthesis and in vitro pharmacology of arpromidine and related phenyl(pyridylalkyl)guanidines, a potential new class of positive inotropic drugs. *J. Med. Chem.* **1989,** 32, 1963-1970.

98. Michel, M. C.; Motulsky, H. J. He 90481: A Competitive Nonpeptidergic Antagonist at Neuropeptide Y Receptors. *Ann. NY Acad. Sci.* **1990,** 611, 392-394.

99. Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Becksickinger, A. G.; Doods, H. N. The First Highly Potent and Selective Nonpeptide Neuropeptide-Y Y-1-Receptor Antagonist - BIBP3226. *Eur. J. Pharm.* **1994,** 271, R11-R13.

100. Wieland, H. A.; Engel, W.; Eberlein, W.; Rudolf, K.; Doods, H. N. Subtype selectivity of the novel nonpeptide neuropeptide Y $Y_1$ receptor antagonist BIBO 3304 and its effect on feeding in rodents. *Br. J. Pharmacol.* **1998,** 125, 549-555.

101. Sit, S. Y.; Huang, Y.; Antal-Zimanyi, I.; Ward, S.; Poindexter, G. S. Novel dihydropyrazine analogues as NPY antagonists. *Bioorg. Med. Chem. Lett.* **2002,** 12, 337-340.

102. Zarrinmayeh, H.; Zimmerman, D. M.; Cantrell, B. E.; Schober, D. A.; Bruns, R. F.; Gackenheimer, S. L.; Ornstein, P. L.; Hipskind, P. A.; Britton, T. C.; Gehlert, D. R. Structure-activity relationship of a series of diaminoalkyl substituted benzimidazole as neuropeptide Y Y1 receptor antagonists. *Bioorg. Med. Chem. Lett.* **1999,** 9, 647-652.

103. Kanatani, A.; Kanno, T.; Ishihara, A.; Hata, M.; Sakuraba, A.; Tanaka, T.; Tsuchiya, Y.; Mase, T.; Fukuroda, T.; Fukami, T.; Ihara, M. The novel neuropeptide Y Y(1) receptor antagonist J-104870: a potent feeding suppressant with oral bioavailability. *Biochem. Biophys. Res. Commun.* **1999,** 266, 88-91.

104. Griffith, D. A.; Hargrove, D. M.; Maurer, T. S.; Blum, C. A.; De Lombaert, S.; Inthavongsay, J. K.; Klade, L. E.; Mack, C. M.; Rose, C. R.; Sanders, M. J.; Carpino, P. A. Discovery and evaluation of pyrazolo[1,5-a]pyrimidines as neuropeptide Y1 receptor antagonists. *Bioorg. Med. Chem. Lett.* **2011,** 21, 2641-2645.

105. Hipskind, P. A.; Lobb, K. L.; Nixon, J. A.; Britton, T. C.; Bruns, R. F.; Catlow, J.; Dieckman-McGinty, D. K.; Gackenheimer, S. L.; Gitter, B. D.; Iyengar, S.; Schober, D. A.; Simmons, R. M.; Swanson, S.; Zarrinmayeh, H.; Zimmerman, D. M.; Gehlert, D. R. Potent and selective 1,2,3-trisubstituted indole NPY Y-1 antagonists. *J. Med. Chem.* **1997,** 40, 3712-3714.

106. Rose, P. M.; Fernandes, P.; Lynch, J. S.; Frazier, S. T.; Fisher, S. M.; Kodukula, K.; Kienzle, B.; Seethala, R. Cloning and functional expression of a cDNA encoding a human type 2 neuropeptide Y receptor. *J. Biol. Chem.* **1995,** 270, 29038.

107. Gehlert, D. R.; Beavers, L. S.; Johnson, D.; Gackenheimer, S. L.; Schober, D. A.; Gadski, R. A. Expression cloning of a human brain neuropeptide Y Y2 receptor. *Mol. Pharm.* **1996,** 49, 224-228.

108. Parker, S. L.; Balasubramaniam, A. Neuropeptide Y Y2 receptor in health and disease. *Br. J. Pharmacol.* **2008,** 153, 420-431.

109. Pedrazzini, T.; Pralong, F.; Grouzmann, E. Neuropeptide Y: the universal soldier. *Cell. Mol. Life Sci.* **2003,** 60, 350-377.

110. Chaki, S.; Kanuma, K. Neuropeptide receptors: novel therapeutic targets for depression and anxiety disorders. *Drugs Future* **2007,** 32, 809-822.

111. DeCarr, L. B.; Buckholz, T. M.; Milardo, L. F.; Mays, M. R.; Ortiz, A.; Lumb, K. J. A long-acting selective neuropeptide $Y_2$ receptor PEGylated peptide agonist reduces food intake in mice. *Bioorg. Med. Chem. Lett.* **2007,** 17, 1916-1919.

112. Dumont, Y.; Fournier, A.; St-Pierre, S.; Quirion, R. Characterization of neuropeptide Y binding sites in rat brain membrane preparations using [125I][Leu31, Pro34] peptide YY and [125I]Peptide YY3-36 as selective Y1 and Y2 radioligands. *J. Pharmacol. Exp. Ther.* **1995,** 272, 673-680.

113. Dumont, Y.; Fournier, A.; St-Pierre, S.; Quiron, R. Autoradiographic distribution of [125I][Leu31,Pro34]PYY and [125I]PYY3-36 binding sites in the rat brain evaluated with two newly developed Y1 and Y2 receptor radioligands. *Synapse (New York)* **1996,** 22, 139-158.

114. Potter, E. K.; Fuhlendorff, J.; Schwartz, T. W. [Pro34]neuropeptide Y selectively identifies postjunctional-mediated actions of neuropeptide Y in vivo in rats and dogs. *Eur. J. Pharmacol.* **1991,** 193, 15-19.

115. Beck, A.; Jung, G.; Gaida, W.; Koppen, H.; Lang, R.; Schnorrenberg, G. Highly potent and small neuropeptide Y agonist obtained by linking NPY 1-4 via spacer to alpha-helical NPY 25-36. *FEBS Lett.* **1989,** 244, 119-122.

116. Beck-Sickinger, A. G.; Wieland, H. A.; Wittneben, H.; Willim, K. D.; Rudolf, K.; Jung, G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* **1994,** 225, 947-958.

117. Balasubramaniam, A.; Joshi, R.; Su, C.; Friend, L. A.; James, J. H. Neuropeptide Y (NPY) $Y_2$ receptor-selective agonist inhibits food intake and promotes fat metabolism in mice: combined anorectic effects of $Y_2$ and $Y_4$ receptor-selective agonists. *Peptides* **2007,** 28, 235-240.

118. Jensen, K. J.; Pedersen, S. L.; Holst, B.; Vrang, N. Modifying the conserved C-terminal tyrosine of the peptide hormone PYY3-36 to improve Y2 receptor selectivity. *J. Pept. Sci.* **2009,** 15, 753-759.

119. Vrang, N.; Jensen, K. J.; Pedersen, S. L.; Chelur, S. S.; Sasikumar, P. G.; Dhakshinamoorthy, S.; Ramachandra, M. Y2 receptor agonists. WO 2009080608 A1, **2011**.

120. Doods, H.; Gaida, W.; Wieland, H. A.; Dollinger, H.; Schnorrenberg, G.; Esser, F.; Engel, W.; Eberlein, W.; Rudolf, K. BIIE0246: a selective and high affinity neuropeptide Y $Y_2$ receptor antagonist. *Eur. J. Pharmacol.* **1999,** 384, R3-5.

121. Grouzmann, E.; Buclin, T.; Martire, M.; Cannizzaro, C.; Dorner, B.; Razaname, A.; Mutter, M. Characterization of a selective antagonist of neuropeptide Y at the Y2 receptor. Synthesis and pharmacological evaluation of a Y2 antagonist. *J. Biol. Chem.* **1997,** 272, 7699-7706.

122. Bonaventure, P.; Nepomuceno, D.; Mazur, C.; Lord, B.; Rudolph, D. A.; Jablonowski, J. A.; Carruthers, N. I.; Lovenberg, T. W. Characterization of N-(1-Acetyl-2,3-dihydro-1H-indol-6-yl)-3-(3-cyano-phenyl)-N-[1-(2-cyclopen tyl-ethyl)-piperidin-4yl]acrylamide (JNJ-5207787), a small molecule antagonist of the neuropeptide Y $Y_2$ receptor. *J. Pharmacol. Exp. Ther.* **2004,** 308, 1130-1137.

123. Andres, C. J.; Antal Zimanyi, I.; Deshpande, M. S.; Iben, L. G.; Grant-Young, K.; Mattson, G. K.; Zhai, W. Differentially functionalized diamines as novel ligands for the NPY2 receptor. *Bioorg. Med. Chem. Lett.* **2003,** 13, 2883-2885.

124. Shoblock, J. R.; Welty, N.; Nepomuceno, D.; Lord, B.; Aluisio, L.; Fraser, I.; Motley, S. T.; Sutton, S. W.; Morton, K.; Galici, R.; Atack, J. R.; Dvorak, L.; Swanson, D. M.; Carruthers, N. I.; Dvorak, C.; Lovenberg, T. W.; Bonaventure, P. In vitro and in vivo characterization of JNJ-31020028 (N-(4-{4-[2-(diethylamino)-2-oxo-1-phenylethyl]piperazin-1-yl}-3-fluorophe nyl)-2-pyridin-3-ylbenzamide), a selective brain penetrant small molecule antagonist of the neuropeptide Y $Y_2$ receptor. *Psychopharmacology (Berl)* **2010,** 208, 265-277.

125. Brothers, S. P.; Saldanha, S. A.; Spicer, T. P.; Cameron, M.; Mercer, B. A.; Chase, P.; McDonald, P.; Wahlestedt, C.; Hodder, P. S. Selective and brain penetrant neuropeptide Y $Y_2$ receptor antagonists discovered by whole-cell high-throughput screening. *Mol. Pharmacol.* **2010,** 77, 46-57.

126. Stanley, B. G.; Magdalin, W.; Seirafi, A.; Nguyen, M. M.; Leibowitz, S. F. Evidence for neuropeptide Y mediation of eating produced by food deprivation and for a variant of the Y1 receptor mediating this peptide's effect. *Peptides* **1992,** 13, 581-587.

127. Quirion, R.; Martel, J. C.; Dumont, Y.; Cadieux, A.; Jolicoeur, F.; St-Pierre, S.; Fournier, A. Neuropeptide Y receptors: autoradiographic distribution in the brain and structure-activity relationships. *Ann. NY Acad. Sci.* **1990,** 611, 58-72.

128. Borowsky, B.; Walker, M. W.; Bard, J.; Weinshank, R. L.; Laz, T. M.; Vaysse, P.; Branchek, T. A.; Gerald, C. Molecular biology and pharmacology of multiple NPY Y5 receptor species homologs. *Regul. Pept.* **1998,** 75-76, 45-53.

129. Lundell, I.; Eriksson, H.; Marklund, U.; Larhammar, D. Cloning and characterization of the guinea pig neuropeptide Y receptor Y5. *Peptides* **2001,** 22, 357-363.

130. Parker, M. S.; Parker, S. L.; Kane, J. K. Internalization of neuropeptide Y Y1 and Y5 and of pancreatic polypeptide Y4 receptors is inhibited by lithium in preference to sodium and potassium ions. *Regul. Pept.* **2004,** 118, 67-74.

131. Balasubramaniam, A.; Sheriff, S.; Johnson, M. E.; Prabhakaran, M.; Huang, Y.; Fischer, J. E.; Chance, W. T. [D-TRP32]neuropeptide Y: a competitive antagonist of NPY in rat hypothalamus. *J. Med. Chem.* **1994,** 37, 811-815.

132. Cabrele, C.; Wieland, H. A.; Koglin, N.; Stidsen, C.; Beck-Sickinger, A. G. Ala31-Aib32: identification of the key motif for high affinity and selectivity of neuropeptide Y at the Y5-receptor. *Biochemistry* **2002,** 41, 8043-8049.

133. Walker, M. W.; Wolinsky, T. D.; Jubian, V.; Chandrasena, G.; Zhong, H.; Huang, X.; Miller, S.; Hegde, L. G.; Marsteller, D. A.; Marzabadi, M. R.; Papp, M.; Overstreet, D. H.; Gerald, C. P.; Craig, D. A. The novel neuropeptide Y Y5 receptor antagonist Lu AA33810 [N-[[trans-4-[(4,5-dihydro[1]benzothiepino[5,4-d]thiazol-2-yl)amino]cycloh exyl]methyl]-methanesulfonamide] exerts anxiolytic- and antidepressant-like effects in rat models of stress sensitivity. *J. Pharmacol. Exp. Ther.* **2009,** 328, 900-911.

134. Kanatani, A.; Ishihara, A.; Iwaasa, H.; Nakamura, K.; Okamoto, O.; Hidaka, M.; Ito, J.; Fukuroda, T.; MacNeil, D. J.; Van der Ploeg, L. H.; Ishii, Y.; Okabe, T.; Fukami, T.; Ihara, M. L-152,804: orally active and selective neuropeptide Y Y5 receptor antagonist. *Biochem. Biophys. Res. Commun.* **2000,** 272, 169-173.

135. Itani, H.; Ito, H.; Sakata, Y.; Hatakeyama, Y.; Oohashi, H.; Satoh, Y. Novel potent antagonists of human neuropeptide Y Y5 receptors. Part 2: substituted benzo[a]cycloheptene derivatives. *Bioorg. Med. Chem. Lett.* **2002,** 12, 757-761.

136. Sakamoto, T.; Moriya, M.; Tsuge, H.; Takahashi, T.; Haga, Y.; Nonoshita, K.; Okamoto, O.; Takahashi, H.; Sakuraba, A.; Hirohashi, T.; Shibata, T.; Kanno, T.; Ito, J.; Iwaasa, H.; Gomori, A.; Ishihara, A.; Fukuroda, T.; Kanatani, A.; Fukami, T. Novel orally active NPY Y5 receptor antagonists: Synthesis and structure-activity relationship of spiroindoline class compounds. *Bioorg. Med. Chem.* **2009,** 17, 5015-5026.

137. Erondu, N.; Gantz, I.; Musser, B.; Suryawanshi, S.; Mallick, M.; Addy, C.; Cote, J.; Bray, G.; Fujioka, K.; Bays, H.; Hollander, P.; Sanabria-Bohorquez, S. M.; Eng, W.; Langstrom, B.; Hargreaves, R. J.; Burns, H. D.; Kanatani, A.; Fukami, T.; MacNeil, D. J.; Gottesdiener, K. M.; Amatruda, J. M.; Kaufman, K. D.; Heymsfield, S. B. Neuropeptide $Y_5$ receptor antagonism does not induce clinically meaningful weight loss in overweight and obese adults. *Cell Metab.* **2006,** 4, 275-282.

138. Lundell, I.; Statnick, M. A.; Johnson, D.; Schober, D. A.; Starback, P.; Gehlert, D. R.; Larhammar, D. The cloned rat pancreatic polypeptide receptor exhibits profound differences to the orthologous receptor. *Proc. Natl. Acad. Sci. USA* **1996,** 93, 5111-5115.

139. Voisin, T.; Goumain, M.; Lorinet, A. M.; Maoret, J. J.; Laburthe, M. Functional and molecular properties of the human recombinant $Y_4$ receptor: resistance to agonist-promoted desensitization. *J. Pharmacol. Exp. Ther.* **2000,** 292, 638-646.

140. Gehlert, D. R.; Schober, D. A.; Beavers, L.; Gadski, R.; Hoffman, J. A.; Smiley, D. L.; Chance, R. E.; Lundell, I.; Larhammar, D. Characterization of the peptide binding requirements for the cloned human pancreatic polypeptide-preferring receptor. *Mol. Pharmacol.* **1996,** 50, 112-118.

141. Douglas A. Schober, A. M. v. A., David L. Smiley, Robert F. Bruns, Donald R. Gehlert. The Neuropeptide Y Y1 Antagonist, 1229U91, A Potent Agonist for the Human Pancreatic Polypeptide-Preferring (NPY Y4) Receptor. *Peptides* **1998,** 19, 537-542.

142. Schober, D. A.; Van Abbema, A. M.; Smiley, D. L.; Bruns, R. F.; Gehlert, D. R. The neuropeptide Y Y1 antagonist, 1229U91, a potent agonist for the human pancreatic polypeptide-preferring (NPY Y4) receptor. *Peptides* **1998,** 19, 537-542.

143. Parker, E. M.; Babij, C. K.; Balasubramaniam, A.; Burrier, R. E.; Guzzi, M.; Hamud, F.; Mukhopadhyay, G.; Rudinski, M. S.; Tao, Z.; Tice, M.; Xia, L.; Mullins, D. E.; Salisbury, B. G. GR231118 (1229U91) and other analogues of the C-terminus of neuropeptide Y are potent neuropeptide Y $Y_1$ receptor antagonists and neuropeptide Y $Y_4$ receptor agonists. *Eur. J. Pharm.* **1998,** 349, 97-105.

144. Gehlert, D. R.; Gackenheimer, S. L.; Schober, D. A.; Beavers, L.; Gadski, R.; Burnett, J. P.; Mayne, N.; Lundell, I.; Larhammar, D. The neuropeptide Y Y1 receptor selective radioligand, [125I][Leu31,Pro34]peptide YY, is also a high affinity radioligand for human pancreatic polypeptide 1 receptors. *Eur. J. Pharmacol.* **1996,** 318, 485-490.

145. Cabrele, C.; Wieland, H. A.; Langer, M.; Stidsen, C. E.; Beck-Sickinger, A. G. Y-receptor affinity modulation by the design of pancreatic polypeptide/neuropeptide Y chimera led to Y(5)-receptor ligands with picomolar affinity. *Peptides* **2001,** 22, 365-378.

146. McCrea, K.; Wisialowski, T.; Cabrele, C.; Church, B.; Beck-Sickinger, A.; Kraegen, E.; Herzog, H. 2-36[K4,RYYSA(19-23)]PP a novel Y5-receptor preferring ligand with strong stimulatory effect on food intake. *Regul. Pept.* **2000,** 87, 47-58.

147. Balasubramaniam, A.; Mullins, D. E.; Lin, S.; Zhai, W.; Tao, Z.; Dhawan, V. C.; Guzzi, M.; Knittel, J. J.; Slack, K.; Herzog, H.; Parker, E. M. Neuropeptide Y (NPY) Y4 receptor selective agonists based on NPY(32-36): development of an anorectic Y4 receptor selective agonist with picomolar affinity. *J. Med. Chem.* **2006,** 49, 2661-2665.

148. Parker, M. S.; Sah, R.; Sheriff, S.; Balasubramaniam, A.; Parker, S. L. Internalization of cloned pancreatic polypeptide receptors is accelerated by all types of Y4 agonists. *Regul. Pept.* **2005,** 132, 91-101.

149. Balasubramaniam, A.; Dhawan, V. C.; Mullins, D. E.; Chance, W. T.; Sheriff, S.; Guzzi, M.; Prabhakaran, M.; Parker, E. M. Highly selective and potent neuropeptide Y (NPY) Y1 receptor antagonists based on [Pro(30), Tyr(32), Leu(34)]NPY(28-36)-NH2 (BW1911U90). *J. Med. Chem.* **2001,** 44, 1479-1482.

150. Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Determination of affinity and activity of ligands at the human neuropeptide Y $Y_4$ receptor by flow cytometry and aequorin luminescence. *J. Recept. Signal Trans. Res.* **2007,** 27, 217-233.

151. Pop, N.; Igel, P.; Brennauer, A.; Cabrele, C.; Bernhardt, G. N.; Seifert, R.; Buschauer, A. Functional reconstitution of human neuropeptide Y (NPY) Y(2) and Y(4) receptors in Sf9 insect cells. *J. Recept. Signal Transduct. Res.* **2011,** 31, 271-285.

152. Silva, A. P.; Cavadas, C.; Grouzmann, E. Neuropeptide Y and its receptors as potential therapeutic drug targets. *Clin. Chim. Acta* **2002,** 326, 3-25.

153. Ingalls, A. M.; Dickie, M. M.; Snell, G. D. Obese, a new mutation in the house mouse. *J Hered* **1950,** 41, 317-318.

154. Stephens, T. W.; Basinski, M.; Bristow, P. K.; Bue-Valleskey, J. M.; Burgett, S. G.; Craft, L.; Hale, J.; Hoffmann, J.; Hsiung, H. M.; Kriauciunas, A.; et al. The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* **1995,** 377, 530-532.

155. Clark, J. T.; Kalra, P. S.; Crowley, W. R.; Kalra, S. P. Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. *Endocrinology* **1984,** 115, 427-429.

156. Stanley, B. G.; Leibowitz, S. F. Neuropeptide Y: stimulation of feeding and drinking by injection into the paraventricular nucleus. *Life Sci.* **1984,** 35, 2635-2642.

157. Kushi, A.; Sasai, H.; Koizumi, H.; Takeda, N.; Yokoyama, M.; Nakamura, M. Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice. *Proc. Natl. Acad. Sci. USA* **1998,** 95, 15659-15664.

158. Marsh, D. J.; Hollopeter, G.; Kafer, K. E.; Palmiter, R. D. Role of the Y5 neuropeptide Y receptor in feeding and obesity. *Nat. Med.* **1998,** 4, 718-721.

159. Naveilhan, P.; Hassani, H.; Canals, J. M.; Ekstrand, A. J.; Larefalk, A.; Chhajlani, V.; Arenas, E.; Gedda, K.; Svensson, L.; Thoren, P.; Ernfors, P. Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y2 receptor. *Nat. Med.* **1999,** 5, 1188-1193.

160. Sainsbury, A.; Schwarzer, C.; Couzens, M.; Jenkins, A.; Oakes, S. R.; Ormandy, C. J.; Herzog, H. Y4 receptor knockout rescues fertility in ob/ob mice. *Genes Dev.* **2002,** 16, 1077-1088.

161. Kuo, L. E.; Czarnecka, M.; Kitlinska, J. B.; Tilan, J. U.; Kvetnansky, R.; Zukowska, Z. Chronic stress, combined with a high-fat/high-sugar diet, shifts sympathetic signaling toward neuropeptide Y and leads to obesity and the metabolic syndrome. *Ann. N Y Acad. Sci.* **2008,** 1148, 232-237.

162. Sato, N.; Ogino, Y.; Mashiko, S.; Ando, M. Modulation of neuropeptide Y receptors for the treatment of obesity. *Expert Opin. Ther. Pat.* **2009,** 19, 1401-1415.

163. Berglund, M. M.; Hipskind, P. A.; Gehlert, D. R. Function, distribution and molecular pharmacology of NPY-family receptors. The NPY Family of Peptides in Immune Disorders, Inflammation, Angiogenesis and Cancer, *Prog. Inflamm. Res.*; **2005**; 29-62.

164. Körner, M.; Reubi, J. C. NPY receptors in human cancer: A review of current knowledge. *Peptides* **2007,** 28, 418-425.

165. Zukowska-Grojec, Z.; Karwatowska-Prokopczuk, E.; Rose, W.; Rone, J.; Movafagh, S.; Ji, H.; Yeh, Y.; Chen, W. T.; Kleinman, H. K.; Grouzmann, E.; Grant, D. S. Neuropeptide Y: a novel angiogenic factor from the sympathetic nerves and endothelium. *Circ. Res.* **1998,** 83, 187-195.

166. Ekstrand, A. J.; Cao, R.; Bjorndahl, M.; Nystrom, S.; Jonsson-Rylander, A. C.; Hassani, H.; Hallberg, B.; Nordlander, M.; Cao, Y. Deletion of neuropeptide Y (NPY) 2 receptor in mice results in blockage of NPY-induced angiogenesis and delayed wound healing. *Proc. Natl. Acad. Sci. USA* **2003,** 100, 6033-6038.

167. Redrobe, J. P.; Dumont, Y.; Quirion, R. Neuropeptide Y (NPY) and depression: from animal studies to the human condition. *Life Sci.* **2002,** 71, 2921-2937.

168. Heilig, M. The NPY system in stress, anxiety and depression. *Neuropeptides (Amsterdam, Neth.)* **2004,** 38, 213-224.

169. Widdowson, P. S.; Ordway, G. A.; Halaris, A. E. Reduced neuropeptide Y concentrations in suicide brain. *J. Neurochem.* **1992,** 59, 73-80.

170. Redrobe, J. P.; Dumont, Y.; Herzog, H.; Quirion, R. Neuropeptide Y (NPY) Y2 receptors mediate behaviour in two animal models of anxiety: evidence from Y2 receptor knockout mice. *Behav. Brain Res.* **2003,** 141, 251-255.

171. Kask, A.; Eller, M.; Oreland, L.; Harro, J. Neuropeptide Y attenuates the effect of locus coeruleus denervation by DSP-4 treatment on social behaviour in the rat. *Neuropeptides* **2000,** 34, 58-61.

172. Rimondini, R.; Thorsell, A.; Heilig, M. Suppression of ethanol self-administration by the neuropeptide Y (NPY) Y2 receptor antagonist BIIE0246: evidence for sensitization in rats with a history of dependence. *Neurosci. Lett.* **2005,** 375, 129-133.

173. Decressac, M.; Wright, B.; David, B.; Tyers, P.; Jaber, M.; Barker, R. A.; Gaillard, A. Exogenous neuropeptide Y promotes in vivo hippocampal neurogenesis. *Hippocampus* **2011,** 21, 233-238.

174. Thiele, T. E.; Marsh, D. J.; Ste Marie, L.; Bernstein, I. L.; Palmiter, R. D. Ethanol consumption and resistance are inversely related to neuropeptide Y levels. *Nature* **1998,** 396, 366-369.

175. Mayfield, R. D.; Lewohl, J. M.; Dodd, P. R.; Herlihy, A.; Liu, J.; Harris, R. A. Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. *J. Neurochem.* **2002,** 81, 802-813.

176. Doods, H. N.; Wieland, H. A.; Engel, W.; Eberlein, W.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Rudolf, K. BIBP 3226, the first selective neuropeptide Y1 receptor antagonist: a review of its pharmacological properties. *Regul. Pept.* **1996,** 65, 71-77.

177. Doods, H. N.; Wienen, W.; Entzeroth, M.; Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A. Pharmacological characterization of the selective nonpeptide neuropeptide Y Y1 receptor antagonist BIBP 3226. *J. Pharmacol. Exp. Ther.* **1995,** 275, 136-142.

178. Kask, A.; Rago, L.; Harro, J. Evidence for involvement of neuropeptide Y receptors in the regulation of food intake: studies with Y1-selective antagonist BIBP3226. *Br. J. Pharmacol.* **1998,** 124, 1507-1515.

179. Erondu, N.; Wadden, T.; Gantz, I.; Musser, B.; Nguyen, A. M.; Bays, H.; Bray, G.; O'Neil, P. M.; Basdevant, A.; Kaufman, K. D.; Heymsfield, S. B.; Amatruda, J. M. Effect of NPY5R antagonist MK-0557 on weight regain after very-low-calorie diet-induced weight loss. *Obesity (Silver Spring)* **2007,** 15, 895-905.

180. Batterham, R. L.; Heffron, H.; Kapoor, S.; Chivers, J. E.; Chandarana, K.; Herzog, H.; Le Roux, C. W.; Thomas, E. L.; Bell, J. D.; Withers, D. J. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. *Cell Metab.* **2006,** 4, 223-233.

181. Adrian, T. E.; Ferri, G. L.; Bacarese-Hamilton, A. J.; Fuessl, H. S.; Polak, J. M.; Bloom, S. R. Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology* **1985,** 89, 1070-1077.

182. Pedersen-Bjergaard, U.; Host, U.; Kelbaek, H.; Schifter, S.; Rehfeld, J. F.; Faber, J.; Christensen, N. J. Influence of meal composition on postprandial peripheral plasma concentrations of vasoactive peptides in man. *Scand. J. Clin. Lab. Invest.* **1996,** 56, 497-503.

183. Adrian, T. E.; Bloom, S. R.; Bryant, M. G.; Polak, J. M.; Heitz, P. H.; Barnes, A. J. Distribution and release of human pancreatic polypeptide. *Gut* **1976,** 17, 940-944.

184. Track, N. S.; McLeod, R. S.; Mee, A. V. Human pancreatic polypeptide: studies of fasting and postprandial plasma concentrations. *Can. J. Physiol. Pharmacol.* **1980,** 58, 1484-1489.

185. Grandt, D.; Schimiczek, M.; Beglinger, C.; Layer, P.; Goebell, H.; Eysselein, V. E.; Reeve, J. R., Jr. Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1-36 and PYY 3-36. *Regul. Pept.* **1994,** 51, 151-159.

186. Keire, D. A.; Mannon, P.; Kobayashi, M.; Walsh, J. H.; Solomon, T. E.; Reeve, J. R., Jr. Primary structures of PYY, [Pro(34)]PYY, and PYY-(3-36) confer different conformations and receptor selectivity. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2000,** 279, G126-131.

187. Nygaard, R.; Nielbo, S.; Schwartz, T. W.; Poulsen, F. M. The PP-fold solution structure of human polypeptide YY and human PYY3-36 as determined by NMR. *Biochemistry* **2006,** 45, 8350-8357.

188. Chelikani, P. K.; Haver, A. C.; Reidelberger, R. D. Ghrelin attenuates the inhibitory effects of glucagon-like peptide-1 and peptide YY(3-36) on food intake and gastric emptying in rats. *Diabetes* **2006,** 55, 3038-3046.

189. Koegler, F. H.; Enriori, P. J.; Billes, S. K.; Takahashi, D. L.; Martin, M. S.; Clark, R. L.; Evans, A. E.; Grove, K. L.; Cameron, J. L.; Cowley, M. A. Peptide YY(3-36) inhibits morning, but not evening, food intake and decreases body weight in rhesus macaques. *Diabetes* **2005,** 54, 3198-3204.

190. Pittner, R. A.; Moore, C. X.; Bhavsar, S. P.; Gedulin, B. R.; Smith, P. A.; Jodka, C. M.; Parkes, D. G.; Paterniti, J. R.; Srivastava, V. P.; Young, A. A. Effects of PYY[3-36] in rodent models of diabetes and obesity. *Int. J. Obes. Relat. Metab. Disord.* **2004,** 28, 963-971.

191. Degen, L.; Oesch, S.; Casanova, M.; Graf, S.; Ketterer, S.; Drewe, J.; Beglinger, C. Effect of peptide YY3-36 on food intake in humans. *Gastroenterology* **2005,** 129, 1430-1436.

192. Gantz, I.; Erondu, N.; Mallick, M.; Musser, B.; Krishna, R.; Tanaka, W. K.; Snyder, K.; Stevens, C.; Stroh, M. A.; Zhu, H.; Wagner, J. A.; Macneil, D. J.; Heymsfield, S. B.; Amatruda, J. M. Efficacy and safety of intranasal peptide YY3-36 for weight reduction in obese adults. *J. Clin. Endocrinol. Metab.* **2007,** 92, 1754-1757.

193. Field, B. C. T.; Chaudhri, O. B.; Bloom, S. R. Obesity treatment: novel peripheral targets. *Br. J. Clin. Pharmacol.* **2009,** 68, 830-843.

194. Berntson, G. G.; Zipf, W. B.; O'Dorisio, T. M.; Hoffman, J. A.; Chance, R. E. Pancreatic polypeptide infusions reduce food intake in Prader-Willi syndrome. *Peptides* **1993,** 14, 497-503.

195. Batterham, R. L.; Le Roux, C. W.; Cohen, M. A.; Park, A. J.; Ellis, S. M.; Patterson, M.; Frost, G. S.; Ghatei, M. A.; Bloom, S. R. Pancreatic polypeptide reduces appetite and food intake in humans. *J. Clin. Endocrinol. Metab.* **2003,** 88, 3989-3992.

196. Jesudason, D. R.; Monteiro, M. P.; McGowan, B. M.; Neary, N. M.; Park, A. J.; Philippou, E.; Small, C. J.; Frost, G. S.; Ghatei, M. A.; Bloom, S. R. Low-dose pancreatic polypeptide inhibits food intake in man. *Br. J. Nutr.* **2007,** 97, 426-429.

197. Cooke, D.; Bloom, S. The obesity pipeline: current strategies in the development of anti-obesity drugs. *Nat. Rev. Drug. Discov.* **2006,** 5, 919-931.

198. Otvos, L., Jr. Peptide-based drug design: here and now. *Methods Mol. Biol. (Totowa, NJ, U. S.)* **2008,** 494, 1-8.

199. McGregor, D. P. Discovering and improving novel peptide therapeutics. *Curr. Opin. Pharmacol.* **2008,** 8, 616-619.

200. McGonigle, P. Peptide therapeutics for CNS indications. *Biochem. Pharmacol.* **2012,** 83, 559-566.

201. Mantzourani, E.; Laimou, D.; Matsoukas, M. T.; Tselios, T. Peptides as Therapeutic Agents or Drug Leads for Autoimmune, Hormone Dependent and Cardiovascular Diseases. *Anti-Allergy Agents in Med. Chem.* **2008,** 7, 294-306.

202. Vagner, J.; Qu, H.; Hruby, V. J. Peptidomimetics, a synthetic tool of drug discovery. *Curr. Opin. Chem. Biol.* **2008,** 12, 292-296.

203. Hruby, V. J. Designing peptide receptor agonists and antagonists. *Nat. Rev. Drug. Discov.* **2002,** 1, 847-858.

204. Hruby, V. J.; Qui, W.; Okayama, T.; Soloshonok, V. A. Design of nonpeptides from peptide ligands for peptide receptors. *Methods Enzymol.* **2002,** 343, 91-123.

205. Seebach, D.; Gardiner, J. β-Peptidic Peptidomimetics. *Acc. Chem. Res.* **2008,** 41, 1366-1375.

# CHAPTER 2

# Scope and Objectives

During the last decades, obesity has become a serious health problem throughout the modern world, associated with the risk of developing chronic diseases such as type II diabetes, coronary heart disease and hypertension. The neuropeptide Y (NPY) hormone family and its four receptor subtypes ($Y_1R$, $Y_2R$, $Y_4R$ and $Y_5R$) have been demonstrated to be involved in the regulation of e.g. feeding behavior and energy homeostasis. However, the role of NPY and NPY receptors in obesity is not completely understood to date due to very complex and in part contradictory effects of receptor subtype stimulation.[1, 2] Besides the regulation of feeding, NPY receptors and their endogenous ligands seem to be implicated in diseases like alcoholism, depression, pain and cancer. Hence, there is a need for small molecules as NPY receptor ligands to assess the physiological and pathophysiological role of NPY as well as to clarify NPY mediated pathways and the therapeutic potential of NPY receptor ligands.[3-5] Unfortunately, to date only few low molecular weight ligands for the NPY $Y_2R$ and $Y_5R$, fulfilling the pharmacodynamics and pharmacokinetic properties for the application as therapeutics, are known. For the $Y_4R$ potent no non-peptidic ligands are described in literature. Even low molecular weight peptide agonists are lacking.[3] Therefore, this work aimed at the design and synthesis of potent $Y_4R$ selective ligands.

Previously, UR-AK49, an antagonist with $Y_4R$ activity in the upper micromolar range ($IC_{50}$ = 60.9 µM), was discovered, when a selection of commercially available drugs and in-house compounds was investigated in a functional $Ca^{2+}$-assay (aequorin assay) developed in our laboratory. This $N^G$-acylated imidazolylpropylguanidine, initially designed as potent $H_2R$ agonist in our workgroup ,was considered a lead structure.[6] Therefore, UR-AK49 should be structurally varied and optimized, hopefully leading to compounds with improved $Y_4R$ affinity and antagonistic activity and devoid of $H_2R$ agonism.
To increase the chance of identifying an $Y_4R$ antagonist, a subproject focusing on argininamides related to BIBP 3326 and BIBO 3304 was devised. Previously, compounds with moderate $Y_4R$ affinities were identified among potent argininamide-type $Y_1R$ antagonists, in particular bivalent ligands from our workgroup.[7-9] Hence, a set of monovalent and bivalent BIBP 3226 and BIBO 3304 analogs should be characterized in $Y_4R$ assays to further explore the structure-activity/selectivity relationships as basis for the development of potent $Y_4R$ antagonists.

Interestingly, in case of the $Y_2R$ and the $Y_4R$, agonists instead of antagonists have been intensively discussed in literature as potential drugs for the treatment of obesity.[2, 3] Therefore, the idea to synthesize potent and selective peptidic $Y_4R$ agonists arose from incidental discoveries within a cooperation project with the workgroup of Prof. Dr. Oliver Reiser (University of Regensburg). Introduction of unnatural amino acids to truncated pNPY and hPP analogs led to the identification of a highly potent and selective $Y_4R$ ligand ([cpen$^{34}$]pNPY(25-36): $K_i$ = 10 nM). Thus, the synthesis of

further truncated analogs, taking into consideration cyclic β- and γ-amino acids as well as D-configured amino acids should harbor the potential to obtain low(er) molecular weight agonists, additional peptidomimetic-like structures and non-peptidic ligands with improved stability for *in vitro* and *in vivo* studies.

To better understand the biological role of the different NPY receptor subtypes, especially that of the NPY $Y_4R$, a promising truncated $Y_4R$ selective peptide should be subjected to fluorescent-labeling to obtain an appropriate pharmacological tool. Additionally, $Y_1R$, $Y_2R$ and $Y_5R$ selective ligands described in literature should be included in fluorescence-labeling for applications in flow cytometry and confocal microscopy.

## 2.1 References


1. Kamiji, M. M.; Inui, A. Neuropeptide Y Receptor Selective Ligands in the Treatment of Obesity. *Endocr. Rev.* **2007,** 28, 664-684.
2. Kamiji, M. M.; Inui, A. NPY Y2 and Y4 receptors selective ligands: promising anti-obesity drugs? *Curr. Top. Med. Chem.* **2007,** 7, 1734-1742.
3. Brothers, S. P.; Wahlestedt, C. Therapeutic potential of neuropeptide Y (NPY) receptor ligands. *EMBO Mol. Med.* **2010,** 2, 429-439.
4. Kamiji, M. M.; Inui, A. Neuropeptide y receptor selective ligands in the treatment of obesity. *Endocr. Rev.* **2007,** 28, 664-684.
5. Sato, N.; Ogino, Y.; Mashiko, S.; Ando, M. Modulation of neuropeptide Y receptors for the treatment of obesity. *Expert Opin Ther Pat* **2009,** 19, 1401-1415.
6. Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Determination of Affinity and Activity of Ligands at the Human Neuropeptide Y Y4 Receptor by Flow Cytometry and Aequorin Luminescence. *J. Recept. Signal. Transduct. Res.* **2007,** 27, 217 - 233.
7. Keller, M.; Erdmann, D.; Pop, N.; Pluym, N.; Teng, S.; Bernhardt, G.; Buschauer, A. Red-fluorescent argininamide-type NPY Y1 receptor antagonists as pharmacological tools. *Bioorg. Med. Chem.* **2011,** 19, 2859-2878.
8. Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Guanidine-acylguanidine bioisosteric approach in the design of radioligands: synthesis of a tritium-labeled $N^G$-propionylargininamide ([$^3$H]-UR-MK114) as a highly potent and selective neuropeptide Y $Y_1$ receptor antagonist. *J. Med. Chem.* **2008,** 51, 8168-8172.

9. Keller, M.; Teng, S.; Bernhardt, G.; Buschauer, A. Bivalent argininamide-type neuropeptide y y(1) antagonists do not support the hypothesis of receptor dimerisation. *ChemMedChem* **2009,** 4, 1733-1745.

# CHAPTER 3

# $N^G$-Acylated Hetarylpropylguanidines as Antagonists of the Human NPY $Y_4$ Receptor: Synthesis and Pharmacological Investigations

## 3.1 Introduction

Due to the lack of non-peptidic ligands for the NPY $Y_4R$, a small library of compounds was screened for antagonistic activity in a new functional assay (aequorin assay) developed in our laboratory.[1, 2] Stimulated by the discovery of the first non-peptidic ligands in the NPY $Y_1R$ field within guanidine-type histamine $H_2R$ ligands by Michel and Motulski,[3] $N^G$-acylated imidazolylpropylguanidines, synthesized as $H_2R$ agonists in our workgroup, were used for this screening approach. Surprisingly, one out of 80 tested compounds, namely UR-AK49, showed weak antagonistic activity and was considered a promising starting point for the design of a first series of acylguanidine-type NPY $Y_4R$ ligands.[2]

The imidazolylpropylguanidine moiety is an essential substructure of $H_2R$ agonists, but it also confers receptor affinity to other histamine receptor subtypes, in particular $H_3$ and $H_4$ receptors. Therefore, starting from the "lead structure" UR-AK49, the aim of this project was to explore the structure-activity relationships with respect to $Y_4R$ antagonist activity by replacing the imidazolyl moiety with different heterocyclic structures, i.e. typical "privileged structures" [4] in medicinal chemistry, or by simple functional groups (-OH, $-NH_2$). As the cyclohexyl ring was supposed to exert cytotoxic effects,[5] it was exchanged by a phenyl ring, which should retain the physicochemical properties of the compounds but reduce undesired effects in cellular assays.

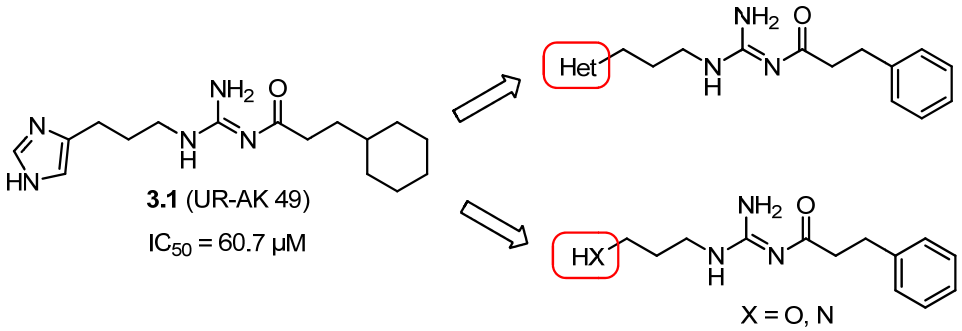
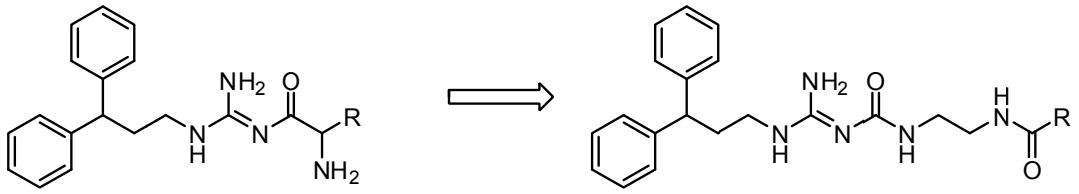



**Figure 3.1**: Overview of the structural modifications of $N^G$-acylated imidazolylpropylguanidine derived from **3.1** as an initial approach to the synthesis of non-peptidic NPY $Y_4R$ ligands.

A second strategy in the search for non-peptidic $Y_4R$ ligands focused on the synthesis of hybrid molecules by combining an acylguanidine or carbamoylguanidine substructure with an amino acid of the C-termini of pNPY and hPP, suggested to play a key role in receptor binding.[6, 7] A similar approach, i.e. mimicking the C-terminal part of NPY, was successfully performed by Boehringer Ingelheim Pharma for the development of the highly potent and selective $Y_1R$ and $Y_2R$ antagonists BIBP 3226 and BIIE 0246, respectively.[8-10] In the design of the acylguanidines the lipophilic benzhydryl moiety was also adapted from the BIBP 3229 motif.




R = amino acid side chain of Tyr, Leu, Pro, Arg, Orn

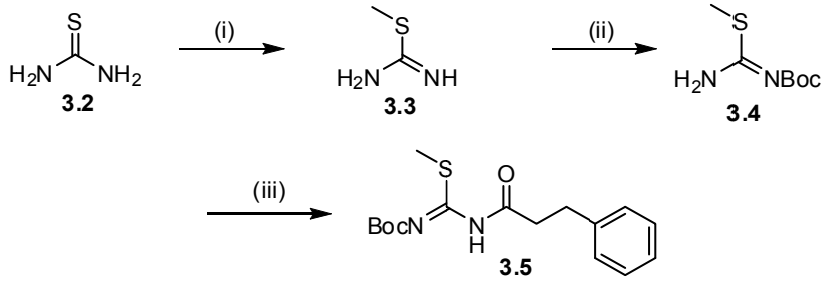
**Figure 3.2:** Structures of hybrid molecules consisting of acylguanidine/carbamoyl substructures and selected amino acids.

The $N^G$-acylated hetarylpropylguanidines (**Figure 3.1**) and hybrid molecules (**Figure 3.2**) were synthesized and pharmacologically characterized for functional activity (agonism, antagonism) at the human $Y_4R$ in an aequorin assay as well as for receptor subtype selectivity in flow cytometric binding studies on human $Y_1$, $Y_2$, $Y_4$ and $Y_5$ receptors.

# 3.2 Chemistry

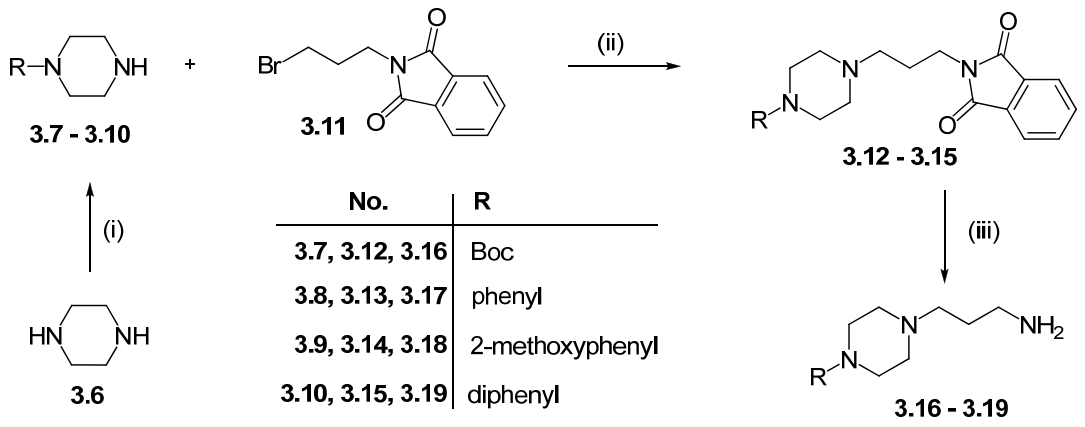
## 3.2.1 Synthesis of the $N^G$-Acylated Arylpropylguanidines

According to **Scheme 3.1,** the preparation of $N^G$-acylated arylpropylguanidines started via a three-step synthesis of *N*-Boc-protected *N'*-(3-phenylpropionyl)-*S*-methylisothiourea (**3.5**). *N*-Boc protected *S*-methylisothiourea **3.4** was synthesized as previously described.[11] Subsequently, **3.4** was treated with 3-phenylpropionic acid under standard conditions for amide coupling**,** using TBTU, HOBt and DIEA.



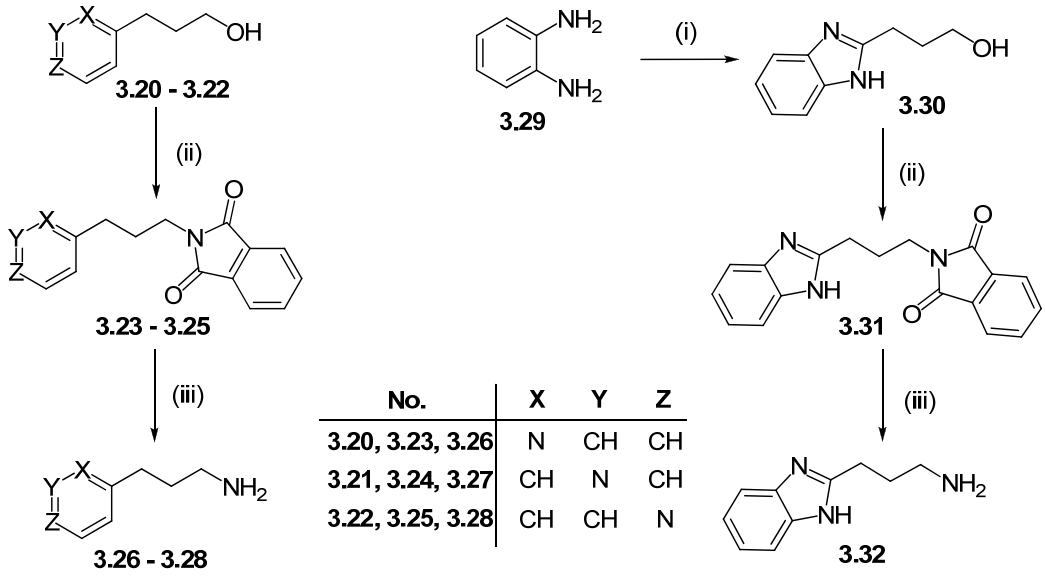

**Scheme 3.1**: Synthetic route for the preparation of the building block **3.5**. Reagents and conditions: (i) MeI (1 eq), MeOH, reflux, 1 h; (ii) $(Boc)_2O$ (1 eq), $NEt_3$, $DCM_{abs}$, rt, overnight; (iii) TBTU (1.2 eq), HOBt (1.2 eq), DIEA (3.0 eq), rt, overnight.

The amines **3.16 – 3.19** were obtained by *N*-alkylation of the pertinent mono-substituted piperazines **3.7 – 3.10**[12, 13] with 3-bromopropylphthalimide (**3.11**) followed by hydrazinolysis[12, 14]. Alkylation of the unsubstituted piperazine (**3.6**) required a mono-Boc-protection[15] first to avoid the formation of di-alkylated products (**Scheme 3.2**).




**Scheme 3.2**: Synthesis of piperazinylpropylamines **3.16 – 3.19**. Reagents and conditions: (i) $Boc_2O$ (0.5 eq), $Et_3N$ (0.5 eq), DMF, 0 °C to rt, overnight; (ii) $K_2CO_3$ (1.5 eq), KI (0.5 eq), acetone, rt, overnight; (iii) $N_2H_4 \cdot H_2O$ (6 eq), EtOH, reflux, overnight.

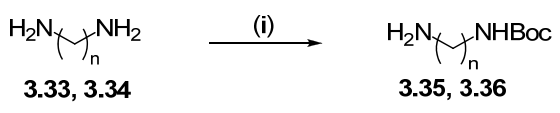
The hetarylpropylalcohols **3.20 – 3.22** and **3.30** were converted to the corresponding phthalimides **3.23 – 3.25** and **3.31** under Mitsunobu conditions[16] and subsequently subjected to hydrazinolysis[14, 17] as depicted in **Scheme 3.3**. 3-(1*H*-Benzimidazol-2-yl)propan-1-ol (**3.30**) had to be prepared by cyclization of ortho-phenylenediamine (**3.29**) and 4-chlorobutanoic acid according to a *Phillips-type*[18] reaction. The cyclisation was performed under acidic conditions (pH 3-4) providing the chloroalkyl intermediate after neutralization with ammonia. This unstable product was transformed to the alcohol **3.30** by treating with 2 M sodium hydroxide solution.[19, 20]




**Scheme 3.3**: Synthesis of hetarylpropylamines **3.26 – 3.28** and **3.32**. Reagents and conditions: (i) a) 5 M HCl, 4-chlorobutanoic acid, reflux, overnight, b) 2 M NaOH, 40 °C; (ii) phthalimide (1.1 eq), TPP (1.1 eq), DIAD (1.1 eq), THF, 0 °C, overnight; (iii) $N_2H_4 \cdot H_2O$ (6 eq), EtOH, reflux, overnight.

The mono Boc-protected diamines **3.35** and **3.36** were synthesized according to Krapcho et. al[21] using a high excess of the diamine (**3.33** or **3.34**), to reduce the formation of the corresponding diprotected

product to a minimum. The di-protected by-product can easily be separated – after precipitation from water – by filtration.

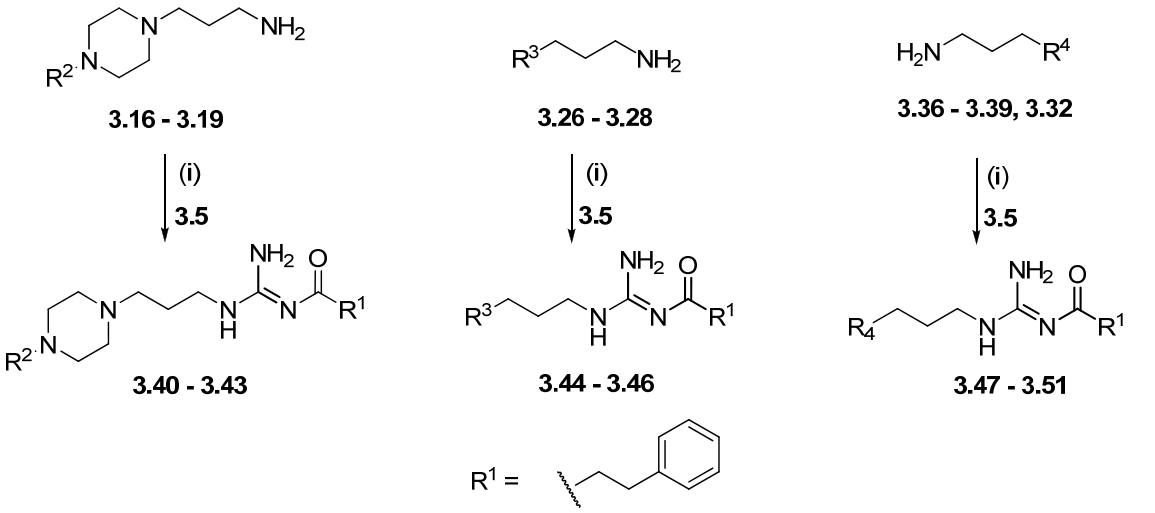



3.33, 3.34 → 3.35, 3.36

| No. | n |
|---|---|
| 3.35 | 2 |
| 3.36 | 3 |

**Scheme 3.4**: Synthesis of mono Boc-protected amines **3.35** and **3.36**. Reagents and conditions: (i) $Boc_2O$ (0.1 eq), dioxane, rt, overnight.

The designated $N^G$-acylated arylpropylguanidines were prepared according to the synthetic pathway outlined in **Scheme 3.5**. Treatment of the amines **3.16 – 3.19**, **3.26 – 3.28**, **3.36 – 3.39** and **3.32** with the guanidinylating reagent **3.5**[22-24] in the presence of $HgCl_2$ – the metal ion reacts as a desulfurizing agent via complex formation[25, 26] – followed by Boc-deprotection with trifluoroacetic acid and purification by flash chromatography or preparative RP-HPLC yielded the acylguanidines **3.40 – 3.51**.



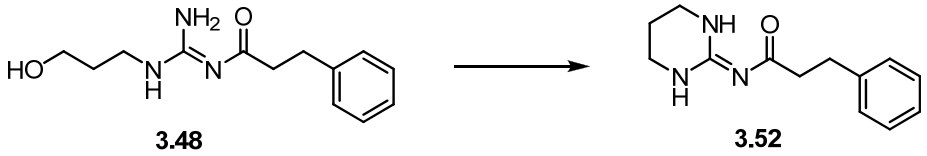

| No. | $R^2$ |
|---|---|
| 3.16, 3.40 | –H |
| 3.17, 3.41 | |
| 3.18, 3.42 | MeO |
| 3.19, 3.43 | |

| No. | $R^3$ |
|---|---|
| 3.26, 3.44 | |
| 3.27, 3.45 | |
| 3.28, 3.46 | |

| No. | $R^4$ |
|---|---|
| 3.36, 3.47 | $NH_2$ |
| 3.37, 3.48 | OH |
| 3.38, 3.49 | |
| 3.39, 3.50 | |
| 3.32, 3.51 | |

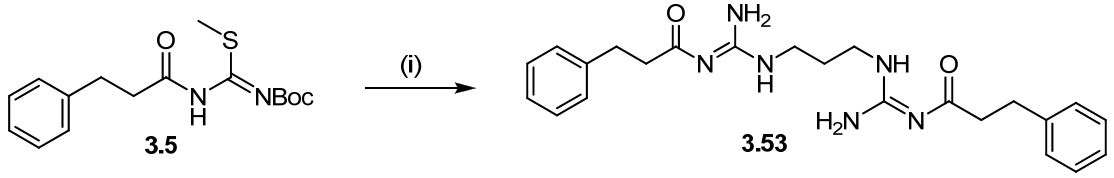
**Scheme 3.5**: Synthesis of the $N^G$-acylated arylpropylguandines **3.40 – 3.51**. Reagents and conditions: (i) a) $HgCl_2$ (2 eq), $Et_3N$ (3 eq), DCM, 36 – 48 h, rt; b) TFA (50 %), DCM, 3 – 8 h.

Attempts to obtain compound **3.48** in high purity failed due to decomposition of the purified product. The by-product was isolated and analyzed by mass spectrometry and NMR. The mass of this compound corresponds to that of the cyclisation product **3.52** (**Scheme 3.6**). By now, the reaction conditions as well as the mechanism leading to the formation of this six-membered ring could not be clarified. Despite of complete conversion to **3.52**, the compound was included in investigations for NPY $Y_4R$ activity.




**Scheme 3.6**: Cyclisation of **3.48** forming a six-membered ring and yielding compound **3.52**.

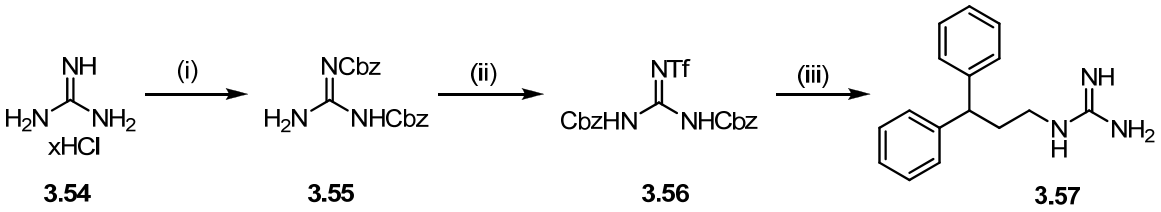
Another "by-product" was formed, when unprotected propane-1,3-diamine was used in a first approach to synthesize **3.47**. The "bisguanidine" **3.53** was obtained as main product as highlighted in **Scheme 3.7**. As compounds with a similar bisguanidine substructure derived from arpromidine were previously reported to have activities at the $Y_1R$ [27], compound **3.53** was included in the screening for NPY $Y_4R$ activity.




**Scheme 3.7**: Formation of byproduct **3.53**. Reagents and conditions: (i) a) 1,3-propanediamine (1 eq), $HgCl_2$ (2 eq), $Et_3N$ (3 eq), DCM, 36 – 48 h, rt; b) TFA (50 %), DCM, 3 – 8 h.

## 3.2.2 Synthesis of Acylguanidine-Type and Carbamoylguanidine-Type Amino Acid Derivatives

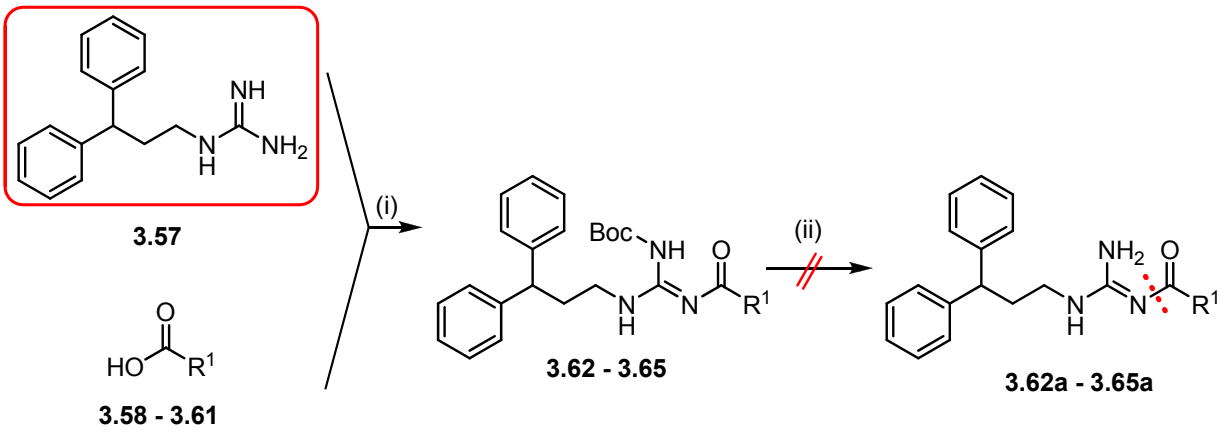
In a first approach, the selected amino acids should directly be coupled to a guanidine building block depicted in **Scheme 3.8**. The building block **3.57** was prepared from guanidine hydrochloride (**3.54**) by Cbz-di-protection (**3.55**), followed by reaction of **3.55** with trifluoromethane sulfonic anhydride to give the *N*,*N'*,*N"*-tri-protected guanidine **3.56** according to Feichtinger et al.[28, 29] Subsequently, 3,3-diphenylpropylamine was treated with the guanidinylating reagent **3.56** under mild basic conditions releasing triflic amide, which could be easily removed by aqueous workup. Finally, cleavage of the Cbz-protection groups by hydrogenolysis gave the building block **3.57**.

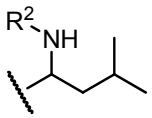
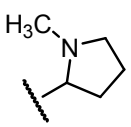


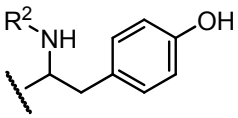
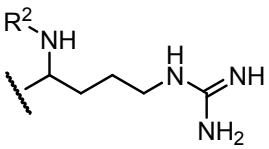

**3.54** **3.55** **3.56** **3.57**

**Scheme 3.8**: Synthesis of the guanidine building block **3.57**. Reagents and conditions: (i) benzyl chloroformate (3 eq), NaOH (5 eq), $H_2O$/DCM, 20 h, 0 °C; (ii) $Tf_2O$ (1 eq), NaH (60 % dispersion in mineral oil, 2 eq), chlorobenzene, overnight, -45 °C; (iii) $Et_3N$, (1 eq), DCM, overnight, rt.

In principle, the compounds **3.62 – 3.65** should be easily accessible by coupling of **3.57** with the pertinent $N^{\alpha}$-Boc-protected amino acids using CDI as activating reagent (**Scheme 3.9**) followed by removal of the protecting group. [30-32] Surprisingly, the Boc-deprotection and isolation of the desired compounds led to unexpected problems as described in the following paragraphs using compound **3.64** as an example.




| No. | $R^1$ | $R^2$ |
|---|---|---|
| **3.62** |  | Boc |
| **3.62a** | | H |
| **3.63** |  | Boc |
| **3.63a** | | H |

| No. | $R^1$ | $R^2$ |
|---|---|---|
| **3.64** |  | Boc |
| **3.64a** | | H |
| **3.65** |  | Boc |
| **3.65a** | | H |

**Scheme 3.9**: Approach to the synthesis of acylguanidine-type amino acid derivatives **3.62a – 3.65a**. Reagents and conditions: (i) CDI, 1.2 eq), NaH (60 % dispersion in mineral oil, 2 eq), THF, 5 h, rt; (ii) TFA (50 %), DCM, rt, 3 – 5 h.

Compound **3.64** – still carrying the Boc-protection group at $N^{\alpha}$ – was isolated in high yield and purity by means of flash chromatography and was clearly identified by mass spectrometry (**Figure 3.3**).

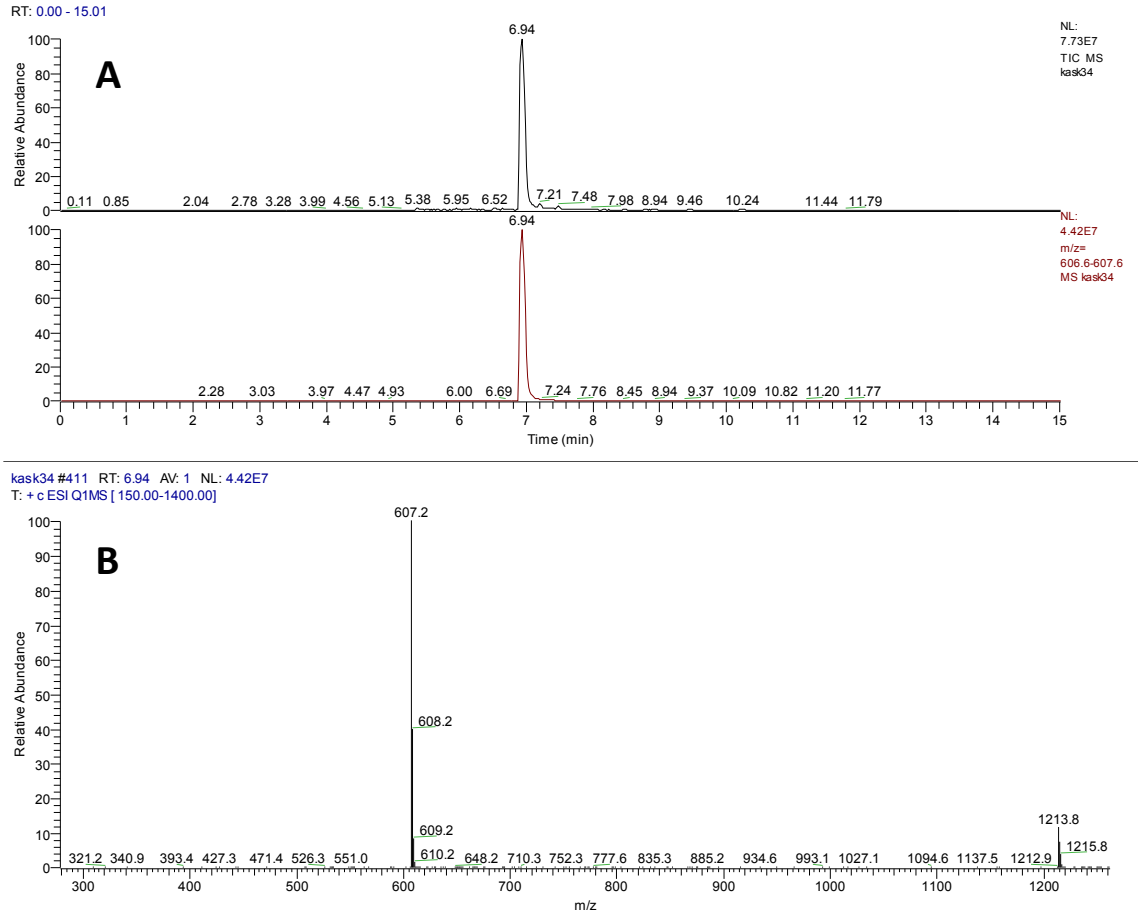



**Figure 3.3**: LC-MS analysis of the intermediate of compound **3.64**. **A**: TIC, **B**: ESI-MS spectrum of compound **3.64.**

However, after Boc-deprotection, purification by RP-HPLC revealed two peaks, which could be easily separated (ratio 2:1) and analyzed by mass spectrometry. Interestingly, besides compound **3.64** (Peak 1, **Figure 3.4: D**), the building block **3.57** (Peak 2, **Figure 3.4: A**+**B**) was identified. Obviously, under the acidic conditions required for the Boc-deprotection, the intermediate slowly decomposed (**Figure 3.4: C, E**). The same holds for the synthesis of compounds **3.62**, **3.63** and **3.65**.

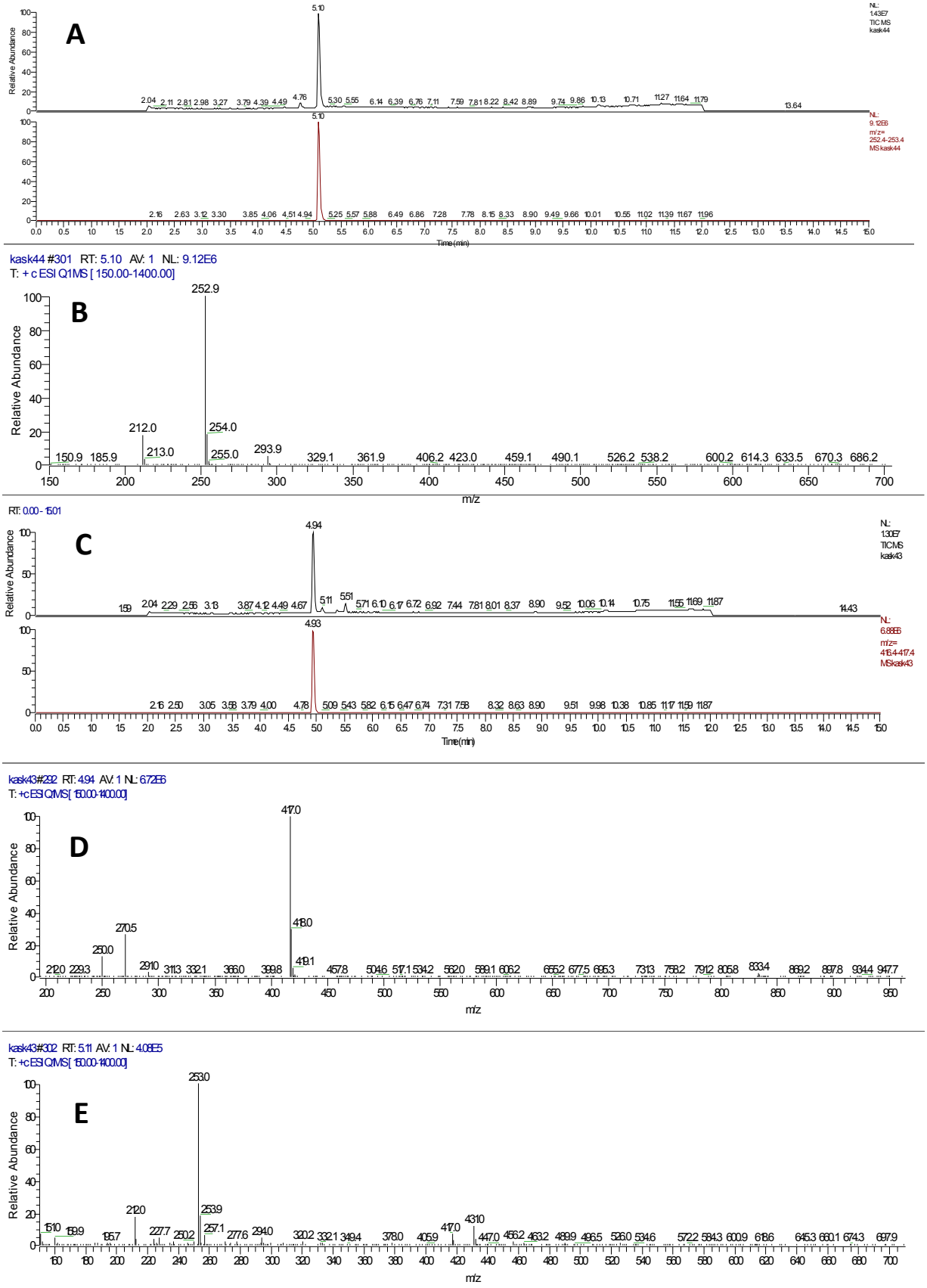
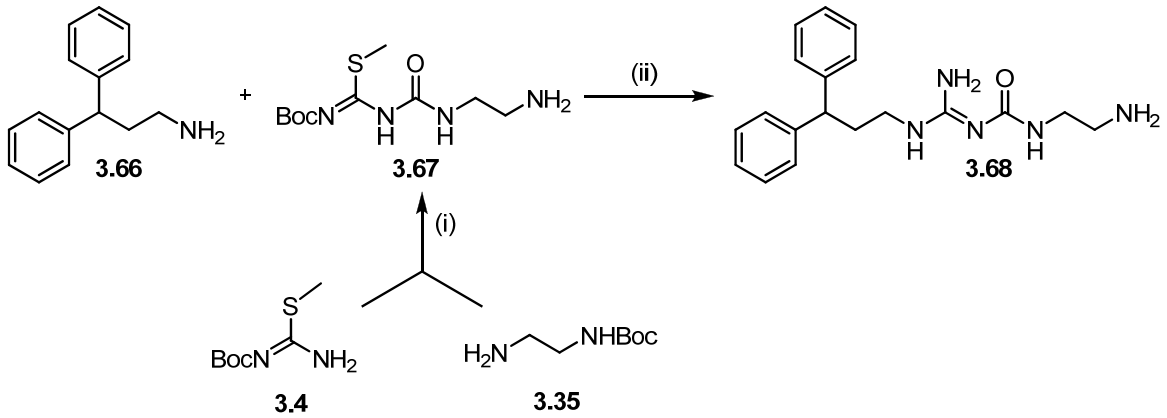



**Figure 3.4:** LC-MS analysis of compound **3.64** (Peak 1(**C**+**D**) and 2(**A**+**B**)) isolated by preparative HPLC. **A:** TIC Peak 2, **B**: ESI-MS spectrum Peak 2, **C:** TIC Peak 1, **D:** ESI-MS spectrum Peak 1 (mass of **3.64**), **E:** ESI-MS spectrum Peak 1 (mass of decomposition product).

The phenomenon of slow cleavage of the acylguanidine substructure was also observed for BIBP 3226 derivatives synthesized in our laboratories. Stability tests for this class of compounds revealed that decomposition was detectable especially under assay-like conditions (aqueous solution, pH 7.4 pH) and during experiments requiring long incubation periods.[33] The decomposition of the acylguanidine substructure in the amino acid derivatives is probably facilitated by neighboring groups even under acidic conditions. The additional primary amine in the vicinity to the acylguanidine could react as a nucleophile, attacking the guanidine moiety and accelerating the cleavage of the acylguanidine bond.

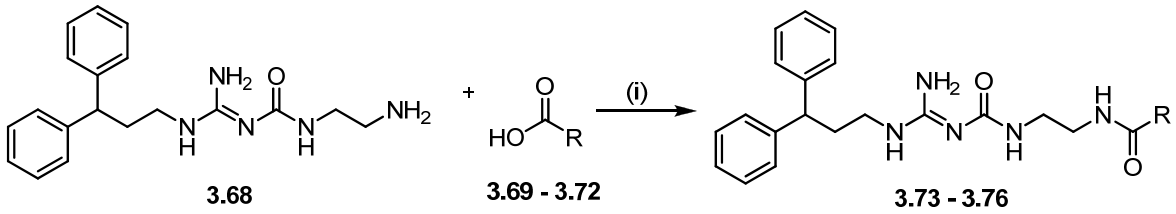
Hence, by analogy with a bioisosteric approach successfully applied to BIBP 3226 derivatives,[33] the acylguanidine substructure was replaced by a carbamoylguanidine moiety for the synthesis of a second series of amino acid derivatives. Furthermore, $N^{\alpha}$-methyl substituted amino acids were used.

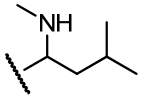
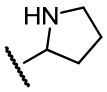
The carbamoylisothiourea building block **3.67** was synthesized as highlighted in **Scheme 3.10**, starting from *S*-methylisothiourea and *N*-Boc-ethane-1,2-diamine (**3.35**), which were allowed to react in the presence of triphosgene, following a protocol for the conversion of amines into isocyanates.[34] Guandinylation of 3,3-diphenylpropylamine (**3.66**) with **3.67** in the presence of $HgCl_2$ (see 3.1.1), followed by Boc-deprotection afforded the building block **3.68**.

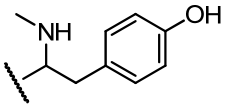
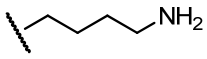



**Scheme 3.10**: Synthesis of the carbamoylguanidine-type building block **3.68**. Reagents and conditions: (i) triphosgene (0.5 eq), DIPEA (2.5 eq), $DCM_{abs}$, rt, 3 h; (ii) a) $HgCl_2$ (2 eq), $Et_3N$ (3 eq), rt, 48 h; b) acetyl chloride, MeOH, rt, 3 h.

Coupling of the primary amine **3.68** with Boc-protected amino acid derivatives **3.69** – **3.72** was performed in the presence of EDAC. Deprotection with TFA and purification by preparative RP-HPLC provided the desired compounds **3.73 – 3.76** in purities > 95 %.

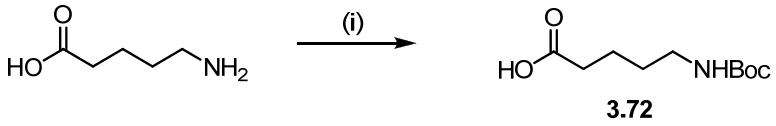



| No. | R |
|---|---|
| 3.69, 3.73 |  |
| 3.70, 3.74 |  |

| No. | R |
|---|---|
| 3.71, 3.75 |  |
| 3.72, 3.76 |  |

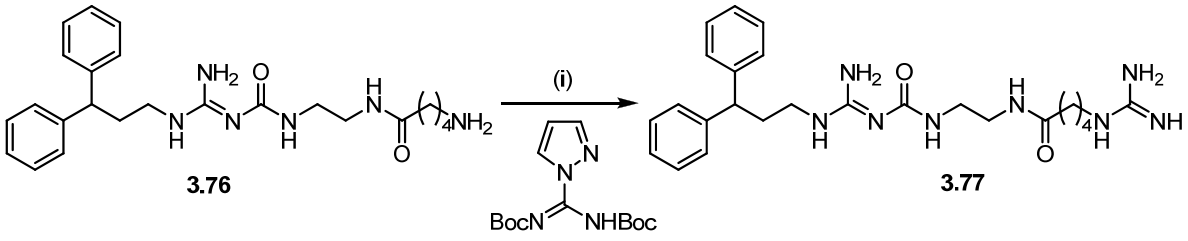
**Scheme 3.11**: Synthesis of carbamoylguanidines **3.73 – 3.76**. Reagents and conditions: (i) a) EDAC (1.2 eq), HOBt (1.2 eq), DIEA (2.0 eq), rt, overnight; b) TFA (50 %), DCM, rt, 3 – 5 h.

As in case of protected arginine the coupling to **3.68** resulted in low yields and deprotection of the guanidine moiety caused problems, a different route was preferred to replace the arginine substructure: Boc-protected 5-aminovaleric acid **3.72** – prepared according to a standard procedure[35] – was used first to synthesize building block **3.76**.




**Scheme 3.12**: Synthesis of Boc-protected 5-aminovaleric acid **3.72**. Reagents and conditions: (i) $Boc_2O$ (1 eq), NaOH (1 eq), dioxane/$H_2O$, 0 °C to rt, overnight.

Afterwards, compound **3.76** was guanidinylated with *N*,*N'*-bis-*tert*-butoxycarbonyl-1*H*-pyrazole-1-carboxamidine to yield the "arginine-like" residue in compound **3.77**.




**Scheme 3.13**: Synthesis of the "arginine-like" amino acid derivative **3.77**. Reagents and conditions: (i) a) $Et_3N$ (2.5 eq), DCM, rt, 20 h; b) TFA (50 %), DCM, rt, 4 h.

## 3.3 Pharmacological Results and Discussion

The synthesized $N^G$-acylated hetarylpropylguanidines as well as the carbamoylguanidine-tpye amino acid derivatives were investigated for functional activity at the NPY $Y_4R$ in a luminescence based $Ca^{2+}$-assay (aequorin assay) using CHO-h$Y_4$-qi5-mtAEQ cells.[2] Additionally, a selection of compounds showing promising results in the functional studies, were analyzed in flow cytometric binding studies using the same h$Y_4$R expressing cell line.[2] In the following, antagonistic potencies are expressed as $IC_{50}$ and $K_B$ values, determined from the concentration-dependent inhibition of the luminescence signal elicited by hPP (100 nM). The $K_B$ values were calculated according to the Cheng-Prusoff equation.[36] Additionally, all compounds were investigated in the agonist mode. For this purpose, the increase in intracellular calcium induced by the investigated compounds at concentrations of 100 µM or 10 µM is given in percent referred to the response to the standard agonist hPP (100 nM). Flow cytometric binding studies were performed in the presence of Cy5-[$K^4$]hPP (c = 3 nM, $K_D$ = 5.62). Results are given as $K_i$ calculated according to the Cheng-Prusoff equation[36].
Furthermore, all synthesized compounds were investigated for binding to the other NPY receptor subtypes in a flow cytometric binding assay using HEL ($Y_1R$), and stably transfected CHO-h$Y_2$ and HEC-1B-$Y_5$ cells, respectively. Compounds were tested at two concentrations (10 µM and 1 µM) in the presence of Cy5-pNPY (c = 5 nM, $K_D$ = 5.2 nM); results are given as $K_i$.[37]

### 3.3.1 Functional Activities and Affinities at the $Y_4R$ and Receptor Subtype Selectivity of the $N^G$-Acylated Hetarylpropylguanidines

As starting point for the synthesis of new $N^G$-acylated arylpropylguanidines the imidazolyl moiety of the "lead structure" UR-AK49 should be replaced by "privileged structures", which are a constituent of high affinity ligands of many GPCR. Savchuck et al.[4] compiled such privileged structures of peptidergic GPCR ligands by searching literature and scanning databases. Several of the suggested hetaryl substructures where used in this work to design and synthesize potential non-peptidic $Y_4R$ antagonists in which the imidazole ring was replaced. The pharmacological data of the synthesized compounds is summarized in **Table 3.1**.

Replacement of the imidazole moiety by simple functional groups such as a primary amine (**3.47**) and a hydroxyl group, which turned out to be unstable forming a completely cyclized product (**3.52**), or by a phenyl moiety (**3.50**) did not improve the activity at the NPY $Y_4R$. Similarly, introduction of a heterocyclic moiety like morpholine (**3.49**) did not result in an increase in potency. By contrast, a

benzimidazole instead of an imidazole moiety was tolerated (**3.51**). Compound **3.51** proved to have 3-fold higher $Y_4$R antagonistic activity than the lead structure UR-AK49.

For the pyridine derivatives poor $Y_4$R antagonistic activity was observed for **3.44** ($IC_{50}$ = 32 µM) and **3.45** ($IC_{50}$ = 35 µM), whereas the 4-pyridyl substituted compound **3.46** was completely inactive.

Finally, the introduction of a phenylpiperazine substructure (**3.41**) led to the most promising result namely a 20-fold increase in antagonistic activity compared to UR-AK49 (**3.1**). Whereas o-methoxyphenyl and phenyl substitution at the piperazine moiety gave equipotent compounds (**3.42** vs. **3.41**), the introduction of a more bulky benzhydryl substituent (**3.43**) was not tolerated. Complete loss of $Y_4$R antagonistic activity was also found for compound **3.40**, in which the 4-position of the piperazine ring was left unsubstituted.

Compound **3.53**, the "bisguanidine"-like compound, which was isolated as a byproduct, also showed moderate $Y_4$R antagonistic activity. This was not a complete surprise, because bisguanidine compounds were previously described by Knieps et al.[27] to possess weak antagonistic activity at NPY receptors, albeit only investigated for the $Y_1$R in more detail. Surprisingly, **3.53** showed also an agonist-like behavior: at a concentration of 100 µM the compound induced a luminescence signal corresponding to 50 % of that elicited by 100 nM hPP. For compound **3.43** even a value higher than 100 % was obtained. A closer look at the assay and the properties of these compounds revealed that this increase in intracellular calcium was produced due to cytotoxic effects at higher concentrations, but not by $Y_4$R agonistic activity. For details cf. **section 4.4**.

Possible cytotoxic effects of the tested compounds had also been taken in consideration when flow cytometric binding studies were performed. Reliable binding data were obtained when high concentrations of the compounds were avoided.

**Table 3.1**: NPY $Y_4R$ activities and affinities of the synthesized $N^G$-acylated arylpropylguanidines **3.1**, **3.40** – **3.53.**

| No. | $hY_4R$ | | | |
|---|---|---|---|---|
| | % Relative Potency[a] | $IC_{50}$ [µM][b] | $K_B$ [µM][c] | $K_i$ [µM][d] |
| **3.1** | n.d. | 61 ± 7.0 | 8.1 ± 1.0 | 68 ± 27 |
| **3.40** | 1.4 ± 0.4 | inactive[f] | - | n.d. |
| **3.41** | 3.0 ± 1.1 | 3.0 ± 1.8 | 0.40 ± 0.16 | inactive[g] |
| **3.42** | 0.8 ± 0.6 | 6.7 ± 2.3 | 0.90 ± 0.31 | inactive[g] |
| **3.43** | 280 ± 13[e] | inactive[f] | - | n.d. |
| **3.44** | 1.7 ± 0.6 | 31.9 ± 9.5 | 4.3 ± 1.3 | n.d. |
| **3.45** | 1.4 ± 0.5 | 35.2 ± 9.4 | 4.7 ± 1.7 | n.d. |
| **3.46** | 3.0 ± 0.8 | inactive[f] | - | n.d. |
| **3.47** | 1.2 ± 0.8 | inactive[f] | - | n.d. |
| **3.49** | 1.6 ± 1.4 | inactive[f] | - | n.d. |
| **3.50** | 10.7 ± 0.9 | inactive[f] | - | n.d. |
| **3.51** | 3.8 ± 0.9 | 18.6 ± 6.8 | 2.5 ± 0.9 | n.d. |
| **3.52** | 1.9 ± 0.9 | inactive[f] | - | n.d. |
| **3.53** | 51 ± 5.5 [e] | 16.4 ± 8.1 | 2.2 ± 1.1 | n.d. |

[a] $[Ca^{2+}]_i$ mobilization by compounds tested at 100 µM concentration (performed in triplicate) in the agonist mode; the response is given in % referred to the $[Ca^{2+}]_i$ mobilization induced by the standard agonist hPP (100 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells. [b] $Y_4R$ antagonistic activity determined by inhibition of 100 nM hPP induced $[Ca^{2+}]_i$ mobilization in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate.[c] $K_B$ calculated from $IC_{50}$ values according to the Cheng-Prusoff equation (c = 100 nM, $K_D$ = 15.5 nM). [d] $Y_4R$ binding data determined by flow cytometry using 3 nM Cy5-[$K^4$]hPP ($K_D$ = 5.62 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [e] no agonistic activity; luminescence caused by cytotoxic effects (see 4.3.2). [f] less than 20 % decrease of the luminescence signal elicited by 100 nM hPP at 100 µM concentration of the test compound; testing at higher concentrations was avoided due to cytotoxic effects. [g] less than 20 % displacement of Cy5-[$K^4$]hPP at 30 µM concentration of the test compound; testing at higher concentrations was avoided due to cytotoxic effects.

Except for the piperazine derivatives **3.42** and **3.44**, none of the synthesized $N^G$-acylated arylpropylguanidines showed affinity to the different NPY receptor subtypes (**Table 3.2**). Surprisingly, the additional methoxy substituent at the phenylpiperazinyl substructure in **3.42** conferred low micromolar affinity to the $Y_5R$, whereas the non-substituted phenylpiperazine **3.41** was devoid of affinity to the $Y_5R$ at 10 µM.

Although both compounds, **3.41** and **3.42**, had comparable $Y_4R$ activity in the low micromolar range, **3.41** should be preferred for further investigations due to its better subtype selectivity profile.

**Table 3.2**: Binding data of synthesized $N^G$-acylated arylpropylguanidines **3.40** – **3.52** at the NPY $Y_1$, $Y_2$ and $Y_5$ receptor.

| No. | $hY_1R$ | $hY_2R$ | $hY_5R$ | No. | $hY_1R$ | $hY_2R$ | $hY_5R$ |
|---|---|---|---|---|---|---|---|
| | $K_i$ [μM][a] | $K_i$ [μM][a] | $K_i$ [μM][a] | | $K_i$ [μM][a] | $K_i$ [μM][a] | $K_i$ [μM][a] |
| **3.40** | inactive* | inactive* | inactive* | **3.46** | inactive* | inactive* | inactive* |
| **3.41** | inactive* | inactive* | inactive* | **3.47** | inactive* | inactive* | inactive* |
| **3.42** | inactive* | inactive* | 7.2 ± 1.3 | **3.49** | inactive* | inactive* | inactive* |
| **3.43** | 1.8 ± 0.4 | inactive* | inactive* | **3.50** | inactive* | inactive* | inactive* |
| **3.44** | inactive* | inactive* | inactive* | **3.51** | inactive* | inactive* | inactive* |
| **3.45** | inactive* | inactive* | inactive* | **3.52** | inactive* | inactive* | inactive* |

[a] Flow cytometric binding assay using 5 nM cy5-pNPY ($K_D$ = 5.2 nM) in ChO-$hY_2$ cells, HEL ($Y_1$) and HEC-1B-$Y_5$ cells, respectively. *less than 20 % displacement of Cy5-pNPY ($hY_1R$, $hY_2R$, $hY_5R$) at 10 μM concentration of the test compound; testing at higher concentrations was avoided due to cytotoxic effects.

## 3.3.2 Functional Activities at the NPY $Y_4$R and Receptor Subtype Selectivity of Carbamoylguanidine-Type Amino Acid Derivatives

The idea to synthesize hybrid molecules consisting of a carbamoylguandine-substructure and amino acids present in the C-terminus of hPP or pNPY was stimulated by a similar concept successfully employed by Boehringer-Ingelheim in the search for potent NPY $Y_1$ receptor ligands.[8] Attempts to mimic the C-terminal part of pNPY emerged when an alanine scan of NPY revealed that, among the C-terminal amino acids, especially $Arg^{35}$ and $Tyr^{33}$ have a great impact on binding at the $Y_1$ and $Y_2$ receptor.[6] Additional studies on single amino acid replacements in pNPY confirmed that the C-terminal part for the peptide is also of major importance for $Y_4$R binding.[7] Taking these results into account, the side chains of Tyr, Leu, Pro and Arg were chosen for the synthesis of hybrid molecules.

Unfortunately, all synthesized amino acid derivatives showed neither antagonistic nor agonistic activity at the NPY $Y_4$R nor affinity to other NPY receptor subtypes (**Table 3.3**).

**Table 3.3**: Potencies and subtype selectivity of the synthesized carbamoylguanidine-type amino acid derivatives **3.62** – **3.66**.

| No. | $hY_4R$ | | $hY_1R$ | $hY_2R$ | $hY_5R$ |
|---|---|---|---|---|---|
| | % Relative Potency[a] | $IC_{50}$ [µM][b] | $K_i$ [µM][c] | $K_i$ [µM][c] | $K_i$ [µM][c] |
| **3.73** | 0.9 ± 0.7 | inactive[d] | inactive[e] | inactive[e] | inactive[e] |
| **3.74** | 4.1 ± 1.4 | inactive[d] | inactive[e] | inactive[e] | inactive[e] |
| **3.75** | 1.0 ± 1.3 | inactive[d] | inactive[e] | inactive[e] | inactive[e] |
| **3.76** | 2.0 ± 0.7 | inactive[d] | inactive[e] | inactive[e] | inactive[e] |
| **3.77** | 3.4 ± 0.9 | inactive[d] | inactive[e] | inactive[e] | inactive[e] |

[a] $[Ca^{2+}]_i$ mobilization by tested compound (agonist mode) at 100 µM concentration (performed in triplicate) given in % referred to the $[Ca^{2+}]_i$ mobilization induced by the standard agonist hPP (100 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells. [b] $Y_4R$ antagonism; inhibition of 100 nM hPP induced $[Ca^{2+}]_i$ mobilization in CHO-$hY_4$-qi5-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [c] Flow cytometric binding assay using 5 nM cy5-pNPY ($K_D$ = 5.2 nM) in CHO-$hY_2$ cells, HEL ($Y_1$) and HEC-1B-$Y_5$ cells (10 µM and 1 µM concentration, duplicates), respectively. [d] less than 20 % decrease of the luminescence signal elicited by 100 nM hPP at 10 µM concentration of the test compound; testing at higher concentrations was avoided due to cytotoxic effects.[e] less than 20 % displacement of Cy5-[$K^4$]hPP ($Y_4R$) and Cy5-pNPY ($hY_1R$, $hY_2R$, $hY_5R$); testing at higher concentrations was avoided due to cytotoxic effects.

## 3.4 Summary and Conclusion

Based on the $N^G$-acylated imidazolylpropyl guanidine UR-AK49, described as the first non-peptidic antagonist for the NPY $Y_4R$ ($IC_{50}$ = 60.7 µM), a series of compounds was synthesized in which the imidazolyl moiety was replaced by different "privileged structures". Most promising results were obtained for the phenylpiperazine **3.41** with $IC_{50}$ values in the low micromolar range ($IC_{50}$ = 3.0 µM) improving the potency towards the NPY $Y_4R$ compared to UR AK-49 ($IC_{50}$ = 60.7 µM) by a factor of 20. Additionally, the unsubstituted phenylpiperazine residue proved to confer some selectivity for the $Y_4R$ compared to the other NPY receptor subtypes. Interestingly, phenylpiperazinyl substructures already played an important role in the identification of novel NPY $Y_1R$ ligands published by Neurogen and Pfizer.[38, 39] Thus the $N^G$-acylated piperazinylpropylguanidines may be considered a good starting point for the design of new $Y_4R$ ligands.

Unfortunately, the design of hybrid molecules consisting of a carbamoylguanidine-type building block and amino acid residues trying to mimic the C-terminal part of hPP or pNPY afforded compounds with a complete lack of NPY $Y_4R$ activity. Due to the disappointing results, the synthesis of compounds combining an acylated guanidine substructure as well as amino acid residues was discontinued within this doctoral project.

# 3.5 Experimental Section

## 3.5.1 Chemistry

### 3.5.1.1 General Experimental Conditions

Commercial reagents and chemicals were purchased from Acros Organics (Geel, Belgium), IRIS Biotech GmbH (Marktredwitz, Germany), Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany), Merck KGaA (Darmstadt, Germany) as well as Sigma-Aldrich Chemie GmbH (Munich, Germany) and were used without further purification unless otherwise stated. *N*,*N'*-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine was a gift from Max Keller (University of Regensburg). Deuterated solvents for NMR spectroscopy were from Deutero GmbH (Kastellaun, Germany). All solvents were of analytical grade or distilled prior to use. Millipore water for the preparation of HPLC eluents and buffers was used throughout. Anhydrous DMF (< 0.01 % $H_2O$) was purchased from Sigma-Aldrich Chemie GmbH. Reactions requiring moisture-free conditions were performed in dried glassware under inert atmosphere (argon or nitrogen). Flash chromatography was performed in glass columns on silica gel (Merck silica gel 60, 40 - 63 µM). The reactions were monitored by TLC on aluminum plates coated with silica gel (Merck silica gel 60 F254, thickness 0.2 mm). The compounds were detected by UV light (254 nm), a 0.3 % solution of ninhydrine in n-butanol (amines) or a solution of bromocresol green (0.04 %) in ethanol (carboxylic acids). All melting points are uncorrected and were measured on a Büchi 530 apparatus (Büchi GmbH, Essen, Germany). For lyophilisation a Christ alpha 2-4 LD equipped with a vaccubrand RZ 6 rotary vane vacuum pump was used.

Nuclear Magnetic Resonance spectra ($^1$H-NMR and $^{13}$C-NMR) were recorded with a Bruker Avance 300 ($^1$H: 300.1 MHz, $^{13}$C: 75.5 MHz) NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Chemical shifts are given in δ (ppm) relative to external standards. Abbreviations for the multiplicities of the signals are specified as follows: s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), m (multiplet), bs (broad singulet) and combinations thereof. The multiplicity of carbon atoms ($^{13}$C-NMR) was determined by DEPT 135 (distortionless enhancement by polarization transfer): "+" primary and tertiary carbon atom (positive DEPT signal), "-" secondary carbon atom (negative DEPT signal), "$C_{quat}$" quaternary carbon atom. Mass spectra (MS) were recorded on a Finnigan MAT 95 (EI-MS 70 eV), Finnigan SSQ 710A (CI-MS ($NH_3$)) or a Finnigan ThermoQuest TSQ 7000 (ES-MS). For high resolution mass spectrometry an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) equipped with an ESI source or a Finnigan MAT 95 with a liquid secondary ionization system (LSI-MS) was used. The peak intensity in % relative to the strongest signal is indicated in parenthesis.

Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Eurospher-100 C18, 250 × 32 mm, 5 mm, Knauer (1) or Nucleodur-100 $C_{18}$, 250 x 21 mm, Macherey-Nagel, Düren, Germany (2)) at a flow rate of 38 mL/min (1) or 18 mL/min (2). Mixtures of acetonitrile and 0.1 % aq. TFA were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure prior to lyophilisation. Analytical HPLC analysis was performed with a Eurospher-100 C18 column (250 x 4 mm, 5 µm, Knauer, Berlin, Germany) on two different Merck Hitachi systems that are listed in detail below. Acetonitrile (A) and 0.05 % TFA (B) was used as mobile phase. Helium degassing was used throughout.

*Analytical HPLC systems for purity control:*

**System 1**: Merck Hitachi, composed of a L-5000 controller, a 655A-12 pump, a 655A-40 autosampler and a L-4250 UV-VIS detector; flow rate: 0.8 mL/min; UV detection 220 nm; applied gradient: 0 to 30 min: A/B 10/90 to 90/10, 30 to 40 min: 95/5

**System 2**: Merck Hitachi, composed of a L-6200-A pump, a AS-2000A autosampler, a L-4000A UV-VIS detector and a F-1050 fluorescence spectrophotometer; flow rate: 0.8 mL/min; UV detection 220 nm; applied gradient: 0 to 30 min: A/B 10/90 to 70/30, 30 to 40 min: 95/5

### 3.5.1.2 Preparation of the Guanidinylating Reagent 3.4 and the $N^G$-Boc Protected Building Block 3.5

**_S_-Methylthiouronium iodide (3.3)[11]**

Thiourea (10 g, 131 mmol, 1 eq) was dissolved in MeOH (100 mL). Methyl iodide (8.21 mL, 131 mmol, 1 eq) was added and the reaction mixture was refluxed for 1 h. After evaporation of the solvent, the crude product was triturated with diethyl ether, filtered and washed twice with diethyl ether to yield the desired product as a white solid (28.1 g, 129.0 mmol, 95 %); mp 110 °C (ref. [40]: 113.5 – 115 °C). $^1$H-NMR (300 MHz, DMSO-d$_6$): δ (ppm) 2.57 (s, 3H, S-C***H***$_3$), 8.88 (bs, 4H, N***H***$_2$); MS (ES, DCM/MeOH + $NH_4OAc$) *m/z*: (%), 91 (100) $M^+$. $C_2H_7IN_2S$ (218.06).

***N-tert*-Butoxycarbonyl-*S*-methylthiourea (3.4)[11]**

As solution of $Boc_2O$ (18.65 g, 124 mmol, 1 eq) in 50 mL DCM was slowly added to a solution of **3.3** (28.0 g, 128.0 mmol, 1 eq) and $Et_3N$ (17.4 mL, 124 mmol, 1 eq) in DCM (200 mL). After stirring for 20 h at rt, the reaction mixture was washed with water and brine and the organic phase was dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was subjected to flash chromatography (PE/EtOAc 90/10 v/v) affording compound **3.4** as a white solid

(20.0 g, 105.0 mmol, 82 %); mp 77 °C (ref.[41]: 77 °C). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.40 (s, 9H, C(C***H***$_3$)$_3$), 2.31 (s, 3H, S-C***H***$_3$), 8.54 (bs, 2H, N***H***$_2$); MS (ESI, MeOH + 0.1 % TFA) *m/z* (%): 191 (100) $[M+H]^+$. $C_7H_{14}N_2O_2S$ (190.26).

**_N-tert_-Butoxycarbonyl-_N'_-(3-phenylpropionyl)-_S_-methylisothiourea (3.5)[42]**

Phenylpropionic acid (4.0 g, 21 mmol, 1 eq) was dissolved in anhydrous DCM (250 mL). DIEA (10.99 mL, 63.1 mmol, 3 eq) and TBTU (8.10 g, 25.2 mmol, 1.2 eq) were added and the reaction mixture was stirred for 20 min at rt under argon atmosphere. Compound **3.4**, dissolved in anhydrous DCM (100 mL), was added slowly and stirring was continued overnight. The mixture was poured into ice-cold water, acidified with 0.1 M HCl (pH 4-5) and extracted with DCM (3x 50 mL). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure yielding a yellow-orange oil. Flash chromatography (DCM/MeOH 100/1 – 95/5 v/v) afforded the product **3.5** as a white solid (5.35 g, 16.6 mmol, 79 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.42 (s, 9H, C(C***H***$_3$)$_3$), 2.27 (s, 3H, S-C***H***$_3$), 2.68 (t, $^3J$ = 7.4 Hz, 2H, Ph-CH$_2$-C***H***$_2$), 2.84 (t, $^3J$ = 7.4 Hz, 2H, Ph-C***H***$_2$), 7.15 – 7.33 (m, 5H, Ph-***H***). $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 13.81 (+, S-***C***H$_3$), 27.62 (+, C(***C***H$_3$)$_3$), 29.81 and 37.32 (-, ***C***H$_2$-***C***H$_2$-CO), 125.92 (+, Ph-***C***-3), 128.13 (+, 2 Ph-***C***), 128.21 (+, 2 Ph-***C***), 140.41 ($C_{quat}$, Ph-***C***-1), 157.70 ($C_{quat}$., ***C***=N), 161.45 ($C_{quat}$, ***C***=O), 170.35 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 323 (100) $[M+H]^+$. $C_{16}H_{22}N_2O_3S$ (322.42).

### 3.5.1.3 Preparation of the 3-(1*H*-Benzimidazol-2-yl)propan-1-ol 3.32

**3-(1*H*-Benzimidazol-2-yl)propan-1-ol (3.30)[43]**

1,2-Phenylenediamine **3.29** (4.0 g, 37.0 mmol, 1 eq) was dissolved in 50 mL 5 M HCl (aq). 4-Chlorobutanoic acid (6.35 g, 51.8 mmol, 1.4 eq) was added slowly through an addition funnel under vigorous stirring. After complete addition, the reaction mixture was kept under reflux overnight. The reaction mixture was allowed to cool down to room temperature and was poured into 50 mL ice-cold water. Careful neutralization with aqueous $NH_3$ afforded the intermediate, 2-(3-chloropropyl)-1*H*-benzimidazol, as a white precipitate, which was filtered off, washed with $H_2O$ and dissolved in 20 mL of 2 M NaOH. After stirring at 40 °C overnight, the reaction mixture was cooled to 5 °C and adjusted to pH 9 by addition of aqueous HCl and extracted with EtOAc (3x 50 mL). The combined organic layers were washed with $H_2O$ and dried over $MgSO_4$. Evaporation of the solvent and purification by flash chromatography (DCM/EtOAc 80/20 v/v) afforded product **3.30** as beige half-solid compound (1.9 g, 30 %); mp 163 – 164 °C (ref.[43]: 165 - 167 °C). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.85 – 1.96 (m, 2H, C***H***$_2$-CH$_2$-OH), 2.84 (t, 2H, $^3J$ = 6.3 Hz,C***H***$_2$-CH$_2$-CH$_2$-OH), 3.48 (t, 2H, $^3J$ = 6.3 Hz, C***H***$_2$-OH), 6.98 – 7.20 (m, 2 H, Ph-***H***), 7.35 – 7.51 (m, 2H, Ph-***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 25.18 and

30.63 (-, (***C***$H_2$)$_2$-$CH_2$-OH), 60.04 (-, $(CH_2)_2$-***C***$H_2$-OH), 121.00 (+, 4 Ph-***C***), 155.03 ($C_{quat}$, 2 Ph-***C***); MS (CI, $NH_3$) *m/z* (%): 177 (100) $[M+H]^+$. $C_{10}H_{12}N_2O$ (176.22).

### 3.5.1.4 Preparation of 2-(3-Piperazinopropyl)isoindoline-1,3-diones 3.12 – 3.15

**General procedure**

The piperazine derivatives **3.7** – **3.10** (1 eq), potassium carbonate (1.5 eq), phthalimide (1 eq) and catalytic amounts of potassium iodide (0.5 eq) were dissolved in acetone and stirred at rt overnight. After removal of the solvent under reduced pressure, the crude product was subjected to column chromatography or crystallized from EtOAc.

***tert*-Butyl 4-[3-(1,3-dioxoisoindolin-2-yl)propyl]piperazine-1-carboxylate (3.12)[44]**

The title compound was prepared from **3.7** (1.05 g, 5.62 mmol), potassium carbonate (1.16 g, 8.42 mmol), phthalimide (1.50 g, 5.6 mmol) and potassium iodide (0.47 g, 2.8 mmol) in 100 mL acetone according to the general procedure. Purification by flash chromatography (DCM/MeOH 100/1- 95/5 v/v) yielded **3.12** as pale yellow oil (1.65 g, 79 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.80 – 1.92 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.26 – 2.34 (m, 4H, Pip-***H***), 2.41 (t, $^3J$ = 6.8 Hz, 2H, Pip-C***H***$_2$), 3.22-3.29 (m, 4H, Pip-***H***), 3.77 (t, $^3J$ = 6.9 Hz, 2H, Pip-$(CH_2)_2$-C***H***$_2$), 7.68 – 7.73 (m, 2H, Phth-***H***), 7.81 – 7.86 (m, 2H, Phth-***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$) 24.96 (-, Pip-$CH_2$-***C***$H_2$), 28.39 (+, C(***C***$H_3$)$_3$), 36.46 (-, Pip-***C***$H_2$), 52.84 (-, 4 Pip-***C***), 56.01 (-, Pip-$(CH_2)_2$-***C***$H_2$), 123.18 (+, 2 Ph-***C***), 132.20 ($C_{quat}$, Ph-***C***), 133.97 (+, 2 Ph-***C***), 154.60 ($C_{quat}$, ***C***=O), 168.49 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 374 (100) $[M+H]^+$. $C_{20}H_{27}N_3O_4$ (373.45).

**2-[3-(4-Phenylpiperazin-1-yl)propyl]isoindoline-1,3-dione (3.13)[45]**

The title compound was prepared from **3.8** (2.42 g, 14.9 mmol), potassium carbonate (3.09 g, 22.4 mmol), phthalimide (4.00 g, 14.9 mmol) and potassium iodide (1.24 g, 7.5 mmol) in 300 mL acetone according to the general procedure. Purification by flash chromatography (DCM/MeOH 100/1- 95/5 v/v) yielded **3.13** as yellow solid (1.65 g, 79 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.86 – 2.00 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.45 – 2.65 (m, 6H, Pip-***H*** + Pip-C***H***$_2$), 3.00 – 3.15 (m, 4H, Pip-***H***), 3.80 (t, $^3J$ =6.9 Hz, 2H, Pip-$(CH_2)_2$-C***H***$_2$), 6.80 – 6.90 (m, 3H, Ph-***H***),7.20 – 7.25 (m, 2H, Ph-***H***), 7.67 – 7.72 (m, 2H, Phth-***H***), 7.81 – 7.86 (m, 2H, Phth-***H***); $^{13}$C-NMR (75 MHz; $CDCl_3$) 25.08 (-, Pip-$CH_2$-***C***$H_2$), 36.51 (-, Pip-***C***$H_2$), 48.78 (-, 2 Pip-***C***), 53.03 (-, 2 Pip-***C***), 56.00 (-, Pip-$(CH_2)_2$-***C***$H_2$), 116.15 (+, 2 Ph-***C***), 119.84 (+, Ph-***C***-4), 123.20 (+, 2 Ph-***C***), 129.10 (+, 2 Ph-***C***), 132.24 ($C_{quat}$, 2 Ph-***C***), 133.94 (+, 2 Ph-***C***), 151.06 ($C_{quat}$, Ph-***C***), 168.51 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 350 (100) $[M+H]^+$. $C_{21}H_{23}N_3O_2$ (349.43).

**2-{3-[4-(2-Methoxyphenyl)piperazin-1-yl]propyl}isoindoline-1,3-dione (3.14)[45]**

The title compound was prepared from **3.9** (1.40 g, 7.2 mmol), potassium carbonate (1.50 g, 10.8 mmol), phthalimide (1.95 g, 7.2 mmol) and potassium iodide (0.60 g, 3.6 mmol) in 150 mL acetone according to the general procedure. Purification by flash chromatography (DCM/MeOH 100/1- 95/5 v/v) yielded **3.14** as colorless oil (1.95 g, 71 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.83 – 2.00 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.44 – 2.72 (m, 6H, Pip-***H*** + Pip-C***H***$_2$), 2.83 – 3.08 (m, 4H, Pip-***H***), 3.80 (t, $^3J$ = 6.9 Hz, 2H, Pip-$(CH_2)_2$-C***H***$_2$), 3.83 (s, 3H, OC***H***$_3$), 6.79 – 7.02 (m, 4H, Ph-***H***), 7.67 – 7.72 (m, 2H, Phth-***H***), 7.82 – 7.86 (m, 2H, Phth-***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$) 25.14 (-, Pip-$CH_2$-***C***$H_2$), 36.65 (-, Pip-***C***$H_2$), 50.34 (-, 2 Pip-***C***), 53.32 (-, 2 Pip-***C***), 55.32 (+, O***C***$H_3$), 56.11 (-, Pip-$(CH_2)_2$-***C***$H_2$), 111.11 (+, Ph-***C***), 118.15 (+, Ph-***C***), 120.93 (+, Ph-***C***), 122.90 (+, Ph-***C***), 123.18 (+, 2 Phth-***C***), 132.33 ($C_{quat}$, 2 Phth-***C***), 133.87 (+, 2 Phth-***C***), 141.19 and 152.23 ($C_{quat}$, Ph-***C***), 168.52 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 380 (100) $[M+H]^+$. $C_{22}H_{25}N_3O_3$ (379.45).

**2-[3-(4-Benzhydrylpiperazin-1-yl)propyl]isoindoline-1,3-dione (3.15)[46]**

The compound was prepared from **3.10** (2.52 g, 10.0 mmol), potassium carbonate (2.07 g, 15.0 mmol), phthalimide (2.68 g, 10.0 mmol) and potassium iodide (0.83 g, 5.0 mmol) in 200 mL acetone according to the general procedure. Purification by flash chromatography (DCM/MeOH 100/1- 95/5 v/v) yielded **3.15** as pale yellow oil (3.90 g, 89 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.79 – 1.92 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.12 – 2.56 (m, 10H, Pip-***H*** + Pip-C***H***$_2$), 3.74 (t, 2H, $^3J$ = 6.9 Hz, Pip-$(CH_2)_2$-C***H***$_2$), 7.10 – 7.19 (m, 2H, Ph-***H***), 7.20 – 7.30 (m, 4H, Ph-***H***), 7.41 – 7.31 (m, 4H, Ph-***H***), 7.68-7.73 (m, 2H, Phth-***H***), 7.81 – 7.84 (m, 2H, Phth-***H***); $^{13}$C-NMR (75 MHz; $CDCl_3$) δ (ppm) 25.33 (-, Pip-$CH_2$-***C***$H_2$), 36.62 (-, Pip-***C***$H_2$), 51.77 (-, 2 Pip-***C***), 53.34 (-, 2 Pip-***C***), 55.96 (-, Pip-$(CH_2)_2$-***C***$H_2$), 76.31 (+, di-Ph-***C***H), 123.17 and 126.89 (+, 2 Phth-***C*** and 2 di-Ph-***C***-4), 127.91 (+, 4 Ph-***C***), 128.44 (+, 4 Ph-***C***), 132.32 ($C_{quat}$, 2 Phth-***C***), 133.81 (+, 2 Phth-***C***), 142.75 ($C_{quat}$, 2 di-Ph-***C***), 168.46 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 440 (100) $[M+H]^+$. $C_{28}H_{29}N_3O_2$ (439.55).

### 3.5.1.5 Preparation of the 2-[3-(Hetaryl)propyl]isoindoline-1,3-diones 3.23 - 3.25 and 3.31

**General procedure**

The hetarylpropylalcohols **3.20** – **3.22** and **3.30** (1 eq), triphenylphosphine (1.1 eq) and phthalimide (1.1 eq) were dissolved in $THF_{abs}$ and cooled to 0 °C. A solution of DIAD (1.1 eq) in $THF_{abs}$ was added dropwise. After complete addition, the mixture was allowed to warm to room temperature and was stirred overnight. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (DCM/MeOH 100/0 – 95/5 v/v). For analytical purposes a small amount was

converted into the picrate by addition of a saturated solution of picric acid in EtOH and recrystallized from EtOH/$H_2O$.

**2-[3-(2-Pyridyl)propyl]isoindoline-1,3-dione (3.23)[14, 47]**

The title compound was prepared from pyridine-2-propanol **3.20** (8.23 g, 60 mmol), phthalimide (9.71, 66 mmol), TPP (17.31 g, 66 mmol) in 160 mL $THF_{abs}$ and DIAD (12.13 g, 66 mmol) in 80 mL $THF_{abs}$ according to the general procedure yielding **3.23** as a brownish oil (10.9 g, 68 %); mp (picrate) 138 – 139 °C. $^1$H-NMR (300 MHz, DMSO-$d_6$, picrate): δ (ppm) 2.00 – 2.12 (m, 2H, Pyr-2-$CH_2$-C***H***$_2$), 3.02 (t, $^3J$ = 8.2 Hz, 2H, Pyr-2-C***H***$_2$), 3.67 (t, 2H, $^3J$ = 6.6 Hz, Pyr-2-$(CH_2)_2$-C***H***$_2$), 7.79 – 7.91 (m, 5H, Pyr-5-***H*** + Phth-***H***), 7.94 (d, 1H, $^3J$ = 8.0 Hz, Pyr-3-***H***), 8.44 – 8.52 (m, 1H, Pyr-4-***H***), 8.57 (s, 2H, picrate-***H***), 8.77 (dd, 1H, $^3J$ = 5.8 Hz, $^4J$ = 10.9 Hz, Pyr-6-***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$, picrate): δ (ppm) 27.38 (-, Pyr-2-$CH_2$-***C***$H_2$), 30.64 (-, Pyr-2-***C***$H_2$), 36.63 (-, Pyr-2-$(CH_2)_2$-***C***$H_2$), 122.92 (-, Phth-***C***), 124.06 ($C_{quat}$, picrate-***C***-4), 124.28 (+, Pyr-***C***-5), 125.10 (+, picrate-***C***-3,5), 126.35 (+, Pyr-***C***-3), 131.55 ($C_{quat}$, Phth-***C***), 134.30 (+, Phth-***C***), 141.71 ($C_{quat}$, picrate-***C***-2,6), 142.17 (+, Pyr-***C***-6), 145.24 (+, Pyr-***C***-4), 156.47 ($C_{quat}$, Pyr-***C***-2), 160.69 ($C_{quat}$, picrate-***C***-1), 167.89 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 267 (100) $[M+H]^+$. $C_{16}H_{14}N_2O_2$ (266.29).

**2-[3-(3-Pyridyl)propyl]isoindoline-1,3-dione (3.24)[14]**

The title compound was prepared from pyridine-3-propanol **3.21** (1.07 g, 7.8 mmol), phthalimide (1.26 g, 8.6 mmol), TPP (2.26 g, 8.6 mmol) in 25 mL $THF_{abs}$ and DIAD (1.74, 8.6 mmol) in 15 mL $THF_{abs}$ according to the general procedure yielding **3.24** as an orange oil (1.52 g, 73%); mp (picrate) 145 – 146 °C. $^1$H-NMR (300 MHz, DMSO-$d_6$, picrate): δ (ppm) 1.92 – 2.04 (m, 2H, Pyr-3-$CH_2$-C***H***$_2$), 2.85 (t, $^3J$ = 7.9 Hz, 2H, Pyr-3-C***H***$_2$), 3.63 (t, $^3J$ = 6.7 Hz, 2H, Pyr-3-$(CH_2)_2$-C***H***$_2$), 7.80 – 7.90 (m, 4H, Phth-***H***), 8.00 (dd, $^3J$ = 8.0 Hz, $^3J$ = 5.7 Hz, 1H, Pyr-5-***H***), 8.52 (m, 1H, Pyr-4-***H***), 8.58 (s, 2H, picrate-***H***), 8.77 (d, $^3J$ = 5.5 Hz, 1H, Pyr-6-***H***), 8.84 (m, 1H, Pyr-2-***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$, picrate): δ (ppm) 28.73 (-, Pyr-3-$CH_2$-***C***$H_2$), 28.94 (-, Pyr-3-***C***$H_2$), 36.67 (-, Pyr-3-$(CH_2)_2$-***C***$H_2$), 122.88 (-, Phth-***C***), 124.06 ($C_{quat}$, picrate-***C***-4), 125.10 (+, picrate-***C***-3,5), 126.60 (+, Pyr-***C***-5), 131.57 ($C_{quat}$, Phth-***C***), 134.25 (+, Phth-***C***), 139.78 (+, Pyr-***C***-6), 141.13 ($C_{quat}$, Pyr-***C***-3), 141.71 (+, Pyr-***C***-2), 141.84 ($C_{quat}$, picrate ***C***-2,6), 145.84 (+, Pyr-***C***-4), 160.68 ($C_{quat}$, picrate-***C***-1), 167.92 ($C_{qua}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 267 (100) $[M+H]^+$. $C_{16}H_{14}N_2O_2$ (266.29).

**2-[3-(4-Pyridyl)propyl]isoindoline-1,3-dione (3.25)[14]**

The title compound was prepared from pyridine-4-propanol **3.22** (1.07 g, 7.8 mmol), phthalimide (1.26 g, 8.6 mmol), TPP (2.26 g, 8.6 mmol) in 25 mL $THF_{abs}$ and DIAD (1.74 g, 8.6 mmol) in 15 mL $THF_{abs}$ according to the general procedure yielding **3.25** as a red oil (1.23 g, 59 %); mp (picrate) 180 –

181 °C. $^1$H-NMR (300 MHz, DMSO-$d_6$, picrate): δ (ppm) 1.95 – 2.07 (m, 2H, Pyr-4-$CH_2$-C***H***$_2$), 2.95 (t, $^3J$ = 7.8 Hz, 2H, Pyr-4-C***H***$_2$), 3.64 (t, $^3J$ = 6.7 Hz, 2H, Pyr-4-$(CH_2)_2$-C***H***$_2$), 7.80 – 7.89 (m, 4H, Phth-***H***), 7.98 (d, $^3J$ = 6.7 Hz, 2H, Pyr-3,5-***H***), 8.57 (s, 2H, picrate-***H***), 8.81 (d, $^3J$ = 6.7 Hz, 2H, Pyr-2,6-***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$, picrate): δ (ppm) 27.96 (-, Pyr-4-$CH_2$-***C***$H_2$), 32.29 (-, Pyr-4-***C***$H_2$), 36.73 (-, Pyr-4-$(CH_2)_2$-***C***$H_2$), 122.87 (-, Phth-***C***), 124.06 ($C_{quat}$, picrate-***C***-4), 125.10 (+, picrate-***C***-3,5), 126.78 (+, Pyr-***C***-3,5), 131.57 ($C_{quat}$, Phth-***C***), 134.24 (+, Phth-***C***), 141.33 (+, Pyr-***C***-2,6), 141.71 ($C_{quat}$, picrate-***C***-2,6), 160.69 ($C_{quat}$, picrate-***C***-1), 162.36 ($C_{quat}$, Pyr-***C***-4), 167.89 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 267 (100) $[M+H]^+$. $C_{16}H_{14}N_2O_2$ (266.29).

**2-[3-(1*H*-Benzimidazol-2-yl)propyl]isoindoline-1,3-dione (3.31)**

The title compound was prepared from **3.30** (1.04 g, 5.9 mmol), phthalimide (0.95 g, 6.5 mmol), TPP (1.70 g, 6.5 mmol) and DIAD (1.31 g, 6.5 mmol) according to the general procedure. Purification by flash chromatography (DCM/MeOH 100/0 – 95/5 v/v) yielded **3.31** as a pale brown oil (0.93 g, 52 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 2.15 – 2.28 (m, 2H, Benzim-$CH_2$-C***H***$_2$), 2.95 (t, $^3J$ = 6.9 Hz, 2H, Benzim-C***H***$_2$), 3.79 (t, $^3J$ = 6.1 Hz, Benzim-$(CH_2)_2$-C***H***$_2$), 7.18 – 7.25 (m, 4H, Benzim-***H***), 7.71 – 7.79 (m, 2H, Phth-***H***), 7.82 – 7.92 (m, 2H, Phth-***H***); MS (CI, $NH_3$) *m/z* (%): 306 (100) $[M+H]^+$. $C_{18}H_{15}N_3O_2$ (305.33).

### 3.5.1.6 Preparation of the 3-Hetarylpropylamines 3.16 – 3.19 and 3.26 – 3.28 and 3.32 by Hydrazinolysis of the Phthalimides

**General procedure**

A mixture of the pertinent hetarylpropylisoindoline-1,3-dione (1 eq) and hydrazine monohydrate (6 eq) in EtOH was stirred at rt overnight. After removal of insoluble material by filtration the solvent was evaporated under reduced pressure and the product subjected to flash chromatography.

***tert*-Butyl 4-(3-aminopropyl)piperazine-1-carboxylate (3.16)[44]**

The title compound was prepared from **3.12** (1.3 g, 3.5 mmol) and hydrazine monohydrate (0.76 mL, 20.9 mmol) in 35 mL EtOH according to the general procedure. Purification by flash chromatography ($CHCl_3$/MeOH/$NH_3$ (aq.) 85/13/2 v/v/v) yielded **3.16** as colorless oil (0.65 g, 76 %). $^1$H-NMR (300 MHz, $CDCl_3$ ): δ (ppm) 1.45 (s, 9H, C(C***H***$_3$)$_3$), 1.58 – 1.70 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.33 – 2.44 (m, 6H, Pip-***H*** + Pip- $(CH_2)_2$-C***H***$_2$), 2.75 (t, 2H, Pip-C***H***$_2$), 3.38 – 3.46 (m, 4H, Pip-***H***). $C_{12}H_{25}N_3O_2$ (243.35).

**3-(4-Phenylpiperazin-1-yl)propan-1-amine (3.17)[45]**

The title compound was prepared from **3.13** (3.50 g, 10.0 mmol) and hydrazine monohydrate (2.19 mL, 60.0 mmol) in 100 mL EtOH according to the general procedure. Purification by flash

chromatography ($CHCl_3$/MeOH/$NH_3$ (aq.) 85/13/2 v/v/v) yielded **3.17** as a pale yellow solid (1.90 g, 65 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 2.10 – 2.15 (m, 2H, Pip-$CH_2$-C***H***$_2$), 3.23 (t, $^3J$ = 7.5 Hz, 2H, Pip-$(CH_2)_2$-C***H***$_2$), 3.32 – 3.65 (m, 8H, Pip-***H***), 6.87 – 6.97 (m, 1H, Ph-***H***-4), 6.98 – 7.05 (m, 2H, Ph-***H***), 7.23 – 7.32 (m, 2H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 23.53 (-, Pip-$CH_2$-***C***$H_2$), 38.28 (-, Pip-***C***$H_2$), 48.38 (-, 2 Pip-***C***), 53.48 (-, 2 Pip-***C***), 55.05 (-, Pip-$(CH_2)_2$-***C***$H_2$), 118.04 (+, 2 Ph-***C***), 122.23 (+, Ph-***C***-4), 130.37 (+, 2 Ph-***C***), 151.37 ($C_{quat}$, Ph-***C***-1); MS (CI, $NH_3$) *m/z* (%): 220 (100) $[M+H]^+$. $C_{13}H_{21}N_3$ (219.33).

**3-[4-(2-Methoxyphenyl)piperazin-1-yl]propan-1-amine (3.18)[45]**

The title compound was prepared from **3.14** (1.85 g, 4.9 mmol) and hydrazine monohydrate (1.07 mL, 29.3 mmol) in 50 mL EtOH according to the general procedure. Purification by flash chromatography ($CHCl_3$/MeOH/$NH_3$ (aq.) 85/13/2 v/v/v) yielded **3.18** as pale brown semi-solid compound (0.93, 76 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.64 – 1.76 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.49 (t, $^3J$ = 7.2 Hz, 2H, Pip-$(CH_2)_2$-C***H***$_2$), 2.60 – 2.75 (m, 4H, Pip-***H***), 2.80 (t, $^3J$ = 6.7 Hz, 2H, Pip-C***H***$_2$), 3.00 – 3.20 (m, 4H, Pip-***H***), 3.86 (s, 3H, OC***H***$_3$), 6.82 – 7.05 (m, 4H, Ph-***H***); MS (CI, $NH_3$) *m/z* (%): 250 (100) $[M+H]^+$. $C_{14}H_{23}N_3O$ (249.35).

**3-(4-Benzhydrylpiperazin-1-yl)propan-1-amine (3.19)[46]**

The title compound was prepared from **3.15** (1.50 g, 3.4 mmol) and hydrazine monohydrate (0.75 mL, 20.5 mmol) in 35 mL EtOH according to the general procedure. Purification by flash chromatography ($CHCl_3$/MeOH/$NH_3$ (aq.) 85/13/2 v/v/v) yielded **3.19** as beige semi-solid compound (0.85 g, 77 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.57 – 1.69 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.25 – 2.60 (m, 6H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$), 2.75 (t, $^3J$ = 6.7 Hz, 2H, Pip-C***H***$_2$), 2.75 (t, $^3J$ =6.7 Hz, 2H, Pip-***H***), 4.20 (s, 1H, di-Ph-C***H***), 7.12-7.21 (m, 2H, Ph-***H***), 7.22-7.31 (m, 4H, Ph-***H***), 7.36-7.45 (m, 4H, Ph-***H***); $^{13}$C-NMR (75 MHz; $CDCl_3$) δ (ppm) 26.74 (-, Pip-$CH_2$-***C***$H_2$), 41.20 (-, Pip-***C***$H_2$), 51.86 (-, 2 Pip-***C***), 53.49 (-, 2 Pip-***C***), 57.19 (-, Pip-$(CH_2)_2$-***C***$H_2$), 76.23 (+, di-Ph-***C***H), 127.00 (+, 2 Ph-***C***-4), 127.88 (+, 4 Ph-***C***), 128.51 (+, 4 Ph-***C***), 142.69 ($C_{quat}$, Ph-***C***); MS (CI, $NH_3$) *m/z* (%): 310 (100) $[M+H]^+$. $C_{20}H_{27}N_3$ (309.45).

**3-(2-Pyridyl)propan-1-amine (3.26)[10, 15]**

The title compound was prepared from **3.23** (6.0 g, 22.5 mmol) and hydrazine monohydrate (4.9 mL, 135.0 mmol) in EtOH (200 mL) according to the general procedure. Purification by flash chromatography ($CHCl_3$/MeOH/$NH_3$ (aq.) 85/13/2 v/v/v) yielded **3.26** as brownish oil (5.15 g, 84 %) . $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.80 – 1.93 (m, 2H, Pyr-2-$CH_2$-C***H***$_2$), 2.73 (t, $^3J$ = 7.0 Hz, 2H, Pyr-2-$(CH_2)_2$-C***H***$_2$), 2.81 (t, $^3J$ = 7.5 Hz, 2H, Pyr-2-C***H***$_2$), 7.04 – 7.16 (m, 2H, Pyr-3,5-***H***), 7.56 (dt, $^4J$ = 1.8 Hz, $^3J$ = 7.7 Hz, 1H, Pyr-4-***H***), 8.45 – 8.52 (m, 1H, Pyr-6-***H***); $^{13}$C-NMR (75 MHz; $CDCl_3$) 33.41 (-, Pyr-2-$CH_2$-***C***$H_2$),

35.57 (-, Pyr-2-***C***$H_2$), 41.58 (-, Pyr-2-$(CH_2)_2$-***C***$H_2$), 121.07 and 122.79 (+, 2 Pyr-***C***-3,5), 136.44 (+, Pyr-***C***-4), 149.17 (+, Pyr-***C***-6), 161.69 ($C_{quat}$, Pyr-***C***-2); MS (ESI, MeCN/0.1% TFA) *m/z* (%): 137 (100) $[M+H]^+$. $C_8H_{12}N_2$ (136.19).

**3-(3-Pyridyl)propan-1-amine (3.27)[47, 48]**

The title compound was prepared from **3.24** (2.0 g, 7.51 mmol) and hydrazine monohydrate (1.64 mL, 45.1 mmol) in 70 mL EtOH according to the general procedure. Purification by flash chromatography ($CHCl_3$/MeOH/$NH_3$ (aq) 85/13/2 v/v/v) yielding **3.27** as brownish oil (0.71 g, 69 %). $^1$H-NMR (300 MHz, DMSO): δ (ppm) 1.77 – 1.90 (m, 2H, Pyr-3-$CH_2$-C***H***$_2$), 2.66 (t, 2H, $^3J$ = 7.2 Hz, Pyr-3-$(CH_2)_2$-C***H***$_2$), 2.72 (t, 2H, $^3J$ = 7.2 Hz, Pyr-3-C***H***$_2$), 7.32 (dd, 1H, $^3J$ = 7.8 Hz, $^3J$ = 4.9 Hz, Pyr-5-***H***), 7.58-7.70 (m, 1H, Pyr-4-***H***), 8.35 – 8.50 (m, 2H, Pyr-2-***H*** + Pyr-6-***H***); $^{13}$C-NMR (75 MHz, DMSO): δ (ppm) 28.88 (-, Pyr-3-$CH_2$-***C***$H_2$), 29.44 (-, Pyr-3-***C***$H_2$), 38.53 (-, Pyr-3-$(CH_2)_2$-***C***$H_2$), 123.40 (+, Pyr-***C***-5), 135.72 (+, Pyr-***C***-6), 136.47 ($C_{quat}$, Pyr-***C***-3), 147.21 (+, Pyr-***C***-2), 149.44 (+, Pyr-***C***-4); MS (CI, $NH_3$) *m/z* (%): 137 (100) $[M+H]^+$. $C_8H_{12}N_2$ (136.19).

**3-(4-Pyridyl)propan-1-amine (3.28)[14, 48]**

The title compound was prepared from **3.25** (1.40 g, 5.26 mmol) and hydrazine monohydrate (1.15 mL, 31.5 mmol) in 50 mL EtOH according to the general procedure. Purification by flash chromatography ($CHCl_3$/MeOH/$NH_3$ (aq.) 85/13/2 v/v/v) yielded **3.28** as pale yellow oil (0.53 g, 74 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.71 – 1.85 (m, 2H, Pyr-4-$CH_2$-C***H***$_2$), 2.65 (t, $^3J$ = 7.0 Hz, 2H, Pyr-4-$(CH_2)_2$-C***H***$_2$), 2.74 (t, $^3J$ = 7.0 Hz, 2H, Pyr-4-C***H***$_2$), 7.11 (d, $^3J$ = 5.0 Hz, 2H, Pyr-3,5-***H***), 8.48 (d, $^3J$ = 5.0 Hz, 2H, Pyr-2,6-***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$) 29.45 (-, Pyr-4-$CH_2$-***C***$H_2$), 32.83 (-, Pyr-4-***C***$H_2$), 40.30 (-, Pyr-4-$(CH_2)_2$-***C***$H_2$), 125.71 (+, Pyr-***C***-3,5), 149.99 (+, Pyr-***C***-2,6), 163.93 ($C_{quat}$, Pyr-***C***-4); MS (CI, $NH_3$) *m/z* (%): 137 (100) $[M+H]^+$. $C_8H_{12}N_2$ (136.19).

**3-(1*H*-Benzimidazol-2-yl)propan-1-amine (3.32)[49]**

The title compound was prepared from **3.31** (0.70 g, 2.29 mmol) and hydrazine monohydrate (0.5 mL, 13.8 mmol) in 25 mL EtOH according to the general procedure. Purification by flash chromatography ($CHCl_3$/MeOH/$NH_3$ (aq.) 85/13/2 v/v/v) yielded **3.32** as brownish oil (0.31 g, 77 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.92 – 2.02 (m, 2H, Benzim-$CH_2$-C***H***$_2$), 2.91 (t, $^3J$ = 6.1 Hz, 2H, Benzim-$(CH_2)_2$-C***H***$_2$), 3.07 (t, $^3J$ = 6.8 Hz, 2H, Benzim-C***H***$_2$), 7.16 – 7.22 (m, 2H, Ph-***H***), 7.50 – 7.58 (m, 2H, Ph-***H***); MS (CI, $NH_3$) *m/z* (%): 176 (100) $[M+H]^+$. $C_{10}H_{13}N_3$ (175.23).

### 3.5.1.7 Preparation of mono-Boc-Protected Diamines 3.35 and 3.36 and mono-Boc-Protected Piperazine 3.7

**General procedure for the synthesis of mono Boc-protected diamines**

A solution of $(Boc)_2O$ (1 eq) in dioxane was added dropwise to a solution of the diamine (10 eq) in dioxane over a period of 2.5 h. After complete addition, the reaction mixture was stirred for 22 h at rt. The solvent was evaporated and the residue was treated with water (300 mL). The di-Boc-protected product precipitated and was removed by filtration. The filtrate was then extracted with DCM (3x 150 mL). Drying of the organic phase over $MgSO_4$ and removal of the solvent *in vacuo* afforded the mono-Boc-protected amine which was used without further purification.

***N*-(*tert*-Butoxycarbonyl)ethane-1,2-diamine (3.35)[21]**

The title compound was prepared from $(Boc)_2O$ (9.82 g, 45.0 mmol) and ethane-1,2-diamine **3.33** (30.0 mL, 450 mmol) according to the general procedure, yielding **3.35** as a pale yellow oil (5.06 g, 70 %). $^1$H-NMR (300 MHz, $CD_3OD$):δ (ppm) 1.44 (s, 9H, C(C***H***$_3$)$_3$), 2.78 (t, $^3J$ = 6.2 Hz, 2H, C***H***$_2$-NH$_2$), 3.17 (t, $^3J$ = 6.2 Hz, 2H, C***H***$_2$-NHBoc); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.77 (+, C(***C***H$_3$)$_3$), 41.91 (-, ***C***H$_2$-N), 42.39 (-, ***C***H$_2$-N), 80.32 ($C_{quat}$, ***C***$(CH_3)_3$), 158.72 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 161 (100) $[M+H]^+$. $C_7H_{16}N_2O_2$ (160.21).

***N*-(*tert*-Butoxycarbonyl)propane-1,3-diamine (3.36)[21]**

The title compound was prepared from $(Boc)_2O$ (3.27 g, 15.0 mmol) and propane-1,3-diamine **3.34** (12.63 mL, 150 mmol) according to the general procedure, yielding **3.36** as pale yellow oil (2.01 g, 80 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.53 – 1.66 (m, 2H, C***H***$_2$-CH$_2$-NH$_2$), 2.64 (t, $^3J$ = 7.0 Hz, 2H, C***H***$_2$-NH$_2$), 3.09 (t, $^3J$ = 6.7 Hz, 2H, C***H***$_2$-NHBoc); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.78 (+, C(***C***H$_3$)$_3$), 31.47 (-, ***C***H$_2$-CH$_2$-NH$_2$), 38.24 (-, ***C***H$_2$-N), 38.93 (-, ***C***H$_2$-N), 80.18 ($C_{quat}$, ***C***$(CH_3)_3$), 158.88 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 175 (100) $[M+H]^+$. $C_8H_{18}N_2O_2$ (174.24).

***N*-(*tert*-Butoxycarbonyl)piperazine (3.7) [15]**

Piperazine (**3.6**, 1.72 g, 20.0 mmol, 1 eq) was dissolved in 60 mL DCM. $Et_3N$ (1.41 mL, 10.0 mmol, 0.5 eq) and $(Boc)_2O$ (2.18 g, 10.0 mmol, 0.5 eq) in 40 mL DCM were slowly added at 0 °C. The reaction mixture was stirred at room temperature overnight. After evaporation of the solvent, the crude product was subjected to flash chromatography (DCM/MeOH 100/0 – 95/5 v/v) to separate the mono- from the di-protected piperazine (ratio 2:1) yielding **3.7** as colorless semi-solid compound (1.24 g, 33 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.45 (s, 9H, C(C***H***$_3$)$_3$), 1.78 (s, 1H, N***H***), 2.76 – 2.83 (m, 4H, Pip-***H***), 3.34 – 3.41 (m, 4H, Pip-***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$): δ (ppm) 28.28 (+, C(***C***H$_3$)$_3$), 43.56

(-, Pip-***C***), 51.25 (-, Pip-***C***), 81.25 ($C_{quat}$, ***C***$(CH_3)_3$), 153.90 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 187 (100) $[M+H]^+$. $C_9H_{18}N_2O_2$ (186.25).

### 3.5.1.8 Preparation of $N^G$-Acylated Aylpropylguanidines 3.40 – 3.52

**General procedure for guanidinylation and Boc-deprotection**

The arylpropylamines **3.16** – **3.19**, **3.26** – **3.28**, **3.36** – **3.39** or **3.32** (1 eq), the guanidinylating reagent **3.5** (1 eq) and $HgCl_2$ (2 eq) were dissolved in anhydrous DCM. $Et_3N$ (3 eq) was added and the mixture was stirred for 36 to 48 h at ambient temperature. Subsequently, EtOAc was added and the precipitate was removed by filtration over Celite. The crude product was purified by flash chromatography (DCM/MeOH 100/1 – 90/10 v/v). The residue was dissolved in DCM, TFA was added and the reaction mixture was stirred until the protection groups were removed (3 – 8 h, TLC control). After evaporation of the solvent *in vacuo*, the crude product was purified by preparative RP-HPLC or flash chromatography (DCM/MeOH 98/2 – 90/10 v/v).

**$N^2$-(3-Phenylpropanoyl)-$N^1$-[3-(piperazin-1-yl)propyl]guanidine (3.40)**

The title compound was prepared from **3.5** (120 mg, 0.37 mmol) and **3.16** (65 mg, 0.37 mmol) in the presence of $HgCl_2$ (202 mg, 0.75 mmol) and $Et_3N$ (157 µL, 1.12 mmol). Deprotection in 3 mL DCM and 3 mL TFA, followed by preparative RP-HPLC (10 – 40 % MeCN in 30 min) afforded **3.40** as pale brown resin (59 mg, 29 %) . $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 1.85 – 1.95 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.71 – 2.84 (m, 4H, Pip-C***H***$_2$ + Ph-$CH_2$-C***H***$_2$), 2.88 – 3.02 (m, 6H, Pip-***H*** + Ph-C***H***$_2$), 3.33 – 3.45 (m, 6H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$), 7.15 – 7.31 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 24.55 (-, Pip-C$H_2$-***C***$H_2$), 31.26 (-, Ph-***C***$H_2$), 39.47 (-, Pip-$(CH_2)_2$-***C***$H_2$), 40.59 (-, Ph-$CH_2$-***C***$H_2$), 43.43 (-, 2 Pip-***C***), 50.48 (-, Pip-***C***$H_2$), 56.04 (-, 2 Pip-***C***), 127.51 (+, Ph-***C***-4), 129.46 (+, 2 Ph-***C***), 129.63 (+, 2 Ph-***C***), 141.36 ($C_{quat}$, Ph-***C***-1), 155.19 ($C_{quat}$, ***C***=N), 176.40 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 100 % ($t_R$ = 11.05 min, *k'* = 2.90); HRMS (ESI): *m/z* calcd. for $[C_{17}H_{27}N_5O + H]^+$ 318.228, found: 318.2291. $C_{17}H_{27}N_5O \cdot 2TFA$ (545.47).

**$N^2$-(3-Phenylpropanoyl)-$N^1$-[3-(4-phenylpiperazin-1-yl)propyl]guanidine (3.41)**

The title compound was prepared from **3.5** (120 mg, 0.37 mmol) and **3.17** (65 mg, 0.37 mmol) in the presence of $HgCl_2$ (202 mg, 0.75 mmol) and $Et_3N$ (157 µL, 1.12 mmol). Deprotection in 3 mL DCM and 3 mL TFA, followed by preparative RP-HPLC (20 – 65 % MeCN in 30 min) afforded **3.41** as pale orange oil (63 mg, 30 %) . $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetic acid): δ (ppm) 2.07 – 2.21 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.80 (t, $^3J$ = 7.4 Hz, 2H, Pip-C***H***$_2$), 2.97 (t, $^3J$ = 7.3 Hz, 2H, Ph-$CH_2$-C***H***$_2$), 3.21 – 3.34 (m, 4H, overlap with solvent, Pip-***H*** + Ph-C***H***$_2$), m 3.35 – 3.75 (m, 8H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$ ), 6.88 – 7.06 (m, 3H, Pip-Ph-***H***), 7.14 – 7.33 (m, 7H, Ph-***H*** + Pip-Ph-***H***); $^{13}$C-NMR (75 Mhz, $CD_3OD$, trifluoroacetate): δ

(ppm) 23.91 (-, Pip-$CH_2$-***C***$H_2$), 30.23 (-, Ph-C***H***$_2$), 38.43 (-, Ph-$CH_2$-***C***$H_2$ + Pip-$(CH_2)_2$-***C***$H_2$), 46.92 (-, Pip-***C***), 52.32 (-, Pip-***C***$H_2$), 54.33 (-, Pip-***C***), 117.33 (+, 2 Pip-Ph-***C***), 122.21 (+, Pip-Ph-***C***-4), 126.48 (+, Ph-***C***-4), 128.49 (+,2 Ph-***C***), 128.59 (+, 2 Ph-***C***), 129.58 (+, 2 Pip-Ph-***C***), 139.64 ($C_{quat}$, Ph-***C***-1 ), 149.15 ($C_{quat}$, Pip-Ph-***C***-1) 154.71 ($C_{quat}$, ***C***=N), 176.22 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 100 % ($t_R$ = 16.13 min, *k'* = 4.70); HRMS (LSI, glycerine): *m/z* calcd. for $[C_{23}H_{31}N_5O + H]^+$ 394.2607, found: 394.2605. $C_{23}H_{31}N_5O \cdot 2TFA$ (621.57).

**$N^1$-{3-[4-(2-Methoxyphenyl)piperazin-1-yl]propyl}-$N^2$-(3-phenylpropanoyl)-guanidine (3.42)**

The title compound was prepared from **3.5** (120 mg, 0.37 mmol) and **3.18** (65 mg, 0.37 mmol) in the presence of $HgCl_2$ (202 mg, 0.75 mmol) and $Et_3N$ (157 µL, 1.12 mmol). Deprotection in 3 mL DCM and 3 mL TFA, followed by preparative RP-HPLC (20 – 65 % MeCN in 30 min) afforded **3.42** as pale orange resin (54 mg, 31 %) . $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 2.07 – 2.21 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.80 (t, $^3J$ = 7.3 Hz, 2H, Pip-C***H***$_2$), 2.97 (t, $^3J$ = 7.3 Hz, 2H, Ph-$CH_2$-C***H***$_2$), 3.21 – 3.32 (m, overlap with solvent, 4H, Pip-***H*** + Ph-C***H***$_2$), 3.33 – 3.72 (m, 6H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$), 6.88 – 7.11 (m, 4H, Pip-Ph-***H***), 7.13 – 7.33 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 Mhz, $CD_3OD$, trifluoroacetate): δ (ppm) 22.98 (-, Pip-$CH_2$-C***H***$_2$), 30.42 (-, Ph-***C***$H_2$), 38.66 and 38.76 (-, Pip-$(CH_2)_2$-***C***$H_2$ + Ph-$CH_2$-***C***$H_2$), 48.15 (-, 2 Pip-***C***), 53.04 (-, Pip-***C***$H_2$), 54.36 (-, 2 Pip-***C***), 55.22 (+, O-***C***$H_3$), 112.18 (+, Pip-Ph-***C***), 119.19 (+, Pip-Ph-***C***), 121.44 (+, Pip-Ph-***C***), 124.89 (+, Pip-Ph-***C***), 126.65 (+, Ph-***C***-4), 128.67 (+, 2 Ph-***C***), 128.77 (+, 2 Ph-***C***), 139.62 ($C_{quat}$, Ph-***C***-1), 140.56 ($C_{quat}$, ***C***-OMe), 153.19 ($C_{quat}$, Pip-Ph-***C***-1), 154.61 ($C_{quat}$, ***C***=N), 175.66 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 96 % ($t_R$ = 15.97 min, *k'* = 4.64); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{24}H_{33}N_5O_2 + H]^+$ 423.2634, found: 423.2632. $C_{24}H_{33}N_5O_2 \cdot 2TFA$ (651.59).

**$N^1$-[3-(4-Benzhydrylpiperazin-1-yl)propyl] $N^2$-(3-phenylpropanoyl)-guanidine (3.43)**

The title compound was prepared from **3.5** (120 mg, 0.37 mmol), **3.19** (65 mg, 0.37 mmol), $HgCl_2$ (202 mg, 0.75 mmol) and $Et_3N$ (157 µL, 1.12 mmol). Deprotection in 3 mL DCM and 3 mL TFA, followed by flash chromatography afforded **3.43** as pale brown resin (75 mg, 36 %) . $^1$H-NMR (300 MHz, $CDCl_3$, trifluoroacetate): δ (ppm) 1.98 – 2.24 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.80 (t, $^3J$ = 7.2 Hz, 2H, Pip-C***H***$_2$), 2.95 (t, $^3J$ = 7.1 Hz, 2H, Ph-$CH_2$-C***H***$_2$) 3.02 – 3.30 (m, 6H, Pip-***H*** + Ph-C***H***$_2$), 3.31 – 3.68 (m, 6H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$), 4.69 (s, 1H, $(Ph)_2$-C***H***), 7.12 – 7.60 (m, 15 H, Ph-***H***), 8.30 (bs, 3H, -N***H***$_3^+$), 9.38 (s, 1H, -N***H***), 10.27 (bs, 1H, -N***H***), 12.65 (bs, 1H, -N***H***); $^{13}$C-NMR (75 Mhz, $CDCl_3$, trifluoroacetate): δ (ppm) 22.89 (-, Pip-$CH_2$-***C***$H_2$), 30.16 (-, Ph-***C***$H_2$), 38.41 (-, Pip-$(CH_2)_2$-***C***$H_2$ + Ph-$CH_2$-***C***$H_2$), 48.45 (-, 2 Pip-***C***), 50.55 (-, Pip-***C***$H_2$), 53.96 (-, 2 Pip-***C***), 76.38 (+, di-Ph-***C***H), 126.51 (+, Ph-***C***-4), 127.81 (+, 4 diPh-***C***), 128.43 (+, 2 Ph-***C***), 128.60 (+, 2 Ph-***C***), 128.99 (+, 2 diPh-***C***-4), 129.56 (+, 4 diPh-***C***), 136.91 ($C_{quat}$, Ph-***C***-1), 139.52 ($C_{quat}$, 2 diPh-***C***-1), 154.64 ($C_{quat}$, ***C***=N), 176.13 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 2): 97 %

($t_R$ = 25.30 min, *k'* = 7.30); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{30}H_{37}N_5O + H]^+$ 483.2998, found: 483.2993. $C_{30}H_{37}N_5O \cdot 2TFA$ (711.69).

**$N^2$-(3-Phenylpropanoyl)-$N^1$-[3-(2-pyridyl)propyl]guanidine (3.44)**

The title compound was prepared from **3.5** (80 mg, 0.25 mmol), **3.26** (34 mg, 0.25 mmol), $HgCl_2$ (135 mg, 0.50 mmol) and $Et_3N$ (105 µL, 0.75 mmol). Deprotection in 2 mL DCM and 2 mL TFA, followed by preparative HPLC (20 – 65 % MeCN in 30 min) afforded product **3.44** as colorless semi-solid compound (39 mg, 32 %). $^1$H-NMR (300 MHz, $CDCl_3$, trifluoroacetate): δ (ppm) 2.00– 2.24 (m, 2H, Pyr-2-$CH_2$-C***H***$_2$), 2.81 (t, 2H, $^3J$ = 7.3 Hz, Ph-$CH_2$-C***H***$_2$), 2.96 (t, $^3J$ = 7.2 Hz, 2H, Ph-C***H***$_2$), 3.06 – 3.25 (m, 2H, Pyr-2-C***H***$_2$), 3.33 – 3.54 (m, 2H, Pyr-2-$(CH_2)_2$-C***H***$_2$), 7.15 – 7.32 (m, 5H, Ph-***H***), 7.68 – 7.77 (m, 1H, Pyr-***H***-5), 7.73 (d, 2H, 7.7 Hz, Pyr-***H***-3), 8.26 – 8.38 (m, 1H, Pyr-***H***-4), 8.52 – 8.73 (m, 2H, Pyr-***H***-6 + NH), 9.29 (bs, 1H, N***H***), 10.46 (bs, 1H, N***H***), 12.48 (bs, 1H, N***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$, trifluoroacetate): δ (ppm) 29.04 (-, Pyr-2-$CH_2$-***C***$H_2$), 30.24 (-, Pyr-2-***C***$H_2$), 30.84 (-, Ph-***C***$H_2$), 38.42 (-, Pyr-2-$(CH_2)_2$-***C***$H_2$), 40.38 (-, Ph-$CH_2$-***C***$H_2$), 124.63 (+, Pyr-***C***-5), 126.47 (+, Ph-***C***-4), 127.47 (+, Pyr-***C***-3), 128.50 (+, 2 Ph-***C***), 128.57 (+, 2 Ph-***C***), 139.69 ($C_{quat}$, Ph-***C***-1), 141.53 (+, Pyr-***C***-6), 145.64 (+, Pyr-***C***-4), 154.50 ($C_{quat}$, ***C***=N), 157.25 ($C_{quat}$, Pyr-***C***-2), 175.99 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 2): 94 % ($t_R$ = 15.63 min, *k'* = 4.12); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{18}H_{22}N_4O + H]^+$ 310.1794, found: 310.1790. $C_{18}H_{22}N_4O \cdot 2TFA$ (538.43).

**$N^2$-(3-Phenylpropanoyl)-$N^1$-[3-(3-pyridyl)propyl]guanidine (3.45)**

The title compound was prepared from **3.5** (80 mg, 0.25 mmol), **3.27** (34 mg, 0.25 mmol), $HgCl_2$ (135 mg, 0.50 mmol) and $Et_3N$ (105 µL, 0.75 mmol). Deprotection in 2 mL DCM and 2 mL TFA, followed by preparative HPLC (20 – 65 % MeCN in 30 min) afforded product **3.45** as colorless oil (45 mg, 33 %). $^1$H-NMR (300 MHz, $CDCl_3$, trifluoroacetate): δ (ppm) 2.00– 2.24 (m, 2H, Pyr-4-$CH_2$-C***H***$_2$), 2.81 (t, 2H, $^3J$ = 7.4 Hz**,** Ph-$CH_2$-C***H***$_2$), 2.92 – 3.08 (m, 2H, Pyr-4-C***H***$_2$ and Ph-C***H***$_2$), 3.40 (t, 2H, $^3J$ = 7.0 Hz, Pyr-4-$(CH_2)_2$-C***H***$_2$), 7.13 – 7.31 (m, 5H, Ph-***H***), 7.75 – 7.87 (m, 1H, Pyr-***H***-5), 8.15 – 8.38 (m, 1H, Pyr-***H***-4), 8.36 (d, $^3J$ = 7.5 Hz, 1H, Pyr-***H***-6), 8.47 – 8.62 (m, 1H, Pyr-***H***-2), 8.78 (bs, 1H, N***H***), 9.38 (bs, 1H, N***H***), 10.23 (bs, 1H, N***H***), 12.85 (bs, 1H, N***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$, trifluoroacetate): δ (ppm) 28.84 (-, Pyr-3-$CH_2$-***C***$H_2$), 29.63 (-, Pyr-3-***C***$H_2$), 30.16 (-, Ph-***C***$H_2$), 38.35 (-, Pyr-3-$(CH_2)_2$-***C***$H_2$), 40.34 (-, Ph-$CH_2$-***C***$H_2$), 127.50 (+, Ph-***C***-4), 126.50 (+, Ph-***C***-4), 126.88 (+, Pyr-***C***-5), 128.41 (+, 2 Ph-***C***), 128.58 (+, 2 Ph-***C***), 139.20 (+, Pyr-***C***-2), 139.61 ($C_{quat}$, Ph-***C***-1), 141.57 (+, Pyr-***C***-3), 145.98 (+, Pyr-***C***-4), 154.50 ($C_{quat}$, ***C***=N), 176.27 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 2): 95 % ($t_R$ = 15.66 min, *k'* = 4.13); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{18}H_{22}N_4O + H]^+$ 310.1794, found: 310.1788. $C_{18}H_{22}N_4O \cdot 2TFA$ (538.43).

**$N^1$-(3-Phenylpropanoyl)-$N^2$-[3-(4-pyridyl)propyl]guanidine (3.46)**

The title compound was prepared from **3.5** (80 mg, 0.25 mmol), **3.28** (34 mg, 0.25 mmol), $HgCl_2$ (135 mg, 0.50 mmol) and $Et_3N$ (105 µL, 0.75 mmol). Deprotection in 2 mL DCM and 2 mL TFA followed by preparative HPLC (20 – 65 % MeCN in 30 min) afforded product **3.46** as colorless half-solid compound (41 mg, 35 %). $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 2.01– 2.14 (m, 2H, Pyr-4-$CH_2$-C***H***$_2$), 2.80 (t, 2H, $^3J$ = 7.4 Hz**,** Ph-$CH_2$-C***H***$_2$), 2.92 – 3.08 (m, 2H, Pyr-4-C***H***$_2$ and Ph-C***H***$_2$), 3.40 (t, 2H, $^3J$ = 7.0 Hz, Pyr-4-$(CH_2)_2$-C***H***$_2$), 7.13 – 7.31 (m, 5H, Ph-***H***), 7.98 (d, 2H, $^3J$ = 6.8 Hz, Pyr-3,5-***H***), 8.74 (d, $^3J$ = 7.0 Hz, 2H, Pyr-2,6-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 29.09 (-, Pyr-4-$CH_2$-***C***$H_2$), 31.28 (-, Pyr-4-***C***$H_2$), 33.94 (-, Ph-***C***$H_2$), 39.51 (Pyr-4-$(CH_2)_2$-***C***$H_2$), 41.87 (-, Ph-$CH_2$-***C***$H_2$), 127.50 (+, Ph-***C***-4), 128.51 (+, Pyr-***C***-3,5), 129.50 (+, 2 Ph-***C***), 129.62 (+, 2 Ph-***C***), 141.38 ($C_{quat}$, Ph-***C***-1), 142.66 (+, Pyr-***C***-2,6), 164.50 ($C_{quat}$, Pyr-***C***-4), 176.50 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 2): 95 % ($t_R$ = 15.03 min, *k'* = 3.93); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{18}H_{22}N_4O + H]^+$ 310.1794, found: 310.1792. $C_{18}H_{22}N_4O \cdot 2TFA$ (538.43).

**$N^2$-(3-Aminopropyl)-$N^1$-(3-phenylpropanoyl)guanidine (3.47)**

The title compound was prepared from **3.5** (120 mg, 0.37 mmol), **3.36** (65 mg, 0.37 mmol), $HgCl_2$ (202 mg, 0.75 mmol) and $Et_3N$ (157 µL, 1.12 mmol). Deprotection in 3 mL DCM and 3 mL TFA followed by preparative RP-HPLC (10 – 40 % MeCN in 30 min) afforded **3.47** as colorless resin (51 mg, 29 %). $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 1.78 – 1.89 (m, 2H, C***H***$_2$-$CH_2$-NH), 2.77 (t, $^3J$ = 7.5 Hz, 2H, Ph-$CH_2$-C***H***$_2$), 2.96 (t, $^3J$ = 7.4 Hz, 2H, C***H***$_2$-$NH_2$), 3.40 (t, $^3J$ = 6.7 Hz, 2H, Ph-C***H***$_2$), 3.66 (t, $^3J$ = 5.8 Hz, 2H, C***H***$_2$-NH), 7.02 – 7.38 8m, 5H, Ph-***H***); $^{13}$C-NMR (75 Mhz, $CD_3OD$, trifluoroacetate): δ (ppm) 31.25 (-, ***C***$H_2$-$CH_2$-$NH_2$), 31.50 (-, Ph-***C***$H_2$), 39.58 (-, NH-***C***$H_2$), 40.21 (-, Ph-$CH_2$-***C***$H_2$), 59.98 (-, ***C***$H_2$-$NH_2$), 127.51 (+, Ph-***C***-4), 129.46 (+, 2 Ph-***C***), 129.64 (+, 2 Ph-***C***), 141.38 ($C_{quat}$, Ph-***C***-1), 155.15 ($C_{quat}$, ***C***=N), 176.49 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 99 % ($t_R$ = 18.00 min, *k*' = 4.90); HRMS (ESI): *m/z* calcd. for $[C_{13}H_{20}N_4O + H]^+$ 249.1710, found: 249.1713. $C_{13}H_{20}N_4O \cdot 2TFA$ (476.36).

**$N^1$-[3-(Morpholin-4-yl)propyl]-$N^2$-(3-phenylpropanoyl)guanidine (3.49)**

The title compound was prepared from **3.5** (150 mg, 0.47 mmol), **3.38** (67 mg, 0.47 mmol), $HgCl_2$ (253 mg, 0.93 mmol) and $Et_3N$ (196 µL, 1.40 mmol). Deprotection in 3 mL DCM and 3 mL TFA followed by flash chromatography afforded product **3.49** as white solid (65 mg, 65 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.79 – 1.91 (m, 2H, Morph-$CH_2$-C***H***$_2$), 2.43 – 2.60 (m, 6H, Morph-***H*** + Morph-C***H***$_2$), 2.78 (t, $^3J$ = 7.6 Hz, 2H, Ph-$CH_2$-C***H***$_2$), 2.96 (t, $^3J$ = 7.4 Hz, 2H, Ph-C***H***$_2$), 3.37 (t, $^3J$ = 6.5 Hz, 2H, Morph-$(CH_2)_2$-C***H***$_2$), 3.73 (t, $^3J$ = 4.7 Hz, 4H, Morph-***H***), 7.14 – 7.32 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 Mhz, $CD_3OD$): δ (ppm) 25.43 (-, Morph-$CH_2$-***C***$H_2$), 31.29 (-, Ph-***C***$H_2$), 39.52 (-, Morph-$(CH_2)_2$-***C***$H_2$), 40.76 (-, Ph-$CH_2$-***C***$H_2$), 54.45 (-, 2 Morph-***C***), 56.16 (-, Morph-***C***$H_2$), 67.40 (-, 2 Morph-***C***), 127.49 (+, Ph-***C***-4),

129.48 (+, 2 Ph-***C***), 129.62 (+, 2 Ph-***C***), 141.42 ($C_{quat}$, Ph-***C***-1), 151.30 ($C_{quat}$, ***C***=N), 176.38 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 2): 93 % ($t_R$ = 28.90 min, *k'* = 8.48); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{17}H_{26}N_4O_2 + H]^+$ 318.2056, found: 318.2059. $C_{17}H_{26}N_4O_2$ (318.41).

**$N^2$-(3-Phenylpropanoyl)-$N^1$-(3-phenylpropyl)guanidine (3.50)**

The title compound was prepared from **3.5** (120 mg, 0.37 mmol), **3.39** (50 mg, 0.37 mmol), $HgCl_2$ (202 mg, 0.75 mmol) and $Et_3N$ (157 µL, 1.12 mmol). Deprotection in 3 mL DCM and 3 mL TFA, followed by flash chromatography afforded **3.50** as colorless foam (55 mg, 53 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.91 – 2.12 (m, 2H, Ph-$CH_2$-C***H***$_2$), 2.71 (t, $^3J$ = 7.6 Hz, 2H, Ph-C***H***$_2$), 2.84 (t, $^3J$ = 7.8 Hz, 2H, Ph-$CH_2$-C***H***$_2$-CO), 2.98 (t, $^3J$ = 7.2 Hz, 2H, Ph-C***H***$_2$-$CH_2$-CO), 3.25 – 3.35 (m, 2H, Ph-$(CH_2)_2$-C***H***$_2$), 7.10 – 7.35 (m, 10H, Ph-***H***), 9.87 (bs, 2H, N***H***$_2$), 13.34 (bs, 1H, N***H***); $^{13}$C-NMR (75 Mhz, $CDCl_3$): δ (ppm) 29.50 (-, Ph-$CH_2$-***C***$H_2$), 30.25 (-, Ph-***C***$H_2$), 32.51 (-, Ph-***C***$H_2$), 38.41 (-, ***C***$H_2$-NH), 40.66 (-, Ph-$CH_2$-***C***$H_2$-CO), 126.47 and 126.52 (+, 2 Ph-***C***-4), 128.32 (+, 2 Ph-***C***), 128.48 (+, 2 Ph-***C***), 128.60 (+, 2 Ph-***C***), 128.72 (+, 2 Ph-***C***), 139.57 and 139.98 ($C_{quat}$, 2 Ph-***C***-1), 154.51 ($C_{quat}$, ***C***=N), 176.88 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1) : 97 % ($t_R$ = 23.56 min, *k'* = 7.33); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{19}H_{23}N_3O + H]^+$ 309.1841, found: 309.1836. $C_{19}H_{23}N_3O$ (309.41).

**$N^1$-[3-(Benzimidazol-2-yl)propyl]-$N^2$-(3-phenylpropanoyl)guanidine (3.51)**

The title compound was prepared from **3.5** (80 mg, 0.25 mmol), **3.32** (43 mg, 0.25 mmol), $HgCl_2$ (135 mg, 0.50 mmol) and $Et_3N$ (105 µL, 0.75 mmol). Deprotection in 2 mL DCM and 2 mL TFA followed by preparative HPLC (15 – 60 % MeCN in 30 min) afforded product **3.51** as pale brown resin (41 mg, 29 %). $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 2.20 – 2.33 (m, 2H, Benzim-$CH_2$-C***H***$_2$), 2.73 (t, $^3J$ = 7.5 Hz, 2H, Ph-$CH_2$-C***H***$_2$), 2.92 (t, $^3J$ = 7.5 Hz, 2H, Ph-C***H***$_2$), 3.25 – 3.24 (m, overlap with solvent, 2H, Benzim-C***H***$_2$), 3.47 (t, $^3J$ = 6.8 Hz, 2H, Benzim-$(CH_2)_2$-C***H***$_2$), 7.11 – 7.34 (m, 5H, Ph-***H***), 7.52 – 7.60 (m, 2H, Benzim-***H***), 7.71 – 7.78 (m, 2H, Benzim-***H***); $^{13}$C-NMR (75 Mhz, $CD_3OD$, trifluoroacetate): δ (ppm) 24.92 (-, Benzim-$CH_2$-***C***$H_2$), 26.47 (-, Benzim-***C***$H_2$), 31.22 (-, Ph-***C***$H_2$), 39.45 (-, Benzim-$(CH_2)_2$-***C***$H_2$), 41.47 (-, Ph-$CH_2$-***C***$H_2$), 114.83 (+, 2 Benzim-***C***), 127.40 (+, Benzim-***C***), 127.48 (+, Ph-***C***-4), 129.49 (+, 2 Ph-***C***), 129.60 (+, 2 Ph-***C***), 132.52 ($C_{quat}$, Benzim-***C***), 141.37 ($C_{quat}$, Ph-***C***-1), 154.46 ($C_{quat}$, ***C***=N), 155.41 ($C_{quat}$, ***C***=N), 176.48 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 99 % ($t_R$ = 14.71 min, *k'* = 4.20); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{20}H_{23}N_5O + H]^{+\cdot}$ 349.1903, found: 349.1904. $C_{20}H_{23}N_5O \cdot 2TFA$ (577.47).

**3-Phenyl-*N*-(tetrahydropyrimidin-2(1*H*)-ylidene)propanamide (3.52)**

The title compound was prepared from **3.5** (120 mg, 0.37 mmol), **3.37** (28 mg, 0.37 mmol), $HgCl_2$ (202 mg, 0.75 mmol) and $Et_3N$ (157 µL, 1.12 mmol). Deprotection in 3 mL DCM and 3 mL TFA,

followed by flash chromatography afforded **3.52** as white solid (41 mg, 48 %). $^{1}$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.76 – 2.02 (m, 2H, C***H***$_2$-$CH_2$-NH), 2.54 (t, $^{3}J$ = 7.6 Hz, 2H, Ph-$CH_2$-C***H***$_2$), 2.96 (t, $^{3}J$ = 7.5 Hz, 4H, Ph-C***H***$_2$), 3.40 (t, $^{3}J$ = 5.8 Hz, 4H, NH-C***H***$_2$-$CH_2$-C***H***$_2$-NH), 7.13 – 7.30 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$): δ (ppm) 20.14 (-, ***C***$H_2$-$CH_2$-NH), 32.01 (-, Ph-***C***$H_2$), 38.48 (-, NH-***C***$H_2$-$CH_2$-***C***$H_2$-NH), 41.88 (-, Ph-$CH_2$-***C***$H_2$), 127.74 (+, Ph-***C***-4), 128.32 (+, 2 Ph-***C***)), 128.34 (+, 2 Ph-***C***), 142.15 ($C_{quat}$, Ph-***C***-1), 158.67 ($C_{quat}$, ***C***=N), 184.26 ($C_{quat}$, ***C***=O); MS (ESI, MeCN/ 0.1 % FA) *m/z* (%): 232 (100) $[M+H]^+$. $C_{13}H_{17}N_3O$ (231.29).

***N*,*N*'-[Propane-1,3-diylbis(azanediyl)]bis(aminomethan-1-yl-1-ylidene)bis(3-phenylpropanamide) (3.53)**

The title compound was prepared from **3.45** (120 mg, 0.37 mmol), 1,3-diaminopropane **3.34** (28 mg, 0.37 mmol), $HgCl_2$ (202 mg, 0.75 mmol) and $Et_3N$ (157 µL, 1.12 mmol). Deprotection in 3 mL DCM and 3 mL TFA, followed by flash chromatography afforded **3.53** as colorless semi-solid compound (49 mg, 19 %). $^{1}$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 1.86 – 2.06 (m, 2H, C***H***$_2$-$CH_2$-NH), 2.78 ( t, $^{3}J$ = 7.5 Hz, 4H, Ph-$CH_2$-C***H***$_2$), 2.96 (t, $^{3}J$ = 7.4 Hz, 4H, Ph-C***H***$_2$), 3.39 (t, $^{3}J$ = 6.9 Hz, 4H, NH-C***H***$_2$-$CH_2$-C***H***$_2$-NH), 7.12 – 7.32 (m, 10, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 27.37 (-, ***C***$H_2$-$CH_2$-NH), 31.27 (-, 2 Ph-***C***$H_2$), 39.55 (-, NH-***C***$H_2$-$CH_2$-***C***$H_2$-NH), 40.15 (-, 2 Ph-$CH_2$-***C***$H_2$), 127.50 (+, 2 Ph-***C***-4), 129.48 (+, 4 Ph-***C***), 129.62 (+, 4 Ph-***C***), 141.37 ($C_{quat}$, 2 Ph-***C***-1), 155.30 ($C_{quat}$, 2 ***C***=N), 176.46 ($C_{quat}$, 2 ***C***=O); anal. RP-HPLC (system 2): 95 % ($t_R$ = 22.44 min, *k*' = 6.36); HRMS (LSI, glycerine): *m/z* calcd. for $[C_{23}H_{30}N_6O_2 + H]^{+\cdot}$ 423.2508, found: 423.2509. $C_{23}H_{30}N_6O_2$ (422.52).

### 3.5.1.9 Preparation of the Guanidine-Type Building Block 3.57

***N*$^{1}$,*N*$^{2}$-Bis(benzyloxycarbonyl)guanidine (3.55)[28]**

DCM (160 mL) was added to a solution of guanidine hydrochloride **3.54** (7.64 g, 80 mmol, 1 eq) and sodium hydroxide (7.64 g, 240 mmol, 5.0 eq) in 80 mL $H_2O$. Benzyl chloroformate (33.8 mL, 240 mmol, 3 eq) was added dropwise at 0 °C over a period of 45 min. After stirring for 20 h at 0 °C, the reaction mixture was diluted with 200 mL DCM and the layers were separated. The aqueous layer was then extracted with DCM (2x 200 mL) and the combined organic layers were washed with $H_2O$ and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the crude product was recrystallized from MeOH affording **3.55** as colorless crystals (18.1 g, 69 %); mp: 146 – 147 °C (ref.[28]: 149 – 150 °C). $^{1}$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 5.07 (s, 4H, Ph-C***H***$_2$), 7.27 – 7.43 (m, 10H, Ph-***H***), 8.20 – 9.40 (m, 2H, N***H***$_2$). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 328 (70) $[M+H]^+$, 369 (100) $[M+H+MeCN]^+$, 655 (50) $[2M+H]^+$. $C_{17}H_{17}N_3O_4$ (327.33).

**$N^1$,$N^3$-Bis(benzyloxycarbonyl)-$N^2$-(trifluoromethanesulfonyl)guanidine (3.56)[28]**

Sodium hydride (60 % dispersion in mineral oil, 2.11 g, 55.0 mmol, 2 eq) was added in portions to a solution of **3.55** (12.86 g, 27.5 mmol, 1 eq) in anhydrous chlorobenzene (50 mL) at 0 °C under an argon atmosphere. After stirring for 1 h at 0 ° C the mixture was cooled to –45 °C and trifluoromethanesulfonic anhydride (7.76 g, 57.5 mmol, 1 eq) was added slowly. The mixture was allowed to come to room temperature and stirred overnight. After evaporation of the solvent, the residue was dissolved in EtOAc (500 mL) and a solution of $KHSO_4$ (2 M, 200 mL). The phases were separated and the organic layer was washed with $H_2O$ and brine before drying over $MgSO_4$. After removing the solvent under reduced pressure the crude product was subjected to flash chromatography (DCM/diethyl ether 95/5 v/v) yielding **3.56** as colorless oil (9.42 g, 75 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 5.26 (s, 4H, Ph-C***H***$_2$), 7.28 – 7.48 (m, 10H, Ph-***H***), 10.00 – 10.48 (bs, 1H, N***H***). MS (ESI, $NH_4OAc$) *m/z* (%): 460 (100) $[M+H]^+$, 477 (70) $[M+NH_4]^+$, 936.2 (80) $[2M+NH_4]^+$. $C_{18}H_{16}F_3N_3O_6S$ (459.40).

**$N^1$-(3,3-Diphenylpropyl)guanidine (3.57)[50]**

**3.56** (5.0 g, 10.9 mmol, 1 eq) was dissolved in 50 mL anhydrous DCM and 3,3-diphenylpropylamine (2.30 g, 10.9 mmol, 1 eq)was added. The reaction mixture was stirred for 6 h at room temperature. After evaporation of the solvent, the residue was directly subjected to flash chromatography (DCM/MeOH 98/2 v/v) affording a pale yellow oil, which was subsequently dissolved in 100 mL MeOH. A 10 % Pd/C catalyst (360 mg) was added and hydrogen was led through the vigorously stirred mixture at room temperature for 5 h. The catalyst was removed by filtration over Celite and the solvent was removed under reduced pressure yielding **3.57** as colorless foam (1.42 g, 60 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 2.27 – 2.42 (m, 2H, $(Ph)_2$-CH-C***H***$_2$), 3.08 (t, $^3J$ = 7.1 Hz, 2H, $(Ph)_2$-CH-C***H***$_2$), 4.04 (t, $^3J$ = 8.0 Hz, 1H, $(Ph)_2$-C***H***), 7.10 – 7.22 (m, 2h, Ph-***H***-4), 7.24 – 7.33 (m, 8H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 35.53 (-, $(Ph)_2$-CH-(***C***$H_2)_2$), 41.10 (-, $(Ph)_2$-C***H***-(***C***$H_2)_2$), 49.65 (+, $(Ph)_2$-***C***H), 127.56 (+, 2 Ph-***C***-4), 128.89 (+, 4 Ph-***C***), 19.71 (+, 4 Ph-***C***), 145.46 ($C_{quat}$, 2 Ph-***C***-1), 159.01 ($C_{quat}$, ***C***=N); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 254 (100) $[M+H]^+$. $C_{16}H_{19}N_3$ (253.34).

### 3.5.1.10 Preparation of the Carbamoylguanidine-Type Building Block 3.68

***tert*-Butyl (Methylsulfanyl)({[2-(tert-butoxycarbonylamino)ethyl]aminocarbonyl}amino)methylen-carbamate (3.67)**

A solution of *N*-(*tert*-butoxycarbonyl)ethane-1,2-diamine **3.35** (0.96 g, 6.0 mmol, 1 eq) and DIEA (2.61 mL, 15.0 mmol, 2.5 eq) in anhydrous DCM (30 mL) was added dropwise to a solution of triphosgene (1.07 g, 3.6 mmol, 0.6 eq) in 15 mL DCM over a period of 30 min under argon atmosphere and ice cooling. *N*-(*tert*-Butoxycarbonyl)-*S*-methylisothiourea **3.4** (1.14 g, 6.0 mmol, 1

eq) was added and stirring was continued for 2.5 h. After removal of the solvent under reduced pressure, the residue was directly subjected to column chromatography (PE/EE 9:1) yielding **3.67** as white solid (1.75 g, 77%); $^{1}$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.49 (s, 9H, C(C***H***$_3$)$_3$), 2.31 (s, 3H, S-C***H***$_3$), 3.12 – 3.27 (m, 4H, C***H***$_2$-C***H***$_2$-NHBoc); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 14.36 (+, S-***C***H$_3$), 28.02 (+, 3 C(***C***H$_3$)$_3$), 28.35 (+, 3 C(***C***H$_3$)$_3$), 79.66 (-, ***C***H$_2$-N), 82.82 (-, ***C***H$_2$-N), 151.00 (C$_{quat}$, ***C***-N), 156.46 (C$_{quat}$, ***C***-N), 162.07 (C$_{quat}$, ***C***=O), 162.12 (C$_{quat}$, ***C***=O); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 377 (100) $[M+H]^+$. $C_{15}H_{28}N_4O_5S$ (376.47).

**$N^1$-[Amino(3,3-diphenylpropylamino)methylene]-3-(2-aminoethyl)urea (3.68)**

3,3-Diphenylpropylamine **3.66** (0.69 g, 3.25 mmol, 1 eq), the guanidinylating reagent **3.67** (1.22 g, 3.25 mmol, 1 eq) and $HgCl_2$ (1.77 g, 6.50 mmol, 2 eq) were dissolved in anhydrous DCM. $Et_3N$ (1.37 mL, 9.75 mmol, 3 eq) was added and the mixture was stirred for 48 h at ambient temperature. Subsequently, EtOAc was added and the precipitate removed by filtration over Celite. Purification by flash chromatography (DCM/MeOH 100/1 – 95/5 v/v) afforded the intermediate as white solid which was dissolved in 30 mL MeOH and cooled to 0 °C. Acetyl chloride (0.34 mL, 4.73 mmol, 3 eq) was added dropwise. The reaction mixture was stirred for 3h. Removal of the solvent under reduced pressure and washing of the crude product with EtOAc yielded **3.68** as a white solid (0.59 g, 91 %). $^{1}$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 2.24 – 2.35 (m, 2H, (Ph)$_2$-CH-C***H***$_2$), 2.68 (t, $^{3}J$ = 5.9 Hz, 2H, C***H***$_2$-NH$_2$), 3.10 (t, $^{3}J$ = 6.6 Hz, 2H, C***H***$_2$-NH), 3.16 (t, $^{3}J$ = 6.1 Hz, 2H, C***H***$_2$-NH), 4.03 (t, $^{3}J$ = 7.9 Hz, 1H, (Ph)$_2$-C***H***), 7.10 – 7.34 (m, 10H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 36.17 (-, (Ph)$_2$-CH-(***C***H$_2$)$_2$ ), 40.39 (-, 2 N-(***C***H$_2$)$_2$-N), 42.70 (-, (Ph)$_2$-CH-(***C***H$_2$)$_2$), 49.80 (+, (Ph)$_2$-***C***H), 127.41 (+, 2 Ph-***C***-4), 128.93 (+, 4 Ph-***C***), 129.62 (+, 4 Ph-***C***), 145.81 (C$_{quat}$, 2 Ph-***C***-1), 161.82 (C$_{quat}$, ***C***=O); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 340 (100) $[M+H]^+$. $C_{19}H_{25}N_5O \cdot 2HCl$ (412.36).

***5*-(*tert*-Butoxycarbonylamino)pentanoic acid (3.72)[35]**

5-Aminopentanoic acid (1.17g, 10.0 mol, 1 eq) was dissolved in 30 mL dioxane/$H_2O$ (20/10 v/v), and 10 mL 1 N NaOH (1 eq) were added. The clear solution was cooled to 0 °C and $Boc_2O$ (2.18 g, 10.00 mmol, 1.0 eq) was added. The reaction mixture was allowed warm to room temperature and was stirred overnight. After evaporation of the solvent, the residue was dissolved in EtOAc. The organic phase was washed with 5 % citric acid (15 mL) and brine (15 mL) then dried over $MgSO_4$. Removal of the solvent afforded the crude product which was treated with pentane yielding **3.72** as a white powder (1.62 g, 75 %). $^{1}$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.59 – 1.58 (m, 2H, C***H***$_2$), 1.59 – 1.71 (m, 2H, C***H***$_2$), 2.35 (t, $^{3}J$ = 7.2 Hz, C***H***$_2$-COOH), 3.00 – 3.30 (m, 2H, C***H***$_2$-NH), 10.62 (bs, 1H, COO***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 23.31 (-, ***C***H$_2$-CH$_2$-COOH), 28.82 (+, C(***C***H$_3$)$_3$), 30.47 (-, ***C***H$_2$-CH$_2$-NH), 34.57 (-, ***C***H$_2$-COOH), 40.98 (-, ***C***H$_2$-NH), 79.89 (C$_{quat}$, ***C***(CH$_3$)$_3$), 158.59 (C$_{quat}$, O-

***C***O-N), 177.43 ($C_{quat}$, ***C***=O); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 218 (25) $[M+H]^+$, 435 (100) $[2M+H]^+$. $C_{10}H_{19}NO_4$ (217.26).

### 3.5.1.11 Preparation of Carbamoylguanidine-Type Amino Acid Derivatives 3.73 – 3.77

**General Procedure**

The *N*-Boc-*N*-Methyl amino acid (1 eq, EDAC (1.2 eq), HOBt-monohydrate (1.2 eq) and DIEA (2.0 eq) were dissolved in $DCM_{abs}$ under argon atmosphere and stirred for 15 min at room temperature. To this mixture a solution of the building block **3.68** (1 eq) in $DCM_{abs}$ was added and stirred overnight. After removal of the solvent under reduced pressure, the crude product dissolved in EtOAc. The organic phase was washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. Subsequently, the Boc-protection group was removed with 50 % TFA in DCM. Therefore, the reaction mixture was stirred for 3 – 5 h (TLC control). After removal of the solvent *in vacuo*, the crude product was purified by preparative RP-HPLC.

***N*-(2-{3-[Amino(3,3-diphenylpropylamino)methylene]ureido}ethyl)-4-methyl-2-(methylamino)-pentanamide (3.73)**

The title compound was prepared from **3.68** (100 mg, 0.30 mmol), *N*-Boc-*N*-methyl-L-leucine **3.69** (72 mg, 0.30 mmol), EDAC (68 mg, 0.35 mmol), HOBt (48 mg, 0.35 mmol) and DIEA (103 µL, 0.59 mmol) according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA followed by preparative RP-HPLC (30 – 60 % MeCN in 30 min) afforded **3.73** as colorless resin (65 mg, 38 %). $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 0.89 – 1.03 (m, 6H, CH-(C***H***$_3$)$_2$), 1.31 – 1.41 (m, 1H, C***H***-$(CH_3)_2$), 1.55 – 1.70 (m, 2H, C***H***$_2$-CH-$(CH_3)_2$), 2.34 – 2.47 (m, 2H, $(Ph)_2$-CH-C***H***$_2$), 2.64 (s, 3H, N-C***H***$_3$), 3.22 (t, $^3J$ = 7.0 Hz, 2H, $(Ph)_2$-CH-$CH_2$-C***H***$_2$), 3.33 – 3.48 (m, 4H, N-C***H***$_2$-C***H***$_2$-N ), 3.72 (t, $^3J$ = 6.9 Hz, 1H, CO-C***H***-NH), 4.06 (t, $^3J$ = 8.0 Hz, 1H, $(Ph)_2$-C***H***), 7.12 – 7.22 (m, 2H, Ph-***H***-4), 7.23 – 7.37 (m, 8H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 22.42 (+, CH(***C***$H_3$)$_2$), 23.11 (+, ***C***H$(CH_3)_2$)) 32.42 (+, N-***C***$H_3$), 34.82 (-, $(Ph)_2$-CH-(***C***$H_2$)$_2$), 40.06 (-,***C***$H_2$), 40.27 (-,***C***$H_2$), 40.72 (-, ***C***$H_2$), 41.10 (-, $(Ph)_2$-CH-(***C***$H_2$)$_2$), 49.72 (+, $(Ph)_2$-***C***H), 61.84 (+, CO-***C***H-NH), 127.67 (+, 2 Ph-***C***-4), 128.84 (+, 4 Ph-***C***), 129.77 (+, 4 Ph-***C***), 145.15 ($C_{quat}$, Ph-***C***-1), 155.80 ($C_{quat}$, ***C***-N), 155.97 ($C_{quat}$, ***C***-N), 169.49 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 2): 95 % ($t_R$ = 21.56 min, *k'* = 6.07); HRMS (LSI, glycerine): *m/z* calcd. for $[C_{26}H_{38}N_6O_2 + H]^+$ 467.3134, found: 467.3118. $C_{26}H_{38}N_6O_2 \cdot$ TFA (580.64).

***N*-(2-{3-[Amino(3,3-diphenylpropylamino)methylene]ureido}ethyl)pyrrolidine-2-carboxamide (3.74)**

The title compound was prepared from **3.68** (100 mg, 0.30 mmol), *N*-Boc-L-proline **3.70** (63 mg, 0.30 mmol), EDAC (68 mg, 0.35 mmol), HOBt (48 mg, 0.35 mmol) and DIEA (103 µL, 0.59 mmol)

according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA followed by preparative RP-HPLC (15 – 60 % MeCN in 30 min) afforded **3.74** as pale brown resin (55 mg, 33 %); $^{1}$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 1.94 – 2.12 (m, 3H, pyrrolidine-H), 2.29 – 2.47 (m, 3H, pyrrolidine-***H*** + $(Ph)_2$-CH-C***H***$_2$), 3.22 (t, $^{3}J$ = 7.0 Hz, 2H, $(Ph)_2$-CH-$CH_2$-C***H***$_2$), 3.30 – 3.54 (m, 6H, overlap with solvent, pyrrolidine-***H*** + N-C***H***$_2$-C***H***$_2$-N), 4.06 (t, $^{3}J$ = 8.0 Hz, 1H, $(Ph)_2$-C***H***), 4.19 – 4.27 (m, 1H, CO-C***H***-NH), 7.14 – 7.22 (m, 2H, Ph-***H***-4), 7.24 – 7.34 (m, 8H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 25.07 (-, Pyrrolidine-***C***), 30.93 (-, Pyrrolidine-***C***), 34.38 (-, $(Ph)_2$-CH-(***C***$H_2)_2$), 40.18 (-, N-(***C***$H_2)_2$-N), 40.45 (-, N-(***C***$H_2)_2$-N), 41.13 (-, $(Ph)_2$-CH-(***C***$H_2)_2$), 47.34 (-, Pyrrolidine-***C***), 49.76 (+, $(Ph)_2$-***C***H), 61.21 (+, CO-***C***H-NH), 127.66 (+, 2 Ph-***C***-4), 128.85 (+, 4 Ph-***C***), 129.77 (+, 4 Ph-***C***), 145.17 ($C_{quat}$, Ph-***C***-1), 155.80 ($C_{quat}$, ***C***-N), 155.97 ($C_{quat}$, ***C***-N), 169.90 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 99 % ($t_R$ = 16.46 min, *k'* = 4.82); HRMS (LSI, glycerine): *m/z* calcd. for $[C_{24}H_{32}N_6O_2 + H]^+$ 437.2665 , found: 437.2678. $C_{24}H_{32}N_6O_2 \cdot$ TFA (550.57).

**N-(2-{3-[Amino(3,3-diphenylpropylamino)methylene]ureido}ethyl)-3-(4-hydroxyphenyl)-2-(methylamino)propanamide (3.75)**

The title compound was prepared from **3.68** (100 mg, 0.30 mmol), *N*-Boc-*N*-methyl-O-benzyl-L-tyrosine **3.71** (87 mg, 0.30 mmol), EDAC (68 mg, 0.35 mmol), HOBt (48 mg, 0.35 mmol) and DIEA (103 µL, 0.59 mmol) according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA followed by preparative RP-HPLC (15 – 60 % MeCN in 30 min) afforded **3.75** as white solid (81 mg, 43 %); mp 59 -62 °C. $^{1}$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 2.31 – 2.44 (m, 2H, $(Ph)_2$-CH-C***H***$_2$), 2.62 (s, 3H, N-C***H***$_3$), 2.99 – 3.08 (m, 1H, Phenol-C***H***$_2$), 3.14 – 3.23 (m, 3H, Phenol-C***H***$_2$ + $(Ph)_2$-CH -$CH_2$-C***H***$_2$), 3.25 – 3.32 (m, overlap with solvent, 4H, NH-C***H***$_2$-C***H***$_2$-NH), 3.88 (t, $^{3}J$ = 7.2 Hz, 1H, CO-C***H***-NH), 4.04 (t, $^{3}J$ = 8.0 Hz, 1H, $(Ph)_2$-C***H***), 6.75 (d, $^{3}J$ = 8.5 Hz, 2H, Phenol-***H***), 7.04 (d, $^{3}J$ = 8.5 Hz, Phenol-***H***), 7.13 – 7.22 (m, 2H, Ph-***H***-4), 7.23 – 7.32 (m, 8H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 32.66 (+, N-***C***$H_3$), 34.79 (-, $(Ph)_2$-CH-(***C***$H_2)_2$), 37.12 (-, Phenol-***C***$H_2$), 40.09 and 40.08 (-, N-(***C***$H_2)_2$-N), 41.15 (-, $(Ph)_2$-CH-(***C***$H_2)_2$), 49.76 (+, $(Ph)_2$-***C***H), 64.48 (CO-***C***H-NH), 116.83 (+, 2 Phenol-***C***), 125.58 ($C_{quat}$, Phenol-***C***-1), 127.65 (+, 2 Ph-***C***-4), 128.84 (+, 4 Ph-***C***), 129.76 (+, 4 Ph-***C***), 131.62 (+, Phenol-***C***), 145.17 ($C_{quat}$, 2 Ph-***C***-1), 155.85 ($C_{quat}$, ***C***-N), 158.39 ($C_{quat}$, Phenol-***C***-4), 168.95 (***C***=O); anal. RP-HPLC (system 1): 96 % ($t_R$ = 17.06 min, *k'* = 5.03); HRMS (LSI, glycerine): *m/z* calcd. for $[C_{29}H_{36}N_6O_3 + H]^+$ 517.2927, found: 517.2924. $C_{29}H_{36}N_6O_3 \cdot$ TFA (630.65).

**5-Amino-N-(2-{3-[amino(3,3-diphenylpropylamino)methylene]ureido}ethyl)pentanamide (3.76)**

The title compound was prepared from **3.68** (140 mg, 0.32 mmol), 5-(Boc-amino)pentanoic acid (**3.72**, 69 mg, 0.32 mmol), EDAC (73 mg, 0.38 mmol), HOBt (52 mg, 0.38 mmol) and DIEA (111 µL, 0.64 mmol) according to the general procedure. Deprotection in 4 mL DCM and 4 mL TFA followed by

preparative RP-HPLC (15 – 60 % MeCN in 30 min) afforded **3.76** as pale brown resin (73 mg, 50 %); $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 1.55 – 1.77 (m, 4H, (C***H***$_2$)$_2$-$CH_2$-$NH_2$), 2.21 – 2-31 (m, 2H, C***H***$_2$-CO), 2.35 – 2.47 (m, 2H, (Ph)$_2$-CH-C***H***$_2$), 2.86 – 2.96 (m, 2H, C***H***$_2$-$NH_2$), 3.22 (t, $^3J$ = 7.0 Hz, 2H, (Ph)$_2$-CH-$CH_2$-C***H***$_2$), 3.28 – 3.34 (m, 4H, NH-C***H***$_2$-C***H***$_2$-NH), 4.06 (t, $^3J$ = 8.0 Hz, 1H, (Ph)$_2$-C***H***), 7.13 – 7.23 (m, 2H, Ph-***H***-4), 7.24 – 7.34 (m, 8H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 23.37 (-, N-$CH_2$-(***C***$H_2$)$_2$-$CH_2$-N), 28.06 (-,N-$CH_2$-(***C***$H_2$)$_2$-$CH_2$-N), 34.81 (-, (Ph)$_2$-CH-(***C***$H_2$)$_2$), 36.00 (-, ***C***$H_2$-N), 39.94 (-, ***C***$H_2$-N), 40.36 (-, ***C***$H_2$-N), 40.41 (-, ***C***$H_2$-N), 41.15 (-, (Ph)$_2$-CH-(***C***$H_2$)$_2$), 49.77 (+, (Ph)$_2$-***C***H), 127.66 (+, 2 Ph-***C***-1), 128.84 (+, 4 Ph-***C***), 129.76 (+, 4 Ph-***C***), 145.18 ($C_{quat}$, 2 Ph-***C***-1), 155.84 ($C_{quat}$, ***C***=N), 175.81 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 2): 99 % ($t_R$ = 19.36 min, *k'* = 5.35); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{24}H_{34}N_6O_2 + H]^+$ 439.2821, found: 439.2826. $C_{24}H_{34}N_6O_2 \cdot 2TFA$ (666.61).

***N*-(2-{3-[Amino(3,3-diphenylpropylamino)methylene]ureido}ethyl)-5-guanidinopentanamide (3.77)**
Compound **3.76** (44 mg, 0.10 mmol, 1eq) was dissolved in anhydrous DCM. *N*,*N'*-Bis(*tert*-Butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine (37 mg, 0.12 mmol, 1 eq) and $Et_3N$ (35 µl, 0.25 mmol, 2.5 eq) were added and the mixture was stirred for 20 h at room temperature. Volatiles were removed under reduced pressure. The residue was taken up in 3 mL DCM and treated with 3 mL TFA. After 4 h stirring at room temperature the Boc-deprotection was completed and the mixture was concentrated under reduced pressure. Purification of the crude product by preparative RP-HPLC (15 – 60 % MeCN in 30 min) afforded product **3.77** as a pale brown resin (32 mg, 45 %); $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 1.51 – 1.72 (m, 4H, (C***H***$_2$)$_2$-$CH_2$-$NH_2$), 2.24 (t, $^3J$ = 7.0 Hz, 2H, C***H***$_2$-CO), 2.36 – 2.47 (m, 2H, (Ph)$_2$-CH-C***H***$_2$), 2.86 – 2.96 (m, 2H, ***C***$H_2$-$NH_2$), 3.22 (t, $^3J$ = 7.0 Hz, 2H, (Ph)$_2$-CH-$CH_2$-C***H***$_2$), 3.27 – 3.33 (m, overlap with solvent, 4H, NH-C***H***$_2$-C***H***$_2$-NH) 4.06 (t, $^3J$ = 8.0 Hz, 1H, (Ph)$_2$-C***H***), 7.13 – 7.23 (m, 2H, Ph-***H***-4), 7.24 – 7.33 (m, 8H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 23.75 (-, ***C***$H_2$), 29.40 (-, ***C***$H_2$), 34.84 (-, (Ph)$_2$-CH-(***C***$H_2$)$_2$), 36.28 (-, ***C***$H_2$-N), 39.92 (-, ***C***$H_2$-N), 40.44 (-, ***C***$H_2$-N), 40.73 (-, ***C***$H_2$-N), 41.17 (-, (Ph)$_2$-CH-(***C***$H_2$)$_2$), 49.76 (+, (Ph)$_2$-***C***H), 127.66 (+, 2 Ph-***C***-1), 128.84 (+, 4 Ph-***C***), 129.76 (+, 4 Ph-***C***), 145.17 ($C_{quat}$, 2 Ph-***C***-1), 155.84 ($C_{quat}$, ***C***=N), 174.18 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 100 % ($t_R$ = 16.72 min, *k'* = 4.91); HRMS (LSI, glycerine): *m/z* calcd. for $[C_{25}H_{36}N_8O_2 + H]^+$ 481.3039, found: 481.3028. $C_{25}H_{36}N_8O_2 \cdot 2TFA$ (708.65).

## 3.5.2 Pharmacological Methods

### 3.5.2.1 Materials and Cell Culture

Porcine NPY (pNPY), human pancreatic polypeptide (hPP), [$K^4$]hPP and GW1229 [51] (also designated GR231118 or 1229U91) were a gift of Prof. Cabrele (University of Bochum, Germany). The cyanine

dye labeled fluorescent peptides cy5-pNPY and cy5-[$K^4$]hPP were prepared in our laboratory as described previously.[52,2]

Unless otherwise indicated, chemicals, buffers and reagents were purchased from Merck (Darmstadt, Germany). Millipore water was used throughout. HEPES and bovine serum albumin (BSA) were purchased from Serva (Heidelberg, Germany). FCS was obtained from Biochrom (Berlin, Germany), cyanine dye Cy5-succinimidyl ester from Amersham Biosciences (Little Chalfont, UK) and Coelenterazine h from Biotrend (Cologne, Germany).

*Cell culture:*

Except for HEL (human erythroleukemia) cells, expressing the NPY $Y_1$R, all other cells – HEC-1-B-$Y_5$ [53] cells and CHO-h$Y_2$/-h$Y_4$ - were genetically engineered to stably express the receptor of interest as described elsewhere.[1, 2]

HEL cells (suspension) were passaged in RPMI with FCS (5%). HEC-1-B-$Y_5$ cells ($Y_5$R) were cultured in EMEM with FCS (10 %) and G418 (400 µg/mL) as described previously.[53] CHO cells, expressing $Y_2$R or $Y_4$R were grown in Ham's F12 medium supplemented with FCS (10 %) and G418 (400 mg/mL), hygromycin (400 mg/mL) and zeocin (200 mg/mL).[2, 54]

### 3.5.2.2 Aequorin Assay

***Preparation of the cell suspension:***

CHO-h$Y_4$-G$q_{i5}$-mtAEQ were grown to 80 – 90 % confluency in a 175-$cm^2$ culture flask, trypsinized and detached with Ham's F12 supplemented with 10 % fetal calf serum. After centrifugation at 300 $g$ for 5 min, the cells were resuspended in DMEM without phenol red containing 1 % fetal calf serum at a density of $10^7$ cells/mL. Coelenterazine h (2 mM stock solution in methanol) was added yielding a final concentration of 2 µM and the cell suspension was kept in the dark under gentle stirring for 2 h (reconstitution of the holoenzyme). After dilution with loading buffer (1:20) the cell suspension was incubated for 3 h in the dark.

***Agonist mode:***

For the characterization of agonists, 162 µL of the cell suspension were injected to 18 µL of agonist (10-fold concentrated) in loading buffer supplemented with 1 % BSA and 100 µg/mL bacitracin in a white 96-well plate (Greiner Bio-One, Frickenhausen, Germany) and the emitted luminescence (peak 1) was recorded in the kinetic mode for 43 s as a series of 200 ms integrations with a GENios Pro plate reader (Tecan, Salzburg, Austria). Subsequently, 20 µL of a 1 % Triton-X-100 solution were injected to discharge the remaining aequorin, and light emission was recorded for additional 22 s (peak 2).

***Antagonist mode:***

For the determination of antagonistic activity, 178 µL of cell suspension were added to 2 µL of test compound (100-fold concentration) in DMSO (100 % or 50 %) in a white 96-well plate and incubated for 15 min. 20 µL of a hPP solution (1 µM) in loading buffer supplemented with 1 % BSA and 100 µg/mL bacitracin were injected and the emitted luminescence was recorded in the kinetic mode for 43 with the GENios Pro plate reader before lysis of the cells by injection of a 1 % Triton-X-100 solution (peak 2).

The experiments were analyzed by using the term of fractional luminescence which is calculated by dividing the area of peak 1 (signal elicited by hPP or agonist) by the total area under peaks 1 and 2 (signal induced by addition of Trition-X-100) with the Sigma Plot 9.0 software. For detailed information on procedures, settings and calculation, cf. ref.[1, 2]

### 3.5.2.3 Flow Cytometric Binding Assay

The compounds were investigated in flow cytometric binding assays with respect to $Y_4R$ affinity and receptor subtype selectivity compared to $Y_1R$, $Y_2R$ and $Y_5R$.

***Competition binding assay:***

All binding assays on the NPY $Y_4R$ were performed using Cy5-[$K^4$]hPP (final concentration 3 nM) as fluorescent receptor ligands. For all experiments, HEPES (25 mM) buffer (pH 7.4), containing $CaCl_2 \cdot 2H_2O$ (2.5 mM) and $MgCl_2$ (1.5 mM), was supplemented with bovine serum albumin (BSA, 1 %) and bacitracin (0.1 g/L). All binding assays were performed in a final volume of 500 µL consisting of 490 µL of cell suspension, 5 µL of Cy5[$K^4$]-hPP and 5 µL of increasing concentrations of test compound (100-fold concentrated). Unspecific binding was determined by addition of an excess (final concentration 1 µM) of GW1229. The samples were incubated in "siliconized" (Sigmacote, Sigma-Aldrich, Taufkirchen, Germany) Eppendorf reaction vessels at room temperature for 90–120 min and analyzed by a FacsCaliburTM flow cytometer from Becton Dickinson (Heidelberg, Germany) equipped with an argon laser (488 nm) and a red diode laser (635 nm) using the following instrument settings: FSC: E-1, SSC: 280 V, Fl-4: 800 V, Flow: HI. The measurement was stopped when 10000 – 15000 gated events were counted. $K_i$ values were obtained from 2 – 3 independent experiments and were calculated by the Cheng-Prusoff equation. The detailed procedure and the instrument settings of flow cytometric binding studies were described previously. [1, 2]

***Selectivity assay:***

The selectivity of the synthesized $Y_4R$ ligands for the human NPY $Y_4R$ over $Y_1$, $Y_2$ and $Y_5$ receptor was determined by flow cytometric binding studies using Cy5-pNPY as described previously.[37] All measurements were performed on a FACSCaliburTM flow cytometer from Becton Dickinson (Heidelberg, Germany) equipped with an argon laser (488 nm) and a red diode laser (635 nm). All compounds were tested at two concentrations (1000 nM and 10000 nM) in duplicate.

## 3.6 References


1. Ziemek, R. Development of binding and functional assays for the neuropeptide Y Y 2 and Y 4 receptors. Doctoral Thesis, Regensburg, **2006**.
2. Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Determination of Affinity and Activity of Ligands at the Human Neuropeptide Y Y4 Receptor by Flow Cytometry and Aequorin Luminescence. *J. Recept. Signal. Transduct. Res.* **2007,** 27, 217 - 233.
3. Michel, M. C.; Motulsky, H. J. He 90481: A Competitive Nonpeptidergic Antagonist at Neuropeptide Y Receptors. *Ann. NY Acad. Sci.* **1990,** 611, 392-394.
4. Savchuk, N. P.; Tkachenko, S. E.; Balakin, K. V. Strategies for the Design of pGPCR-targeted Libraries. Ligand Design for G Protein-coupled Receptors, **2006**; 137-164.
5. Ertel, M., Personal Communication, University of Regensburg, Regensburg, **2008**.
6. Beck-Sickinger, A. G.; Wieland, H. A.; Wittneben, H.; Willim, K. D.; Rudolf, K.; Jung, G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* **1994,** 225, 947-958.
7. Eckard, C.; Cabrele, C.; Wieland, H.; Beck-Sickinger, A. Characterisation of Neuropeptide Y Receptor Subtypes by Synthetic NPY Analogues and by Anti-receptor Antibodies. *Molecules* **2001,** 6, 448-467.
8. Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Becksickinger, A. G.; Doods, H. N. The First Highly Potent and Selective Nonpeptide Neuropeptide-Y Y-1-Receptor Antagonist - BIBP3226. *Eur. J. Pharm.* **1994,** 271, R11-R13.
9. Doods, H.; Gaida, W.; Wieland, H. A.; Dollinger, H.; Schnorrenberg, G.; Esser, F.; Engel, W.; Eberlein, W.; Rudolf, K. BIIE0246: a selective and high affinity neuropeptide Y Y(2) receptor antagonist. *Eur. J. Pharm.* **1999,** 384, R3-5.
10. Dumont, Y.; Cadieux, A.; Doods, H.; Pheng, L. H.; Abounader, R.; Hamel, E.; Jacques, D.; Regoli, D.; Quirion, R. BIIE0246, a potent and highly selective non-peptide neuropeptide Y Y(2) receptor antagonist. *Br. J. Pharmacol.* **2000,** 129, 1075-1088.

11. Kraus, A.; Ghorai, P.; Birnkammer, T.; Schnell, D.; Elz, S.; Seifert, R.; Dove, S.; Bernhardt, G.; Buschauer, A. NG-acylated aminothiazolylpropylguanidines as potent and selective histamine H2 receptor agonists. *ChemMedChem* **2009,** 4, 232-240.
12. Curtet, S.; Soulier, J.-L.; Zahradnik, I.; Giner, M.; Berque-Bestel, I.; Mialet, J.; Lezoualc'h, F.; Donzeau-Gouge, P.; Sicsic, S.; Fischmeister, R.; Langlois, M. New Arylpiperazine Derivatives as Antagonists of the Human Cloned 5-HT4 Receptor Isoforms. *J. Med. Chem.* **2000,** 43, 3761-3769.
13. Dutta, A. K.; Venkataraman, S. K.; Fei, X.-S.; Kolhatkar, R.; Zhang, S.; Reith, M. E. A. Synthesis and biological characterization of novel hybrid 7-{[2-(4-phenyl-piperazin-1-yl)-ethyl]-propyl-amino}-5,6,7,8-tetrahydro-naphthalen-2-ol and their heterocyclic bioisosteric analogues for dopamine D2 and D3 receptors. *Bioorg. Med. Chem.* **2004,** 12, 4361-4373.
14. Allen, J. R.; Hitchcock, S. A.; Liu, B.; Turner, W. W., Jr. Preparation of 2-hydroxyindanylamide derivatives as muscarinic receptor agonists. WO2005009941A1, **2005**.
15. Park, J. H.; Choi, J. K.; Lee, E.; Lee, J. K.; Rhim, H.; Seo, S. H.; Kim, Y.; Doddareddy, M. R.; Pae, A. N.; Kang, J.; Roh, E. J. Lead discovery and optimization of T-type calcium channel blockers. *Bioorg. Med. Chem.* **2007,** 15, 1409-1419.
16. Mitsunobu, O.; Yamada, M.; Mukaiyam.T. Preparation of Esters Pf Phosphoric Acid by Reaction of Trivalent Phosphorus Compounds with Diethyl Azodicarboxylate in Presence of Alcohols. *Bull. Chem. Soc. Jpn.* **1967,** 40, 935-&.
17. Igel, P. Synthesis and structure-activity relationships of NG-acylated arylalkylguanidines and related comopounds as histamine receptor ligands: Searching for seletive H4R agonists. Doctoral Thesis, Regensburg, **2008**.
18. Wright, J. B. The Chemistry of the Benzimidazoles. *Chem. Rev.* **1951,** 48, 397-541.
19. Preston, P. N. Synthesis, reactions, and spectroscopic properties of benzimidazoles. *Chem. Rev.* **1974,** 74, 279-314.
20. Selms, R. C. D. Benzimidazoles. I. 2-(Heterocyclic Substituted)benzimidazoles. *J. Org. Chem.* **1962,** 27, 2163-2165.
21. Krapcho, P. K., Christopher,S. Mono-Protected Diamines. N-tert-Butoxycarbonylalkanediamines from Alkanediamines. *Synth. Commun.* **1990,** 20, 2559-2564.
22. Iwanowicz, E. J.; Poss, M. A.; Lin, J. Preparation of N,N'-Bis-Tert-Butoxycarbonylthiourea. *Synth. Commun.* **1993,** 23, 1443-1445.
23. Poss, M. A.; Iwanowicz, E.; Reid, J. A.; Lin, J.; Gu, Z. X. A Mild and Efficient Method for the Preparation of Guanidines. *Tetrahedron Lett.* **1992,** 33, 5933-5936.

24. Rasmussen, C. R.; Villani, F. J.; Reynolds, B. E.; Plampin, J. N.; Hood, A. R.; Hecker, L. R.; Nortey, S. O.; Hanslin, A.; Costanzo, M. J.; Howse, R. M.; Molinari, A. J. A Versatile Synthesis of Novel N,N,N'-Trisubstituted Guanidines. *Synthesis* **1988**, 460-466.

25. DeMong, D. E.; Williams, R. M. The asymmetric synthesis of (2S,3R)-capreomycidine. *Tetrahedron Lett.* **2001,** 42, 3529-3532.

26. Kim, K. S.; Qian, L. G. Improved Method for the Preparation of Guanidines. *Tetrahedron Lett.* **1993,** 34, 7677-7680.

27. Knieps, S.; Dove, S.; Michel, M. C.; Rottmeier, K.; Werner, W.; Bernhardt, G.; Buschauer, A. ω-Phenyl-ω-(2-pyridyl)alkyl-substituted bisguanidines are moderate neuropeptide Y antagonists. *Pharm. Pharmacol. Lett.* **1996,** 6, 27-30.

28. Feichtinger, K.; Zapf, C.; Sings, H. L.; Goodman, M. Diprotected Triflylguanidines: A New Class of Guanidinylation Reagents. *J. Org. Chem.* **1998,** 63, 3804-3805.

29. Feichtinger, K.; Sings, H. L.; Baker, T. J.; Matthews, K.; Goodman, M. Triurethane-Protected Guanidines and Triflyldiurethane-Protected Guanidines: New Reagents for Guanidinylation Reactions. *J. Org. Chem.* **1998,** 63, 8432-8439.

30. Ghorai, P.; Kraus, A.; Keller, M.; Gotte, C.; Igel, P.; Schneider, E.; Schnell, D.; Bernhardt, G.; Dove, S.; Zabel, M.; Elz, S.; Seifert, R.; Buschauer, A. Acylguanidines as bioisosteres of guanidines: NG-acylated imidazolylpropylguanidines, a new class of histamine H2 receptor agonists. *J. Med. Chem.* **2008,** 51, 7193-7204.

31. Paul, R.; Anderson, G. W. N,N'-Carbonyldiimidazole, a New Peptide Forming Reagent1. *J. Am. Chem. Soc.* **1960,** 82, 4596-4600.

32. Paul, R.; Anderson, G. W. N,N'-Carbonyldiimidazole in Peptide Synthesis. III.1 A Synthesis of Isoleucine-5 Angiotensin II Amide-I. *J. Org. Chem.* **1962,** 27, 2094-2099.

33. Keller, M.; Bernhardt, G.; Buschauer, A. [3H]UR-MK136: A Highly Potent and Selective Radioligand for Neuropeptide Y Y1 Receptors. *ChemMedChem* **2011,** 6, 1566-1571.

34. Majer, P.; Randad, R. S. A Safe and Efficient Method for Preparation of N,N'-Unsymmetrically Disubstituted Ureas Utilizing Triphosgene. *J. Org. Chem.* **1994,** 59, 1937-1938.

35. Salmon-Chemin, L.; Buisine, E.; Yardley, V.; Kohler, S.; Debreu, M.-A.; Landry, V.; Sergheraert, C.; Croft, S. L.; Krauth-Siegel, R. L.; Davioud-Charvet, E. 2- and 3-Substituted 1,4-Naphthoquinone Derivatives as Subversive Substrates of Trypanothione Reductase and Lipoamide Dehydrogenase from Trypanosoma cruzi: Synthesis and Correlation between Redox Cycling Activities and in Vitro Cytotoxicity. *J. Med. Chem.* **2001,** 44, 548-565.

36. Yung-Chi Cheng, W. H. P. Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzymatic reaction. *Biochem. Pharm.* **1973,** 22, 3099-3108.

37. Schneider, E.; Mayer, M.; Ziemek, R.; Li, L.; Hutzler, C.; Bernhardt, G.; Buschauer, A. A simple and powerful flow cytometric method for the simultaneous determination of multiple parameters at G protein-coupled receptor subtypes. *ChemBioChem* **2006,** 7, 1400-1409.

38. Peterson, J. M., Blum, C. A., Cai G., Hutchison, A. Certain Substituted Benzylamine Derivatives: A New Class Of Neuropeptide Y1 Specific Ligands WO9640660 **1996**.

39. Blum, C. A., Desimone, R., Hutchison, A., Peterson, J. M. Certain amido- and amino-substituted benzylamine derivatives: a new class of neuropeptide Y1 specific ligands. US613365, **2000**.

40. Chen, X.; Wang, J.; Sun, S.; Fan, J.; Wu, S.; Liu, J.; Ma, S.; Zhang, L.; Peng, X. Efficient enhancement of DNA cleavage activity by introducing guanidinium groups into diiron(III) complex. *Bioorg. Med. Chem. Lett.* **2008,** 18, 109-113.

41. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; Koenig, B. Modular synthesis of non-peptidic bivalent NPY Y1 receptor antagonists. *Bioorg. Med. Chem.* **2008,** 16, 9858-9866.

42. Pluym, N.; Brennauer, A.; Keller, M.; Ziemek, R.; Pop, N.; Bernhardt, G.; Buschauer, A. Application of the Guanidine-Acylguanidine Bioisosteric Approach to Argininamide-Type NPY Y2 Receptor Antagonists. *ChemMedChem* **2011,** 6, 1727-1738.

43. Woudenberg, R. C.; Coughlin, E. B. Selective nitrogen protection of hydroxyalkylbenzimidazoles using 2,2,2-trichloroethylchloroformate. *Tetrahedron Lett.* **2005,** 46, 6311-6313.

44. Jones, C. D.; Luke, R. W. A.; McCoull, W. Preparation of 2-aminopyrimidine derivatives as inhibitors of Tie2 receptor tyrosine kinases. WO2005060970A1, **2005**.

45. Yoo, E.; Yoon, J.; Pae, A. N.; Rhim, H.; Park, W.-K.; Kong, J. Y.; Park Choo, H.-Y. Synthesis and biological evaluation of (phenylpiperazinyl-propyl)arylsulfonamides as selective 5-HT2A receptor antagonists. *Bioorg. Med. Chem.* **2010,** 18, 1665-1675.

46. Huang, T.-N.; Liang, G.; Liu, W.; Przewloka, T.; Shen, M.; Zhang, S. Preparation of cyanoguanidines as antitumor agents. WO2000061561A1, **2000**.

47. Kroth, H.; Froestl, W.; Pfeifer, A.; Muhs, A. Preparation of 2,6-diaminopyridine compounds for treating diseases associated with amyloid or amyloid-like proteins, especially ocular diseases. WO2011045383A2, **2011**.

48. Igel, P.; Geyer, R.; Strasser, A.; Dove, S.; Seifert, R.; Buschauer, A. Synthesis and Structure-Activity Relationships of Cyanoguanidine-Type and Structurally Related Histamine H4 Receptor Agonists. *J. Med. Chem.* **2009,** 52, 6297-6313.

49. Botta, A. Ring-opening aminolysis of lactams.-A convenient route to five- and six-membered 2-(ω-aminoalkyl)-substituted heterocycles. *Justus Liebigs Ann. Chem.* **1976**, 336-347.

50. Rekka, E.; Sterk, G. J.; Timmerman, H.; Bast, A. Identification of structural characteristics of some potential H2-receptor antagonists that determine the interaction with rat hepatic P-450. *Chem. Biol. Interact.* **1988,** 67, 117-127.

51. Balasubramaniam, A.; Mullins, D. E.; Lin, S.; Zhai, W.; Tao, Z.; Dhawan, V. C.; Guzzi, M.; Knittel, J. J.; Slack, K.; Herzog, H.; Parker, E. M. Neuropeptide Y (NPY) $Y_4$ receptor selective agonists based on NPY(32-36): development of an anorectic $Y_4$ receptor selective agonist with picomolar affinity. *J. Med. Chem.* **2006,** 49, 2661-2665.

52. Schneider, E.; Keller, M.; Brennauer, A.; Hoefelschweiger, B. K.; Gross, D.; Wolfbeis, O. S.; Bernhardt, G.; Buschauer, A. Synthesis and characterization of the first fluorescent nonpeptide NPY Y1 receptor antagonist. *ChemBioChem* **2007,** 8, 1981-1988.

53. Moser, C.; Bernhardt, G.; Michel, J.; Schwarz, H.; Buschauer, A. Cloning and functional expression of the hNPY $Y_5$ receptor in human endometrial cancer (HEC-1B) cells. *Can. J. Physiol. Pharmacol.* **2000,** 78, 134-142.

54. Ziemek, R.; Brennauer, A.; Schneider, E.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Fluorescence- and luminescence-based methods for the determination of affinity and activity of neuropeptide Y2 receptor ligands. *Eur. J. Pharm.* **2006,** 551, 10-18.

# CHAPTER 4

# $N^G$-Acylated Phenylpiperazinylguanidines as $Y_4R$ Ligands: Synthesis, Molecular Pharmacology and Toxicity

## 4.1 Introduction

As outlined in Chapter 3, a first approach in searching for non-peptidic NPY $Y_4R$ ligands was stimulated by the discovery of UR-AK 49 as a "lead structure" (**Figure 4.1**). A bioisosteric replacement of the imidazolyl moiety by different privileged structures was most successful in case of phenylpiperazine substructures resulting in a 20-fold increase in $Y_4R$ antagonistic activity.

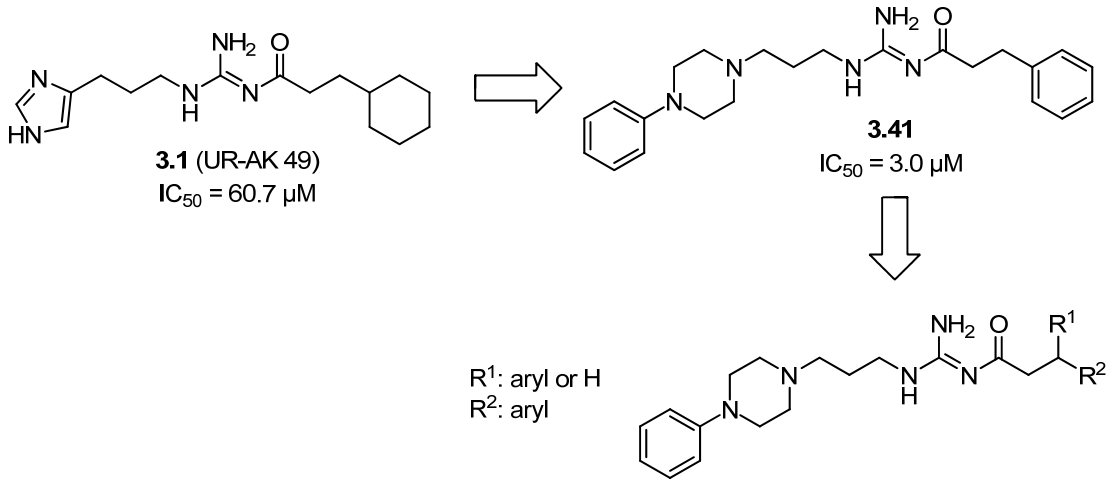
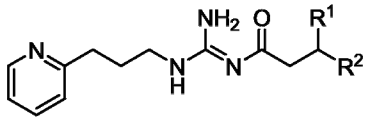



**Figure 4.1**: Structural modifications of the "lead structure" UR-AK 49 aiming at new non-peptidic NPY $Y_4R$ ligands.

This successful approach prompted us to continue the acylguanidine project. Moreover, upon screening of additional acylguanidines, which were initially designed as histamine $H_4$ receptor ligands, UR-PI 284 revealed one-digit $Y_4R$ antagonist activity ($IC_{50}$ = 5.0 µM). This compound shows a high degree of structural similarity with the lately developed **3.44**, but 6-fold higher activity at the NPY $Y_4R$ (**Figure 4.2**).[1] Obviously, the additional phenyl ring in the acyl residue accounts for the increased activity. Consequently, the study presented in this chapter was initiated to prepare a small set of analogs of **3.41,** modified in the acyl moiety by introduction of similarly lipophilic residues as in UR-PI284 as well as more polar and basic substructures.



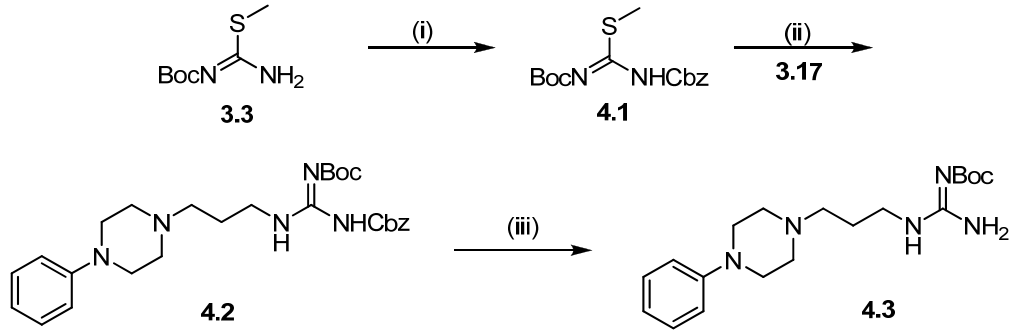
| No. | $R^1$ | $R^2$ | $IC_{50}$ [µM] |
|---|---|---|---|
| UR-PI284 | Ph | Ph | 5.0 ± 0.7 |
| 3.44 | H | Ph | 32 ± 9.5 |

**Figure 4.2:** Antagonistic activity of UR-PI284 and **3.44**, determined in the aequorin assay using CHO-h$Y_4$-qi5-mtAEQ cells.

The synthesized compounds (**Figure 4.1**) were characterized pharmacologically in functional cellular assays and in flow cytometric binding studies at the different NPY receptor subtypes. Furthermore, the new compounds were assessed with respect to potential cytotoxicitiy.

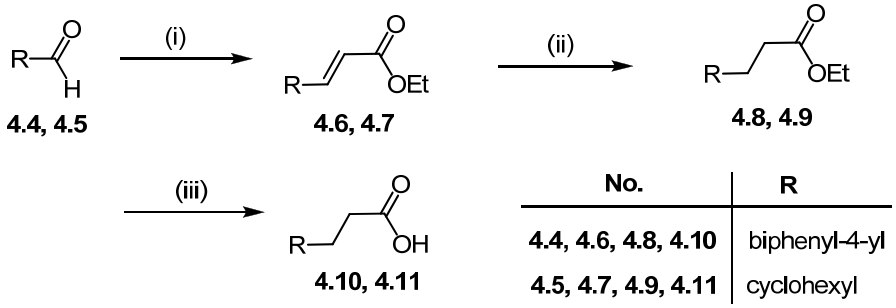
## 4.2 Chemistry

The preparation of the $N^G$-acylated piperazinylpropylguanidines was started with a three step synthesis affording the 3-(4-phenylpiperazin-1-yl)propylguanidine building block **4.3** according to **Scheme 4.1**. The *N*-Boc-protected *S*-methylisothiourea was synthesized as described in chapter 3 (see 3.2). Prior to the coupling of 3-(4-phenylpiperazin-1-yl)propylamine (**3.17**) with the guanidinylating reagent **3.3**, an additional Cbz-protection group was introduced according to a standard protocol. Finally, the Cbz group was removed by hydrogenation yielding the Boc-protected 3-(phenylpiperazin-1-yl)propylguandine building block **4.3**.




**Scheme 4.1**: Synthesis of the phenylpiperazinylpropylguanidine building block **4.3**. Reagents and conditions: (i) benzylsuccinimidyl carbonate (1 eq), $DCM_{abs}$, rt, 20 h; (ii) $HgCl_2$ (2 eq), $Et_3N$ (3 eq), DCM, rt, 48 h; (iii) 10 % Pd/C catalyst, MeOH, rt, 4-5 h.

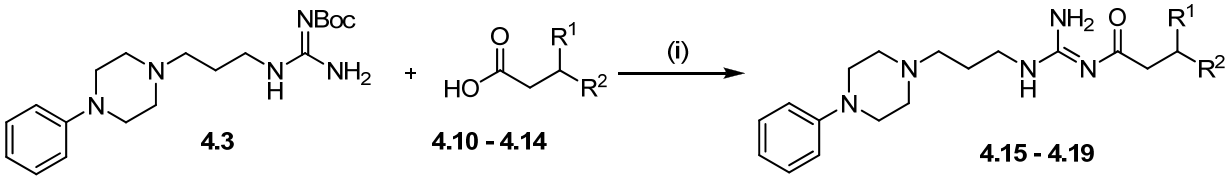
Most of the arylpropionic acids used for coupling with **4.3** were commercially available. Only biphenyl-4-yl- and cyclohexylpropionic acid (**Scheme 4.2**) had to be synthesized *via Horner Wadsworth-Emmons* reaction[2] from biphenyl-4-yl- or cyclohexylcarboxaldehyd with triethyl phosphonoacetate followed by hydrogenation of the C=C double bond. Finally, the ester was treated with LiOH yielding the carboxylic acids **4.10** and **4.11**.

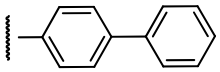
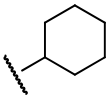
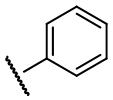
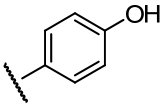
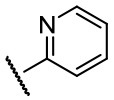



**Scheme 4.2:** Synthesis of biphenylyl- and cyclohexylpropionic acid **4.10** and **4.11**. Reagents and conditions: (i) triethyl phosphonoacetate (1.5 eq), NaH (1.5 eq), $THF_{abs}$, reflux, overnight; (ii) 10 % Pd/C catalyst, MeOH, 5 bar, rt, 4-5 h; (iii) LiOH (5 eq), $EtOH/H_2O$, rt, overnight.

The coupling of the carboxylic acids **4.10** – **4.14** was performed under standard conditions using EDAC, HOBt and DIEA. Finally, the Boc-protection group was removed with TFA (50 %) in DCM within

3 to 8 h affording the $N^G$-acylated 3-(4-phenylpiperazin-1-yl)propylguanidines **4.15** – **4.19** after separation by preparative RP-HPLC in purities > 95 %.




| No | 4.10, 4.15 | 4.11, 4.16 | 4.12, 4.17 | 4.13, 4.18 | 4.14, 4.19 |
|---|---|---|---|---|---|
| $R^1$ | H | H | Ph | H | H |
| $R^2$ |  |  |  |  |  |

**Scheme 4.3**: Synthesis of $N^G$-acylated 3-(4-phenylpiperazin-1-yl)propylguanidines **4.15** – **4.19**: Reagents and conditions: (i) a) EDC (1.2 eq), HOBt, (1.2 eq), DIEA (2.0 eq), $DCM_{abs}$, rt, overnight, b) TFA (50 %), DCM, rt, 3 – 8 h.

## 4.3 Pharmacological Results and Discussion

### 4.3.1 Potencies and Subtype Selectivity of the Synthesized $N^G$-Acylated Piperazinylpropylguanidines at the NPY $Y_4$R

The synthesized $N^G$-acylated phenylpiperazinylpropylguanidines were investigated for functional activity at the NPY $Y_4$R in a luminescence based $Ca^{2+}$-assay (aequorin assay) using CHO-h$Y_4$-qi5-mtAEQ cells. In the following, antagonistic potencies are expressed as $IC_{50}$ and $K_B$ values, determined from the concentration-dependent inhibition of the luminescence signal elicited by hPP (100 nM). $K_B$ values were calculated according to the Cheng-Prusoff equation[3]. Additionally, all compounds were investigated in the agonist mode. Results were given in percent by referring the measured luminescence signal of the compounds (100 µM or 10 µM) to the maximal response of the standard hPP (100 nM). Flow cytometric binding studies were performed in presence of Cy5-[$K^4$]hPP (c = 3 nM, $K_D$ = 5.62).[4, 5] Results are given as $K_i$ calculated according to the Cheng-Prusoff equation[3].

Furthermore, all synthesized compounds were investigated for binding to the other NPY receptor subtypes in a flow cytometric binding assay using HEL ($Y_1$R), and stably transfected CHO-$Y_2$ ($Y_2$R) and HEC-1B-$Y_5$ cells ($Y_5$R), respectively. Compounds were tested at two concentrations (10 µM and 1 µM, in duplicate) in the presence of Cy5-pNPY (c = 5 nM, $K_D$ = 5.2 nM); results are given as $K_i$ (**Table 4.1**).[6]

Obviously, the introduction of different lipophilic moieties (**4.15** – **4.17**) instead of the phenyl ring in the phenylpropanoyl portion in **3.41** does not influence the NPY $Y_4$R antagonistic activity. The $K_B$

values were in the same range as that of **3.41**. Interestingly, the introduction of a more polar (**4.18**) or a basic (**4.19**) residue was not tolerated. Concerning subtype selectivity, all investigated compounds were inactive at 10 µM at the other NPY receptor subtypes except for **4.18**, bearing a hydroxyphenyl substructure, and **4.16**, containing a cylcohexyl moiety, which showed affinity to the $Y_5R$ in the low micromolar range.

It is striking that only highly lipophilic substructures afforded promising results. Due to cytotoxic properties depending on the lipophilicity of the compounds (cf. **section 4.4**), the results from cell-based assays have to be analyzed very carefully in order to avoid misinterpretation of false-positives.

**Table 4.1:** Potencies, affinities and subtype selectivities of the synthesized $N^G$-acylated 3-(4-phenylpiperazin-1-yl)propylguanidines.

| No. | $hY_4R$ | | | | $hY_1R$ | $hY_2R$ | $hY_5R$ |
|---|---|---|---|---|---|---|---|
| | % Relative Potency[a] | $IC_{50}$ [µM][b] | $K_B$ [µM][c] | $K_i$ [µM][d] | $K_i$ [µM][e] | $K_i$ [µM][e] | $K_i$ [µM][e] |
| **3.41** | 3.0 ± 1.1 | 3.01 ± 1.18 | 0.40 ± 0.16 | n.d. | inactive[g] | inactive[g] | inactive[g] |
| **4.15** | 3.8 ± 1.6 | 2.99 ± 1.11 | 0.40 ± 0.15 | n.d. | inactive[g] | inactive[g] | inactive[g] |
| **4.16** | 3.7 ± 1.5 | 2.74 ± 0.28 | 0.37 ± 0.04 | inactive[g] | inactive[g] | inactive[g] | 3.7 ± 1.5 |
| **4.17** | 3.6 ± 1.0 | 3.10 ± 1.81 | 0.42 ± 0.24 | 4.08 ± 3.45 | inactive[g] | inactive[g] | inactive[g] |
| **4.18** | 4.3 ± 2.0 | inactive[f] | - | n.d. | inactive[g] | inactive[g] | 5.8 ± 3.6 |
| **4.19** | 2.9 ± 1.5 | inactive[f] | - | n.d. | inactive[g] | inactive[g] | inactive[g] |

[a] $[Ca^{2+}]_i$ mobilization of tested compound (agonist mode) at 100 µM concentration (performed in triplicate) given in % referred to the $[Ca^{2+}]_i$ mobilization induced by the standard hPP (100 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells. [b] Inhibition of 100 nM hPP induced $[Ca^{2+}]_i$ mobilization in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [c] $K_B$ calculated according to the Cheng-Prusoff equation[3] (c = 100 nM, $K_D$ = 15.5 nM). [d] Flow cytometric binding assay using 3 nM Cy5-[$K^4$]hPP ($K_D$ = 5.62 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate [e] Flow cytometric binding assay using 5 nM cy5-pNPY ($K_D$ = 5.2 nM) in CHO-$hY_2$ cells, HEL ($Y_1$) and HEC-1B-$Y_5$ cells, respectively. [f] less than 20 % decrease of the luminescence signal elicited by 100 nM hPP at 10 µM concentration of the test compound; testing at higher concentrations was avoided due to cytotoxic effects. [g] less than 20 % displacement of Cy5-[$K^4$]hPP ($Y_4R$) and Cy5-pNPY ($hY_1R$, $hY_2R$, $hY_5R$); testing at higher concentrations was avoided due to cytotoxic effects.

## 4.3.2 Cytotoxicity of the Synthesized $N^G$-Acylated Piperazinylpropyl-guanidines

### 4.3.2.1 Introduction

The variability of data from functional and binding assays with UR-AK49 and the synthesized $N^G$-acylated hetarylpropylguanidines in cell-based $Y_4R$ assays prompted us to take toxic effects into consideration. As depicted in **Figure 4.3,** in case of UR-AK49, reproducibility problems became

obvious in the aequorin assay ($IC_{50}$ = 178 µM, **Figure 4.3**: b), when comparing the $IC_{50}$ value of 60.7 µM obtained by Ralf Ziemek[5] (**Figure 4.3 a**). In addition, the shapes of the concentration-response curves differed (**Figure 4.3**).

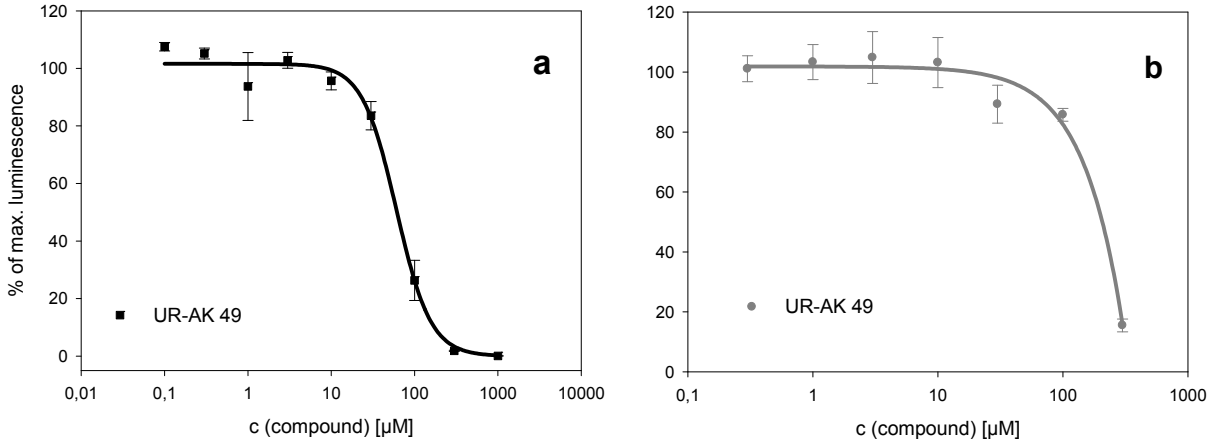



**Figure 4.3**: Variability of functional data obtained with UR-AK49: a) aequorin assay performed by Ralf Ziemek[5] ($IC_{50}$ = 60.7 µM); b) aequorin assay performed to reproduce data of Ralf Ziemek ($IC_{50}$ = 178 µM).

A closer analysis of the data, considering in particular concentrations higher than 30 µM (**Figure 4.4 a**), reveals that the luminescence signal, resulting from the addition of Triton-X (**Figure 4.4 b**), is reduced (100 µM) or even completely vanished (300 µM) compared to the standard hPP.

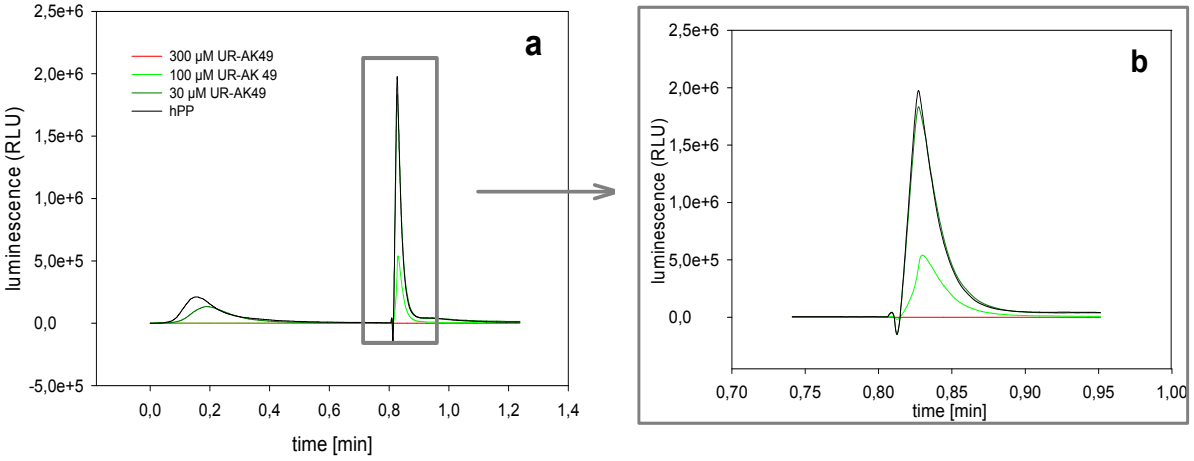
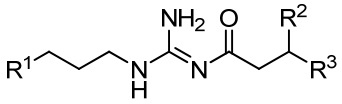


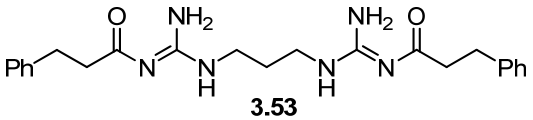

**Figure 4.4**: Aequorin assay with CHO-$hY_4$-qi5-mtAEQ cells: a) UR-AK49 tested at 300 µM, 100 µM and 30 µM concentrations; b) magnification of the luminescence signal induced by Triton-X-100.

One may speculate that due to toxic effects of the tested compounds, especially at high concentrations, cell rupture occurs, resulting in calcium release and partial or complete consumption of the aequorin substrate coelenterazin. Luminescence would be mainly emitted during incubation prior to the registration of the signal. This hypothesis is supported by the chemical nature of the synthesized $N^G$-acylated hetarylpropylguanidines, considering their amphiphilic character due to a

basic moiety (hetarylpropylguanidine) combined with a lipophilic substructure (alkyl and aryl residues). According to Schreier et al.[7] such amphiphilic compounds can interact with biological membranes, causing membrane disruption or solubilization by analogy with classical detergents.

To verify this hypothesis, a selection of $N^G$-acylated hetarylpropylguanidines (cf. sections **3.2** and **4.2**), summarized in **Figure 4.5**, was investigated for cytotoxicity and with respect to their potential to induce hemolysis.





**3.53**

| No. | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **3.40** | piperazin-1-yl | H | Ph |
| **3.41** | 4-phenylpiperazin-1-yl | H | Ph |
| **3.42** | 4-(2-methoxyphenyl)piperazin-1-yl | H | Ph |
| **3.43** | 4-benzhydrylpiperazin-1-yl | H | Ph |
| **3.44** | pyridin-2-yl | H | Ph |
| **4.15** | 4-phenylpiperazin-1-yl | H | biphenyl-4-yl |
| **4.16** | 4-phenylpiperazin-1-yl | H | cyclohexyl |
| **4.17** | 4-phenylpiperazin-1-yl | Ph | Ph |
| **4.18** | 4-phenylpiperazin-1-yl | H | 4-hydroxyphenyl |
| **4.19** | 4-phenylpiperazin-1-yl | H | pyridin-2-yl |

**Figure 4.5**: Overview of compounds, selected for investigations in a crystal violet based chemosensitivity assay and hemolytic assay, respectively.

### 4.3.3 Hemolytic Properties of Selected Ligands

Lysis of red blood cells is a well-known readout to study amphiphilic compounds such as classic surfactants. As often described in literature, interactions of amphiphilic substances with the erythrocyte membrane can lead to hemolysis induced by an increase in osmotic pressure followed by cell swelling or by partial solubilization of membrane lipids and proteins, e.g. by formation of mixed micelles.[8] Complete disruption of red blood cells leads to a release of hemoglobin into the surrounding fluid. This hemolytic effect can be determined spectrophotometrically using two different wavelengths (580 nm and 485 nm). **Figure 4.6** shows the percentage of hemolysis induced by the selected compounds referred to the effect of the reference compound Trition-X 100 (10 %), which is known to induce strong hemolysis at high concentrations.[9]

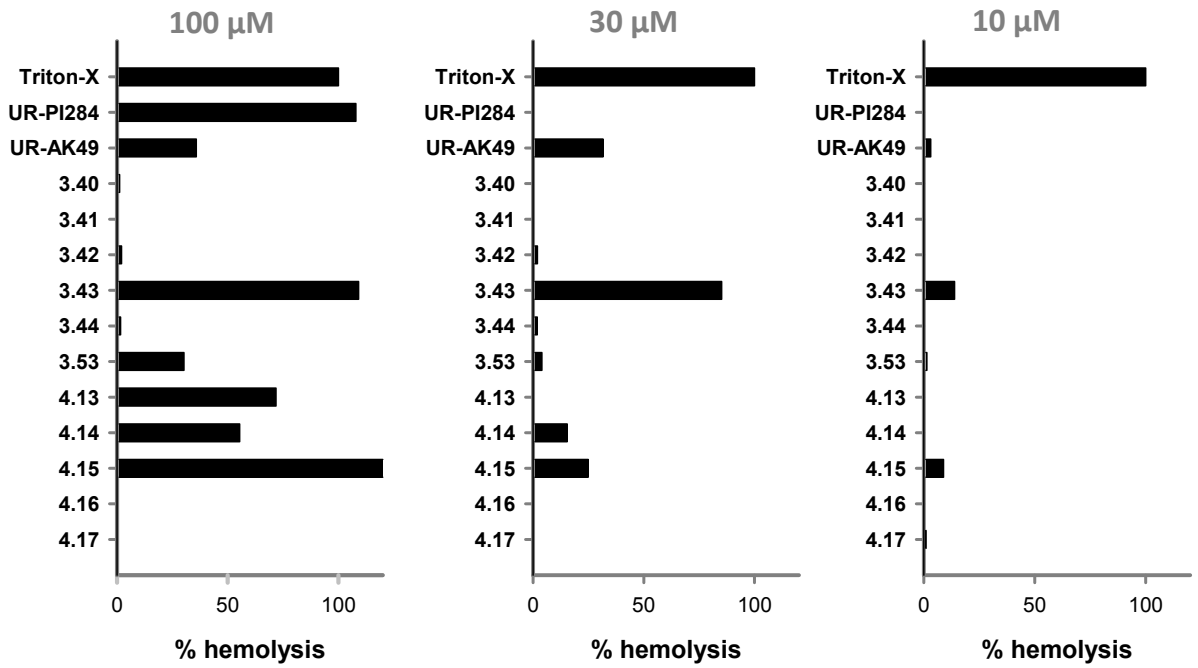



**Figure 4.6**: Hemolysis induced by acylguanidine- and carbamoylguanidine-type ligands at concentrations of 10 µM, 30 µM and 100 µM; Triton-X 100 was used as positive control.

At a concentration of 100 µM compounds **3.43**, **4.15**, **4.16**, **4.17** and **UR-PI284** induced severe hemolysis (> 50 %). Moreover, a significant hemolytic effect was observed for **3.43** and **UR-AK49** (30 – 50 %). All other investigated substances revealed no hemolytic effect. Interestingly, all compounds causing severe damage of red blood cell have a cyclohexyl or benzhydryl moiety in common. These highly lipophilic residues combined with the polar hetarylpropylguanidine substructure obviously results in a critical amphiphilic character inducing severe cell damaging effects. In contrast, compounds exhibiting a less tenside-like character and those containing less lipophilic residues (e.g. **3.40**, **3.41**, **3.42**, **3.44**, **4.18**, **4.19**) showed no evidence for solubilization of cell membranes even at 100 µM. At 30 µM, the hemolytic effect in particular for **4.15**, **4.16**, **4.17** and **UR-PI284** was drastically decreased. Only **3.43** still caused severe hemolysis.

Altogether, one should be aware of the cell damaging effects induced by highly hydrophobic and amphiphilic $N^G$-hetarylpropylguanidines with respect to cell-based *in vitro* experiments. With regard to future design and synthesis of NPY receptor ligands, such tenside-like structures should be avoided.

### 4.3.4 Cytotoxicity of Selected Ligands

The cytotoxic properties of selected acylguanidine-type compounds were investigated in a kinetic crystal violet based chemosensitivity assay over a period of approximately 200 h using proliferating

HT-29 colon carcinoma cells.[10] Cisplatin was taken as reference compound. The results are depicted in **Figure 4.7**.

The reference compound cisplatin was ineffective at a concentration of 0.3 µM, whereas at increasing concentrations the drug was weakly cytotoxic (1 µM) or cytocidal (3 µM). On the contrary, incubation of the cells with the "lead structure" UR-AK 49 at a concentration of 3 µM led to a recovery of the initially impaired cell population. At a concentration of 10 µM, UR-AK 49 was cytostatic. All other compounds (**3.41**, **3.43**, **4.16** – **4.18**) produced severe cell damage, immediately leading to complete cell lysis at concentrations higher than 10 µM. Only compound **3.44,** comprising a pyridyl instead of a phenylpiperazinyl moiety in the western part of the acylguanidine substructure was non-toxic up to a concentration of 30 µM. At a concentration of 10 µM the cell proliferation strongly depended on the lipophilic nature of both the acyl residue and the substituent at the piperazinyl moiety. Whereas the more polar hydroxyphenyl comprising compound **4.18** and the phenylpiperazinylpropylguanidine **3.44** with a phenyl substituent in the eastern part lead to a recovery of the cell population, compounds **4.16, 4.17** and **3.43** bearing highly lipophilic cyclohexyl or diphenyl residues still revealed a distinct cytotoxic potential presumably due to the highest extent of amphiphilicity. These observations are in good agreement with the hemolytic properties, except for compounds **3.41** and **4.18**, comprising a phenyl and a hydroxyphenyl moiety, respectively. In spite of their cytotoxic effects at 100 µM and 30 µM in the crystal violet assay, no hemolysis these could be observed at these concentrations.

In conclusion, these results should be taken into account when cell based *in vitro* investigations (and *in vivo* studies) are performed with $N^G$-acylated hetarylpropylguanidines at concentrations in the micromolar range. Thus, in the aequorin assay, the cytotoxicity of several title compounds, e.g. **3.43**, could be misinterpreted as NPY $Y_4R$ agonism.

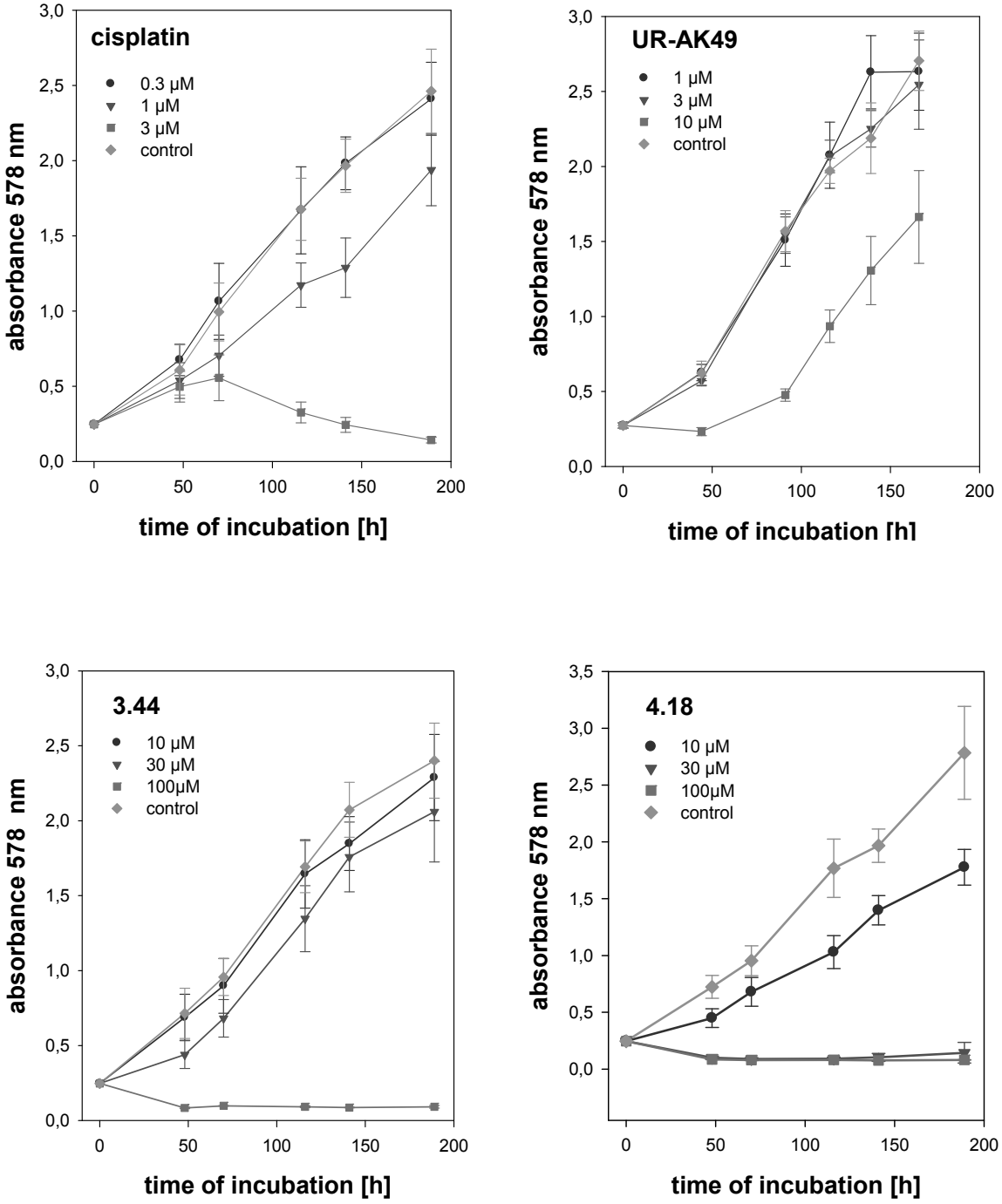



**Figure 4.7**: continued on page 93

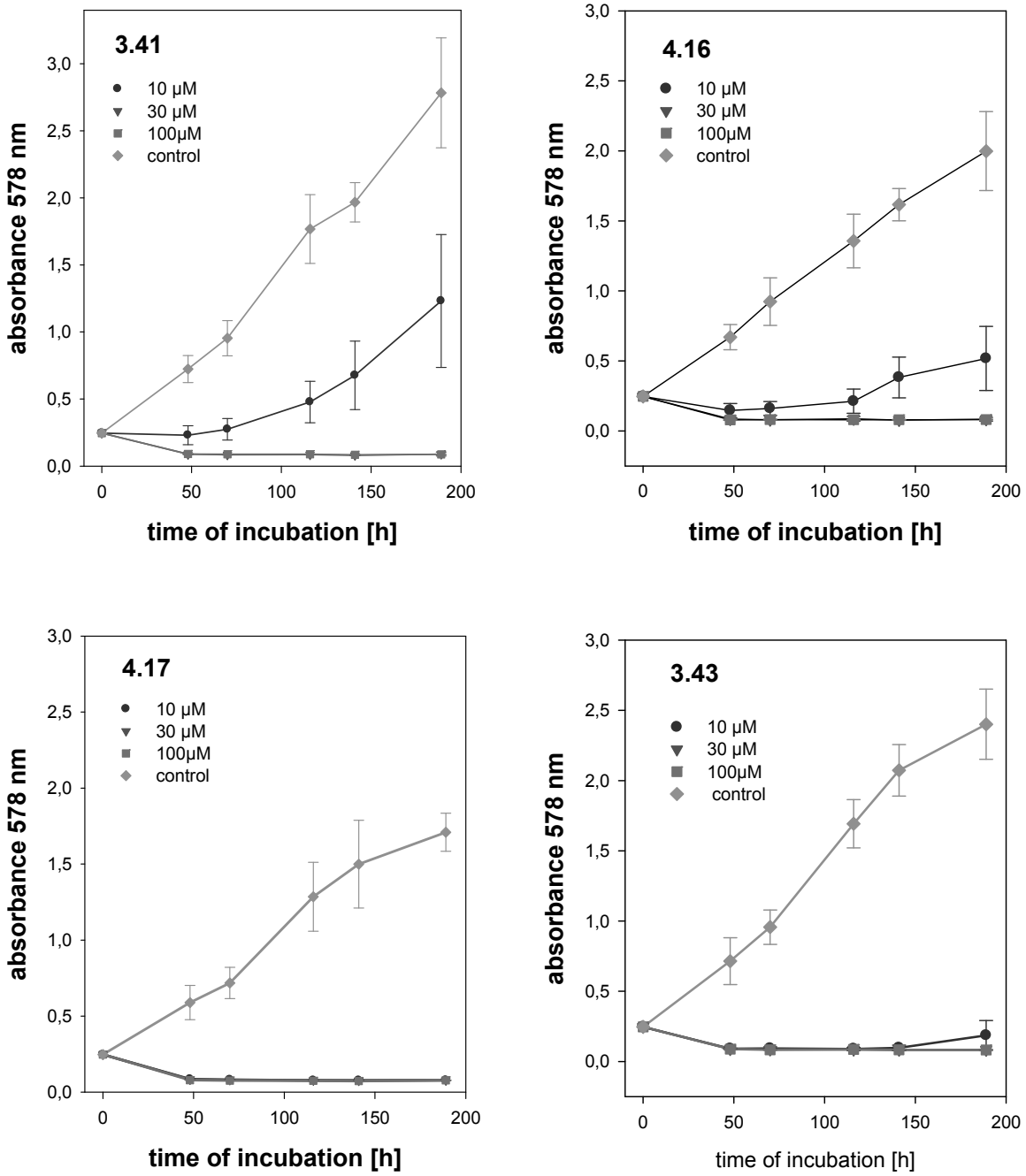



**Figure 4.7:** Proliferation of HT-29 cells in the presence of various concentrations of cisplatin or selected $hY_4R$ ligands.

## 4.4 Summary and Conclusion

Essentially, the results of the investigations on $N^G$-acylated piperazinylpropylguanidines provided quite disillusioning results. On the one hand, replacement of the phenyl moiety in the "eastern part" of **3.41**, used as a starting point, didn't result in increased NPY $Y_4R$ activity. The $IC_{50}$ values of compounds bearing lipophilic moieties were similar to that of **3.41**. On the other hand, investigations

on hemolysis and chemotoxicity revealed that many of the synthesized compounds caused severe hemolytic and cell-damaging effects at the tested concentrations. This raises questions, if the observed calcium signal actually resulted from NPY $Y_4R$ receptor binding or if it was caused by cell lysis due to the toxicity of the test compounds. Thus, this class of strongly amphiphilic compounds with only weak activities at the NPY $Y_4R$ seems not suitable for whole cell based test systems at concentrations higher than 10 µM. Indeed, activity of the lead structure UR-AK49 was confirmed in a steady state GTPase assay, using membranes instead of whole cells. Additionally, some other acylguanidine-type compounds, originally designed as histamine receptor ligands, were identified as weak antagonists at the NPY $Y_4R$.[1]

In conclusion, the $N^G$-acylated hetarylpropylguanidines turned out to be unsuitable as model compounds for the synthesis of potent NPY $Y_4R$ ligands. Attempts to increase the NPY $Y_4R$ activity and to reduce the toxic potential by structural variations failed. The introduction of more polar or basic residues like hydroxyphenyl or pyridyl which showed a quite better toxicological profile resulted in a negligible or even a complete loss of activity. Hence, the synthesis of acylguanidine-type compounds as ligands for the NPY $Y_4R$ was discontinued.

# 4.5 Experimental Section

## 4.5.1 Chemistry

### 4.5.1.1 General Experimental Conditions

See section 3.4.1.1

### 4.5.1.2 Preparation of the $N^G$-Boc Protected Phenylpiperazinylguanidine Building Block 4.3

**$N^1$-Benzyloxycarbonyl-$N^2$-*tert*-butoxycarbonyl-*S*-methylisothiourea (4.1)[11]**

To a solution of **3.3** (5.71 g, 30 mmol, 1 eq) in 75 mL $DCM_{abs}$ was added benzyl succinimidyl carbonate (7.48 g, 30 mmol, 1 eq) under argon atmosphere and stirred for 20 h at room temperature. The mixture was subsequently extracted with DCM and basified with $Na_2CO_3$ (pH 9 – 10). After washing the organic phase with water and drying over $MgSO_4$, the solvent was evaporated. The crude product was subjected to flash chromatography (PE/EtOAc 90/10 v/v) yielding **4.1** as white solid (9.04 g, 93 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.50 (s, 9H, C(C***H***$_3$)$_3$), 2.41 (s, 3H, S-C***H***$_3$), 5.20 (s, 2H, Ph-C***H***$_2$), 7.28 – 7.47 (m, 5H, Ph-***H***). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 325 (100) $[M+H]^+$. $C_{15}H_{20}N_2O_4S$ (324.40).

***N*$^1$-Benzyloxycarbonyl-*N*$^2$-*tert*-Butoxycarbonyl-*N*$^3$-[3-(4-phenylpiperazin-1-yl)propyl]guanidine (4.2)**

**3.14** (1.65 g, 7.5 mmol, 1 eq), the guanidinylating reagent **4.1** (2.43 g, 7.5 mmol, 1 eq) and $HgCl_2$ (4.07 g, 15.0 mmol, 2 eq) were dissolved in anhydrous DCM. $Et_3N$ (5.27 ml, 37.5 mmol, 3 eq) was added and the mixture was stirred for 48 h at ambient temperature. Subsequently, EtOAc was added and the precipitate filtered over Celite. Purification by flash chromatography (DCM/MeOH 100/0 – 95/5 v/v) afforded the product as white solid (3.13 g, 84 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.73 – 1.85 (m, 2H, Pip-CH$_2$-C***H***$_2$), 2.47 (t, $^3J$ = 6.7 Hz, 2H, Pip-C***H***$_2$), 2.54 – 2.65 (m, 4H, Pip-***H***), 3.15 – 3.27 (m, 4H, Pip-***H***), 3.15 – 3.27 (m, 2H, Pip-(CH$_2$)$_2$-C***H***$_2$), 5.14 (s, 2H, Ph-C***H***$_2$), 6.80 – 6.88 (m, 1H, Pip-Ph-***H***-4), 6.89 – 6.98 (m, 2H, Pip-Ph-***H***), 7.20 – 7.44 (m, 7H, Pip-Ph-***H*** + Ph-***H***), 8.74 (t, $^3J$ = 4.8 Hz, 1H, N***H***), 11.36 (bs, 1H, N***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$): δ (ppm) 25.72 (-, Pip-CH$_2$-***C***H$_2$), 28.03 (+, C(***C***H$_3$)$_3$), 40.14 (-, Pip-(CH$_2$)$_2$-***C***H$_2$), 48.84 (-, 2 Pip-***C***), 53.38 (-, 2 Pip-***C***), 56.55 (-, Pip-***C***H$_2$), 66.97 (-, Ph-***C***H$_2$), 82.99 (C$_{quat}$, ***C***(CH$_3$)$_3$), 119.57 (+, 2 Pip-Ph-***C***), 127.86 (+, Pip-Ph-***C***-4), 128.02 (+, 2 Ph-***C***), 128.41 (+, 2 Ph-***C***), 129.04 (+, 2 Pip-Ph-***C***), 137.04 (C$_{quat}$, Ph-***C***-1), 151.39 (C$_{quat}$, Pip-Ph-***C***-1), 152.83 (C$_{quat}$, ***C***=O), 156.39 (C$_{quat}$, ***C***=O) 163.72 (C$_{quat}$, ***C***=N); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 496 (100) $[M+H]^+$. $C_{27}H_{37}N_5O_4$ (495.61).

***N*$^1$-*tert*-Butoxycarbonyl-*N*$^2$-(3-(4-phenylpiperazin-1-yl)propyl)guanidine (4.3)**

**4.2** (2.90 g, 5.85 mmol, 1 eq) was dissolved in 150 mL MeOH. A 10 % Pd/C catalyst (300 mg) was added and hydrogen was led through the vigorously stirred mixture at room temperature at 5 bar for 4 – 5 h (TLC control). The catalyst was removed by filtration over Celite and the solvent was concentrated under reduced pressure prior to purification by flash chromatography (DCM/MeOH 100/0 – 90/10 v/v) yielding a pale yellow solid (1.66, 78 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.44 (s, 9H, C(C***H***$_3$)$_3$), 1.69 – 1.89 (m, 2H, Pip-CH$_2$-C***H***$_2$), 2.48 (t, $^3J$ = 7.1 Hz, 2H, Pip-C***H***$_2$), 2.56 – 2.75 (m, 4H, Pip-***H***), 3.12 – 3.30 (m, 6H, Pip-***H*** + Pip-(CH$_2$)$_2$-C***H***$_2$), 6.79 – 6.88 (m, 1H, Ph-***H***-4), 6.92 – 7.02 (m, 2H, Ph-***H***), 7.18 – 7.28 (m, 2H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 21.09 (-, Pip-CH$_2$-***C***H$_2$), 28.79 (+, C(***C***H$_3$)$_3$), 39.96 (-, Pip-(CH$_2$)$_2$-***C***H$_2$), 50.34 (-, 2 Pip-***C***), 54.19 (+, 2 Pip-***C***), 56.29 (-, Pip-***C***H$_2$), 79.02 (C$_{quat}$, ***C***(CH$_3$)$_3$), 117.56 (+, 2 Pip-Ph-***C***), 121.69 (+, Pip-Ph-***C***-4), 130.09 (+, 2 Pip-Ph-***C***), 152.73 (C$_{quat}$, ***C***=O), 157.44 (C$_{quat}$, ***C***=N); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 362 (100) $[M+H]^+$. $C_{19}H_{31}N_5O_2$ (361.48).

### 4.5.1.3 Preparation of Biphenyl- and Cyclohexylpropanoic acid 4.10 and 4.11

**General Procedure for the preparation of (*E,Z*)-alkenoates**

To a solution of triethylphosphonoacetate (1.5 eq) in 100 mL $THF_{abs}$, NaH (60 % dispersion in mineral oil, 1.5 eq) was added in portions. After stirring for 1 h at room temperature, a solution of the aldehyde (1 eq) in $THF_{abs}$ (50 mL) was added dropwise. When addition was complete, the mixture was refluxed overnight. The solvent was removed under reduced pressure and the residue was

diluted with EtOAc (50 mL) and washed with water (3x 20 mL). The organic layer was dried over $MgSO_4$, evaporated and directly subjected to flash chromatography.

**(*E,Z*)-Ethyl 3-(biphenyl-4-yl)acrylate (4.6)[12]**

The title compound was prepared from triethylphosphonoacetate (3.36 g, 15 mmol), NaH (0.58 g, 15 mmol) and 4-biphenylcarboxaldehyde **4.4** (1.82 g, 10 mmol) according to the general procedure. Purification by flash chromatography (PE/EE 90/10) afforded **4.6** as a colorless oil (1.82 g, 7.2 mmol, 72 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.35 (t, 3H, $^3J$ = 7.1 Hz, C***H***$_3$), 4.28 (q, 2H, $^3J$ = 7.1 Hz, C***H***$_2$), 6.48 (d, 1H, $^3J$ = 16.0 Hz, C***H***CO),7.34-7.51 (m, 3H, Biphenyl-***H***), 7.56-7.67 (m, 6H, Biphenyl-***H***), 7.73 (d, 1H, $^3J$ = 16.0 Hz, Biphenyl-C***H***); $^{13}$C-NMR (75 MHz; $CDCl_3$): δ (ppm) 14.37 (+, ***C***H$_3$), 60.55 (-, ***C***H$_2$), 118.14 (+, ***C***HCO) , 127.06 (+, 2 Ph-***C***), 127.55 (+, 2 Ph-***C***), 127.85 (+, Ph-***C***-4), 128.57 (+, 2 Ph-***C***), 128.92 (+, 2 Ph-***C***), 133.44 ($C_{quat.}$, Ph-***C***), 140.18 ($C_{quat.}$, Ph-***C***), 143.00 ($C_{quat.}$, Ph-***C***), 144.14 (Biphenyl-***C***H), 167.07 ($C_{quat}$, ***C***=O); MS (CI, $NH_3$) *m/z* (%): 252 (100) $M^{+\cdot}$. $C_{17}H_{16}O_2$ (252.31).

**(*E,Z*)-Ethyl 3-cyclohexylacrylate (4.7)[13]**

The title compound was prepared from triethyl phosphonoacetate (6.73 g, 30 mmol), NaH (1.15 g, 30.0 mmol) and cyclohexane carboxaldehyde **4.5** (2.42 mL, 20.0 mmol) according to the general procedure. Purification by flash chromatography (PE/EE 100/0 – 90/10 v/v) yielded **4.7** as colorless fluid (2.81 g, 77 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.10 – 1.36 (m, 5H, Cyclohexyl-***H***), 1.30 (t, $^3J$ = 7.1 Hz, 3H, $CH_2$-C***H***$_3$), 1.66 – 1.71 (m, 1H, Cyclohexyl-***H***), 1.73 – 1.81 (m, 4H, Cyclohexyl-***H***), 2.08 – 2.19 (m, 1H, Cyclohexyl-***H***), 4.08 (q, $^3J$ = 7.1 Hz, C***H***$_2$-$CH_3$), 5.75 (dd, $^3J$ = 15.8 Hz, $^4J$ = 1.5 Hz, 1H, CH=C***H***-CO), 6.90 (dd, $^3J$ = 15.8 Hz, 6.8 Hz, C***H***=CH-CO). $C_{11}H_{18}O_2$ (182.26).

**General procedure for the preparation of the alkanoates 4.8 und 4.9**

The ethyl (*E*,*Z*)-alkenoates were dissolved in MeOH. A 10 % Pd/C catalyst was added and hydrogen was led through the vigorously stirred mixture at room temperature for 4 – 5 h (TLC control). The catalyst was removed by filtration over Celite and the solvent was concentrated under reduced pressure. The product was used without further purification.

**Ethyl 3-(biphenyl-4-yl)propanoate (4.8)[12]**

The title compound was prepared from **4.6** (1.45 g, 5.75 mmol) by hydrogenation over Pd/C (150 mg) in 50 mL MeOH according to the general procedure yielding **4.8** as a colorless semi-solid compound (1.42 g, 97 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.26 (t, 3H, $^3J$ = 7.1 Hz, $CH_2$-C***H***$_3$), 2.67 (t, $^3J$ = 7.8 Hz, $CH_2$-C***H***$_2$-CO), 3.01 (t, 2H, $^3J$ = 7.8, Hz, C***H***$_2$-$CH_2$-CO), 4.16 (q, 2H, $^3J$ = 7.1 Hz, C***H***$_2$-$CH_3$), 7.25-7.38 (m, 3H, Biphenyl-***H***), 7.41-7.48 (m, 2H, Biphenyl-***H***), 7.51- 7.62 (m, 4H, Biphenyl-***H***); $^{13}$C-NMR (75 MHz; $CDCl_3$):

δ (ppm) 14.26 (+, ***C***$H_3$), 30.63 (-, ***C***$H_2$-$CH_2$-CO), 35.92 (-, $CH_2$-***C***$H_2$-CO), 60.50 (-, ***C***$H_2$-$CH_3$), 127.03 (+,2 Ph-***C***), 127.15 (+, Ph-***C***-4), 127.25 (+, 2 Ph-***C***), 128.77 (+, 4 Ph-***C***), 139.23 ($C_{quat}$, Ph-***C***), 139.71 ($C_{quat}$, Ph-***C***), 140.97 ($C_{quat}$, Ph-***C***), 172.95 ($C_{quat}$, ***C***=O). MS (CI, $NH_3$) *m/z* (%): 254 (60) $[M]^{+\cdot}$, 180 (100) $[C_{14}H_{12}]^{+\cdot}$. $C_{17}H_{18}O_2$ (254.32).

**Ethyl 3-cyclohexylpropanoate 4.9**[14]

The title compound was prepared from **4.7** (2.7g, mmol) by hydrogenation over Pd/C (270 mg) in 100 mL MeOH according to the general procedure yielding **4.9** as a colorless fluid (2.5 g, 92 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.24 (t, $^3J$ = 7.2 H, $CH_2$-C***H***$_3$), 1.10 – 1.30 (m, 5H, Cyclohexyl-***H***), 1.50 – 1.85 (m, 8H, Cyclohexyl-***H*** + C***H***$_2$-$CH_2$-CO), 2.24 – 2.32 (m, 2H, C***H***$_2$-CO) 4.11 (q, $^3J$ = 7.2 Hz, C***H***$_2$-$CH_3$). $C_{11}H_2O_2$ (184.28).

**General procedure for the preparation of the propanoic acids 4.10 und 4.11**

The alkanoates **4.8** and **4.9** (1 eq) were dissolved in EtOH. LiOH (5 eq) in $H_2O$ was added and the reaction mixture was stirred overnight. After evaporation of the ethanol, the clear solution was adjusted to pH 3 with 1N HCl and extracted with EtOAc (3x). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The obtained product was used without further purification.

**3-(Biphenyl-4-yl)propanoic acid (4.10)**[12]

The title compound was prepared from **4.8** (1.0 g, 3.9 mmol) and LiOH (0.47 g, 19.7 mmol) according to the general procedure to give **4.10** as a white solid (0.83 g, 93 %)**.** $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 2.62 (t, 2H, $^3J$ = 7.7 Hz, $CH_2$-C***H***$_2$-CO), 2.94 (t, 2H, $^3J$ = 7.7 Hz, C***H***$_2$-$CH_2$-CO), 7.24-7.33 (m, 3H, Biphenyl-***H***), 7.35-7.44 (m, 2H, Biphenyl-***H***), 7.47-7.60 (m, 4H, Biphenyl-***H***); $^{13}$C-NMR (75 MHz; $CD_3OD$): δ (ppm) 33.11 (-, ***C***$H_2$-$CH_2$-CO), 40.09 (-, $CH_2$-***C***$H_2$-CO), 127.85 (+,2 Ph-***C***), 127.96 (+,2 Ph-***C***), 128.07 (+, 4 Ph-***C***), 129.83 (+,2 Ph-***C***), 129.88 (+,2 Ph-***C***), 140.11 ($C_{quat}$, Ph-***C***), 142.48 ($C_{quat}$,Ph-***C***), 142.56 ($C_{quat}$,Ph-***C***), 180.69 ($C_{quat}$, ***C***=O). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 271 (40) $[M+HCOO]^-$, 451 (70) $[2M-H]^-$, 677 (100) $[3M-H]^-$. $C_{15}H_{14}O_2$ (226.27).

**3-Cyclohexylpropanoic acid (4.11)**[15]

The title compound was prepared from **4.9** (2.50 g, 13.6 mmol) and LiOH according to the general procedure to give **4.11** as a colorless fluid (2.05 g, 97 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.80-1.00 (m, 2H, Cyclohexyl-***H***), 1.10- 1.35 (m, 4H, Cyclohexyl-***H***), 1.47-1.57 (m, 2H, C***H***$_2$-$CH_2$-CO), 1.58-1.77 (m, 5H, Cyclohexyl-***H***), 2.37 (t, 2H, $^3J$ = 11.3 Hz, $CH_2$-C***H***$_2$-CO), 10.45 (bs, 1H, COO***H***). $^{13}$C-NMR (75 MHz; $CDCl_3$): δ (ppm) 26.19 (-, 2 Cyclohexyl-***C***-3), 26.50 (-, 2 Cyclohexyl-***C***-4), 31.71 (-, ***C***$H_2$-$CH_2$-CO),

32.04 (-, $CH_2$-***C***$H_2$-CO), 32.93 (-, 2 Cyclohexyl-***C***-2), 37.11 (+, Cyclohexyl-***C***-1), 180.91 ($C_{quat}$, ***C***=O). $C_9H_{16}O_2$ (156.22).

### 4.5.1.4 Preparation of Acylguanidines 4.15 – 4.19

**General procedure**

The carboxylic acids **4.10** – **4.14** (1 eq) were dissolved in anhydrous DCM. EDAC (1.2 eq), HOBt (1.2 eq) and DIEA (2.0 eq) were added, and the reaction mixture was stirred at room temperature under argon atmosphere. After 15 min, the building block **4.3** (1 eq), dissolved in anhydrous DCM was added in one portion. The clear solution was stirred at room temperature overnight. Subsequently, the solvent was removed under reduced pressure and the crude product was taken up in EtOAc and water. The aqueous phase was separated and extracted additional two times with EtOAc. The combined organic phase was dried over $MgSO_4$, evaporated and subjected to flash chromatography. The purified product was dissolved in DCM. TFA (50 % final concentration) was added and the reaction mixture was stirred until the deprotection was complete (3 – 8 h, TLC control). After evaporation of the solvent *in vacuo*, the crude product was purified by preparative RP-HPLC.

**$N^2$-[3-(Biphenyl-4-yl)propanoyl]-$N^1$-[3-(4-phenylpiperazin-1-yl)propyl]guanidine (4.15)**

The title compound was prepared from **4.10** (68 mg, 0.30 mmol), EDAC (69 mg, 0.36 mmol), HOBt (49 mg, 0.36 mmol), DIEA (105 µL, 0.60 mmol) in $DCM_{abs}$ (10 mL) and **4.3** (108 mg, 0.30 mmol) in $DCM_{abs}$ (5 mL) according to the general procedure. Deprotection with a mixture of 3 mL DCM and 3 mL TFA, followed by preparative RP-HPLC (20 – 50 % MeCN in 30 min) afforded product **4.15** as beige solid (53 mg, 25 %). $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 2.03 – 2.22 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.84 (t, $^3J$ = 7.2 Hz, Pip-C***H***$_2$), 3.02 (t, $^3J$ = 7.3 Hz, 2H, $CH_2$-C***H***$_2$-CO), 3.19 – 3.33 (m, overlap with solvent, 4H, Pip-***H*** + C***H***$_2$-$CH_2$-CO), 3.34 – 3.45 (m, 4H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$), 3.46 – 3.77 (bs, 4H, Pip-***H***), 6.88 – 6.96 (m, 1H, Ph-***H***-4), 6.97 – 7.03 8m, 2H, Ph-***H***), 7.23 – 7.35 (m, 5H, Ph-***H,*** + biPh-***H***), 7.37 – 7.45 (m, 2H, biPh-***H***), 7.50 – 7.60 (m, 4H, biPh-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 25.58 (-, Pip-$CH_2$-***C***$H_2$), 30.89 (-, ***C***$H_2$-$CH_2$-CO), 39.49 and 39.63 (-, Pip-$(CH_2)_2$-***C***$H_2$ + $CH_2$-***C***$H_2$-CO), 48.17 (-, Pip-***C***$H_2$), 53.34 (-, 2 Pip-***C***), 55.11 (-, 2 Pip-***C***), 118.09 (+, 2 Ph-***C***), 122.50 (+, Ph-***C***-4), 127.88 (+, 2 biPh-***C***), 128.16 (+, 2 biPh-***C***), 128.29 (+, 2 biPh-***C***), 128.29 (+, biPh-***C***-4), 129.91 (+, 2 biPh-***C***), 130.06 (+, 2 biPh-***C***), 130.41 (+, 2 Ph-***C***), 140.53 ($C_{quat}$, biPh-***C***), 140.70 ($C_{quat}$, biPh-***C***), 142.19 ($C_{quat}$, biPh-***C***), 151.07 ($C_{quat}$, Ph-***C***-1), 155.41 ($C_{quat}$, ***C***=N), 176.43 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 2): 99 % ($t_R$ = 25.55 min, *k*' = 7.38 HRMS (LSI, glycerine): *m/z* calcd. for $[C_{29}H_{35}N_5O + H]^+$ 470.2920, found: 470.2925. $C_{29}H_{35}N_5O \cdot 2TFA$ (697.66).

***N*²-(3-Cyclohexylpropanoyl)-*N*¹-[3-(4-phenylpiperazin-1-yl)propyl]guanidine (4.16)**

The title compound was prepared from **4.11** (47 mg, 0.30 mmol), EDAC (69 mg, 0.36 mmol), HOBT (49 mg, 0.36 mmol), DIEA (105 µL, 0.60 mmol in $DCM_{abs}$ (10 mL) and **4.3** (108 mg, 0.30 mmol) in $DCM_{abs}$ (5 mL) according to the general procedure. Deprotection with a mixture of 3 mL DCM and 3 mL TFA followed by preparative RP-HPLC (20 – 50 % MeCN in 30 min) afforded product **4.16** as pale brown resin (63 mg, 34 %).$^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 0.83 – 1.00 (m, 2H, Cyclohexyl-***H***), 1.08 – 1.37 (m, 4H, Cyclohexyl-***H***), 1.48 – 1.59 (m, 2H, C***H***$_2$-$CH_2$-CO), 1.60 – 1.78 (m, 5H, Cyclohexyl-***H***), 2.03 – 2.25 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.50 (t, $^3J$ = 7.9 Hz, 2H, $CH_2$-C***H***$_2$-CO), 2.95- 3.33 (m, overlap with solvent, 4H, Pip-***H*** + Pip-C***H***$_2$), 3.34 – 3.49 (m, 4H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$), 3.50 – 3.95 (bs, 4H, Pip-***H***), 6.88 – 6.96 (m, 1H, Ph-***H***-4), 6.98 – 7.04 (m, 2H, Ph-***H***), 7.23 – 7.32 (m, 2H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): 23.92 (-, Pip-$CH_2$-***C***$H_2$), 27.36 (-, 2 Cyclohexyl-***C***), 27.63 (-, Cyclohexyl-***C***), 32.91 (-, ***C***$H_2$-$CH_2$-CO), 34.14 (-, 2 Cyclohexyl-***C***), 35.46 (-, $CH_2$-***C***$H_2$-CO), 38.40 (+, Cyclohexyl-***C***-1), 39.57 (-, Pip-$(CH_2)_2$-***C***$H_2$), 48.13 (-, Pip-***C***$H_2$), 53.32 (-, 2 Pip-***C***), 55.11 (-, 2 Pip-***C***), 118.11 (+, 2 Ph-***C***), 122.51 (+, Ph-***C***-4), 130.41 (+, 2 Ph-***C***), 151.16 ($C_{quat}$, Ph-***C***-1), 155.56 ($C_{quat}$, ***C***=N), 177.68 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 99 % ($t_R$ = 18.84 min, *k*' = 5.66); HRMS (LSI, glycerine): *m/z* calcd. for $[C_{23}H_{37}N_5O + H]^+$ 400.3076, found: 400.3070. $C_{23}H_{37}N_5O \cdot 2TFA$ (627.61).

***N*²-[3,3-Diphenylpropanoyl)-*N*¹-[3-(4-phenylpiperazin-1-yl)propyl]guanidine (4.17)**

The title compound was prepared from **4.12** (68 mg, 0.30 mmol), EDC (69 mg, 0.36 mmol), HOBt (49 mg, 0.36 mmol), DIEA (105 µL, 0.60 mmol in $DCM_{abs}$ (10 mL) and **4.3** (108 mg, 0.30 mmol) in $DCM_{abs}$ (5 mL) according to the general procedure. Deprotection with a mixture of 3 mL DCM and 3 mL TFA followed by preparative RP-HPLC (25 – 55 % in 30 min) afforded product **4.17** as beige solid (90 mg, 43 %). $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 2.03 – 2.16 (m, 2H, Pip-$CH_2$-C***H***$_2$), 3.18 – 3.33 (m, overlap with solvent, 6H, Pip-***H*** +Pip-C***H***$_2$ +CH-C***H***$_2$), 3.34 – 3.41 (m, 4H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$), 3.42 – 3.85 (bs, 4H, Pip-***H***), 4.60 (t, $^3J$ = 8.0 Hz, 1H, $(Ph)_2$-C***H***), 6.89 – 6.97 (m, 1H, Ph-***H***-4), 6.98 – 7.04 (m, 2H, Ph-***H***), 7.13 – 7.21 (m, 2H, diPh-***H***-4), 7.23 – 7.34 (m, 10H, diPh-***H*** + Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 23.85 (-, Pip-$CH_2$-***C***$H_2$), 39.63 (-, Pip-$(CH_2)_2$-***C***$H_2$), 43.76 (-, CH-***C***$H_2$-CO), 47.97 (+, $(Ph)_2$-***C***H), 48.16 (-, Pip-***C***$H_2$), 53.35 (-, 2 Pip-***C***), 55.10 (-, 2 Pip-***C***), 118.10 (+, 2 Ph-***C***), 122.52 (+, Ph-***C***-4), 127.80 (+, 2 diPh-***C***), 128.82 (+, 4 diPh-***C***), 129.71 (+, 4 diPh-***C***), 130.42 (+, 2 Ph-***C***), 144.54 (+, 2 diPh-***C***-1), 151.16 ($C_{quat}$, Ph-***C***-1), 155.29 ($C_{quat}$, ***C***=N), 175.43 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 100 % ($t_R$ = 20.12 min, *k*' = 6.11); HRMS (LSI, glycerine): *m/z* calcd. for $[C_{29}H_{35}N_5O + H]^+$ 470.2920, found: 470.2925. $C_{29}H_{35}N_5O \cdot 2TFA$ (697.66).

**$N^2$-[3-(4-Hydroxyphenyl)propanoyl)-$N^1$-[3-(4-phenylpiperazin-1-yl)propyl]guanidine (4.18)**

The title compound was prepared from **4.13** (50 mg, 0.30 mmol), EDAC (69 mg, 0.36 mmol), HOBt (49 mg, 0.36 mmol), DIEA (105 µL, 0.60 mmol) in $DCM_{abs}$ (10 mL) and **4.3** (108 mg, 0.30 mmol) in $DCM_{abs}$ (5 mL) according to the general procedure. Deprotection with 3 mL DCM and 3 mL TFA followed by preparative RP-HPLC (15-45 % MeCN in 30 min) afforded product **4.18** as colorless resin (49 mg, 26 %). $^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 2.04 – 2.21 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.73 (t, $^3J$ = 7.6 Hz, 2H, Pip-C***H***$_2$), 2.87 (t, $^3J$ = 7.1 Hz, 2H, $CH_2$-C***H***$_2$-CO), 2.92 – 3.33 (m, overlap with solvent, 4H, C***H***$_2$-$CH_2$-CO + Pip-***H***), 3.34 – 3.46 (m, 4H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$), 3.47 – 3.87 (bs, 4H, Pip-***H***), 6.69 (d, $^3J$ = 8.6 Hz, 2H, Phenol-***H***), 6.89 – 7.03 (m, 1H, Ph-***H***-4), 6.98 – 7.08 (m, 4H, Ph-***H*** + Phenol-***H***), 7.24 – 7.33 (m, 2H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 23.90 (-, Pip-$CH_2$-***C***$H_2$), 30.62 (-, ***C***$H_2$-$CH_2$-CO), 39.57 and 39.95 (-, Pip-$(CH_2)_2$-***C***$H_2$ + $CH_2$-***C***$H_2$-O), 48.15 (-, Pip-***C***$H_2$), 53.34 (-, 2 Pip-***C***), 55.11 (-, 2 Pip-***C***), 116.32 (+, 2 Phenol-***C***), 118.11 (+, 2 Ph-***C***), 122.52 (+, Ph-***C***-4), 130.42 (+, 2 Ph-***C***), 130.49 (+, 2 Phenol-***C***), 151.16 ($C_{quat}$, Ph-***C***-1), 155.44 ($C_{quat}$, ***C***=N), 157.00 ($C_{quat}$, Phenol-***C***-4), 176.61 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 99 % ($t_R$ = 13.94 min, *k*' = 3.93); HRMS (LSI, glycerine): *m/z* calcd. for $[C_{23}H_{31}N_5O_2 + H]^+$ 410.2556, found: 410.2564. $C_{23}H_{31}N_5O_2 \cdot 2TFA$ (637.56).

**$N^1$-[3-(4-Phenylpiperazin-1-yl)propyl]-$N^2$-[3-(2-pyridyl)propanoyl)guanidine (4.19)**

The title compound was prepared from **4.14** (45 mg, 0.30 mmol), EDAC (69 mg, 0.36 mmol), HOBt (49 mg, 0.36 mmol), DIEA (105 µL, 0.60 mmol) in $DCM_{abs}$ (10 mL) and **4.3** (108 mg, 0.30 mmol) in $DCM_{abs}$ (5 mL) according to the general procedure. Deprotection with 3 mL DCM and 3 mL TFA followed by preparative RP-HPLC (15 – 45 % MeCN in 30 min) afforded product **4.19** as pale brown resin (73 mg, 33 %).$^1$H-NMR (300 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 2.05 – 2.20 (m, 2H, Pip-$CH_2$-C***H***$_2$), 2.93 (t, $^3J$ = 7.2 Hz, 2H, Pip-C***H***$_2$), 3.12 (t, $^3J$ = 7.2 Hz, 2H, $CH_2$-C***H***$_2$-CO), 3.22 – 3.33 (m, overlap with solvent, 4H, -$CH_2$-C***H***$_2$-CO + Pip-***H***), 3.34 – 3.60 (m, 8H, Pip-***H*** + Pip-$(CH_2)_2$-C***H***$_2$), 6.90 – 6.97 (m, 1H, Ph-***H***-4), 6.98 – 7.05 (m, 2H, Ph-***H***), 7.23 – 7.34 (m, 2H, Ph-***H***), 7.64 – 7.74 (m, 1H, Pyr-***H***-5), 8.18 (d, $^3J$ = 8.0 Hz, 1H, Pyr-***H***-3), 8.51 – 8.59 (m, 1H, Pyr-***H***-4), 8.61 – 8.70 (m, 1H, Pyr-***H***-6); $^{13}$C-NMR (75 MHz, $CD_3OD$, trifluoroacetate): δ (ppm) 23.87 (-, Pip-$CH_2$-***C***$H_2$), 27.90 (-, ***C***$H_2$-$CH_2$-CO), 38.05 (-, Pip-$(CH_2)_2$-***C***$H_2$), 39.60 (-, $CH_2$-***C***$H_2$-CO), 48.16 (-, Pip-***C***$H_2$), 53.36 (-, 2 Pip-***C***), 55.11 (-, 2 Pip-***C***), 118.10 (+, 2 Ph-***C***), 122.54 (+, Ph-***C***-4), 126.75 (+, Pyr-***C***-5), 130.41 (+, 2 Ph-***C***), 131.58 (+, Pyr-***C***-4), 143.25 (+, Pyr-***C***-6), 146.76 (+, Pyr-***C***-4), 151.16 ($C_{quat}$, Ph-***C***-1), 154.65 ($C_{quat}$, ***C***=N), 157.05 ($C_{quat}$, Pyr-***C***-2), 175.42 ($C_{quat}$, ***C***=O); anal. RP-HPLC (system 1): 94 % ($t_R$ = 10.72 min, *k*' = 2.79); HRMS (EI, 70 eV): *m/z* calcd. for $[C_{22}H_{30}N_6O^+ H]^+$ 394.2481, found: 394.2480. $C_{22}H_{30}N_6O \cdot 3TFA$ (736.57).

## 4.5.2 Pharmacological Methods and Toxicological Investigations

### 4.5.2.1 Aequorin Assay

See section 3.3.2.1

### 4.5.2.2 Flow Cytometric Binding Assay

See section 3.3.2.2

### 4.5.2.3 Determination of Hemolytic Properties Using Mouse Erythrocytes

The hemolytic properties of the selected compounds were determined as described previously.[16]

**General procedure for the isolation and preparation of erythrocytes**

Murine blood was collected in deep anesthesia by cardiac puncture into 200 µL of Alsever's buffer (0.8 % trisodium citrate; 0.05 % citric acid monohydrate; 2.0 % glucose; 0.42 % NaCl) yielding a total volume of 0.5 to 1 mL. The resulting suspension was centrifuged for 15 min (70 g, 4 °C). After removal of the supernatant plasma and the leukocyte-layer, the obtained erythrocytes were re-suspended in isotonic saline (1 mL) prior to centrifugation (2000 g, 10 in, 4 °C). The supernatant was discarded, and the washing procedure was repeated twice. Finally, the isolated erythrocytes were stored on ice before use on the same day.

**Determination of hemolysis**

Stock solutions (10 mM) of selected NPY $Y_4R$ ligands were prepared in 20 %, 50 % or 100 % DMSO prior to dilution to concentrations of 0.5 mM, 1.5 mM and 5 mM in 70 % EtOH for determination of the hemolytic properties.

500 µL of freshly prepared erythrocytes were suspended in 9.5 mL of isotonic saline and a volume of 50 µL of this suspension was pipetted into each well of a 96-well flat-bottomed microtiter plate (Greiner, Frickenhausen, Germany). Subsequently, 1 µL of the respective test compound stock solution was added to obtain the desired final concentrations of 10 µM, 30 µM and 100 µM. As positive control (100 % hemolysis) a solution of Triton-X (10 % w/w) in water was used. For the blank values (0 % hemolysis) solvents without ligand were added. Each sample was prepared in duplicate. After careful mixing, the plate was incubated for 1 h at 37 °C and shaken every 20 min. The plate was centrifuged at 200 g for 3 min and 30 µL of the supernatant was transferred to a new microtiter plate and every well was diluted with 100 µL of isotonic NaCl solution. Absorbance of the samples was

measured at 485 nm and 580 nm using a GENios microplate reader (TECAN Group Ltd, Salzburg, Austria). The hemolytic activity (percentage) was calculated according to the following equation:

$$\%\,\text{hemolysis} = \left( \frac{A_{580nm}}{A_{485nm}} - \frac{A_{580nm(0\%)}}{A_{485nm(0\%)}} \right) - \left( \frac{A_{580nm(100\%)}}{A_{485nm(100\%)}} - \frac{A_{580nm(0\%)}}{A_{485nm(0\%)}} \right) \cdot 100$$

**Equation 4.1**: $A_{485nm}$, $A_{580nm}$ = measured absorbance at 580 nm and 485 nm, respectively; $A_{485nm(0\%)}$, $A_{580nm(0\%)}$ = measured absorbance of the blank (solvent) at 485 nm and 580 nm, respectively; $A_{485nm(100\%)}$, $A_{580nm(100\%)}$ = measured absorbance of the positive control (Triton-X) at 485nm and 580 nm, respectively

#### 4.5.2.4 Chemosensitivity Assay

The chemosensitivtiy assay was performed as previously reported by Bernhardt et al. [10]: Tumor cells (HT-29, passage 98 and 135, respectively) were seeded (100 µL/well) into 96-well flat bottom plates (Greiner, Frickenhausen, Germany) at a density of ca. 15 cells/microscopic field (magnificaiton 320-fold). After 48 – 72 h of incubation (dependent on the confluency of the cells) at 37 °C and 5 % $CO_2$, the culture medium (McCoy's + 5 % FCS) was removed and replaced by fresh medium containing the NPY $Y_4R$ ligands at varying concentrations. On every plate, 16 wells served as controls and 16 wells were used for each compound concentration. After various periods of incubation, the medium was discarded, the cells were fixed with 1 % glutardialdehyd solution in PBS and the plates were stored at 4 °C. At the end of the experiment, all plates were simultaneously stained with 0.02 % aqueous crystal violet (Serva, Heidelberg, Germany) solution. Then, the excess of the dye was removed by washing with water prior to dissolving the cell-bound crystal violet with 70 % ethanol. The absorbance was measured at 580 nm using a GENios Pro microplate reader (TECAN Group Ltd, Salzburg, Austria). Growth curves were constructed with Sigma plot 11.0 analysis software (Systat Software GmbH, Erkrath, Germany).

## 4.6 References

1. Pop, N.; Igel, P.; Brennauer, A.; Cabrele, C.; Bernhardt, G. N.; Seifert, R.; Buschauer, A. Functional reconstitution of human neuropeptide Y (NPY) Y(2) and Y(4) receptors in Sf9 insect cells. *J. Recept. Signal Transduct. Res.* **2011,** 31, 271-285.
2. Wadsworth, W. S.; Emmons, W. D. The Utility of Phosphonate Carbanions in Olefin Synthesis. *J. Am. Chem. Soc.* **1961,** 83, 1733-1738.
3. Yung-Chi Cheng, W. H. P. Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzymatic reaction. *Biochem. Pharm.* **1973,** 22, 3099-3108.

4. Ziemek, R. Development of binding and functional assays for the neuropeptide Y Y 2 and Y 4 receptors. Doctoral Thesis, Regensburg, **2006**.
5. Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Determination of Affinity and Activity of Ligands at the Human Neuropeptide Y Y4 Receptor by Flow Cytometry and Aequorin Luminescence. *J. Recept. Signal. Transduct. Res.* **2007,** 27, 217 - 233.
6. Schneider, E.; Mayer, M.; Ziemek, R.; Li, L.; Hutzler, C.; Bernhardt, G.; Buschauer, A. A simple and powerful flow cytometric method for the simultaneous determination of multiple parameters at G protein-coupled receptor subtypes. *ChemBioChem* **2006,** 7, 1400-1409.
7. Schreier, S.; Malheiros, S. V. P.; de, P. E. Surface active drugs: self-association and interaction with membranes and surfactants. Physicochemical and biological aspects. *Biochim. Biophys. Acta, Biomembr.* **2000,** 1508, 210-234.
8. Thoren, P. E. G.; Soederman, O.; Engstroem, S.; Von, C. C. Interactions of Novel, Nonhemolytic Surfactants with Phospholipid Vesicles. *Langmuir* **2007,** 23, 6956-6965.
9. Tragner, D.; Csordas, A. Biphasic interaction of Triton detergents with the erythrocyte membrane. *Biochem. J.* **1987,** 244, 605-609.
10. Bernhardt, G.; Reile, H.; Birnbock, H.; Spruss, T.; Schoenenberger, H. Standardized Kinetic Microassay to Quantify Differential Chemosensitivity on the Basis of Proliferative Activity. *J. Cancer Res. Clin.* **1992,** 118, 35-43.
11. Kraus, A.; Ghorai, P.; Birnkammer, T.; Schnell, D.; Elz, S.; Seifert, R.; Dove, S.; Bernhardt, G.; Buschauer, A. NG-acylated aminothiazolylpropylguanidines as potent and selective histamine H2 receptor agonists. *ChemMedChem* **2009,** 4, 232-240.
12. Hardouin, C.; Kelso, M. J.; Romero, F. A.; Rayl, T. J.; Leung, D.; Hwang, I.; Cravatt, B. F.; Boger, D. L. Structure-Activity Relationships of α-Ketooxazole Inhibitors of Fatty Acid Amide Hydrolase. *J. Med. Chem.* **2007,** 50, 3359-3368.
13. Claridge, T. D. W.; Davies, S. G.; Lee, J. A.; Nicholson, R. L.; Roberts, P. M.; Russell, A. J.; Smith, A. D.; Toms, S. M. Highly (E)-Selective Wadsworth-Emmons Reactions Promoted by Methylmagnesium Bromide. *Org. Lett.* **2008,** 10, 5437-5440.
14. Davies, S. J.; Ayscough, A. P.; Beckett, R. P.; Bragg, R. A.; Clements, J. M.; Doel, S.; Grew, C.; Launchbury, S. B.; Perkins, G. M.; Pratt, L. M.; Smith, H. K.; Spavold, Z. M.; Thomas, S. W.; Todd, R. S.; Whittaker, M. Structure-activity relationships of the peptide deformylase inhibitor BB-3497: modification of the methylene spacer and the P1' side chain. *Bioorg. Med. Chem. Lett.* **2003,** 13, 2709-2713.
15. Zhang, Q.; Jiang, Z.-Y.; Luo, J.; Ma, Y.-B.; Liu, J.-F.; Guo, R.-H.; Zhang, X.-M.; Zhou, J.; Niu, W.; Du, F.-F.; Li, L.; Li, C.; Chen, J.-J. Anti-HBV agents. Part 3: Preliminary structure-activity

relationships of tetra-acylalisol A derivatives as potent hepatitis B virus inhibitors. *Bioorg. Med. Chem. Lett.* **2009,** 19, 6659-6665.

16. Müller, M. *N, N*-Disubstituierte ω-Guanidino und ω-Aminocarbonsäureamide als neue Leitstrukturen zur Entwicklung nichtpeptidischer Neuropeptid Y Y1-Antanogisten. Synthese, i*n vitro*-Pharmakologie und Molecular Modelling. Doctoral Thesis, Regensburg, **1995**.

# CHAPTER 5

# From Monovalent and Bivalent Argininamide-Type NPY $Y_1R$ Ligands to $Y_4R$ Antagonists

# 5.1 Introduction

Surprisingly, the determination of NPY receptor selectivity data of bivalent, $N^G$-acylated BIBP-3226-derived $Y_1R$ antagonists (prepared by Max Keller, University of Regensburg)[1] revealed $Y_4R$ affinities in the low micromolar range for some of these compounds. In functional investigations these ligands showed moderate $Y_4R$ antagonistic activity. This type of argininamides (cf. compound **5.1** in **Figure 5.1**) offered a new starting point for the synthesis of non-peptidic ligands for the NPY $Y_4R$. After discontinuation of the project on $N^G$-acylated hetarylpropylguanidines (Chapter 3 and 4), the arginine derivatives were considered much more promising, because adverse effects have not been observed to the same extent, when such compounds were studied in cellular assays. Moreover, the activity of **5.1** at the $Y_4R$ was already in the one-digit micromolar range.

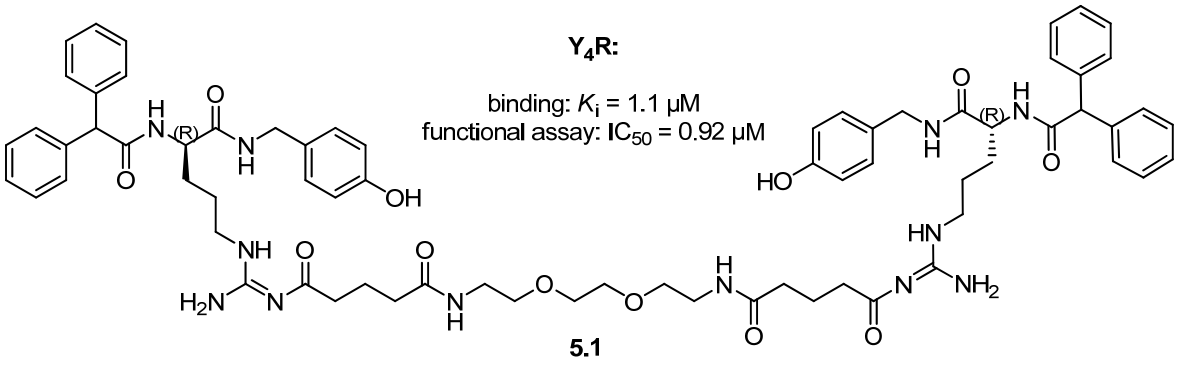



**Figure 5.1**: Bivalent $N^G$-acylated argininamide **5.1**, initially designed as NYP $Y_1R$ ligand.

In 1995, homology-based approaches, including DNA primers as well as oligonucleotides derived from different NPY $Y_1R$ species, led to the identification of a novel NPY receptor subtype with binding preference for hPP, later named PP1/$Y_4$ receptor, independently discovered by Lundell et al. and Bard et. al.[2, 3] Subsequent studies clearly demonstrated that these two receptor subtypes share the highest sequence homology within the NPY receptor family with a 42 % overall identity.[4, 5] As a consequence, compounds formerly developed as agonists for the NPY $Y_1R$ and considered selective for this subtype, e.g. [Leu$^{31}$,Pro$^{34}$]NPY [6] or GW1229 [7], turned out to bind to the $Y_4R$ in nanomolar range, too.[8, 9] In spite of different binding affinity of the endogenous ligands NPY, PYY and PP, both receptors obviously share a similar binding mode for peptidic ligands. Hence, $Y_4R$ affinity of non-peptidic compounds such as **5.1** appears comprehensible. Otherwise, investigations of NPY $Y_1R$ antagonists BIBP 3226 and BIBO 3304, developed by Boehringer Ingelheim Pharma, as well as of numerous especially monovalent argininamide-type $Y_1R$ antagonists, designed in our workgroup, resulted in high $Y_1R$ subtype selectivity to date. So might this $Y_4R$ affinity be reserved to bivalent ligands? To answer this question the monovalent and bivalent ligand approach was revisited, finally leading to the identification of the first $Y_4R$ antagonist with affinity in the nanomolar range ($K_i$ =

132 nM). The discovery of this highly potent $Y_4R$ ligand was only possible by stepwise identification of structure-activity relationships as well as properties considered necessary for $Y_4R$ binding as outlined in **Figure 5.2**.

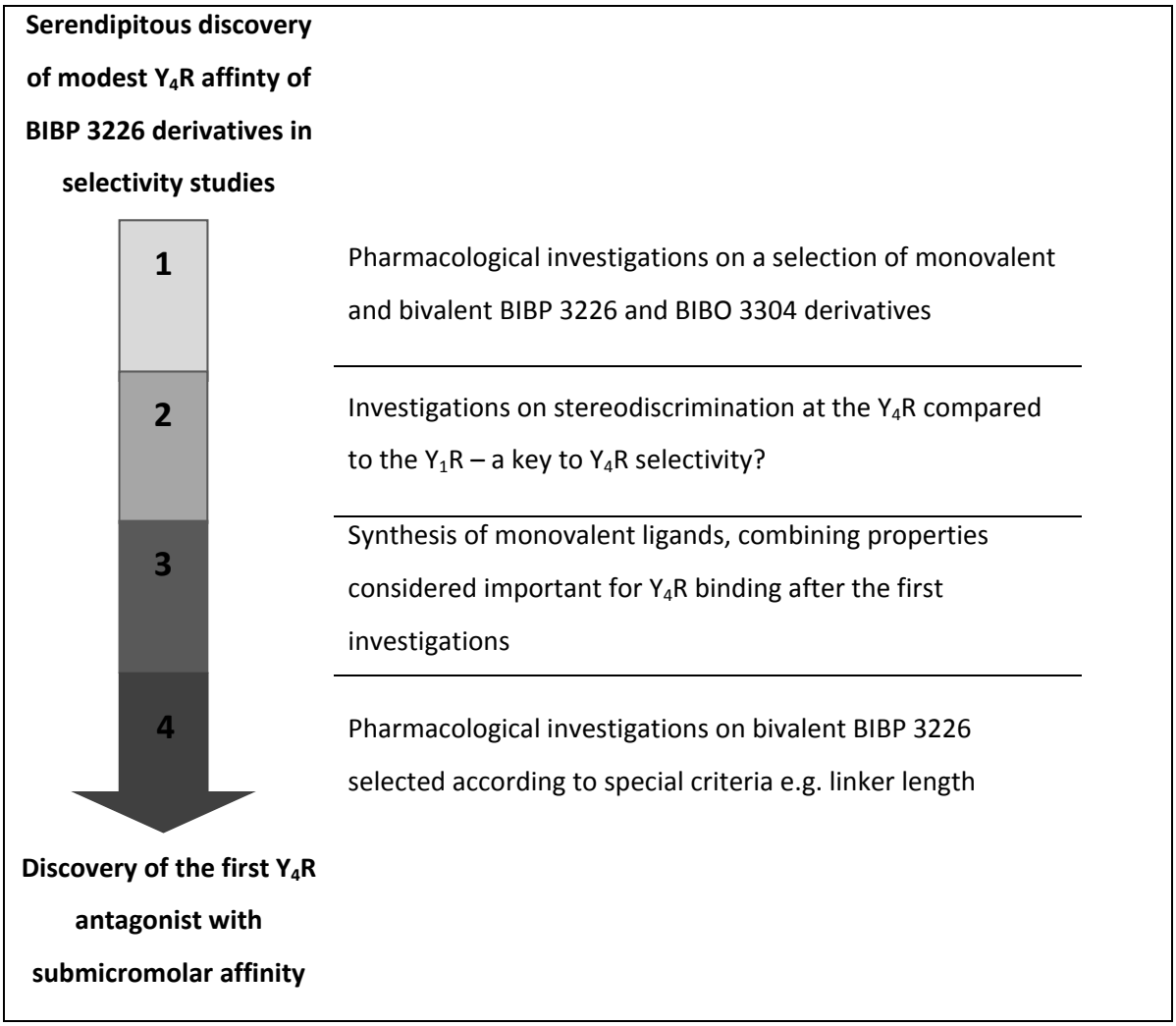



**Figure 5.2:** Procedure for the identification of the first $Y_4R$ antagonist with submicromolar affinity.

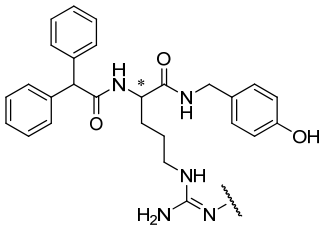
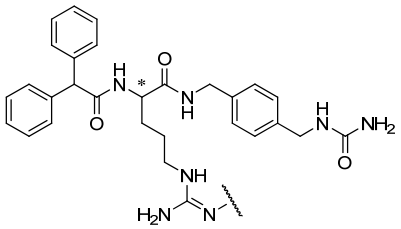
# 5.2 Pharmacological Investigations of Argininamide-Type $Y_1R$ Ligands for Activity at the $Y_4R$

## 5.2.1 Criteria for the Selection of Appropriate Monovalent and Bivalent Argininamide-Type Structures

In a first approach a set of monovalent and bivalent BIBP 3226 and BIBO 3304 derivatives was investigated for $Y_4R$ affinity in flow cytometric binding studies as well as the antagonistic activity in the aequorin assay. The synthesis of the investigated ligands was already described elsewhere.[1, 10-13]

The selection of monovalent argininamide-type compounds, summarized in **Table 5.1**, aimed at covering a wide range of different functional groups (hydroxyl, carboxyl, amine, guanidine, ester, pyridyl) as well as spacers different in chemical nature and length. Additionally, with respect to the $Y_4R$ activity of the bivalent ligand **5.1** (**Figure 5.1**), one may speculate if the second pharmacophoric entity is crucial for $Y_4R$ receptor binding or if it can be replaced by a much simpler moiety such as a guanidine group. Therefore, numerous structures bearing guanidine, amino and pyridyl moieties (**5.3 – 5.8**, **5.12**, **5.14** – **5.18, Table 5.1**) with different spacer lengths between argininamide and functional group were chosen. Furthermore, the structures of the spacers, comprising simple alkanediyl chains, less hydrophobic glycol or amide substructures as well as more rigid moieties such as triazoles (**5.11 – 5.14** and **5.16, Table 5.1**) was varied.

**Table 5.1**: Structures of (*R*)-configured monovalent argininamide-type ligands, which were selected for pharmacological investigations at the NPY $Y_4$R.

$R^1$ =  $R^2$ = 

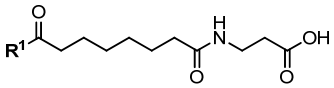
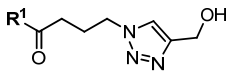
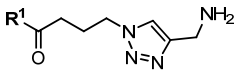
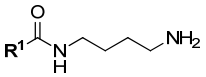
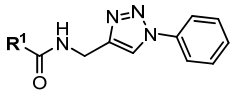
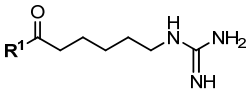
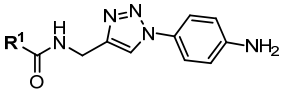
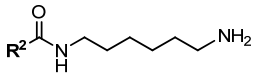
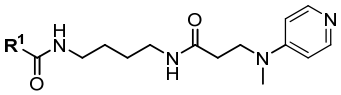
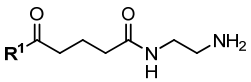
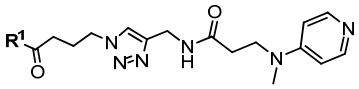
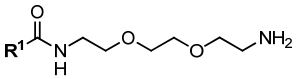
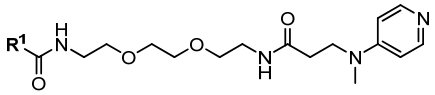
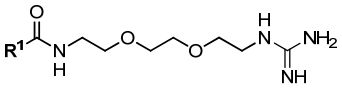
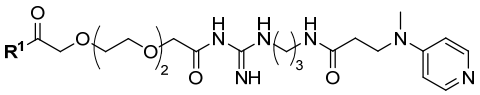
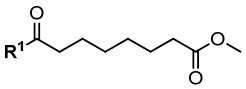
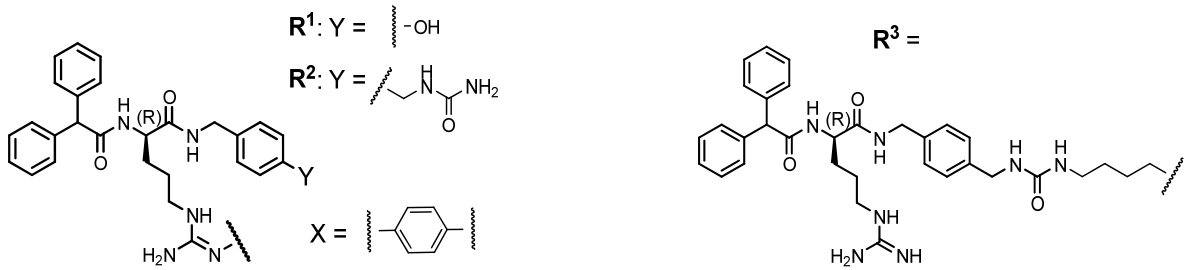
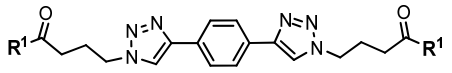
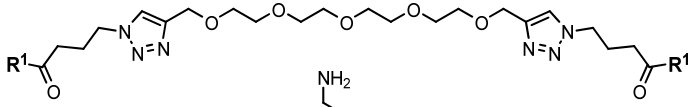
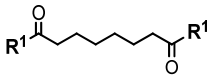
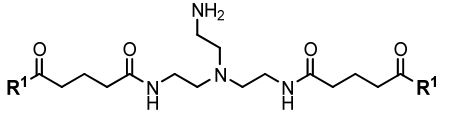
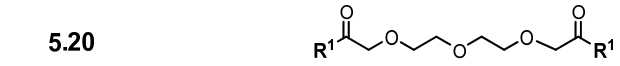
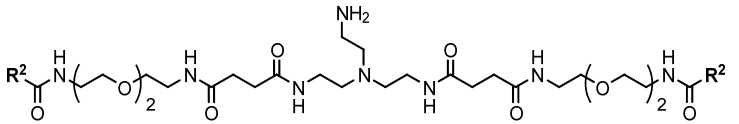
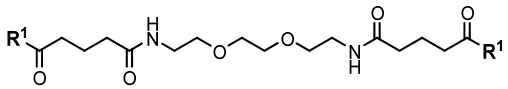
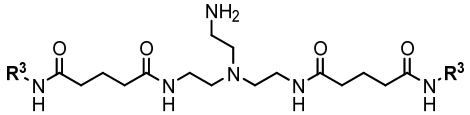
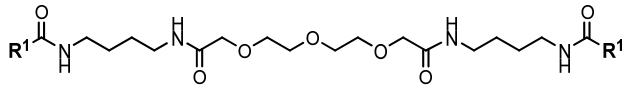
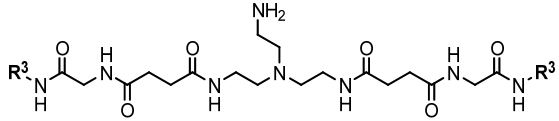
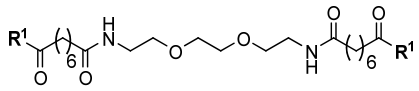
| No. | spacer | No. | spacer |
|---|---|---|---|
| **BIBP 3226** | $R^1$-H | **5.10** |  |
| **BIBO 3304** | $R^2$-H | **5.11** |  |
| **5.2** |  | **5.12** |  |
| **5.3** |  | **5.13** |  |
| **5.4** |  | **5.14** |  |
| **5.5** |  | **5.15** |  |
| **5.6** |  | **5.16** |  |
| **5.7** |  | **5.17** |  |
| **5.8** |  | **5.18** |  |
| **5.9** |  | | |

**Table 5.2** shows the structures of bivalent BIBP 3226 and BIBO 3304 derivatives, which were selected for the determination of $Y_4$R affinity. The essential selection criterion was the length of the linker separating the pharmacophoric moieties (and the guanidine groups, respectively), in order to identify possible correlations between spacer length and $Y_4$R affinity. The influence of the basic amino group attached to a nitrogen atom in the center of the linker on receptor binding should be analyzed, too. Furthermore, bivalent ligands derived from BIBO 3304 in which the argininamide portion is linked via the urea moiety (**5.28** and **5.29**), should be investigated.

**Table 5.2**: Structures and linker lengths of (*R*,*R*)-configured bivalent argininamide-type ligands investigated at the NPY $Y_4R$.




| No. | linker | linker length[a] | No. | linker | linker length[a] |
|---|---|---|---|---|---|
| BIBP | $R^1$-H | 0 | 5.24 |  | 18 |
| BIBO | $R^2$-H | 0 | 5.25 |  | 29 |
| 5.19 |  | 8 | 5.26 |  | 17 |
| 5.20 |  | 11 | 5.27 |  | 37 |
| 5.21 |  | 20 | 5.28 |  | 27 |
| 5.22 |  | 25 | 5.29 |  | 31 |
| 5.23 |  | 26 | | | |

[a] distance between guanidine moieties given in number of atoms

## 5.2.2 Pharmacological Results and Discussion

The selected monovalent and bivalent BIBP 3226 and BIBO 3304 derivatives were investigated in flow cytometric binding studies for determination of $Y_4R$ affinity using CHO-h$Y_4$-qi5-mtAEQ cells. First, the displacement of Cy5-[$K^4$]hPP (c = 3 nM, $K_D$ = 5.62) at concentrations of 1 and 10 µM was determined. For compounds showing more than 50 % displacement of Cy5-[$K^4$]hPP at 10 µM, additional data were collected to construct competition binding curves. Results are given as $K_i$ values calculated according to the Cheng-Prusoff equation [14] (**Table 5.3**). Additionally, all compounds were investigated for functional activity at the NPY $Y_4R$ in a luminescence based $Ca^{2+}$-assay (aequorin assay) using the same cell line. By analogy with the binding studies, all compounds were first screened at 10 µM for antagonistic activity. Only for the most promising candidates $IC_{50}$ values were determined. In the following (**Table 5.3**), antagonistic activities are expressed as $IC_{50}$ and $K_B$ values, determined from the concentration-dependent inhibition of the luminescence signal elicited by hPP (100 nM).[15] $K_B$ values were calculated according to the Cheng-Prusoff equation[14]. Agonistic activity of the compounds was checked for some candidates; the corresponding results are given as percentage of calcium signal (luminescence) elicited by the compounds (10 µM) referred to the response induced by the standard agonist hPP at a concentration of 100 nM (**Table 5.3**). The $Y_1R$ affinities of the studied compounds are given as $K_i$ values determined by radioligand competition binding experiments using [$^3$H]UR-MK114 as a $Y_1R$ selective radioligand (**Table 5.3**).[16]

Unfortunately, most of the investigated monovalent argininamides (**5.2**, **5.3**, **5.10** – **5.14, Table 5.3**) showed neither affinity nor functional activity at the NPY $Y_4R$. This was comparable to the results for the parent compounds BIBP 3226 and BIBO 3304. In particular, ligands carrying a carboxyl (**5.10**), hydroxyl (**5.11**) or simple phenyl (**5.12**) moiety were completely inactive. Only, compounds bearing an amino, guanidine or pyridyl functionality in appropriate distance (> 10 atoms) to the guanidine moiety of the building block, showed moderate affinity at high micromolar concentrations (compounds **5.7**, **5.8**, **5.15** – **5.17**, Table 5.3). Accordingly, these compounds displayed only low antagonistic activities in the aequorin assay. Therefore, the determination of an $IC_{50}$ value wasn't possible for all compounds due to cell-damaging effects at concentrations higher than 10 µM (**5.4**, **5.15**, **5.16**). For these compounds $IC_{50}$ values are estimated to be in the range between 10 and 20 µM. In conclusion, the data collected for the monovalent argininamides give rise to speculate about structural requirements for $Y_4R$ binding of BIBP 3226 derivatives bearing a basic moiety, which is attached to the acyl- or carbamoylguanidine in a distance longer than 10 atoms with regard to the guanidine moiety. Otherwise, in view of the investigations performed to date the structural and chemical properties of the spacers in monovalent argininamides do not seem to play an important

role in receptor binding. This is supported by examples **5.15**, **5.16** and **5.17**, which show similar binding properties and have spacers of comparable spacer length but quite different substructures.

The screening of the bivalent BIBP 3226 and BIBO 3304 derivatives afforded more promising results. Investigation of compounds **5.19 – 5.27** revealed substantially higher affinities than those of the monovalent ligands, with $K_i$ values in the upper nanomolar range. The activities of the bivalent argininamide-type ligands are more sensitive to changes of the chemical nature and length of the linker compared to the monovalent ligands. Considering compounds **5.20** – **5.22**, sharing a similar diethyleneglycol substructure, a significant increase in affinity by a factor of 20 results from the extension of the linker length from 11 (**5.20,** $K_i$ = 8210 nM) to 25 (**5.22,** $K_i$ = 370 nM) atoms. Interestingly, compound **5.23** ($K_i$ = 997 nM) with very similar structural features as **5.22** ($K_i$ = 370 nM) showed a slightly decreased (3-fold) $Y_4R$ affinity, which may be caused by exchange of the carbamoylguanidine (**5.22**) by a bioisosteric acylguanidine (**5.23**) moiety. The superiority of the carbamoyl- compared to the acylguanidine substructure was already reported for argininamide-type antagonists at the $Y_1R$ by Keller et al. and Brennauer et al.[16, 17] The increased affinity of carbamoylguanidines in comparison with the corresponding acylguanidines at $Y_1R$ and $Y_4R$ might be due to slightly higher basicity, i.e. a higher percentage of positively charged molecules capable of interacting with a key aspartate, and an additional H-donor group enabling accessory affinity-enhancing interactions.

Surprisingly, ligand **5.26** binds to the $Y_4R$ in a similar submicromolar range ($K_i$ = 434 nM) as compound **5.22** ($K_i$ = 370 nM) in spite of a reduced linker length (17 vs. 25 atoms). The peculiarity of **5.26** compared to **5.22** is an additional free amino group placed in the center of the linker and positively charged under physiological conditions. In contrast, a short linker with reduced flexibility – p-phenylene and triazole moieties – as in **5.24** (19 atoms, $K_i$ = 2320 nM) seems less tolerated. A similarly unfavorable effect ($K_i$ = 3135 nM) despite a rather long spacer (29 atoms) was observed for **5.25**, which includes a triazole moiety in vicinity to the guanidine groups of the building block. Obviously, conformational constraints in this position lead to a decrease of affinity. Compound **5.23**, which has a more flexible linker (26 atoms, glycol-substructure) compared to **5.25** (29 atoms, triazole-phenylene substructure, $K_i$ = 3135 nM) reveals an increased $K_i$ value of 434 nM. Highest affinity for the $Y_4R$ within the first set of tested compounds was obtained for **5.27** with a $K_i$ of 287 nM. Unlike the other investigated compounds, **5.27** is a bivalent argininamide derived from BIBO 3304, which differs from BIBP 3226 by a urea moiety instead of hydroxyl group. In addition, compound **5.27** provides the longest spacer (37 atoms) of all bivalent compounds tested to date as well as a free amino group in the center of the linker. This basic moiety might confer additional binding as already suggested for compound **5.26** ($K_i$ = 434 nM)**.** By then, it seemed impossible to

consider special structural features responsible for the increase of $Y_4$R affinity. Interestingly, the linkage of two BIBO 3304 building blocks via the urea moiety instead of the guanidine substructures led to a tremendous decrease in affinity (cf. **5.28**: 27 atoms, $K_i$ = 3615 nM and **5.29**: 31 atoms, $K_i$ = 2176 nM) compared to the bivalent compound **5.22** (Ki = 370 nM, 25 atoms) with comparable linker length but connection via the guanidine moieties. Taking into consideration the suggested binding mode of the argininamides at the $Y_1$R, where the substituted benzyl residue is supposed to be oriented between the transmembrane domains, and assuming similar interactions at the $Y_4$R to be possible, obviously, the urea group is an inappropriate position to attach such an extended spacer as in case of the $Y_1$R. By contrast, the guanidino group of the argininamides is suggested to interact with an acidic amino acid close to the extracellular loop regions of the NPY receptor, i. e., there should be more space left to attach a bulky linker.

All functional studies performed with the monovalent and bivalent BIBP3226 and BIBO3304 derivatives revealed antagonistic activity in good correlation with the determined $Y_4$R affinities (**Table 5.3**).

**Table 5.3**: Pharmacological data for selected (*R*)-configured monovalent and (*R*,*R*)-configured bivalent BIBP 3226 and BIBO 3304 derivatives, investigated for NPY $Y_4$R and $Y_1$R binding and $Y_4$R antagonistic activity.

| No. | **$hY_4R$** | | | | **$hY_1R$** |
|---|---|---|---|---|---|
| | % Relative Potency[a] | $IC_{50}$ [nM][b] | $K_B$ [nM][c] | $K_i$ [nM][d] | $K_i$ [nM][e] |
| **BIBP 3226** | 7.9 ± 1.6 | > 10000 | - | > 10000 | 1.3 ± 0.1 |
| **BIBO 3304** | 0.3 ± 0.7 | > 10000 | - | > 10000 | 0.25 ± 0.01 |
| **5.2** | 1.4 ± 2.2 | > 10000 | - | > 10000 | 7.8 ± 3.6 |
| **5.3** | 0.8 ± 3.7 | > 10000 | - | > 10000 | 4.3 ± 0.3 |
| **5.4** | n.d. | > 10000 | - | 5500 ± 390 | 51 ± 26 |
| **5.5** | 1.6 ± 2.0 | > 10000 | - | > 10000 | 203 ± 90 |
| **5.6** | 1.2 ± 3.9 | > 10000 | - | > 10000 | n.d. |
| **5.7** | n.d. | 7100 ± 1700 | 0.95 ± 0.23 | 8200 ± 3200 | 7.3 ± 1.0 |
| **5.8** | n.d. | 3300 ± 100 | 0.46 ± 0.01 | 3800 ± 100 | 96 ± 11[f] |
| **5.9** | 172 ± 26 [g] | 1100 ± 200 | 0.28 ± 0.41 | 22200 ± 3500 | 0.94 ± 0.06 |
| **5.10** | n.d. | > 10000 | - | > 10000 | 73 ± 16 |

**Table 5.3** continued

| No. | $hY_4R$ | | | | $hY_1R$ |
|---|---|---|---|---|---|
| | % relative potency[a] | $IC_{50}$ [nM][b] | $K_B$ [nM][c] | $K_i$ [nM][d] | $IC_{50}$ [nM][b] |
| **5.11** | n.d. | > 10000 | - | > 10000 | 2.6 ± 0.9 |
| **5.12** | n.d. | > 10000 | - | > 10000 | 6.7 ± 2.0 |
| **5.13** | n.d. | > 10000 | - | > 10000 | 3.1 ± 8.9 |
| **5.14** | n.d. | > 10000 | - | > 10000 | 4.0 ± 0.3 |
| **5.15** | n.d. | > 10000 | - | 3400 ± 1400 | 96 ± 28 |
| **5.16** | n.d. | > 10000 | - | 7200 ± 900 | 36 ± 0.3 |
| **5.17** | n.d. | 10000 ± 500 | | 1900 ± 100 | 90 ± 6.0 |
| **5.18** | n.d. | > 10000 | - | > 10000 | 20 ± 1.2 |
| **5.19** | 7.6 ± 2.2 | 732 ± 431 | 98 ± 57 | 1266 ± 462 | 62 ± 13 |
| **5.20** | n.d. | > 10000 | - | 8210 ± 2810 | 6.1 ± 2.7 |
| **5.21** | n.d. | 919 ± 175 | 123 ± 23 | 1070 ± 296 | 8.6 ± 2.2 |
| **5.22** | n.d. | 878 ± 215 | 118 ± 28 | 370 ± 7.0 | 28 ± 6.9 |
| **5.23** | n.d. | 1358 ± 698 | 182 ± 93 | 997 ± 90 | 41 ± 5 |
| **5.24** | n.d. | > 10000 | - | 2320 ± 280 | 43 ± 17 |
| **5.25** | n.d. | 1718 ± 1149 | 230 ± 154 | 3135 ± 1460 | 58 ± 17 |
| **5.26** | 15.6 ± 1.6 | 542 ± 86 | 73 ± 11 | 434 ± 139 | 30 ± 4 |
| **5.27** | 3.7 ± 0.3 | 713 ± 40 | 95 ± 1.0 | 286 ± 67 | 230 ± 24 |
| **5.28** | 0.9 ± 1.9 | > 10000 | - | 3615 ± 784 | 330 ± 95 |
| **5.29** | n.d. | 6737 ± 2956 | 904 ± 396 | 2176 ± 160 | 200 ± 8 |

[a] $[Ca^{2+}]_i$ mobilization determined at a concentration of 10 µM test compound (performed in triplicate) given in % referred to the $[Ca^{2+}]_i$ mobilization induced by the standard agonist hPP (100 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells. [b] $Y_4R$ antagonism: inhibition of 100 nM hPP induced $[Ca^{2+}]_i$ mobilization in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate.[c] $K_B$ calculated according to the Cheng Prusoff equation[14] (c = 100 nM, $EC_{50}$ = 15.5 nM). [d] Flow cytometric binding assay using 3 nM Cy5-[$K^4$]hPP ($K_D$ = 5.6 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [e] $K_i$ from radioligand binding studies, calculated according to the Cheng-Prusoff equation[14] ($K_D$ ([$^3$H]-UR-MK114) = 1.2 nM, c = 1.5 nM) on SK-N-MC cells. [f] Determined by flow cytometry on HEL cells using 5 nM Cy5-pNPY ($K_D$ = 5.2). [g] This value does not represent $Y_4R$ agonistic activity; instead the "calcium signal" is elicited by cytotoxic effects comparable to those described in Chapter 4.

# 5.3 Enantiomers of Argininamide Derivatives: Stereodiscrimination at $Y_4R$ and $Y_1R$

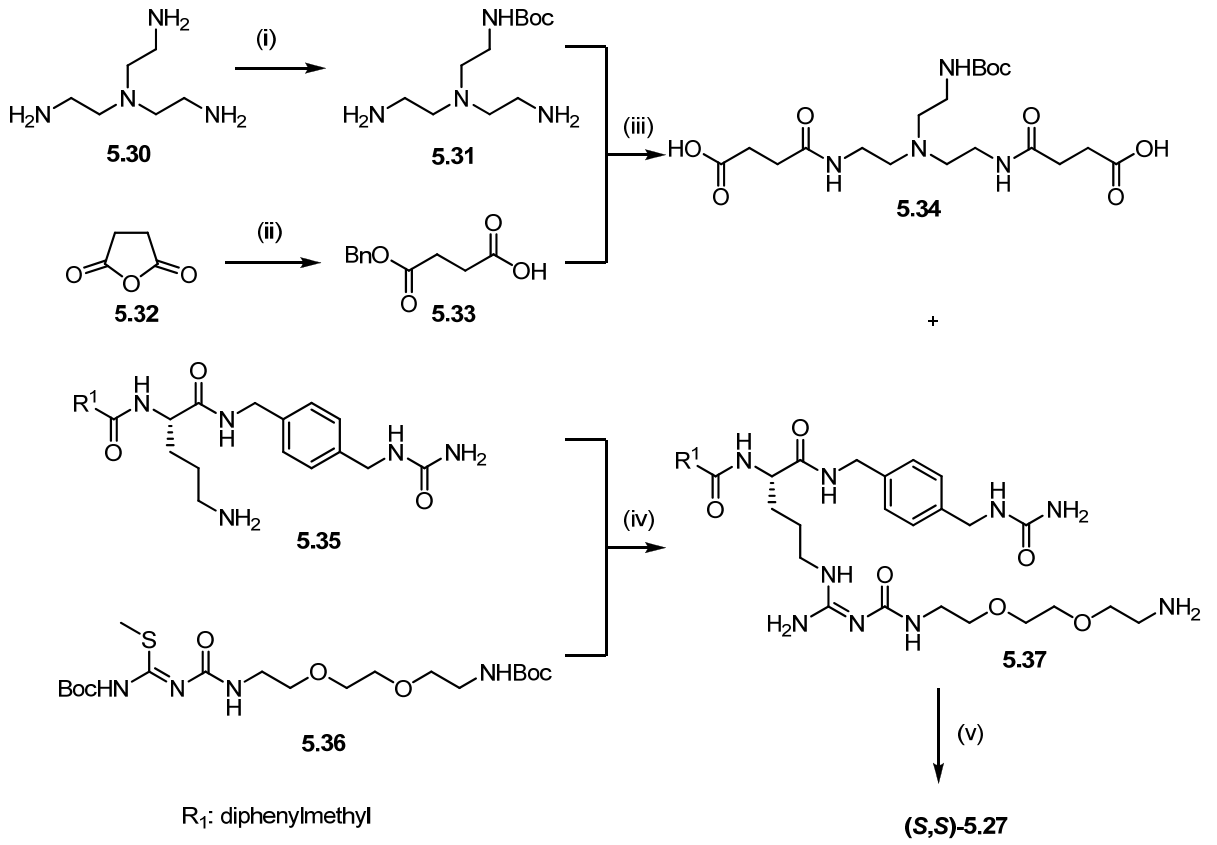
## 5.3.1 Introduction

As the bivalent $Y_1R$ antagonist **5.27**, which is derived from BIBO 3304, exhibits $Y_4R$ affinity in the high nanomolar range, this result was regarded a promising starting point for the development of the first non-peptidic $Y_4R$ ligands. However, one had to be aware that the $Y_4R$ antagonistic properties were more or less a side-effect of the investigated compounds, as these were initially designed as NPY $Y_1R$ antagonists and have $Y_1R$ affinities in the lower nanomolar range as outlined in **Table 5.3**.

As often experienced in drug discovery, biological targets such as enzymes and receptors discriminate between different stereochemical configurations. While one stereoisomer reveals a high affinity for a specified biological target, the other turns out to be inactive or even less potent. In case of argininamide-type antagonists at the $Y_1R$, highest activity resides in the (*R*)-configured enantiomers, as demonstrated, e.g., for the parent compound BIBP 3226 itself, which is at least by a factor of 1000 more potent than its distomer, the (*S*)-argininamide BIBP 3435.[18] If no stereodiscrimination could be observed in case of the $Y_4R$, this could be considered a good starting point for the synthesis of $Y_4R$ selective compounds derived from BIBP 3226 or BIBO 3304 derivatives. Fortunately, several monovalent and bivalent BIBP 3226 derivatives were also available in the (*S*)-configured form and could be tested for $Y_4R$ affinity as summarized in **Table 5.4**. Additionally, the (*S*,*S*)-configured enantiomer of compound **5.27** identified as most potent bivalent $Y_4R$ antagonists in the previous investigations, was synthesized as it was considered most promising as a "lead structure" for the development of potent $Y_4R$ ligands.

## 5.3.2 Synthesis of the Bivalent BIBO 3304 Derivative (*S*,*S*)-5.27

The bivalent ligand **(*S*,*S*)-5.27** was accessible by a five step synthesis (**Scheme 5.1**) starting from the preparation of the linker **5.34** by amide coupling of mono-Boc-protected *N*,*N*'-bis(2-minoethyl)ethane-1,2-diamine (**5.31**) and mono-benzyl protected succinic acid (**5.33**), affording **5.34**. The pertinent mono-Boc-protected amine **5.30** was prepared according to a published protocol[19] whereas the mono-benzyl-protected dicarboxylic acid **5.33** was obtained by refluxing succinic anhydride in benzyl alcohol. Besides, the BIBO 3304 building block **5.35** (for synthesis cf. section 5.4) was treated with the guanidinylating reagent **5.36** (provided by Max Keller, University of Regensburg) in the presence of $HgCl_2$. After Boc-deprotection in TFA (20 %) compound **5.37** was coupled with the linker **5.34**, yielding **(*S*,*S*)-5.27**.




**Scheme 5.1:** Synthesis of the (*S,S*)-configured enantiomer of the bivalent ligand **5.27**. Reagents and conditions: (i) $(Boc)_2O$, DCM, -78 °C to rt, overnight; (ii) benzyl alcohol (1.2 eq), reflux, overnight; (iii) EDAC (2.2 eq), DIEA (2.2 eq), DCM, rt, overnight; (iv) $HgCl_2$ (2 eq), $Et_3N$ (3 eq), DCM, rt, 24 h; (v) a)TBTU (2.4 eq), HOBt (2.4 eq), DIEA (4 eq), DMF, rt, overnight; b) TFA (20 %), DCM, rt, 5 h.

## 5.3.3 Pharmacological Results and Discussion

The stereoisomeric monovalent and bivalent BIBP 3226 and BIBO 3304 derivatives were analyzed for $Y_4R$ binding affinity by flow cytometry and functional activity in a luminescence based $Ca^{2+}$-assay (aequorin assay) at the NPY $Y_4R$ as described in section 5.2.1.2.

The investigation of (*R*)- and (*S*)-configured monovalent ligands (data summarized in **Table 5.4**) revealed quite promising results, stimulating the search for non-peptidic selective $Y_4R$ ligands. As expected, the (*R*)-enantiomers were the eutomers at the $Y_1R$. The (*R*)-configured compounds **5.9**, **5.7** and **5.8** showed 15 to 90 times higher $Y_1R$ affinities than the corresponding (*S*)-enantiomers (**Table 5.4**). By contrast, no discrimination of stereoisomers could be observed for the $Y_4R$. This tendency was also confirmed for the bivalent BIBP 3226 derivative **5.21** as visualized in **Figure 5.3**.

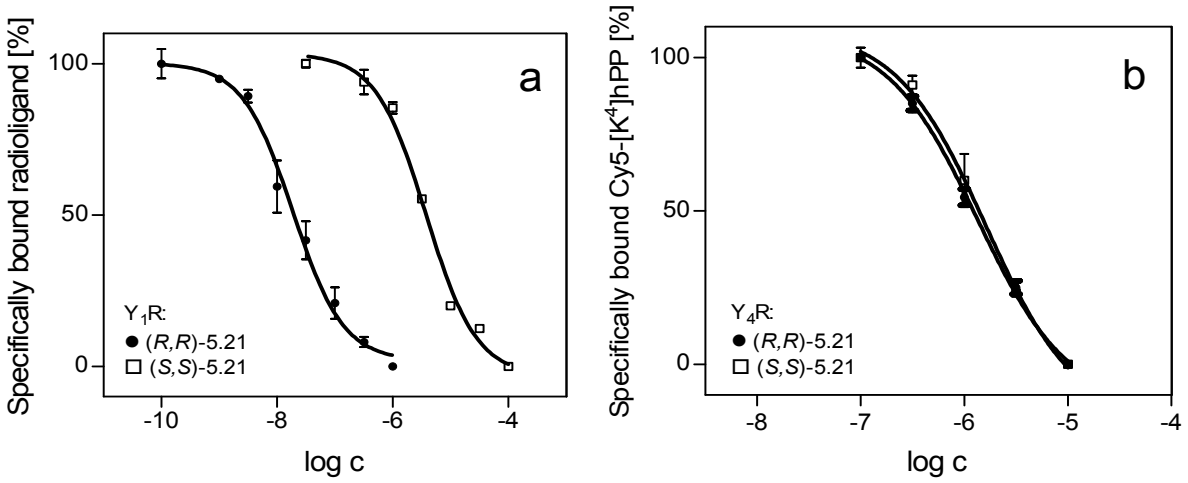
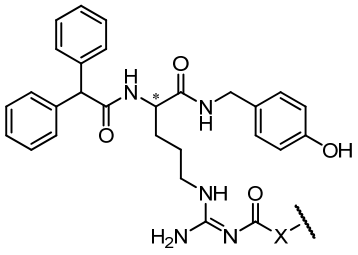
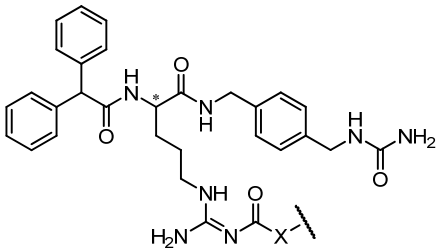


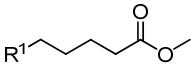
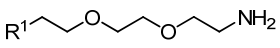
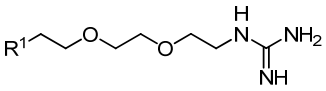
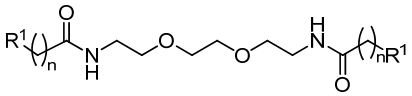
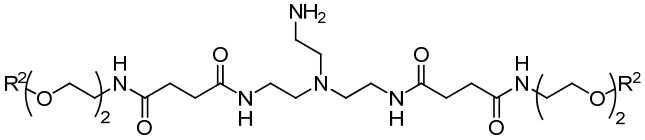

**Figure 5.3**: $Y_1R$ and $Y_4R$ binding properties of the (*R,R*)- and (*S,S*)-configured stereoisomer of argininamide **5.21**. a) Radioligand competition binding studies of (*R,R*)- and (*S,S*)-**5.21** ($K_i$ = 9 nM vs. 1780 nM) at the $Y_1R$ (displacement of [$^3$H]-UR-MK114 at SK-N-MC cells); b) Flow cytometric competition binding studies with (*R,R*)- and (*S,S*)-**5.21** (a $K_i$ value of 1.1 µM was determined for both enantiomers) at CHO-h$Y_4$-qi5-mtAEQ cells (displacement of Cy5-[K$^4$]hPP).

The comparative pharmacological evaluation of the (*S,S*)-enantiomer of compound **5.27** at the $Y_4R$revealed nearly identical $K_i$ values for the two enantiomers ($K_i$ = 290 and 310 nM, respectively, **Table 5.4**). Thus, by switching to the (*S*)-enantiomers the $Y_1R$ binding of argininamide-type NPY receptor ligands listed in **Table 5.4** is substantially reduced, but not – or not significantly - in favor of $Y_4R$ affinity.

**Table 5.4**: $Y_4R$ and $Y_1R$ binding data of (*R*)- and (*S*)-configured monovalent as well as (*R*,*R*)- and (*S*,*S*)-configured bivalent BIBP 3226 and BIBO 3304 derivatives.

**R¹** = 

**R²** = 

| No. | n | X | *config. | $Y_4R$ $IC_{50}$ [nM][a] | $Y_4R$ $K_B$[nM][b] | $Y_1R$ $K_i$ [nM][c] | $Y_1R$ $K_i$ [nM][d] |
|---|---|---|---|---|---|---|---|
|  | | | | | | | |
| **(*R*)-5.9** | - | -$CH_2$- | *R* | 2100 ± 3100 | 280 ± 410 | 23000 ± 3500 | 0.94 ± 0.06 |
| **(*S*)-5.9** | - | | *S* | 1100 ± 200 | 150 ± 30 | 28000 ± 3300 | **82 ± 7** [e] |
|  | | | | | | | |
| **(*R*)-5.7** | - | -NH- | *R* | 7100 ± 1700 | 950 ± 230 | 3800 ± 100 | 112 ± 34 |
| **(*S*)-5.7** | - | | *S* | 3100 ± 3000 | 420 ± 40 | 1900 ± 300 | **5650 ± 160** [e] |
|  | | | | | | | |
| **(*R*)-5.8** | - | -NH- | *R* | 3400 ± 100 | 460 ± 10 | 8200 ± 3200 | 96 ± 11 [e] |
| **(*S*)-5.8** | - | | *S* | 7200 ± 6000 | 960 ± 810 | 1900 ± 100 | **1505 ± 230** [e] |
|  | | | | | | | |
| **(*R,R*)-5.21** | 2 | -$CH_2$- | *R* | 920 ± 170 | 120 ± 50 | 1100 ± 300 | 9 ± 3 |
| **(*S,S*)-5.21** | 2 | | *S* | 970 ± 400 | 130 ± 50 | 1100 ± 100 | **1780 ± 310** |
| **(*R,R*)-5.23** | 5 | -$CH_2$- | *R* | 1400 ± 700 | 180 ± 90 | 1000 ± 100 | 41 ± 5 |
| **(*S,S*)-5.23** | 5 | | *S* | 2000 ± 1100 | 270 ± 150 | 1900 ± 300 | **2540 ± 90** |
|  | | | | | | | |
| **(*R,R*)-5.27** | - | -NH- | *R* | 720 ± 40 | 100 ± 10 | 290 ± 60 | 230 ± 24 |
| **(*S,S*)-5.27** | - | | *S* | 1010 ± 10 | 140 ± 20 | 310 ± 10 | **> 10000** [e] |

[a] Inhibition of 100 nM hPP induced $[Ca^{2+}]_i$ mobilization in CHO-$hY_4$-qi5-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [b] $K_B$ calculated according to the Cheng Prusoff equation (c = 100 nM, $EC_{50}$ = 15.5 nM). [c] Flow cytometric binding assay using 3 nM Cy5-[$K^4$]hPP ($K_D$ = 5.62 nM) in CHO-$hY_4$-qi5-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in duplicate. [d] $K_i$ from radioligand binding studies, calculated by Cheng-Prusoff equation ($K_D$ ([$^3$H]UR-MK114) = 1.2 nM, c = 1.5 nM) on SK-N-MC cells. [e] Determined by flow cytometry on HEL cells using 5 nM Cy5-pNPY ($K_D$ = 5.2).

# 5.4 Monovalent BIBO 3304 Derivatives with Varying $N^G$-Substituents

## 5.4.1 Introduction

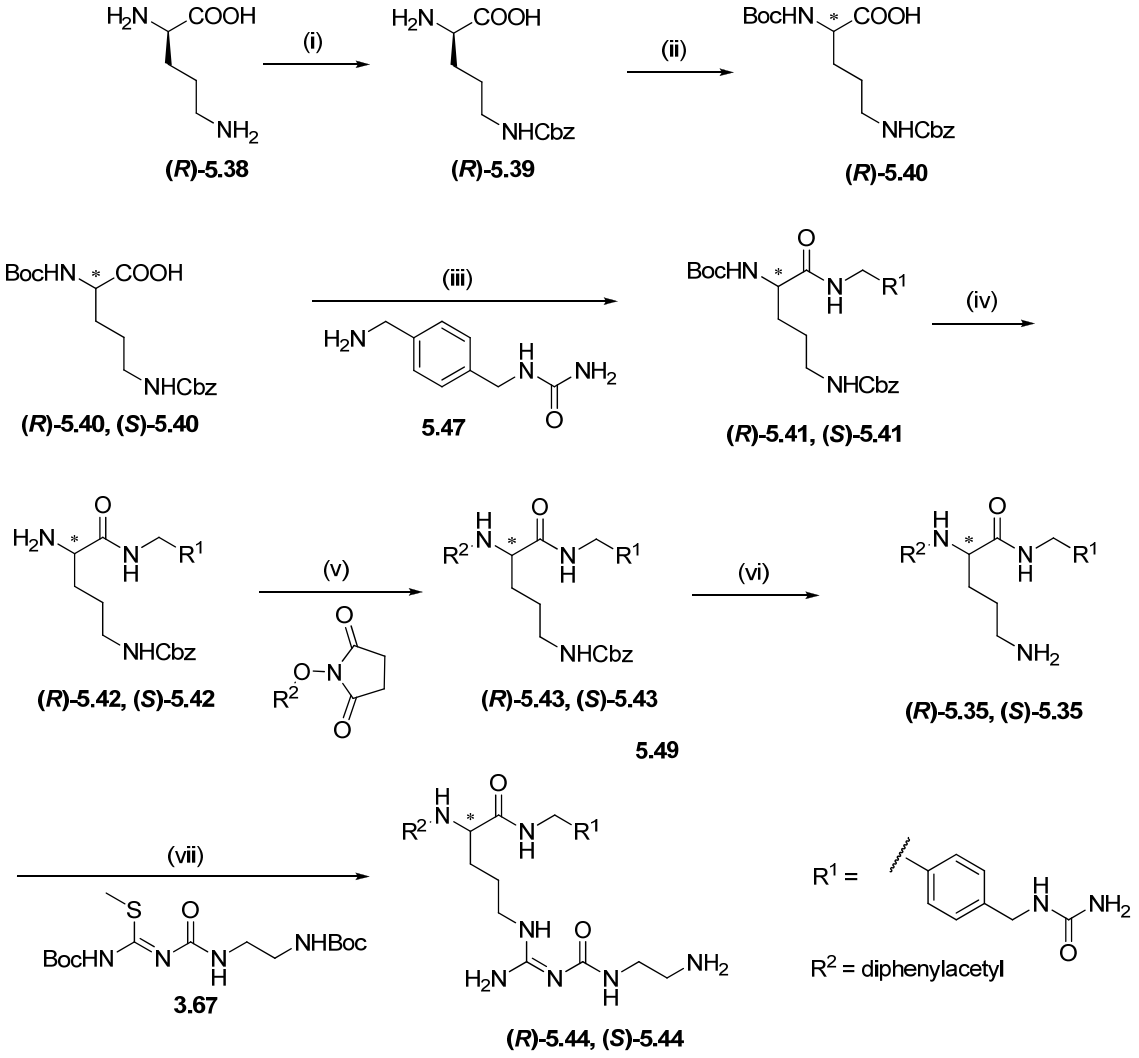
The investigation of monovalent and bivalent BIBP 3226 and BIBO 3304 derivatives led to the identification of compounds with submicromolar $Y_4R$ affinity. However, in contrast to the $Y_1R$, stereodiscrimination was not detected. Highest $Y_4R$ affinity resided in compounds with a spacer length larger than 10 atoms (see results for monovalent ligands, **Table 5.3**). The linker should be attached to the guanidine moiety. Substitution of the urea moiety of the BIBO derivatives **5.28** and **5.29** (**Table 5.3**) resulted in a substantial decrease in affinity. Furthermore, a basic moiety at a certain distance from the crucial acylguanidine or carbamoylguandine of the BIBP 3226 or BIBO 3304 building block is favorable, as becomes obvious from the results for the monovalent ligands (cf. **Table 5.3**). In case of the bivalent compounds, one may speculate if an amino group in the center of the linker is sufficient, or if the guanidine group of the second attached building block is required to play such a role in binding. Possibly, both basic substructures are needed. To clarify the structural requirements, monovalent argininamides were designed, connecting the BIBO 3304 building block with a free amine group via spacers comprising amide and glycol substructures of different lengths (5 – 24 atoms). The synthesis and pharmacological results will be presented within the following sections.

## 5.4.2 Chemistry

The preparation of the presented monovalent BIBO 3304 derivatives aimed at compounds of high purity rather than at the optimization of yields and synthetic routes. Standard orthogonal protecting groups and amide coupling protocols well known from peptide chemistry were applied. The building block **5.35** for the synthesis of $N^G$-carbamoylated argininamides including different spacer length was synthesized as previously described and summarized in **Scheme 5.2.**[12]

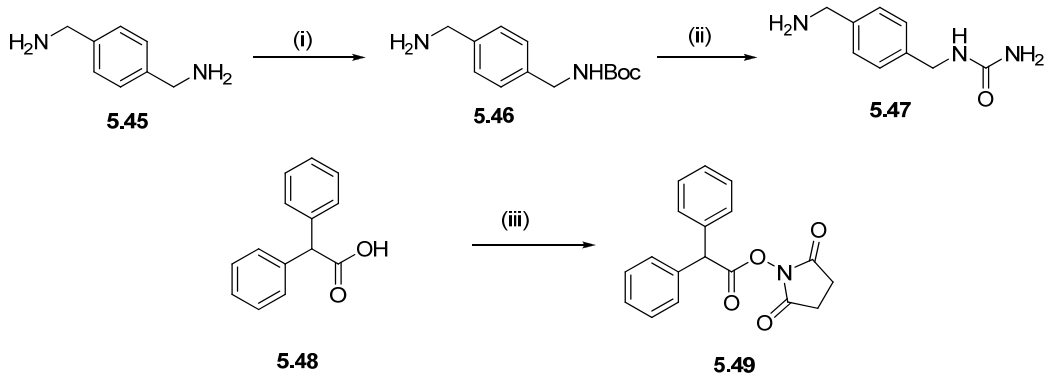
While (*S*)-$N^\alpha$-Boc-$N^\omega$-Cbz-ornithine (**(*S*)-5.40**) was commercially available, the corresponding (*R*)-enantiomer (**(*R*)-5.40**) was synthesized from D-ornithine (**5.38**) by introduction of a Boc-protection group orthogonally to Cbz. Subsequently, the urea moiety (**5.47**, **Scheme 5.2**) was introduced to both enantiomers using CDI as activation reagent. Boc-deprotection and acylation of the α-amino group with succinimidyl diphenylacetate (**5.49**) yielded compound **5.43**. Hydrogenolysis of the Cbz group in the presence of Pd/C catalyst gave the primary amine **5.35**, which was treated with the

guanidinylation reagent **3.67** and Boc-deprotected, yielding building block **5.44**. Despite severe solubility problems after introduction of the urea moiety, the enantiomers of building block **5.44** were obtained in acceptable yields.



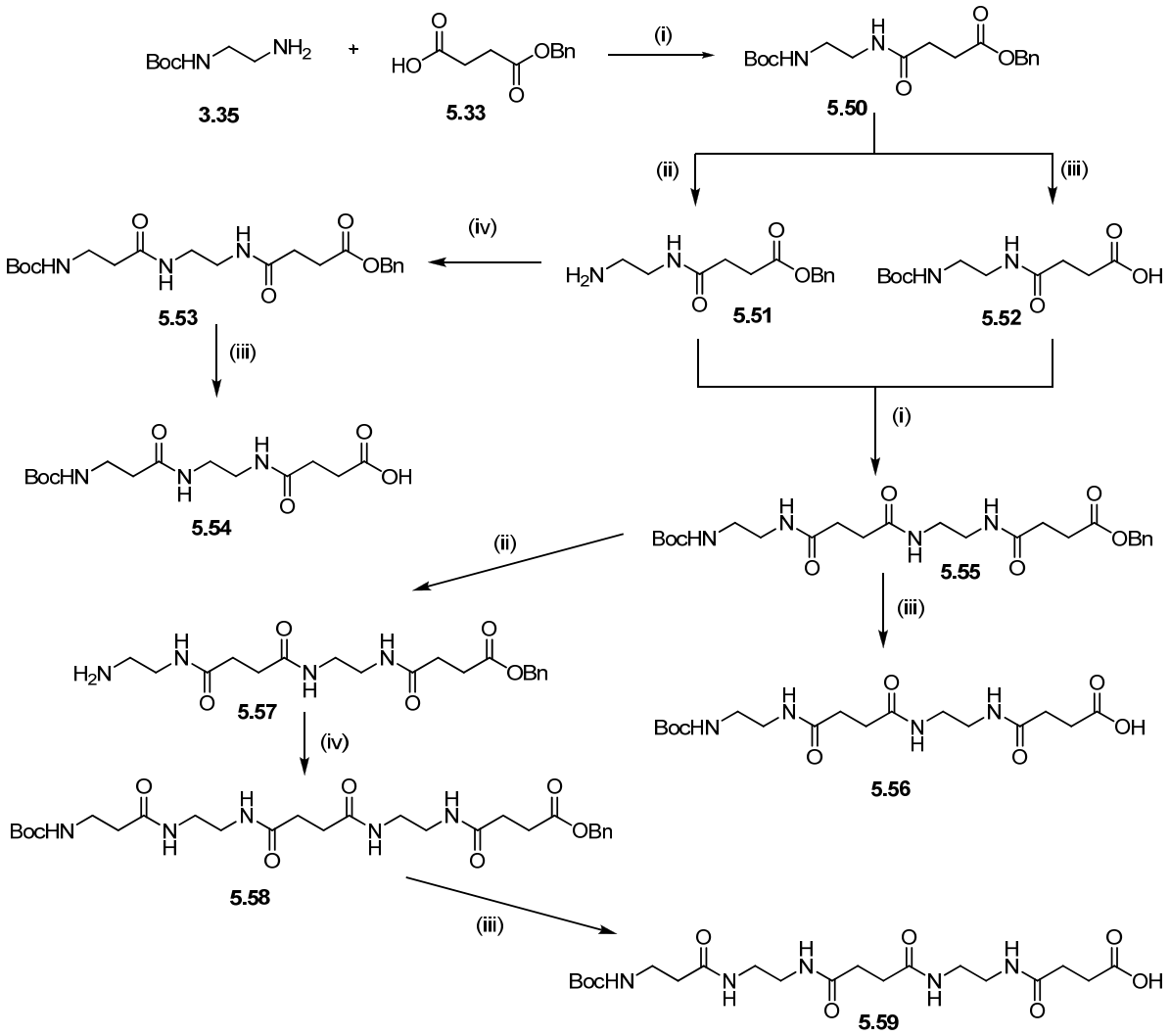

**Scheme 5.2**: Synthetic route for the synthesis of the argininamide **5.44**. Reagents and conditions: (i) a.) Cbz-Cl (45 % in toluene, eq), NaOH, 0.5 N, $K_2CO_3$, $CuSO_4$, 4 °C, 20 h, b.) titriplex III, $H_2O$, 100 °C, 1 h; (ii) $(Boc)_2O$, $K_2CO_3$, dioxane, $H_2O$, rt, 20 h; (iii) CDI, $Et_3N$, , DMAP, DMF, 0 °C/45 °C/rt, 20 h; (iv) AcCl, MeOH, rt, 2 h; (v) $Et_3N$, DMAP, DMF, rt, 20 h; (vi) 10 % Pd/C, $H_2$, acetic acid, MeOH, 45 °C, 3 h; (vii) a) $HgCl_2$ (2 eq), $Et_3N$, (3 eq), DMF, rt, 24 h, b) TFA (20 %), DCM, 5 h.

The urea **5.47** was easily accessible from mono Boc-protected 1,4-bis(aminomethyl)benzene (**5.46**) and potassium cyanate according to a general procedure for the synthesis of *N*-alkylated ureas (**Scheme 5.3**). [20-23] Compound **5.46** was obtained from the corresponding diamine as a mixture of mono and di-protected 1,4-bis(aminomethyl)benzene (ratio 55:45) that was used without purification in the next step. Subsequently, the di-protected product, which remained unchanged during conversion of **5.46** to the urea, could be separated by flash chromatography prior to the Boc-deprotection yielding **5.47**.[12] 2,2-Diphenylacetic acid (**5.45**) was converted to **5.49** with *N*-hydroxysuccinimide and DCC as coupling reagent according to standard procedures.[16]



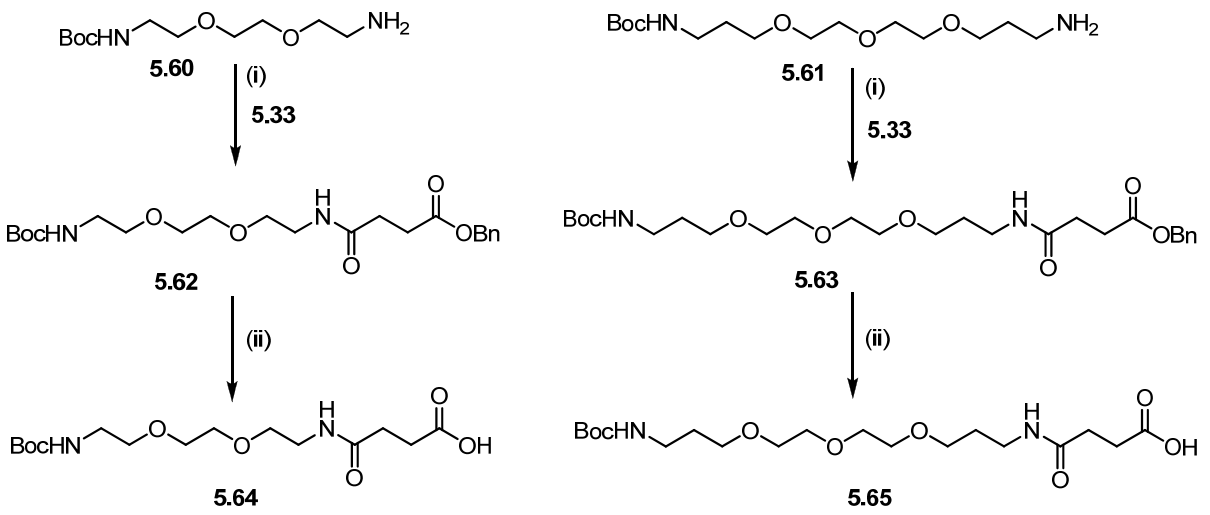

**Scheme 5.3**: Synthesis of *N*-(4-aminomethylbenzyl)urea (**5.47**) and succinimidyl 2,2-diphenylacetate (**5.49**). Reagents and conditions: (i) $(Boc)_2O$, 1M aq. NaOH, dioxane, 0 °C to rt, 20 h; (ii) potassium cyanate, $H_2O$, EtOH, 1M aq. HCl, reflux, 2h; (iii) *N*-hydroxysuccinimide, DCC, THF, rt, 20 h.

The amide spacers **5.52**, **5.54**, **5.56** and **5.59** were synthesised following standard protocols for amide coupling adapted from peptide chemistry using EDAC as an activation reagent (in **Scheme 5.4**).



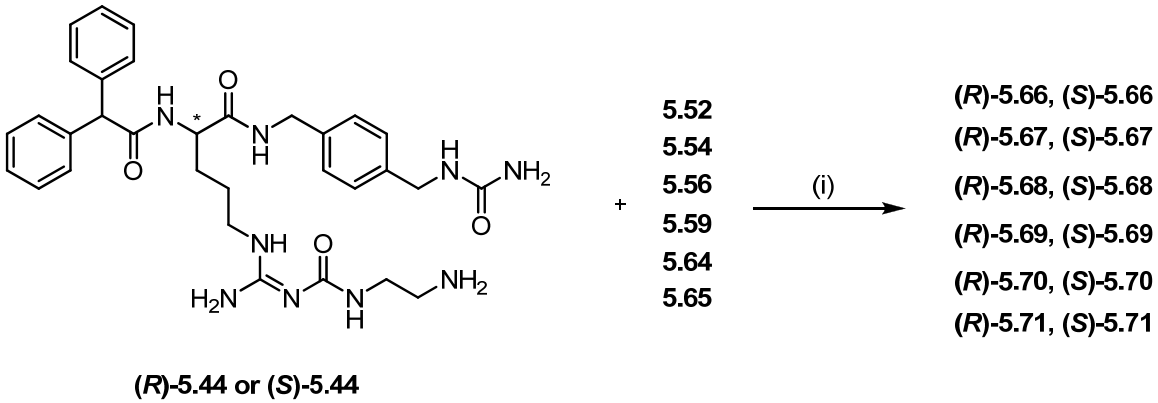

**Scheme 5.4**: Synthetic route for the preparation of the amide spacers **5.52**, **5.54**, **5.56** and **5.59**. Reagents and conditions: (i) EDAC (1.2 eq), HOBt (1.2 eq), DIEA (2.0 eq), DCM or DMF, rt, overnight; (ii) Pd/C, $H_2$, MeOH, rt (iii) TFA (50%), DCM, rt, 3 – 5 h; (iv) Boc-β-Ala-OH (1 eq), EDAC (1.2 eq), HOBt (1.2 eq), DIEA (2.0 eq), DCM or DMF rt, overnight.

The commercially available glycol entities for the glycol spacers were acylated with mono-benzyl-protected succinic acid according to a common procedure. Cleavage of the benzyl ester by hydrogenation over Pd/C catalyst afforded the building blocks **5.64** and **5.65** (**Scheme 5.5**)




**Scheme 5.5**: Preparation of the building blocks **5.64** and **5.65**. Reagents and conditions: (i) EDAC (1.2 eq), HOBt (1.2 eq), DIEA (2.0 eq), rt, overnight; (ii) Pd/C, $H_2$, MeOH, rt, 2 – 5 h.

Finally, the coupling of the spacers to the building blocks **(*R*)-5.44** and **(*S*)-5.44** was performed using TBTU as activating reagent. Subsequent Boc-deprotection afforded the desired monovalent BIBO 3304 derivatives with various spacer lengths (**5.66** – **5.71**, **Scheme 5.6**).




**Scheme 5.6**: Synthesis of the monovalent BIBO 3304 derivatives **5.66** – **5.71**. Reagents and conditions: (i) a) TBTU (1.2 eq), HOBt (1.2 eq), DIEA (2.0 eq), DMF, rt, overnight; b) TFA (20 %), DCM/$H_2O$, rt, 3 – 5 h.

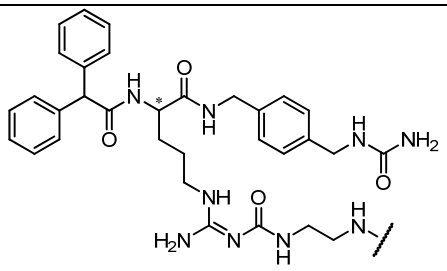
## 5.4.3 Pharmacological Results and Discussion

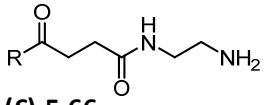
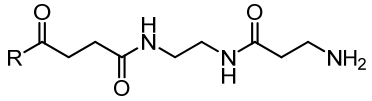
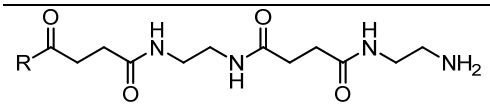
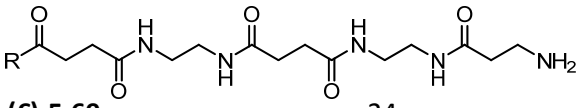
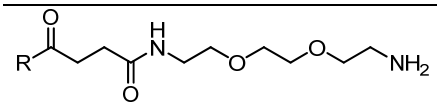
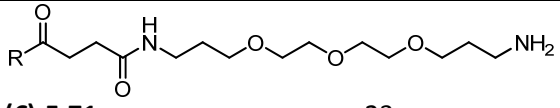
The monovalent BIBO 3304 derivatives were investigated for $Y_4R$ binding by flow cytometry and functional activity in a luminescence based $Ca^{2+}$-assay (aequorin assay) at the NPY $Y_4R$ as described in section 5.2.1.2. The results are summarized in **Table 5.5**.

The data of the synthesized monovalent ligands **5.44** and **5.66 – 5.71** (summarized in **Table 5.5**) do not confirm the working hypothesis that the combination of a BIBO 3304 building block with a basic moiety at a certain distance of the guanidine substructure of the building block results in high affinity NPY $Y_4R$ antagonists. Firstly, as demonstrated for several bivalent ligands, an additional amino group placed in the center of the linkers does obviously not play a key role in $Y_4R$ binding. The monovalent compounds **5.66** (12 atoms) and **5.70** (18 atoms), in which the distance to the additional basic group was kept comparable to that in the tested bivalent ligands (e.g. **5.26**: 11 atoms, **5.27**: 18 atoms), showed only moderate affinities in the micromolar range, whereas a $K_i$ value of 287 nM was determined for **5.27**. Secondly, monovalent ligands with longer spacers (**5.69**: 24 atoms, **5.72**: 23 atoms), comparable to the linkers between the guanidine groups of bivalent ligands (e.g. **5.22**: 24 atoms, $K_i$ = 370 nM) and therefore, in principle, capable of mimicking the two basic moieties of twin compounds such as **5.22**, had about 10-fold lower $Y_4R$ affinity. Most strikingly, the affinity of all monovalent ligands presented in **Table 5.5** is almost identical, independent from the spacer length. One may speculate that due to the high flexibility of the spacers the basic group can always adopt a conformation enabling binding at a similar distance, regardless of the spacer length. Possibly, with regard to bivalent ligand **5.27** ($K_i$ = 267 nM, 37 atoms) the spacer length of the corresponding monovalent ligands was still too short to mimic the guanidine group of the second BIBO 3304 building block.

These results suggest that the monovalent ligands described in this chapter do not fulfill the structural requirements for effective $Y_4R$ binding. Obviously, the second pharmacophoric entity is much more involved in $Y_4R$ binding than assumed on the basis of the previous data. Furthermore, interactions of the second argininamide could induce conformations of the linker which are more favorable for $Y_4R$ binding. Therefore, the synthesis of monovalent derivatives with further elongated spacers would most probably not result in improved affinity. Instead, conformationally constrained spacers and more bulky basic moieties might be useful to further explore the structure-activity relationships of $Y_4R$ preferring argininamides.

**Table 5.5**: Structures, $Y_4R$ antagonistic activities as well as $Y_4R$ and $Y_1R$ binding data of the monovalent ligands with varying spacer length (compounds **5.44** and **5.66-5.71**).

**R** = 

| No. | spacer[a] | $Y_4R$ | | $Y_1R$ |
|---|---|---|---|---|
| | | $K_i$ [nM][b] | $IC_{50}$ [nM][c] | $K_i$ [nM][d] |
| R-H | | | | |
| **(*S*)-5.44** | 5 | 3850 ± 1510 | > 10000 | inactive |
|  | | | | |
| **(*S*)-5.66** | 12 | 4060 ± 760 | > 10000 | inactive |
| **(*R*)-5.66** | 12 | 15350 ± 5590 | n.d. | 1000 |
|  | | | | |
| **(*S*)-5.67** | 16 | 3250 ± 50 | > 10000 | inactive |
| **(*R*)-5.67** | 16 | 15330 ± 8440 | n.d. | 600 |
|  | | | | |
| **(*S*)-5.68** | 20 | 6400 ± 590 | > 10000 | inactive |
| **(*R*)-5.68** | 20 | 5880 ± 2140 | n.d. | 4400 |
|  | | | | |
| **(*S*)-5.69** | 24 | 3520 ± 820 | > 10000 | inactive |
| **(*R*)-5.69** | 24 | 13700 ± 9760 | n.d. | 6000 |
|  | | | | |
| **(*S*)-5.70** | 18 | 3220 ± 1630 | > 10000 | inactive |
| **(*R*)-5.70** | 18 | > 10000 | n.d. | 300 |
|  | | | | |
| **(*S*)-5.71** | 23 | 3660 ± 1240 | > 10000 | inactive |
| **(*R*)-5.71** | 23 | > 10000 | n.d. | 500 |

[a] number of atoms which contribute to spacer length. [b] Inhibition of 100 nM hPP induced [$Ca^{2+}$] mobilization in CHO-h$Y_4$-qi5-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate [c] Flow cytometric binding assay using 3 nM cy5-[$K^4$]hPP ($K_D$ = 5.62 nM) in CHO-h$Y_4$-qi$_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in duplicate.[d] Dissociation constant of [$^3$H]UR-MK114 ($K_D$ = 2.9 nM, c = 2.5 nM) on MCF-7-$Y_1$ cells; estimated by logit-log-plot.

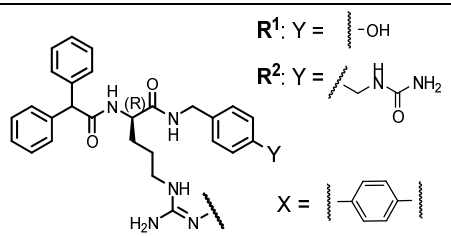
## 5.5 Pharmacological Investigations on Bivalent BIBP 3326 Derivatives as Lead Structures for the NPY $Y_4$R Research

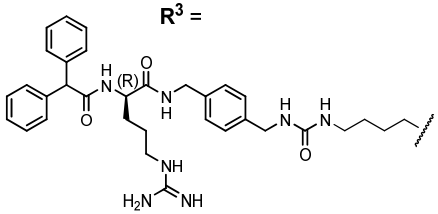
### 5.5.1 Criteria for the Selection of Investigated Structures

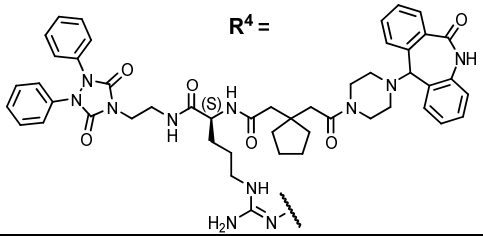
Based on the previous results and the structural features preferred by the $Y_4$R a set of new bivalent ligands (**5.72** – **5.82, Table 5.6**) was selected for further pharmacological investigations.

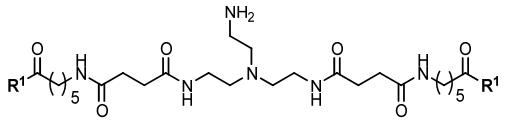
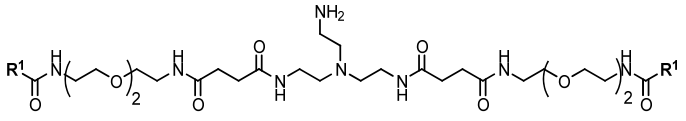
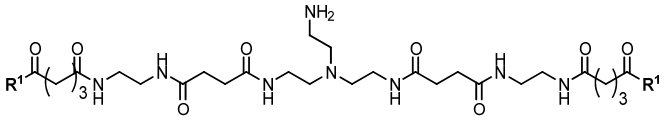
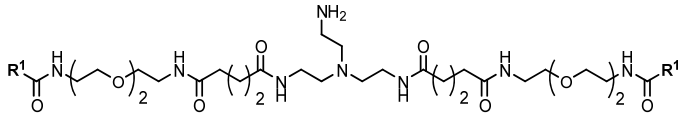
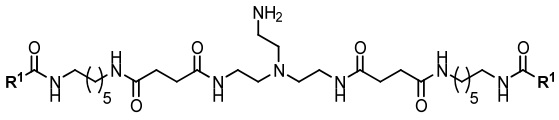
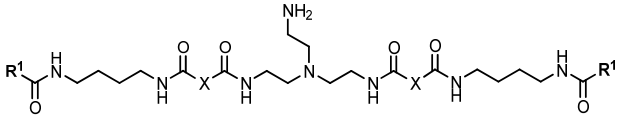
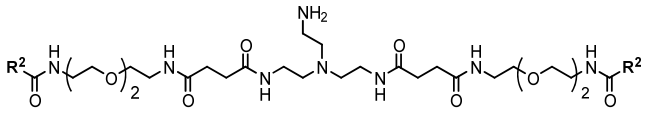
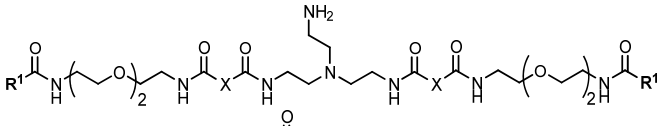
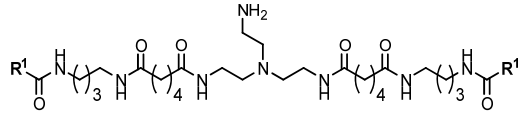
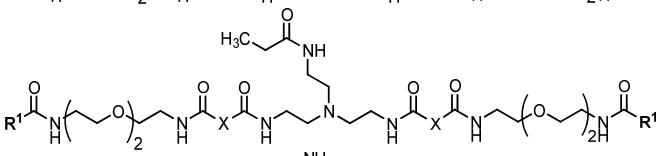
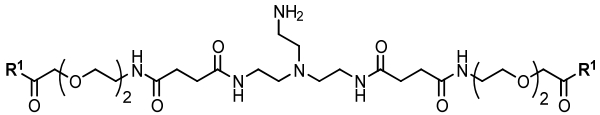
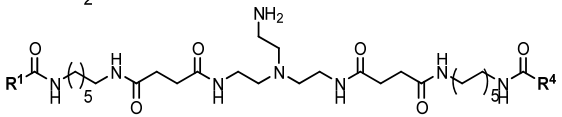
In this test series the focus was on bivalent BIBP 3226 derivatives as all investigated monovalent argininamide-type ligands showed only low $Y_4$R affinities. As the substitution of the urea moiety led to a substantial decrease in affinity, the focus lay on bivalent compounds where the linker was attached to the guanidine group of BIBP 3226 or BIBO 3304. Additionally, spacers of more than 29 atoms in length were chosen as compounds with longer linkers resulted in an increase of $Y_4$R binding and led to the identification of **(*R,R*)-5.27**, a bivalent ligand with submicromolar $Y_4$R affinity ($K_i$ = 287 nM, 37 atoms). Furthermore, compounds providing an additional amino group placed in the middle as well as diverse substructures were selected to further explore the structure-activity relationships with respect to optimization of the chemical nature of the linker. The linkers were composed of simple alkyl chains (**5.72** – **5.75**), glycol derivatives (**5.76**, **5.78**) or p-phenylene moieties (**5.80**, **5.81**) to investigate the impact of rigidization of the linker on $Y_4$R binding, either. Additionally, the propionamide analogyl substitution of the central free amino group (**5.81**) of **5.80**, was investigated to verify if a basic amino group in central position of the molecule effectively contributes to receptor binding at all. Furthermore, $Y_4$R affinity of a bivalent ligand composed of two different argininamide-type pharmacophoric groups, namely a BIBP 3226 entity a BIIE 0246 moiety, which is known for high affinity at the $Y_2$R, was determined.

**Table 5.6**: Structures and linker lengths of (*R*,*R*)-configured bivalent argininamide-type ligands investigated at the NPY $Y_4R$




| No. | linker | linker length[a] | No. | linker | linker length[a] |
|---|---|---|---|---|---|
| 5.72 |  | 29 | 5.77 |  | 37 |
| 5.73 |  | 33 | 5.78 |  | 39 |
| 5.74 |  | 33 | 5.79 |  | 33 |
| 5.27 |  | 37 | 5.80 |  | 41 |
| 5.75 |  | 33 | 5.81 |  | 41 |
| 5.76 |  | 33 | 5.82 |  | 33 |

[a] distance between guanidine moieties given in number of atoms

## 5.5.2 Pharmacological Results and Discussion

The stereoisomeric bivalent BIBP 3226 and BIBO 3304 derivatives were analyzed for $Y_4R$ affinity in flow cytometric binding studies and for functional activity in a $Ca^{2+}$-mobilization assay (aequorin assay) as described in section 5.2.2.1 and were summarized in **Table 5.7**.

With respect to the raised question if the linker length goes along with improved receptor binding the obtained results, in principle, support this hypothesis. For instance compound **5.80** ($K_i$ = 132 nM, 41 atom spacer) is superior to **5.72** ($K_i$ = 470 nM, 29 atom spacer) and **5.21** ($K_i$ = 1070 nM, 20 atom spacer, **Table 5.3**). In addition, the chemical nature of the spacer seems to have an impact on receptor binding. This becomes obvious from compounds **5.74** ($K_i$ = 267 nM), **5.75** ($K_i$ = 549 nM) and **5.76** ($K_i$ = 1520 nM), which share the same linker length (33 atoms), but differ in their $Y_4R$ affinities by a factor of two (**5.75**) or even five (**5.76**) related to **5.74**. Apparently, a long and flexible alkyl chain (6 atoms) in the vicinity to the carbamoylguanidine moiety combined with a more rigid structure in the middle of the linker obtained by introduction of several amide groups as in case of **5.74** ($K_i$ = 267 nM) is preferred over shorter alkyl chains located at various position within the linker (**5.76**, $K_i$ = 1520 nM) or a glycol derived linker (**5.77**, $K_i$ = 391 nM). In case of compound **5.77** the decrease in affinity may also be attributed to the replacement of the carbamoylguanidine by an acylguanidine substructure. Previous investigations have always demonstrated a higher activity of carbamoylguanidines (cf. **section 5.2.2**). The contribution of the linker to receptor binding becomes most obvious from **5.79** and **5.80**. Compound **5.79** ($K_i$ = 214 nM), which shares equal spacer length (33 atoms) with **5.74** ($K_i$ = 267 nM) and **5.75** ($K_i$ = 549 nM) but contains a more rigid phenylene moiety, showed a further slightly increased receptor binding. Highest NPY $Y_4R$ affinity was finally obtained for compound **5.80**, combining a very long spacer (41 atoms, $K_i$ = 132 nM) with phenylene moieties as rigid structural elements, possibly favoring a conformation that results in improved $Y_4R$ binding. In contrast, the acylation of the free amino group placed in the center of the linker (**5.81**, $K_i$ = 409 nM) didn't affect the $Y_4R$ binding that much. The decrease in affinity amounted to a factor of three compared to the non-acylated analogue **5.80** ($K_i$ = 132 nM). Surprisingly, the replacement of one BIBP 3226 building block by the argininamide derivative BIIE 0246 – a highly potent $Y_2R$ antagonist – led to a decrease in affinity (**5.82**, $K_i$ = 1135 nM) by only a factor of four compared to the structurally closely related homobivalent BIBP 3226 derivative **5.74** ($K_i$ = 267 nM). This raises questions about the contribution of the second pharmacophoric group in bivalent ligands to $Y_4R$ binding, although highest affinities were determined for twin compounds. A preference for the BIBP 3226 versus the BIBO 3304 building block was not detectable (cf. **5.27**: $K_i$ = 287 nM, vs. **5.77**: $K_i$ = 391 nM).

**Table 5.7**: Pharmacological data of selected (*R*)-configured monovalent and (*R*,*R*)-configured bivalent BIBP 3226 and BIBO 3304 derivatives determined for NPY $Y_4R$ and $Y_1R$ binding and $Y_4R$ antagonistic activity.

| No. | $hY_4R$ | | | | $hY_1R$ |
|---|---|---|---|---|---|
| | % Relative Potency[a] | $IC_{50}$ [nM][b] | $K_B$ [nM][c] | $K_i$ [nM][d] | $K_i$ [nM][e] |
| **BIBP 3226** | 7.9 ± 1.6 | > 10000 | - | > 10000 | 1.3 ± 0.1 |
| **BIBO 3304** | 0.3 ± 0.7 | > 10000 | - | > 10000 | 0.25 ± 0.01 |
| **5.27** | 3.7 ± 0.3 | 713 ± 40 | 95 ± 1.0 | 286 ± 67 | 230 ± 24 |
| **5.72** | n.d. | 235 ± 67 | 31 ± 0.9 | 469 ± 10 | 47 ± 7 |
| **5.73** | n.d. | 372 ± 17 | 50 ± 0.2 | 574 ± 16 | 13 ± 0.1 |
| **5.74** | n.d. | 247 ± 59 | 33 ± 1.0 | 267 ± 117 | 40 ± 0.1 |
| **5.75** | 162 ± 7.1 | 237 ± 21 | 32 ± 0.3 | 549 ± 89 | 42 ± 1 |
| **5.76** | n.d. | 3565 ± 290 | 478 ± 39 | 1520 ± 480 | 12 ± 0.1 |
| **5.77** | n.d. | 302 ± 20 | 41 ± 0.3 | 391 ± 35 | 25 ± 2 |
| **5.78** | n.d. | 249 ± 57 | 33 ± 0.7 | 290 ± 53 | 28 ± 2 |
| **5.79** | n.d. | 254 ± 24 | 34 ± 0.3 | 214 ± 75 | 53 ± 13 |
| **5.80** | n.d. | 146 ± 60 | 20 ± 0.7 | 132 ± 7 | 24 ± 1 |
| **5.81** | n.d. | 269 ± 6 | 36 ± 0.1 | 409 ± 90 | 64 ± 1 |
| **5.82** | n.d. | 534 ± 33 | 72 ± 0.4 | 1135 ± 489 | 160 ± 19 |

[a] $[Ca^{2+}]_i$ mobilization observed at conc. of 10 µM test compound (performed in triplicate) given in % referred to the $[Ca^{2+}]_i$ mobilization of the standard hPP (100 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells. [b] Inhibition of 100 nM hPP induced $[Ca^{2+}]_i$ mobilization in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [c] $K_B$ calculated according to the Cheng Prusoff equation[14] (c = 100 nM, $EC_{50}$ = 15.5 nM). [d] Flow cytometric binding assay using 3 nM cy5-[$K^4$]hPP ($K_D$ = 5.6 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [e] $K_i$ from radioligand binding studies, calculated by Cheng-Prusoff equation[14] ($K_D$ ([$^3$H]-UR-MK114) = 1.2 nM, c = 1.5 nM) on SK-N-MC cells.

## 5.6 Summary and Outlook

Among the bivalent ligands having quite extended linkers containing different structural elements (glycol derivatives, polymethylene, phenylene substructures), compound **5.80** was identified as the most potent $Y_4R$ antagonist known so far ($K_i$ = 132 nM). An additional basic moiety placed in the center of the linker of most investigated bivalent compounds is not essential. This was clarified by N-propionylation of this central basic position (cf. compound **5.81**), resulting in only a minor (3-fold)

decrease in affinity. Obviously, the free amino group contributes only marginally to $Y_4R$ binding. This suggests that equipotent monovalent ligands might be obtained by further elongation of the spacer to mimic the second guanidine group of the bivalent ligands. However, considering the high flexibility of such spacers this strategy would most probably fail due to unfavorable entropic contributions. First investigations on monovalent ligands support this theory. All synthesized (*S*) and (*R*)-configured monovalent compounds showed only moderate $Y_4R$ affinity independent from the linker. In fact, the bivalent structure seems preferred due to its higher stabilization most likely attributed to the second attached building block. Additionally, other functional groups present in the second argininamide portion may also be involved in receptor binding. There was no preference for BIBO 3304 or BIBP 3226 moieties detected. Even the replacement of the second pharmacophoric group by different argininamide-type building block (cf. BIIE0246) was tolerated.
Moreover, investigations of the enantiomer, **(*S,S*)-5.27** together with other revealed lack of stereodiscrimination at the $Y_4R$. However, this could be advantageous with respect to NPY receptor subtype selectivity as the $Y_1R$ clearly prefers (*R*)-configured compounds.

Indeed, most striking in terms of structure-activity relationships is the fact that linker elongation in bivalent ligands goes along with an increase in affinity. In particular, compounds with a linker length of 37 atoms showed a further increase in affinity compared to shorter analogs. This finding is reminiscent of the structural properties of the C-terminal part of hPP, the endogenous ligand of the NPY $Y_4R$, which holds a distance of 37 atoms between the guanidine groups of $Arg^{25}$ and $Arg^{35}$. In case of the high affinity bivalent ligand **5.80** the spacer comprising 41 atoms spacer seems, at first glance, a bit too long compared to the C-terminus of hPP. However, the conformational constraints achieved through the introduction of phenylene moieties appear to be advantageous. As demonstrated in **Figure 5.4** this less flexible structure contains structural similarities to the amide backbone of the C-terminal part of hPP.

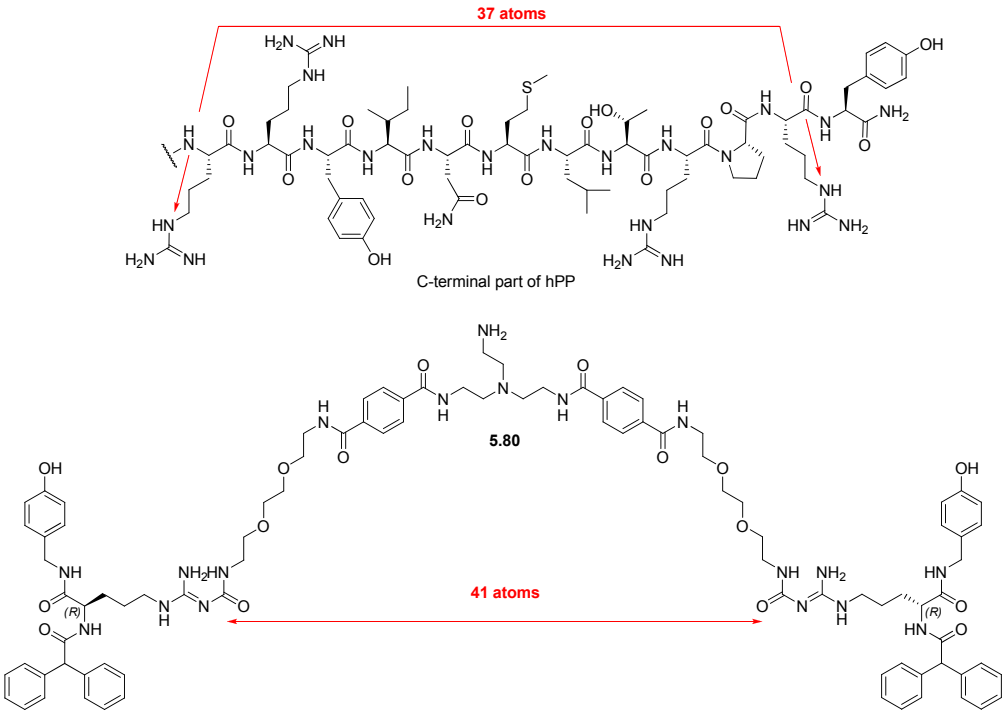



**Figure 5.4**: Comparison of the C-terminal fragment of hPP with the structure of the NPY $Y_4R$ antagonist **5.36**, suggesting that comparable distances between the guanidine moieties are possible.

In conclusion, the discovery of the first potent $Y_4R$ antagonist combined with the results of the stereochemical investigations and the first studies on structure-activity relationships can be considered a good starting point for the design of new NPY $Y_4R$ ligands with improved affinity. The bivalent BIBP 3226 derivative (**5.80**) represents a lead structure leaving a wide range of options for optimization. The investigations on the impact of length and chemical nature of the linker revealed that rigidification of the central part of the spacer has a positive effect on $Y_4R$ affinity, whereas conformational constraints in vicinity of the guanidine groups are less tolerated. Furthermore, due to lack of stereodiscrimination, the switch to achiral compounds is an obvious approach. Additionally, more detailed investigations on the functional groups present in the building blocks could be performed, using for example the amino acids in the vicinity to the arginine residues in hPP as models. Special attention should be paid to the identification and elimination of non-required structural elements to reduce of the molecular weight of the $Y_4R$ ligands and to leave more options for structural modifications.

# 5.7 Experimental Section

## 5.7.1 Chemistry

### 5.7.1.1 General Conditions

See section 3.3.1.1

Nuclear Magnetic Resonance spectra ($^1$H-NMR and $^{13}$C-NMR) were recorded with a Bruker Avance 300 ($^1$H: 300.1 MHz, $^{13}$C: 75.5 MHz) or a Bruker Avance 600 ($^1$H: 600.1 MHz, $^{13}$C: 150.9 MHz) NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Chemical shifts are given in δ (ppm) relative to external standards. Abbreviations for the multiplicities of the signals are specified as follows: s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), m (multiplet), bs (broad singulet) and combinations thereof. The multiplicity of carbon atoms ($^{13}$C-NMR) was determined by DEPT 135 (distortionless enhancement by polarization transfer): "+" primary and tertiary carbon atom (positive DEPT) signal, "-" secondary carbon atom (negative DEPT signal), "$C_{quat}$" quaternary carbon atom. In certain cases 2D-NMR techniques (COSY, HMQC, HSQC, HMBC) were used to assign $^1$H and $^{13}$C chemical shifts

Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Gemini-NX C18, 250 × 21 mm, 5 µm, AXIA Packed, Phenomenex, Aschaffenburg, Germany) at a flow rate 18 mL/min. Mixtures of acetonitrile and 0.1 % aq. TFA were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure prior to lyophilisation. Analytical HPLC analysis was performed with a Gemini-NX C18 (250 x 4.6 mm, 5 µm, 110 Å, Phenomenex, Aschaffenburg, Germany) on a Merck Hitachi system composed of a L-6200-A pump, a AS-2000A autosampler, a L-4000A UV-VIS detector and a F-1050 fluorescence spectrophotometer (flow rate: 0.8 mL/min; UV detection 220 nm). Acetonitrile (A) and 0.05 % TFA (B) was used as mobile phase. Helium degassing was used throughout.

## 5.7.2 Preparation of (*S*)-Configured Bivalent Compound (S,*S*)-5.27

***N*-Boc-nitrilotriethanamine (5.31)[1]**

A solution of $(Boc)_2O$ (1.50 g, 6.9 mmol, 1 eq) in DCM (300 mL) was added dropwise to a cooled solution (- 78 °C) of nitrilotriethanamine **5.30** (10.0 g, 68.7 mmol, 10 eq) over a period of 2 h. The mixture was allowed to warm up to ambient temperature and stirring was continued overnight. The solvent was removed under reduced pressure and the product was isolated by column chromatography (DCM/MeOH/NH3 90/9/1 – 80/19/1) yielding a yellow oil (1.24 g. 73 %). $^1$H-NMR

(300 MHz, $CD_3OD$): δ (ppm) 1.39 (s, 9H, C(C***H***$_3$)$_3$), 2.44 – 2.53 (m, 6H, N-C***H***$_2$), 2.71 (t, $^3J$ = 6.0 Hz, 4H, C***H***$_2$-$NH_2$), 3.08 – 3.22 (m, 2H, N-$CH_2$-C***H***$_2$-NHBoc); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 247 $[M+H]^+$. $C_{11}H_{26}N_4O_2$ (246.35).

**4-Benzyloxy-4-oxobutanoic acid (5.33)[24]**

Succinic anhydride **5.32** (5.00 g, 50.0 mmol, 1 eq) was treated with benzyl alcohol (6.24 mL, 60.0 mmol, 1.2 eq) at 60 °C overnight. The reaction mixture was subjected to flash chromatography (DCM/MeOH 100/0 – 95/5) yielding **5.33** as a white solid (7.48 g, 72 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 2.62 – 2.70 (m, 4H, C***H***$_2$-C***H***$_2$), 5.15 (s, 2H, Ph-C***H***$_2$), 7.20 – 7.42 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$): δ (ppm) 28.89 (-, ***C***$H_2$), 28.95 (-, ***C***$H_2$), 66.69 (-, Ph-***C***$H_2$), 128.24 (+, 2 Ph-***C***), 128.33 (+, Ph-***C***-4), 128.61 (+, 2 Ph-***C***), 135.69 ($C_{quat}$, Ph-***C***-1), 172.02 ($C_{quat}$, ***C***OOH), 178.40 ($C_{quat}$, ***C***=O). MS (EI, 70 eV) *m/z* (%): 208.1 (10) $[M]^{+\cdot}$, 108.1 (100) $[Ph\text{-}CH_2\text{-}OH]^{+\cdot}$. $C_{11}H_{12}O_4$ (208.21).

**8-[2-(3-Carboxypropanamido)ethyl]-2,2-dimethyl-4,12-dioxo-3-oxa-5,8,11-triazapentadecan-15-oic acid (5.34)**

Compound **5.33** (0.69 g, 3.3 mmol, 2.2 eq), EDAC (0.63 g, 3.3 mmol, 2.2 eq), and DIEA (0.58 mL, 3.3 mmol, 2.2 eq) were dissolved in $DCM_{abs}$ under argon atmosphere and stirred for 20 min at room temperature., A solution of the **5.31** (0.37 g, 1.5 mmol, 1 eq) in $DCM_{abs}$ was added and the mixture stirred overnight. After removal of the solvent under reduced pressure, the crude product was dissolved in EtOAc and washed with water. The organic phase was then dried over $MgSO_4$ and concentrated *in vacuo*. Purification by flash chromatography (DCM/MeOH 100/0 – 95/5) afforded the intermediate as a pale yellow which was dissolved in MeOH (50 mL). A 10 % Pd/C catalyst (45 mg) was added and hydrogen was led through the vigorously stirred mixture at room temperature for 3 h (control by TLC). The catalyst was removed by filtration over Celite and the solvent was removed under reduced pressure yielding **5.34** as pale yellow oil (0.31 g, 97 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 2.50 (t, $^3J$ = 6.2 Hz, 4H, CO-C***H***$_2$), 2.58 (t, $^3J$ = 6.3 Hz, 4H, CO-C***H***$_2$), 2.65 – 2.80 (m, 6H, N-C***H***$_2$), 3.26 – 3.33 (m, overlap with solvent, 4H, C***H***$_2$-NH-CO); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.85 (+, C(***C***$H_3$)$_3$), 29.19 (-, 2 ***C***$H_2$-CO), 29.24 (-, 2 ***C***$H_2$-CO), 38.10 (-, ***C***$H_2$-NH), 38.55 (-, 2 ***C***$H_2$-NH) 52.90 (-, ***C***$H_2$-N), 54.37 (-, 2 ***C***$H_2$-N) 80.19 ($C_{quat}$, ***C***$(CH_3)_3$), 174.96 ($C_{quat}$, 2 ***C***=O)180.31 ($C_{quat}$, 2 ***C***OOH). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 447 (100) $[M+H]^+$. $C_{19}H_{34}N_4O_8$ (446.50).

**(*S*)-1-Amino-17-(2,2-diphenylacetamido)-12-imino-10-oxo-N-[4-(ureidomethyl)benzyl]-3,6-dioxa-9,11,13-triazaoctadecan-18-amide (5.37)**

BIBO 3304 building block **5.35** (0.21 g, 0.43 mmol, 1 eq), the guanidinylating reagent **5.36** (0.20 g, 0.43 mmol, 1 eq) and $HgCl_2$ (0.23 g, 6.50 mmol, 2 eq) were dissolved in anhydrous DCM. $Et_3N$

(0.23 µL, 1.29 mmol, 3 eq) was added and the mixture was stirred for 24 h at ambient temperature. Subsequently, EtOAc was added and the precipitate filtered over Celite. Purification by flash chromatography (DCM/MeOH 100/1 – 90/10 v/v) afforded the intermediate as white solid. Boc-deprotection with 5 mL TFA (20 % in DCM) followed by lyophilisation yielded **5.37** as a white solid (0.28 g, 92 %)**.** $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.46 – 1.62 (m, 1H, CH-$CH_2$-C***H***$_2$), 1.65 – 1.76 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.77 – 1.93 (m, 1H, CH-C***H***$_2$-$CH_2$), 3.09 (t, $^3J$ = 4.8 Hz, 2H, C***H***$_2$-$NH_2$), 3.16 – 3.23 (m, 2H, 2x CH-$(CH_2)_2$-C***H***$_2$-NH), 3.39 (t, $^3J$ = 5.7 Hz, 2H, NH-CO-C***H***$_2$-$CH_2$-O), 3.58 (t, $^3J$ = 5.7 Hz, 2H, CO-NH-CO-$CH_2$-C***H***$_2$-O), 3.62 – 3.671 (m, 6H, O-C***H***$_2$-C***H***$_2$-O+O-C***H***$_2$-$CH_2$-$NH_2$), 4.26 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.07 (s, 1H, $(Ph)_2$-C***H***), 7.17 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC): δ (ppm) 25.71 (CH-$CH_2$-***C***$H_2$), 30.25 (CH-***C***$H_2$-$CH_2$), 40.57 (CO-NH-***C***$H_2$-$CH_2$-O), 40.64 (O-$CH_2$-***C***$H_2$-$NH_2$), 41.70 ($(CH_2)_2$-***C***$H_2$-NH-), 43.79 (NH-***C***$H_2$-Ar), 44.31 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.33 (***C***H-$CH_2$-$CH_2$), 58.68 ($(Ph)_2$-***C***H), 67.87 (O-***C***$H_2$-$CH_2$-$NH_2$), 70.54 (CO-NH-$CH_2$-***C***$H_2$-O), 71.35 (O-***C***$H_2$), 71.36 (O-***C***$H_2$), 128.13 (diPh-***C***-4), 128.22 (diPh-***C***-4), 128.35 (2 Ph-***C***), 128.65 (2 Ph-***C***), 129.50 (2 diPh-***C***), 129.55 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.90 (2 diPh-***C***), 138.40 (Ph-***C***-1), 140.84 (Ph-***C***-4), 140.96 (2 diPh-***C***-1), 155.71 (NH-***C***O-NH), 162.12 (Ar-$CH_2$-NH-***C***O-$NH_2$), 173.54 (***C***O-NH-$CH_2$-Ar), 174.84 ($(Ph)_2$-CH-***C***O); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 704 (100) $[M+H]^+$ . $C_{42}H_{59}N_{11}O_8 \cdot 2TFA$ (1074.03).

**$N^1$-[(*S*)-29-(2-Aminoethyl)-4-(2,2-diphenylacetamido)-9-imino-3,11,22,25-tetraoxo-1-(4-(ureidomethyl)phenyl)-15,18-dioxa-2,8,10,12,21,26,29-heptaazahentriacontan-31-yl]-$N^4$-{(S)-4-(2,2-diphenylacetamido)-9-imino-3,11-dioxo-1-[4-(ureidomethyl)phenyl]-15,18-dioxa-2,8,10,12-tetraazaicosan-20-yl}succinamide ((*S,S*)-5.27)**

Compound **5.34** (55 mg, 0.130 mmol, 1 eq), TBTU (1.2 eq), HOBt-monohydrate (50 mg, 0.156 mmol, 1.2 eq) and DIEA (68 µL, 0.390 mmol, 2.0 eq) were dissolved in $DMF_{abs}$ under argon atmosphere and stirred for 20 min at room temperature. To this mixture a solution **5.37** (80 mg, 0.130 mmol, 1 eq) in $DMF_{abs}$ was added and stirred overnight. After removal of the solvent under reduced pressure, the crude product was taken up in EtOAc and washed with water. The organic phase was then dried over $MgSO_4$ and concentrated *in vacuo*. Subsequently, the Boc-protection group was removed with 50 % TFA. Therefore the residue was dissolved in DCM. TFA (50% final concentration) was added and the reaction mixture was stirred until the protection groups were removed (3 – 5 h, TLC control). After removal of the solvent *in vacuo*, the crude product was purified by preparative RP-HPLC (column: Gemini-NX C18, 250 × 21 mm, 5 µm, AXIA Packed; flow: 18 mL/min; gradient: A/B 10/90 to 60/40 in 30 min). Acetonitrile was removed under reduced pressure from the eluate. Lyophilisation afforded the product as a white solid (18 mg, 16 %). $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.46 – 1.63 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.66 – 1.76 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.79 – 1.88 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.45 (t,

$^3J$ = 6.6 Hz 4H, CO-C***H***$_2$), 2.52 (t, $^3J$ = 6.6 Hz, 4H, CO-CH$_2$), 2.70 – 2.92 (bm, 4H, C***H***$_2$-N-C***H***$_2$ ), 2.94 – 3.14 (m, 4H, C***H***$_2$-C***H***$_2$-NH$_2$), 3.16 – 3.25 (m, 4H, CH-(CH$_2$)$_2$-C***H***$_2$-NH), 3.30 – 3.35 (m, overlap with solvent, 8H, 4x C***H***$_2$-NH-CO), 3.38 (t, $^3J$ = 5.0 Hz, 4H, 2x NH-CO-NH-C***H***$_2$-CH$_2$-CO), 3.51 (t, $^3J$ = 6.1 Hz, 4H, 2x O-C***H***$_2$-CH$_2$-NH), 3.56 (t, $^3J$ = 5.1 Hz, 4H, 2x O-C***H***$_2$-CH$_2$-NH-CO-NH), 3.58 – 3.65 (m, 8H, 2x O-(C***H***$_2$)$_2$-O) 4.26 (s, 4H, Ar-C***H***$_2$-NH-CO-NH$_2$), 4.26 – 4.38 (m, 4H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 2H, C***H***-CH$_2$-CH$_2$), 5.08 (s, 2H, (Ph)$_2$-C***H***), 7.17 – 7.22 (m, 8H, CH$_2$-C$_6$***H***$_4$-CH$_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, CD$_3$OD, HSQC): δ (ppm) 25.75 (2x CH-CH$_2$-***C***H$_2$), 30.26 (2x CH-***C***H$_2$-CH$_2$), 31.51 (2x CO-(***C***H$_2$)$_2$-CO), 31.87 (2x CO-***C***H$_2$), 37.73 (CH$_2$-NH$_2$), 40.46 (2x ***C***H$_2$-NH-CO), 40.64 (2x NH-CO-NH-***C***H$_2$-CH$_2$-CO), 41.73 ((CH$_2$)$_2$-***C***H$_2$-NH-), 43.79 (NH-***C***H$_2$-Ar), 44.31 (Ar-***C***H$_2$-NH-CO-NH$_2$), 52.20 (***C***H$_2$-N), 54.40 (***C***H-CH$_2$-CH$_2$), 58.66 ((Ph)$_2$-***C***H), 70.46 (O-***C***H$_2$-CH$_2$-NH-CO-NH), 70.62 (O-***C***H$_2$-CH$_2$-NH), 71.31 (O-(***C***H$_2$)$_2$-O), 71.34 (O-(***C***H$_2$)$_2$-O), 128.16 (diPh-***C***-4), 128.25 (diPh-***C***-4), 128.31 (4 Ph-***C***), 128.64 (4 Ph-***C***), 129.52 (4 diPh-***C***), 129.58 (4 diPh-***C***), 129.88 (4 diPh-***C***), 129.91 (4 diPh-***C***), 138.42 (Ph-***C***-1), 140.86 (Ph-***C***-4), 140.96 (2 diPh-***C***-1), 155.57 (NH-***C***NH$_2$-NH), 155.63 (NH-***C***O-NH), 162.13 (Ar-CH$_2$-NH-***C***O-NH$_2$), 173.58 (***C***O-NH-CH$_2$-Ar), 174.85 ((Ph)$_2$-CH-***C***O), 174.89 (***C***=O); anal. RP-HPLC: 97 % ($t_R$ = 14.45 min, *k*' = 3.74); HRMS (ESI): *m/z* calcd. for [C$_{86}$H$_{120}$N$_{22}$O$_{16}$+2H]$^{2+}$ 859.4699, found: 859.4701.5194. C$_{86}$H$_{120}$N$_{22}$O$_{16}$ · 3TFA (2060.07).

### 5.7.2.1 Synthesis of the (*R*)- and (*S*)-Argininamide-Type Building Blocks (*R*)- and (*S*)-5.44

**(*R*)-*N*$^\delta$-Benzyloxycarbonylornithine (5.39)**[25]

Benzyloxycarbonyl chloride (45 % in toluene, 43.1 mL, 116.0 mmol, 1.1 eq) was added to an ice-cold solution of *D*-ornithine hydrochloride **5.38**, potassium carbonate (14.5 g, 105.0 mmol, 1.0 eq) and copper(II)sulfate pentahydrate (13.11 g, 52.5 mmol, 0.5 eq) in aqueous NaOH (0.5 N, 210 mL, 105 mmol, 1eq) over a period of 15 min. The ice-bath was removed and the reaction was stirred at rt overnight. The resulting solid was filtered off, washed twice with ice-cold water (50 mL), ice-cold MeOH (50 mL) and water (50 mL) again. Lyophilisation yielded a blue-violet powder which was suspended together with Titriplex III (19.6 g, 52.53 mmol, 0.5 eq) in water (175 mL) under vigorous stirring and heated to 100 °C for 60 min. The solid was separated after cooling to 0 °C, washed with ice-cold water (3 × 40 mL) and dried *in vacuo*. Compound **5.39** was obtained as a white solid (23.5 g, 84 %). $^1$H-NMR (300 MHz, DMSO-d$_6$): δ (ppm) 1.35 – 1.83 (m, 4H, CH-C***H***$_2$-C***H***$_2$), 2.89 – 3.07 (m, 2H, C***H***$_2$-NH), 3.09 – 3.13 (m, 1H, C***H***$^\alpha$), 5.0 (s, 2H, Ph-C***H***$_2$), 7.16 – 7.52 (m, 5H, Ph-***H***); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 267 (100) [M+H]$^+$, 533 (70) [2M+H]$^+$; C$_{13}$H$_{18}$N$_2$O$_4$ (266.3).

**(*R*)-$N^{\delta}$-Benzyloxycarbonyl-$N^{\alpha}$-(*tert*-butoxycarbonyl)ornithine (5.40)[12, 26]**

The Cbz-protected *D*-ornithine **5.39** (25.5 g, 96.1 mmol, 1.0 eq) was suspended in an aqueous solution (290 mL) of potassium carbonate (14.6 g, 105 mmol, 1.1 eq) prior to the addition of 1,4-dioxane (120 mL). Subsequently, di-*tert*-butyl dicarbonate (21.9 g, 100.4 mmol, 1.05 eq) dissolved in 1,4-dioxane (230 mL) was added dropwise over a period of 60 min and the reaction mixture was stirred at room temperature overnight. After concentrating the solvent under reduced pressure to a volume of about 150 mL, water (60 mL) was added and the pH was adjusted to 2 – 3 with 1 M aq. hydrochloric acid (about 170 mL). The product was extracted with ethyl acetate (3 × 300 mL) and the combined organic phases were treated with 10 mM aq. hydrochloric acid (100 mL), saturated aq. $NH_4Cl$ (200 mL), water (100 mL) as well as brine (250 mL) prior to drying over $MgSO_4$. The solvent was evaporated and the residue dried *in vacuo*. The crude product was obtained as a yellowish hard foam (28.4 g, 81 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.38 (s, 9H, C(C***H***$_3$)$_3$), 1.39 – 1.59 (m, 3H, CH-C***H***$_2$-C***H***$_2$), 1.60 – 1.76 (m, 1H, CH-C***H***$_2$-CH$_2$), 2.92 – 3.04 (m, 2H, C***H***$_2$-NH), 3.77 – 3.87 (m, 1H, C***H***$^{\alpha}$), 5.0 (s, 2H, Ph-C***H***$_2$), 7.07 (d, $^3J$ = 8.0 Hz, 1H, CO-N***H***-CH), 7.25 (t, $^3J$ = 5.5 Hz, 1H, CO-N***H***-CH$_2$), 7.34 (m, 5H, Ph-***H***), 12.44 (s, 1H, COO***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 26.08 (-, CH-(***C***H$_2$)$_2$), 28.03 (-, CH-(***C***H$_2$)$_2$), 28.09 (+, C(***C***H$_3$)$_3$), 39.80 (-, ***C***H$_2$-NH), 53.07 (+, ***C***H-(CH$_2$)$_2$), 65.00 (-, Ph-***C***H$_2$), 77.85 (C$_{quat}$, ***C***(CH$_3$)$_3$), 127.59 (+, 2 Ph-***C***), 127.63 (+, Ph-***C***-4), 128.24 (+, 2 Ph-***C***), 137.16 (C$_{quat}$, Ph-***C***-1), 155.47 (C$_{quat}$, ***C***=O), 155.98 (C$_{quat}$, ***C***=O). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 367 (50) $[M+H]^+$, 733 (100) $[2M+H]^+$. $C_{18}H_{28}N_2O_6$ (366.41).

**General procedure for the synthesis of (*R*)- and (*S*)-$N^{\delta}$-Benzyloxycarbonyl-$N^{\alpha}$-tert-butoxycarbonyl-*N*-(4-ureidomethylbenzyl)ornithinamide (5.41)**

Compound **(*R*)-5.40** or **(*S*)-5.40** (1 eq) was activated with carbonyldiimidazole (1.1 eq) in DMF at 0 °C for 60 min. $Et_3N$ (1.5 eq), DMAP (0.5 eq) and **5.47** (eq) in DMF were added as suspension. The mixture was slowly warmed up to 45 °C yielding an almost clear solution. After 30 min, heating was finished and the mixture was stirred at rt overnight. The next day, glacial acetic acid (2 mL, about 1.5 eq) was added and DMF was removed under reduced pressure yielding an oily residue which was dried *in vacuo* followed by purification with flash chromatography (DCM/MeOH 90/10).

**(*S*)-$N^{\delta}$-Benzyloxycarbonyl-$N^{\alpha}$-*tert*-butoxycarbonyl-*N*-(4-ureido-methylbenzyl)ornithinamide ((*S*)-5.41)**

The title compound was prepared from **(*S*)-5.40** (5.00 g, 16.7 mmol) and CDI (2.43 g, 15.0 mmol) in 80 mL DMF and **5.47** (2.94 g, 13.7 mmol), DMAP (0.83 g, 6.8 mmol) and $Et_3N$ (2.88 mL, 20.5 mmol) in 250 mL DMF. Purification by flash chromatography yielded **(*S*)-5.41** as yellowish hard solid (6.53 g, 91 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.47 – 1.67 (m, 3H, CH-C***H***$_2$-C***H***$_2$),

1.68 – 1.84 (m, 1H, CH-C***H***$_2$-CH$_2$), 3.05 – 3.17 (m, 2H, CH$_2$-C***H***$_2$-NH), 3.99 – 4.09 (m, 1H, C***H***$^{\alpha}$), 4.25 (s, 2H, C***H***$_2$-NH-CO-NH$_2$), 4.27 – 4.42 (m, 2H, Ar-C***H***$_2$-NH), 5.04 (s, 2H, Ph-C***H***$_2$), 7.19 – 7.24 (m, 4H, CH$_2$-C$_6$***H***$_4$-CH$_2$), 7.26 – 7.38 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, DMSO-d$_6$): δ (ppm) 27.44 (-, CH-(***C***H$_2$)$_2$), 28.78 (+, C(***C***H$_3$)$_3$), 30.72 (-, CH-(***C***H$_2$)$_2$), 41.42 (-, ***C***H$_2$-NH), 43.76 (-, Ar-***C***H$_2$-NH), 44.45 (Ar-***C***H$_2$-NH), 55.98 (+, ***C***H-(CH$_2$)$_2$), 67.41 (-, Ph-***C***H$_2$), 80.69 (C$_{quat}$, ***C***(CH$_3$)$_3$), 128.46 (+, 2 Ph-***C***), 128.67 (+, Ph-***C***-4), 128.81 (+, 2 Ph-***C***), 129.02 (+, 2 Ph-***C***), 129.54 (+, 2 Ph-***C***), 136.38 (C$_{quat}$, Ph-***C***), 138.48 (C$_{quat}$, Ph-***C***), 138.67 (C$_{quat}$, Ph-***C***), 157.90 (C$_{quat}$, ***C***=O), 159.01 (C$_{quat}$, ***C***=O), 162.12 (C$_{quat}$, NH-***C***O-NH), 175.14 (C$_{quat}$, CH-***C***O-NH); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 528 (100) [M + H]$^+$, 1056 (50) [2M+H]$^+$; C$_{27}$H$_{37}$N$_5$O$_6$ (527.61).

**(*R*)-*N*$^{\delta}$-Benzyloxycarbonyl-*N*$^{\alpha}$-*tert*-butoxycarbonyl-*N*-(4-ureidomethylbenzyl)ornithinamide ((*R*)-5.41)** [11]

The title compound was prepared from **(*R*)-5.40** (7.69 g, 21.0 mmol) and CDI (3.75 g, 23.1 mmol) in 80 mL DMF and **5.47** (4.53 g, 21.0 mmol), DMAP (1.28 g, 10.5 mmol) and Et$_3$N (4.43 mL, 31.5 mmol) in 250 mL DMF. Purification by flash chromatography yielded **(*R*)-5.41** as yellowish hard solid (8.35 g, 75 %). $^1$H-NMR (300 MHz, CD$_3$OD): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.47 – 1.66 (m, 3H, CH-C***H***$_2$-C***H***$_2$), 1.67 – 1.83 (m, 1H, CH-C***H***$_2$-CH$_2$), 3.06 – 3.16 (m, 2H, CH$_2$-C***H***$_2$-NH), 3.98 – 4.09 (m, 1H, C***H***$^{\alpha}$), 4.25 (s, 2H, C***H***$_2$-NH-CO-NH$_2$), 4.26 – 4.42 (m, 2H, Ar-C***H***$_2$-NH), 5.04 (s, 2H, Ph-C***H***$_2$), 7.17 – 7.24 (m, 4H, CH$_2$-C$_6$***H***$_4$-CH$_2$), 7.26 – 7.38 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, DMSO-d$_6$): δ (ppm) 27.52 (-, CH-(***C***H$_2$)$_2$), 28.78 (+, C(***C***H$_3$)$_3$), 30.70 (-, CH-(***C***H$_2$)$_2$), 41.23 (-, ***C***H$_2$-NH), 43.75 (-, Ar-***C***H$_2$-NH), 44.44 (Ar-***C***H$_2$-NH), 55.99 (+, ***C***H-(CH$_2$)$_2$), 67.41 (-, Ph-***C***H$_2$), 80.69 (C$_{quat}$, ***C***(CH$_3$)$_3$), 128.45 (+, 2 Ph-***C***), 128.66 (+, Ph-***C***-4), 128.81 (+, 2 Ph-***C***), 129.01 (+, 2 Ph-***C***), 129.53 (+, 2 Ph-***C***), 136.37 (C$_{quat}$, Ph-***C***), 138.48 (C$_{quat}$, Ph-***C***), 138.67 (C$_{quat}$, Ph-***C***), 157.90 (C$_{quat}$, ***C***=O), 159.02 (C$_{quat}$, ***C***=O), 162.13 (C$_{quat}$, NH-***C***O-NH), 175.16 (C$_{quat}$, CH-***C***O-NH); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 528 (100) [M + H]$^+$, 1056 (40) [2M+H]$^+$; C$_{27}$H$_{37}$N$_5$O$_6$ (527.61).

**General procedure for the synthesis of (*R*)- and (*S*)-*N*$^{\delta}$-Benzyloxycarbonyl-*N*-(4-ureidomethyl-benzyl)ornithinamide (5.42)**

**(*R*)-5.41** or **(*S*)-5.41** (1 eq) was dissolved in MeOH and acetyl chloride (25 eq) was added dropwise under water cooling (20 °C) over a period of 1.5 h. Stirring was continued for 30 min, volatiles were removed under reduced pressure and the residue was suspended in water (100 mL). Lyophilisation afforded the product as a white solid.

**(*S*)-*N*$^{\delta}$-Benzyloxycarbonyl-*N*-(4-ureidomethylbenzyl)ornithinamide ((*S*)-5.42)**

The title compound was prepared from **(*S*)-5.41** (6.00 g, 11.4 mmol) and acetyl chloride (20.2 mL, 284.0 mmol) in 200 mL MeOH. Lyophilisation afforded the product as a white solid (5.01 g, 95 %). $^1$H-NMR (300 MHz, CD$_3$OD): δ (ppm) 1.43 – 1.61 (m, 3H, CH-C***H***$_2$-C***H***$_2$), 1.62 – 1.78 (m, 1H, CH-C***H***$_2$-CH$_2$),

3.04 – 3.16 (m, 2H, $CH_2$-C***H***$_2$-NH), 3.30 – 3.35 (m, overlap with solvent, 1H, C***H***$^{\alpha}$), 4.25 (s, 2H, C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.42 (m, 2H, Ar-C***H***$_2$-NH), 5.04 (s, 2H, Ph-C***H***$_2$), 7.20-7.26 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.27 – 7.38 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 27.23 (-, CH-(***C***$H_2)_2$), 33.76 (-, CH-(***C***$H_2)_2$), 41.47 (-, ***C***$H_2$-NH), 43.80 (-, Ar-***C***$H_2$-NH), 44.45 (-, Ar-***C***$H_2$-NH), 55.82 (+, ***C***H-$(CH_2)_2$), 67.38 (-, Ph-***C***$H_2$), 128.50 (+, 2 Ph-***C***), 128.80 (+, 2 Ph-***C***), 128.87 (+, 2 Ph-***C***), 129.00 (+, Ph-***C***), 129.52 (2 Ph-***C***), 138.50 ($C_{quat}$, Ph-***C***), 138.69 ($C_{quat}$, Ph-***C***), 140.20 ($C_{quat}$, Ph-***C***), 158.98 ($C_{quat}$, O-***C***O-NH), 162.09 ($C_{quat}$, NH-***C***O-NH), 177.37 ($C_{quat}$, CH-***C***O-NH). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 428 (100) $[M+H]^+$. $C_{22}H_{29}N_5O_4 \cdot$ HCl (463.96).

**(*R*)-$N^{\delta}$-Benzyloxycarbonyl-*N*-(4-ureidomethylbenzyl)ornithinamide ((*R*)-5.42)[12]**

The title compound was prepared from **(*R*)-5.41** (8.00 g, 15.2 mmol) and acetyl chloride (27.0 mL, 379.0 mmol) in 250 mL MeOH. Lyophilisation afforded the product as a white solid (6.4 g, 91 %)**.** $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.42 – 1.62 (m, 3H, CH-C***H***$_2$-C***H***$_2$), 1.62 – 1.77 (m, 1H, CH-C***H***$_2$-$CH_2$), 3.03 – 3.16 (m, 2H, $CH_2$-C***H***$_2$-NH), 3.29 – 3.35 (m, overlap with solvent, 1H, C***H***$^{\alpha}$), 4.24 (s, 2H, C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.43 (m, 2H, Ar-C***H***$_2$-NH), 5.04 (s, 2H, Ph-C***H***$_2$), 7.20-7.26 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.27 – 7.39 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 27.24 (-, CH-(***C***$H_2)_2$), 33.75 (-, CH-(***C***$H_2)_2$), 41.47 (-, ***C***$H_2$-NH), 43.80 (-, Ar-***C***$H_2$-NH), 44.43 (-, Ar-***C***$H_2$-NH), 55.82 (+, ***C***H-$(CH_2)_2$), 67.38 (-, Ph-***C***$H_2$), 128.49 (+, 2 Ph-***C***), 128.81 (+, 2 Ph-***C***), 128.88 (+, 2 Ph-***C***), 129.01 (+, Ph-***C***), 129.52 (2 Ph-***C***), 138.50 ($C_{quat}$, Ph-***C***), 138.69 ($C_{quat}$, Ph-***C***), 140.24 ($C_{quat}$, Ph-***C***), 158.97 ($C_{quat}$, O-***C***O-NH), 162.13 ($C_{quat}$, NH-***C***O-NH), 177.33 ($C_{quat}$, CH-***C***O-NH). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 428 (100) $[M+H]^+$. $C_{22}H_{29}N_5O_4 \cdot$ HCl (463.96).

**General procedure for the synthesis of (*R*)- and (*S*)-$N^{\delta}$-Benzyloxycarbonyl-$N^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-ureidomethylbenzyl)ornithinamide (5.43)**

**(*R*)-5.42** or **(*S*)-5.42** (1 eq) was dissolved in anhydrous DMF (100 mL). $Et_3N$ (1.5 eq), DMAP (0.5 eq) and **5.49** (1.2 eq) were added and the mixture was kept under stirring for 20 h at rt. After addition of glacial acetic acid (3 mL) DMF was removed under reduced pressure yielding a light yellow-brown solid which was dried *in vacuo*. The solid was insoluble or poorly soluble in most organic solvents. Therefore the solid material was crushed to small pellets and suspended in acetonitrile (700 mL). After heating (70 °C) and sonication, the insoluble crude product turned into a white fluffy solid. Separation by filtration and washing with acetonitrile (2 × 250 mL) and water (3× 150 mL) yielded the product as a white solid.

**(*S*)-$N^{\delta}$-Benzyloxycarbonyl-$N^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-ureidomethylbenzyl)ornithinamide ((*S*)-5.43)**

The title compound was prepared from **(*S*)-5.42** (2.80 g, 6.6 mmol), **5.49** (2.23 g, 7.2 mmol), DMAP (0.40 g, 3.3 mmol) and $Et_3N$ (1.38 mL, 9.8 mmol) in 40 mL DMF according to the general procedure yielding the product as a white solid (3.5 g, 86 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.20 – 1.45 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.46 – 1.76 (m, 2H, CH-C***H***$_2$-$CH_2$), 2.87 – 3.05 (m, 2H, $CH_2$-C***H***$_2$-NH), 4.10 – 4.26 (m, 4H, C***H***$_2$-Ar-C***H***$_2$), 4.27 – 4.38 (m, 1H, C***H***$^{\alpha}$), 5.01 (s, 2H, Ph-C***H***$_2$), 5.12 (s, 1H, $(Ph)_2$-C***H***), 5.53 (bs, 2H, N***H***$_2$), 6.38 (t, $^3J$ = 5.9 Hz, 1H, N***H***-CO), 7.10 – 7.18 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.19 – 7.41 (m, 16H, Ph-***H*** + N***H***-CO), 8.38 – 8.52 (m, 2H, 2× N***H***-CO); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 25.92 (-, ***C***$H_2$), 29.56 (-, ***C***$H_2$), 39.80 (-, ***C***$H_2$-NH), 41.65 (-, Ar-***C***$H_2$-NH), 42.44 (-, Ar-***C***$H_2$-NH), 52.39 (+, $(Ph)_2$-***C***H) 55.81 (+, ***C***H-$(CH_2)_2$), 65.03 (-, Ph-***C***$H_2$), 126.47 (+, 2 diPh-***C***-4), 126.85 (+, 4 $CH_2$-$C_6$***H***$_4$-$CH_2$), 127.61 (+, 2 Ph-***C***), 127.65 (+, Ph-***C***-4), 128.06 (+, 2 diPh-***C***), 128.09 (+, 2 diPh-***C***), 128.25 (+, 2 Ph-***C***), 128.37 (+, 2 diPh-***C***), 128.45 (+, 2 diPh-***C***), 137.15 ($C_{quat}$, Ph-***C***), 137.29 ($C_{quat}$, Ph-***C***), 139.30 ($C_{quat}$, Ph-***C***), 140.24 ($C_{quat}$, Ph-***C***), 140.43 ($C_{quat}$, Ph-***C***), 155.97 ($C_{quat}$, O-***C***O-NH), 158.54 ($C_{quat}$, NH-***C***O-NH), 170.83 ($C_{quat}$, ***C***=O), 171.31 ($C_{quat}$, ***C***=O). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 622 (100) $[M+H]^+$. $C_{36}H_{39}N_5O_5$ (621.73).

**(*R*)-$N^{\delta}$-Benzyloxycarbonyl-$N^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-ureidomethylbenzyl)ornithinamide ((*R*)-5.43)[12, 27]**

The title compound was prepared from **(*R*)-5.42** (5.00 g, 11.7 mmol), **5.49** (4.34 g, 14.0 mmol), DMAP (0.71 g, 5.9 mmol) and $Et_3N$ (2.47 mL, 17.5 mmol) in 70 mL DMF according to the general procedure yielding the product as a white solid (5.65 g, 78 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.22 – 1.45 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.46 – 1.74 (m, 2H, CH-C***H***$_2$-$CH_2$), 2.88 – 3.02 (m, 2H, $CH_2$-C***H***$_2$-NH), 4.10 – 4.26 (m, 4H, C***H***$_2$-Ar-C***H***$_2$), 4.27 – 4.39 (m, 1H, C***H***$^{\alpha}$), 5.00 (s, 2H, Ph-C***H***$_2$), 5.12 (s, 1H, $(Ph)_2$-C***H***), 5.53 (bs, 2H, N***H***$_2$), 6.38 (t, $^3J$ = 5.9 Hz, 1H, N***H***-CO), 7.09 – 7.17 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.19 – 7.42 (m, 16H, Ph-***H*** + N***H***-CO), 8.37 – 8.54 (m, 2H, 2× N***H***-CO); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 25.92 (-, ***C***$H_2$), 29.56 (-, ***C***$H_2$), 39.80 (-, ***C***$H_2$-NH), 41.65 (-, Ar-***C***$H_2$-NH), 42.44 (-, Ar-***C***$H_2$-NH), 52.39 (+, $(Ph)_2$-***C***H) 55.81 (+, ***C***H-$(CH_2)_2$), 65.03 (-, Ph-***C***$H_2$), 126.46 (+, 2 diPh-***C***-4), 126.85 (+, 4 $CH_2$-$C_6$***H***$_4$-$CH_2$), 127.61 (+, 2 Ph-***C***), 127.65 (+, Ph-***C***-4), 128.06 (+, 2 diPh-***C***), 128.09 (+, 2 diPh-***C***), 128.25 (+, 2 Ph-***C***), 128.37 (+, 2 diPh-***C***), 128.45 (+, 2 diPh-***C***), 137.15 ($C_{quat}$, Ph-***C***), 137.30 ($C_{quat}$, Ph-***C***), 139.30 ($C_{quat}$, Ph-***C***), 140.24 ($C_{quat}$, Ph-***C***), 140.44 ($C_{quat}$, Ph-***C***), 155.97 ($C_{quat}$, O-***C***O-NH), 158.54 ($C_{quat}$, NH-***C***O-NH), 170.83 ($C_{quat}$, ***C***=O), 171.31 ($C_{quat}$, ***C***=O). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 622 (100) $[M+H]^+$; $C_{36}H_{39}N_5O_5$ (621.73).

**General procedure for the synthesis (*R*)- and (*S*)-$N^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-ureidomethylbenzyl)-ornithinamide (5.35)**

Compound **(*R*)-5.43** or **(*S*)-5.43** was suspended in MeOH. The amount of solid could be reduced by heating the suspension to 60 °C. Glacial acetic acid (1.2 mL) and a 10 % Pd/C catalyst were added and hydrogen was lead through the vigorously stirred mixture at 45 °C for 2 - 4 h (TLC control). At this time the white solid had disappeared. After 3 h the catalyst was removed by filtration over Celite, the solvent was removed under reduced pressure and the residue was suspended in water (50 mL) at 40 °C prior to lyophilisation.

**(*S*)-$N^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-ureidomethylbenzyl)ornithinamide ((*S*)-5.35)**

The hydrogenation of **(*S*)-5.43** (2.8 g, 4.5 mmol) was carried out in 300 mL MeOH using 300 mg of 10 % Pd/C catalyst according to the general procedure. The title compound **(*S*)-5.35** was obtained as a white solid (2.37 g, 96 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.32 – 1.62 (m, 3H, CH-C***H***$_2$-C***H***$_2$), 1.63 – 1.78 (m, 1H, CH-C***H***$_2$-CH$_2$), 2.56 – 2.68 (m, 2H, C***H***$_2$-NH$_2$), 4.10 – 4.26 (m, 4H, C***H***$_2$-Ar-C***H***$_2$), 4.27 – 4.38 (m, 1H, C***H***$^{\alpha}$), 5.14 (s, 1H, (Ph)$_2$-C***H***), 5.59 (bs, 2H, N***H***$_2$), 6.53 (t, $^3J$ = 5.7 Hz, 1H, N***H***-CO), 7.10 – 7.18 (m, 4H, C***H***$_2$-C$_6$H$_4$-C***H***$_2$), 7.19 – 7.40 (m, 11H, Ph-***H*** + N***H***), 8.62 – 8.76 (m, 2H, N***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 25.46 (-, ***C***H$_2$), 29.15 (-, ***C***H$_2$), 38.72 (-, ***C***H$_2$-NH$_2$), 41.65 (-, Ar-***C***H$_2$-NH), 42.42 (-, Ar-***C***H$_2$-NH), 52.13 (+, (Ph)$_2$-***C***H), 55.78 (+, ***C***H-(CH$_2$)$_2$), 126.46 (+, 2 diPh-***C***-4), 126.82 (+, 4 Ph-***C***), 128.05 (+, 2 diPh-***C***), 128.09 (+, 2 diPh-***C***), 128.40 (+, 2 diPh-***C***), 128.43 (+, 2 diPh-***C***), 137.35 (C$_{quat}$, Ph-***C***), 139.29 (C$_{quat}$, Ph-***C***), 140.27 (C$_{quat}$, Ph-***C***), 140.44 (C$_{quat}$, Ph-***C***), 158.65 (C$_{quat}$, NH-***C***O-NH$_2$), 170.94 (C$_{quat}$, ***C***=O), 171.34 (C$_{quat}$, ***C***=O). MS (ESI, MeCN/0.1 % FA) m/z (%): 488 (100) $[M+H]^+$; $C_{28}H_{33}N_5O_3 \cdot C_2H_4O_2$ (547.65).

**(*R*)-$N^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-ureidomethylbenzyl)ornithinamide ((*R*)-5.35)[12, 27]**

The hydrogenation of **(*R*)-5.43** (2.0 g, 3.2 mmol) was carried out in 220 mL MeOH using 200 mg of 10 % Pd/C catalyst according to the general procedure. The title compound **(*R*)-5.35** was obtained as a white solid (1.64 g, 93 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.31 – 1.63 (m, 3H, CH-C***H***$_2$-C***H***$_2$), 1.64 – 1.74 (m, 1H, CH-C***H***$_2$-CH$_2$), 2.54 – 2.67 (m, 2H, C***H***$_2$-NH$_2$), 4.10 – 4.26 (m, 4H, C***H***$_2$-Ar-C***H***$_2$), 4.27 – 4.38 (m, 1H, C***H***$^{\alpha}$), 5.14 (s, 1H, (Ph)$_2$-C***H***), 5.57 (bs, 2H, N***H***$_2$), 6.51 (t, $^3J$ = 6.0 Hz, 1H, N***H***-CO), 7.10 – 7.18 (m, 4H, C***H***$_2$-C$_6$H$_4$-C***H***$_2$), 7.19 – 7.38 (m, 11H, Ph-***H*** + N***H***), 8.62 – 8.76 (m, 2H, N***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 26.08 (-, ***C***H$_2$), 29.23 (-, ***C***H$_2$), 39.12 (-, ***C***H$_2$-NH$_2$), 41.65 (-, Ar-***C***H$_2$-NH), 42.43 (-, Ar-***C***H$_2$-NH), 52.18 (+, (Ph)$_2$-***C***H), 55.79 (+, ***C***H-(CH$_2$)$_2$), 126.46 (+, 2 diPh-***C***-4), 126.82 (+, 4 Ph-***C***), 128.05 (+, 2 diPh-***C***), 128.09 (+, 2 diPh-***C***), 128.39 (+, 2 diPh-***C***), 128.43 (+, 2 diPh-***C***), 137.36 (C$_{quat}$, Ph-***C***), 139.30 (C$_{quat}$, Ph-***C***), 140.28 (C$_{quat}$, Ph-***C***), 140.45 (C$_{quat}$, Ph-***C***), 158.62 (C$_{quat}$, NH-***C***O-NH$_2$), 170.90

($C_{quat}$, ***C***=O), 171.36 ($C_{quat}$, ***C***=O); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 488 (100) $[M+H]^+$; $C_{28}H_{33}N_5O_3 \cdot C_2H_4O_2$ (547.65).

**General procedure for the synthesis of (*R*)- and (*S*)-5-[2-(2-aminoethylcarbamoyl)guanidine]-2-(2,2-diphenylacetamido)-*N*-[4-(ureidomethyl)benzyl]pentanamide (5.44)**

The compound **(*S*)-5.35** or **(*R*)-5.35** (1 eq), the guanidinylating reagent **3.67** (1 eq) and $HgCl_2$ (2 eq) were dissolved in anhydrous DMF. DIEA (3 eq) was added and the mixture was stirred for 24 to 30 h at ambient temperature. Subsequently, the precipitate was removed by centrifugation. The crude product was purified by flash chromatography (DCM/MeOH 100/1 – 95/5 v/v). The residue was dissolved in DCM. TFA (50 % final concentration) was added and the reaction mixture was stirred until the protection groups were removed (3 – 8 h, TLC control). After evaporation of the solvent *in vacuo*, the crude product was purified by flash chromatography.

**(*S*)-5-[2-(2-Aminoethylcarbamoyl)guanidine]-2-(2,2-diphenylacetamido)-N-[4-(ureidomethyl)-benzyl]pentanamide ((*S*)-5.45)**

The title compound was prepared from **(*S*)-5.35** (1.10 g, 2.3 mmol), **3.67** (0.85 g, 2.3 mmol), $Hg_2Cl_2$ (0.92 g, 3.4 mmol) and DIEA (0.79 mL, 4.5 mmol). Deprotection in 20 mL DCM and 20 mL TFA followed by flash chromatography afforded the product as pale yellow foam (1.43 g, 78 %). $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.47 – 1.63 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.66 – 1.75 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.78 – 1.89 (m, 1H, CH-C***H***$_2$-$CH_2$), 3.07 (t, $^3J$ = 5.0 Hz, 2H, NH-$CH_2$-C***H***$_2$-$NH_2$), 3.17 – 3.27 (m, 2H, CH-$(CH_2)_2$-C***H***$_2$-NH), 3.49 (t, $^3J$ = 5.1 Hz, 2H, NH-C***H***$_2$-$CH_2$-$NH_2$), 4.26 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.07 (s, 1H, $(Ph)_2$-C***H***), 7.16 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.32 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC) 25.61 (CH-$CH_2$-***C***$H_2$), 30.27 (CH-***C***$H_2$-$CH_2$), 38.53 (NH-$CH_2$-***C***$H_2$-$NH_2$), 40.77 (NH-***C***$H_2$-$CH_2$-$NH_2$), 41.81 ($(CH_2)_2$-***C***$H_2$-NH-), 43.80 (NH-***C***$H_2$-Ar), 44.32 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.32 (***C***H-$CH_2$-$CH_2$), 58.69 ($(Ph)_2$-***C***H), 128.15 (diPh-***C***-4), 128.22 (diPh-***C***-4), 128.36 (2 Ph-***C***), 128.66 (2 Ph-***C***), 129.51 (2 diPh-***C***), 129.55 (2 diPh-***C***), 129.85 (2 diPh-***C***), 129.90 (2 diPh-***C***), 138.40 (Ph-***C***-1), 140.84 (Ph-***C***-4), 140.96 (2 diPh-***C***-1), 155.64 (NH-***C***$NH_2$-NH), 156.45 (NH-***C***O-NH), 162.13 (Ar-$CH_2$-NH-***C***O-$NH_2$), 173.51 (***C***O-NH-$CH_2$-Ar), 174.85 ($(Ph)_2$-CH-***C***O); HRMS (ESI) *m/z* calcd. for $[C_{32}H_{41}N_9O_4+H]^+$ 616.3354, found 616.3360. $C_{32}H_{41}N_9O_4 \cdot 2$TFA (985.92).

**(*R*)-5-[2-(2-Aminoethylcarbamoyl)guanidine]-2-(2,2-diphenylacetamido)-N-[4-(ureidomethyl)-benzyl]pentanamide ((*R*)-5.44)**

The title compound was prepared from **(*R*)-5.35** (0.79 g, 1.6 mmol), **3.67** (0.61 g, 1.6 mmol), $HgCl_2$ (0.66 g, 2.4 mmol) and DIEA (0.56 mL, 3.2 mmol). Deprotection in 15 mL DCM and 15 mL TFA followed by flash chromatography afforded the product as pale yellow foam (1.43 g, 78 %). $^1$H-NMR

(300 MHz, $CD_3OD$): δ (ppm) 1.30 – 1.60 (m, 3H, CH-C***H***$_2$-C***H***$_2$), 1.60 – 1.76 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.84 (t, $^3J$ = 5.7 Hz, 2H, NH-$CH_2$-C***H***$_2$-$NH_2$), 3.03 – 3.15 (m, 2H, CH-$(CH_2)_2$-C***H***$_2$-NH), 3.17 – 3.28 (m, 2H, NH-C***H***$_2$-$CH_2$-$NH_2$), 4.10 -4.19 (m, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.19 – 4.27 (m, 2H, Ar-C***H***$_2$-NH), 4.27 – 4.40 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.13 (s, 1H, $(Ph)_2$-C***H***), 5.58 (bs, NH-CO-N***H***$_2$), 6.48 (t, $^3J$ = 6.0 Hz, 1H, N***H***), 7.08 – 7.17 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.18 – 7.34 (m, 10 H, Ph-***H***), 8.44 – 8.57 (m, 2H, 2x N***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$) 25.44 (-, CH-$CH_2$-***C***$H_2$), 29.54 (-, CH-***C***$H_2$-$CH_2$), 37.28 (-, NH-$CH_2$-***C***$H_2$-$NH_2$), 39.37 (-, NH-***C***$H_2$-$CH_2$-$NH_2$), 41.65 ($(CH_2)_2$-***C***$H_2$-NH-), 42.40 (-, NH-***C***$H_2$-Ar), 44.32 (-, Ar-***C***$H_2$-NH-CO-$NH_2$), 52.42 (+, ***C***H-$CH_2$-$CH_2$), 55.80 (+, $(Ph)_2$-***C***H), 126.50 (+, 2 diPh-***C***-4), 126.82 (+, 4 Ph-***C***), 128.07 (+, 2 diPh-***C***), 128.11 (+, 2 diPh-***C***), 128.38 (+, 2 diPh-***C***), 128.41 (+, 2 diPh-***C***), 137.27 (C#, Ph-***C***-1), 140.20 ($C_{quat}$, Ph-***C***-4), 140.39 ($C_{quat}$, 2 diPh-***C***-1), 158.06 ($C_{quat}$, NH-***C***$NH_2$-NH), 158.46 ($C_{quat}$, NH-***C***O-NH), 158.47 ($C_{quat}$, Ar-$CH_2$-NH-***C***O-$NH_2$), 170.88 ($C_{quat}$, ***C***O-NH-$CH_2$-Ar), 171.22 ($C_{quat}$, $(Ph)_2$-CH-***C***O); MS (ESI, MeCN/0.1% FA) *m/z* (%): 308 (50) $[M+2H]^{2+}$, 329 (100) $[M+MeCN+2H]^{2+}$, 616 (75) $[M+H]^+$. $C_{32}H_{41}N_9O_4 \cdot 2TFA$ (985.92).

**Succinimidyl 2,2-diphenylacetate (5.49)** [16]

To a solution of 2,2-diphenylacetic acid **5.48** (25.0 g, 118.0 mmol, 1.0 eq) in anhydrous THF (500 mL) was added DCC (25.5 g, 124.0 mmol, 1.05 eq) and *N*-hydroxysuccinimide (14.2 g, 124 mmol, 1.05 eq). The reaction mixture was stirred at room temperature overnight. The white precipitate (DCU) was removed by filtration and the filtrate was concentrated under reduced pressure. Purification by flash chromatography (PE/EtOAc 70/30 – 30/70 v/v) yielded the product as a white solid (33.9 g, 93 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 2.76 (s, 4H, C***H***$_2$), 5.36 (s, 2H, $(Ph)_2$-C***H***), 7.13 – 7.58 (m, 10 H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$) δ (ppm) 25.60 (-, 2 ***C***$H_2$), 54.04 (+, $(Ph)_2$-***C***H), 127.93 (+, 2 Ph-***C***-4), 128.71 (+, 4 Ph-***C***), 128.89 (+, 4 Ph-***C***), 136.71 ($C_{quat}$, 2 Ph-***C***-1), 168.15 ($C_{quat}$, CH-***C***O-O), 168.95 ($C_{quat}$, 2 ***C***=O). MS (EI, 70 eV) *m/z* (%): 309 (5) $[M]^{+\cdot}$, 167(100) $[(Ph)_2\text{-}CH]^{+\cdot}$. $C_{18}H_{15}NO_4$ (309.32).

***N-tert*-Butoxycarbonyl-1,4-bis(aminomethyl)benzene (5.46)**[11, 28]

1,4-Bis(aminomethyl)benzene **5.45** (30 g, 220.0 mmol, 1 eq) was suspended in 1 M aq. NaOH (220 mL, 220.0 mmol, 1 eq). 1,4-dioxane was added (250 mL) and the solution was cooled in an ice-water bath. Subsequently, di-*tert*-butyl dicarbonate (40.1 g, 184.0 mmol, 1 eq) dissolved in 1,4-dioxane (150 mL) was added dropwise over a period of 2 h. The ice-water bath was removed and the mixture was allowed to stir at rt overnight. The reaction mixture was reduced to about 250 mL under reduced pressure. The white precipitate was separated by filtration, washed twice with ice-cold water (2 × 100 mL) and dried *in vacuo*. **5.46** was obtained as a white solid (33.7 g of a mixture of **5.46** and di-Boc-protected 1,4-bis(aminomethyl)benzene (≈ 55:45), 18.5 g (78.0 mmol, 36 %) related to **5.46**; $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.44 (s, 24H, C(C***H***$_3$)$_3$, **5.46** and "di-Boc"), 3.77 (s, 2H, C***H***$_2$-$NH_2$), 4.19 (s, 5.3H, C***H***$_2$-NH, "di-Boc" + C***H***$_2$-NH, **5.46**), 7.22 (s, 3.3H, Ph-***H***, "di-Boc"), 7.23 – 7.33 (m,

4H, Ph-***H***, 4.7); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 278 (100) $[M+MeCN+H]^+$, 354 (100) ["di-Boc" + $NH_4]^+$; $C_{13}H_{20}N_2O_2$ (236.31).

**_N_-(4-Aminomethylbenzyl)urea (5.47)** [12, 27]

Compound **5.46** (18.5 g, 46.0 mmol, 1 eq; used as 33.7 g of a mixture with di-Boc-protected 1,4-bis(aminomethyl)benzene) was suspended in water (500 mL) and ethanol (400 mL). The suspension was heated to 60 °C and 1 M aq. hydrochloric acid (100 mL) was added yielding a pH of about 3. Potassium cyanate (6.60 g, 81.0 mmol, 1.04 eq) was added and the mixture was refluxed for 75 min. 1 M aq. hydrochloric acid (6-8 mL) was added to adjust the pH to 7, followed by the addition of 4.67 g potassium cyanate (4.42 g, 54.6 mmol, 0.7 eq). Reflux was continued for 2 h, then the mixture was stirred at rt overnight (final pH ≈ 8). Subsequently, the reaction mixture was concentrated under reduced pressure to a volume of about 200 mL. The white solid was separated by filtration, washed twice with water (2 × 150 mL) and dried *in vacuo*. Because of the poor solubility of the crude product dry loading was applied for the purification by flash chromatography ($CH_2Cl_2$/MeOH 20/1 to 7.5/1). The isolated Boc-protected intermediate was dissolved in MeOH (300 mL) under moderate warming. Acetyl chloride (30 mL) was added dropwise over a period of 2 h and stirring was continued for 30 min. Volatiles were removed under reduced pressure and the residue was suspended in water (150 mL) prior to lyophilisation. The product was obtained as a white solid (10.8 g, 77 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 4.12 (s, 2H, C***H***$_2$-$NH_2$), 4.32 (s, 2H, C***H***$_2$-NH), 7.37 (d, $^3J$ = 8.3 Hz, 2H, Ph-***H***), 7.44 (d, $^3J$ = 8.3 Hz, 2H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 44.19 (-, ***C***$H_2$-NH), 44.48 (-, ***C***$H_2$-NH), 129.06 (+ 2 Ph-***C***), 130.45 (+, 2 Ph-***C***), 133.07 ($C_{quat}$, Ph-***C***), 141.87 ($C_{quat}$, Ph-***C***), 162.38 ($C_{quat}$, ***C***=O). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 221 (100) $[M+MeCN+H]^+$, 359 (50) $[2M+MeCN+H]^+$; $C1_4H_{21}N_3O_3$ · HCl (215.68).

### 5.7.2.2 Synthesis of the Spacers 5.52, 5.54, 5.56, 5.59, 5.64 and 5.65

**General procedure for the amide coupling**

A solution of the carboxylic acid (1 eq), EDAC (1.2 eq), HOBt-monohydrate (1.2 eq) and DIPEA (2 eq) was dissolved in $DCM_{abs}$ or $DMF_{abs}$ and stirred under argon atmosphere for 15 minutes. To this mixture a solution of the amine in DCM/abs was added and stirred overnight at room temperature. After removal of the solvent under reduced pressure the crude product was diluted with EtOAc and washed with water. The organic phase was dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was recrystallized from ether or EtOAc or purified by flash chromatography.

**Benzyl 4-[2-(*tert*-butoxycarbonylamino)ethylamino]-4-oxobutanoate (5.50)**

The title compound was prepared from **5.33** (2.60 g, 12.5 mmol), EDAC (2.87, 15.0 mmol), HOBt (2.02, 15.0 mmol), DIEA (4.35 mL, 25.0 mmol) and **3.35** (2.00 g, 12.5 mmol) in DCM afforded the product as white solid after recrystallization from ether (3.70 g, 85 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.42 (s, 9H, C(C***H***$_3$)$_3$), 2.49 (t, $^3J$ = 6.9 Hz, 2H, CO-C***H***$_2$-CH$_2$-CO-NH), 2.66 (t, $^3J$ = 6.8 Hz, 2H, CO-CH$_2$-C***H***$_2$-CO-NH), 3.11 (t, $^3J$ = 5.8 Hz, 2H, NH-CH$_2$-C***H***$_2$-NHBoc), 3.21 (t, $^3J$ = 5.8 Hz, 2H, NH-C***H***$_2$-CH$_2$-NHBoc), 5.11 (s, 2H, Ph-C***H***$_2$), 7.09 – 7.44 (m, 5H, Ph-***H***). $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.81 (+, C(***C***H$_3$)$_3$), 30.49 (-, CO-(***C***H$_2$)$_2$-CO), 31.48 (-, CO-(***C***H$_2$)$_2$-CO), 40.61 (NH-(***C***H$_2$)$_2$-NH), 40.89 (-, NH-(***C***H$_2$)$_2$-NH), 67.46 (-, Ph-***C***H$_2$), 80.18 (C$_{quat}$, ***C***(CH$_3$)$_3$), 129.24 (+, 3 Ph-***C*** + Ph-***C***-4), 129.58 (+, 2 Ph-***C***), 137.60 (+, Ph-***C***-1), 174.18 (C$_{quat}$, ***C***=O), 174.61 (C$_{quat}$, ***C***=O); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 351 (100) [M+H]$^+$, 701 (60) [2M+H]$^+$. $C_{18}H_{26}N_2O_5$ (350.41).

**Benzyl 2,2-dimethyl-4,8,13-trioxo-3-oxa-5,9,12-triazahexadecan-16-oate (5.53)**

The title compound was prepared from Boc-β-Ala-OH (0.38 g, 2.0 mmol), EDAC (0.46 g, 2.4 mmol), HOBt (0.32 g, 2.4 mmol), DIEA (0.70 mL, 4.0 mmol) and **5.51** (0.50 g, 4.0 mmol) in DCM yielding **5.53** as a white solid after recrystallization from diethyl ether (0.53 g, 63 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.41 (s, 9H, C(C***H***$_3$)$_3$), 2.33 (t, $^3J$ = 6.7 Hz, 2H, CO-C***H***$_2$-CH$_2$-CO-NH), 2.50 (t, $^3J$ = 6.8 Hz, 2H, CO-CH$_2$-C***H***$_2$-CO-NH), 2.67 (t, $^3J$ = 6.7 Hz, 2H, CO-C***H***$_2$-CH$_2$-NH), 3.20 – 3.30 (m, overlap with solvent, 6H, NH-C***H***$_2$-C***H***$_2$-NH + CO-CH$_2$-C***H***$_2$-NH), 5.11 (s, 2H, Ph-C***H***$_2$), 7.15 – 7.48 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.80 (+, C(***C***H$_3$)$_3$), 30.43 (-, CO-***C***H$_2$ ), 31.48 (-, CO-***C***H$_2$), 37.52 (-, CO-***C***H$_2$-CH$_2$-NH), 38.09 (-, NH-***C***H$_2$), 39.99 (-, NH-***C***H$_2$), 40.06 (-, NH-***C***H$_2$), 67.48 (-, Ph-***C***H$_2$), 80.18 (C$_{quat}$, ***C***(CH$_3$)$_3$), 129.22 (+, 3 Ph-C + Ph-***C***-4), 129.58 (+, 2 Ph-***C***), 137.60 (C$_{quat}$, Ph-***C***-1), 174.25 (C$_{quat}$, ***C***=O), 174.31 (C$_{quat}$, ***C***=O), 174.81 (C$_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 422 (100) [M+H]$^+$, 439 (70) [M+NH$_4$]$^+$. $C_{21}H_{31}N_3O_6$ (421.49).

**Benzyl 2,2-dimethyl-4,9,12,17-tetraoxo-3-oxa-5,8,13,16-tetraazaicosan-20-oate (5.55)**

The title compound was prepared from **5.52** (0.26 g, 1.0 mmol), EDAC (0.23 g, 1.2 mmol), HOBt (0.16 g, 1.2 mmol), DIEA (0.52 mL, 3.0 mmol) and **5.51** (0.25 g, 1.0 mmol) in DMF yielding **5.55** as a white solid after recrystallization from EtOAc (0.31 g, 63 %). $^1$H-NMR (300 MHz, DMSO-d$_6$): δ (ppm) 1.37 (s, 9H, C(C***H***$_3$)$_3$), 2.24 – 2.31 (m, 4H, CO-(C***H***$_2$)$_2$-CO) 2.37 (t, $^3J$ = 6.9 Hz, 2H, CO-CH$_2$-C***H***$_2$-COO), 2.56 (t, $^3J$ = 6.9 Hz, 2H, CO-C***H***$_2$-CH$_2$-COO), 2.88 – 2.98 (m, 2H, NH-CH$_2$-C***H***$_2$-NHBoc), 2.99 – 3.10 (m, 6H, NH-(C***H***$_2$)$_2$-NH + NH-C***H***$_2$-CH$_2$-NHBoc), 5.08 (s, 2H, Ph-C***H***$_2$), 6.71 – 6.86 (m, 1H, N***H***), 7.29 – 7.41 (m, 5H, Ph-***H***), 7.74 – 7.91 (m, 3H, 3x N***H***). $^{13}$C-NMR (DMSO-d$_6$): δ (ppm) 28.11 (+, C(***C***H$_3$)$_3$), 28.90 (-, 2x CO-***C***H$_2$), 29.74 (-, CO-***C***H$_2$), 30.70 (-, CO-***C***H$_2$), 38.19 (-, NH-***C***H$_2$), 38.24 (-, NH-***C***H$_2$), 38.61 (-, NH-***C***H$_2$), 39.50 (-, NH-***C***H$_2$), 65.27 (-, Ph-***C***H$_2$), 77.52 (C$_{quat}$, ***C***(CH$_3$)$_3$), 127.69 (+, 2 Ph-***C***), 127.81 (+, Ph-***C***-4), 136.12 (C$_{quat}$,

Ph-***C***-1), 155.47 ($C_{quat}$, NH-***C***O-O), 170.66 ($C_{quat}$, ***C***=O), 171.41 ($C_{quat}$, ***C***=O), 172.15 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 493 (100) $[M+H]^+$, 510 (50) $[M+NH_4]^+$. $C_{24}H_{36}N_4O_7$ (492.57).

**Benzyl 2,2-dimethyl-4,8,13,16,21-pentaoxo-3-oxa-5,9,12,17,20-pentaazatetracosan-24-oate (5.58)**

The title compound was prepared from Boc-β-Ala-OH (0.22 g, 1.1 mmol), EDAC (0.26 g, 1.4 mmol), HOBt (0.19 g, 1.4 mmol), DIEA (0.60 mL, 3.4 mmol) and **5.57** (0.45 g, 1.1 mmol) in DMF yielding **5.58** as a white solid after recrystallization from EtOAc (0.39 g, 60 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.36 (s, 9H, C($CH_3$)$_3$), 2.20 (t, $^3J$ = 7.3 Hz, 2H, CO-C***H***$_2$), 2.25 – 2.31 (m, 4H, CO-(C***H***$_2$)$_2$-CO), 2.37 (t, $^3J$ = 6.8 Hz , 2H, CO-C***H***$_2$), 2.57 (t, $^3J$ = 6.9 Hz, 2H, CO-C***H***$_2$), 2.98 – 3.18 (m, 10H, 2x NH-(C***H***$_2$)$_2$-NH + CO-$CH_2$-C***H***$_2$-NHBoc), 5.08 (s, 2H, Ph-C***H***$_2$), 6.75 (t, $^3J$ =, 1H, NH), 7.12 – 1.59 (m, 5H, Ph-H), 7.71 – 8.06 (m, 3H, 3x NH); 13C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 28.11 (+, C(***C***$H_3$)$_3$), 28.89 (-, ), 29.74 (-, CO-***C***$H_2$), 30.69 (-, CO-***C***$H_2$), 35.74 (-, CO-***C***$H_2$-***C***$H_2$-CO), 36.59 (-, CO-(***C***$H_2$)$_2$-NH), 38.18 (-, 2x NH-***C***$H_2$), 38.22 (-, 2x NH-***C***$H_2$), 65.27 (-, Ph-***C***$H_2$), 77.46 ($C_{quat}$, ***C***($CH_3$)$_3$), 127.69 (+, 2 Ph-***C***), 127.82 (+, Ph-***C***-4), 128.29 (+, 2 Ph-***C***), 136.12 ($C_{quat}$, Ph-***C***-1), 170.35 ($C_{quat}$, ***C***=O), 170.68 ($C_{quat}$, ***C***=O), 171.45 ($C_{quat}$, ***C***=O), 172.16 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 564 (100) $[M+H]^+$. $C_{27}H_{41}N_5O_8$ (563.64).

**Benzyl 2,2-dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazaoctadecan-18-oate (5.62)**

The title compound was prepared from **5.33** (0.34 g, 1.6 mmol), EDAC (0.37 g, 1.9 mmol), HOBt (0.26 g, 1.9 mmol), DIEA (0.56 mL, 3.1 mmol) and **5.60** (0.40 g, 1.6 mmol) in DCM yielding **5.62** as a colourless oil (0.42 g, 75 %) after purification by flash chromatography (DCM/MeOH 100/0 – 95/5 v/v). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.42 (s, 9H, C(C***H***$_3$)$_3$), 2.51 (t, $^3J$ = 6.5 Hz, 2H, CO-C***H***$_2$), 2.66 (t, $^3J$ = 6.6 Hz, 2H, CO-C***H***$_2$), 3.21 (t, $^3J$ = 5.6 Hz, 2H, C***H***$_2$-NH), 3.34 (t, $^3J$ = 5.4 Hz, 2H, C***H***$_2$-NH), 3.44 – 3.54 (m, 4H, O-C***H***$_2$-C***H***$_2$-O), 3.55 – 3.63 (m, 4H, 2x O-C***H***$_2$-$CH_2$-NH), 5.11 (s, 2H, Ph-C***H***$_2$), 7.19 – 7.43 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.81 (+, C(***C***$H_3$)$_3$), 30.50 (-, CO-***C***$H_2$), 31.42 (-, CO-***C***$H_2$), 40.43 (-, NH-***C***$H_2$), 41.27 (-, NH-***C***$H_2$), 67.41 (-, Ph-***C***$H_2$), 70.62 (-, CO-***C***$H_2$-$CH_2$-NH), 71.12 (-, CO-***C***$H_2$-$CH_2$-NH), 71.33 (-, CO-***C***$H_2$-***C***$H_2$-CO), 80.13 ($C_{quat}$, ***C***($CH_3$)$_3$), 129.20 (+, 2 Ph-***C*** + Ph-***C***-4), 129.57 (+, Ph-***C***), 137.65 ($C_{quat}$, Ph-***C***-1), 174.09 ($C_{quat}$, ***C***=O), 174.37 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 439 (70) $[M+H]^+$, 456 (100) $[M+NH_4]^+$ . $C_{16}H_{30}N_2O_6$ (438.51).

**Benzyl 2,2-dimethyl-4,20-dioxo-3,9,12,15-tetraoxa-5,19-diazatricosan-23-oate (5.63)**

The title compound was prepared from **5.33** (0.33 g, 1.6 mmol), EDAC (0.36 g, 1.9 mmol), HOBt (0.25 g, 1.9 mmol), DIEA (0.54 mL, 3.1 mmol) and **5.61** (0.50 g, 1.6 mmol) in DCM yielding **5.75** as a colourless oil (0.49 g, 62 %) after purification by flash chromatography (DCM/MeOH 100/0 – 95/5 v/v). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) ; $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.85 (+, C(***C***$H_3$)$_3$), 30.38 (-, O-$CH_2$-***C***$H_2$-$CH_2$-NH), 30.52 (-, CO-***C***$H_2$), 30.96 (-, O-$CH_2$-***C***$H_2$-$CH_2$-NH), 31.52 (-, CO-***C***$H_2$), 37.91

(-, NH-***C***$H_2$), 38.76 (-, NH-***C***$H_2$), 67.40 (-, Ph-***C***$H_2$), 69.91 (-, 2x O-***C***$H_2$-$CH_2$-$CH_2$-NH), 71.27 (-, 2x O-***C***$H_2$), 71.59 (-, 2x O-***C***$H_2$), 79.90 ($C_{quat}$, ***C***$(CH_3)_3$), 129.21 (+, 2 Ph-***C*** + Ph-***C***-4), 129.58 (+, Ph-***C***), 137.65 ($C_{quat}$, Ph-***C***-1), 174.09 ($C_{quat}$, ***C***=O), 174.18 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 511 (65) $[M+H]^+$, 528 (100) $[M+NH_4]^+$. $C_{26}H_{42}N_2O_8$ (510.62).

**General procedure for the Boc-deprotection group**

Compound **5.50** or **5.55** was dissolved in DCM prior to the addition of TFA yielding a concentration of 50 % TFA. The reaction mixture was stirred at room temperature for 3- 5 h until deprotection was complete (control by TLC). Subsequently, the solvent was removed under reduced pressure and the product was dried *in vacuo*.

**Benzyl 4-(2-aminoethylamino)-4-oxobutanoate (5.52)[10]**

The deprotection of **5.50** (3.70 g, 10.6 mmol) was carried out in 20 mL DCM adding 20 mL TFA. Drying *in vacuo* afforded the product as a pale yellow oil (3.80 g, 99 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 2.54 (t, $^3J$ = 6.6 Hz, 2H, CO-C***H***$_2$), 2.69 (t, $^3J$ = 6.5 Hz, 2H, CO-C***H***$_2$), 3.02 (t, $^3J$ = 5.9 Hz, 2H, C***H***$_2$-$NH_2$), 3.37 – 3.50 (m, 4H, 2x NH-C***H***$_2$), 5.12 (s, 2H, Ph-C***H***$_2$), 7.20 – 7.45 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 30.20 (-, CO-***C***$H_2$), 31.19 (-, CO-***C***$H_2$), 38.18 (-, C***H***$_2$-$NH_2$), 40.94 (-, NH-C***H***$_2$), 67.54 (-, Ph-***C***$H_2$), 129.21 (+, 2 Ph-***C***), 129.27 (+, Ph-***C***-4), 129.59 (2 Ph-***C***), 137.54 ($C_{quat}$, Ph-***C***-1), 174.45 ($C_{quat}$, ***C***=O), 175.72 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1% FA) m/z (%): 251 (100) $[M+H]^+$, 501 (50) $[2M+H]^+$. $C_{13}H_{18}N_2O_3$ (250.29).

**Benzyl 4-{2-[4-(2-aminoethylamino)-4-oxobutanamido]ethylamino}-4-oxobutanoate (5.57)**

The deprotection of **5.55** (0.55 g, 1.1 mmol) was carried out in 5 mL DCM adding 20 mL TFA. Drying *in vacuo* afforded the product as a pale yellow oil (0.56 g, 99 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 2.39 – 2.54 (m, 6H, CO-C***H***$_2$-C***H***$_2$-CO + CO-C***H***$_2$), 2.67 (t, $^3J$ = 6.7 Hz, 2H, O-CO-C***H***$_2$), 3.05 (t, $^3J$ = 5.3 Hz, 2H, C***H***$_2$-$NH_2$), 3.20 – 3.29 (m, 4H, 2x NH-C***H***$_2$), 3.45 (t, $^3J$ = 5.4 Hz, 2H, NH-C***H***$_2$-$CH_2$-$NH_2$) 5.12 (s, 2H, Ph-C***H***$_2$), 7.28 – 7.37 (m, 5H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 30.40 (-, CO-***C***$H_2$), 31.43 (-, CO-***C***$H_2$), 31.84 (-, CO-***C***$H_2$), 32.09 (-, CO-***C***$H_2$), 37.99 (-, C***H***$_2$-$NH_2$), 39.82 (-, NH-C***H***$_2$), 40.13 (-, NH-C***H***$_2$), 41.21 (-, NH-C***H***$_2$), 67.48 (-, Ph-***C***$H_2$), 129.18 (+, 2 Ph-***C***), 129.25 (+, Ph-***C***-4), 129.59 (2 Ph-***C***), 137.59 ($C_{quat}$, Ph-***C***-1), 174.34 ($C_{quat}$, ***C***=O), 174.71 ($C_{quat}$, ***C***=O), 175.12 ($C_{quat}$, ***C***=O), 176.35 ($C_{quat}$, ***C***=O).MS (ESI, MeCN/0.1% FA) *m/z* (%): 393 (100) $[M+H]^+$. $C_{19}H_{28}N_4O_5$ · TFA (506.47).

**General procedure for the cleavage of the benzyl ester group**

The compound **5.50**, **5.53**, **5.55**, **5.58**, **5.62** and **5.63** were dissolved in MeOH. A 10 % Pd/C catalyst was added and hydrogen was led through the vigorously stirred mixture at room temperature for 2 –

5 h (control by TLC). The catalyst was removed by filtration over Celite and the solvent was removed under reduced pressure. The obtained products were used for the next step without further purification.

**4-[2-(*tert*-Butoxycarbonylamino)ethylamino]-4-oxobutanoic acid (5.52)**

The title compound was prepared by hydrogenolysis of **5.50** (1.50 g, 4.3 mmol) in 100 mL MeOH in the presence of 10 % Pd/C (150 mg) catalyst yielding **5.52** as white solid (1.10 g, 99 %).$^{1}$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 2.45 (t, $^{3}J$ = 7.1 Hz, 2H, CO-C***H***$_2$), 2.58 (t, $^{3}J$ = 7.0 Hz, 2H, CO-C***H***$_2$), 3.13 (t, $^{3}J$ = 5.6 Hz, 2H, NH-C***H***$_2$), 3.23 (t, $^{3}J$ = 5.8 Hz, 2H, NH-C***H***$_2$); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.78 (+, C(***C***H$_3$)$_3$), 30.45 (-, CO-***C***H$_2$), 31.70 (-, CO-***C***H$_2$), 40.61 (NH-***C***H$_2$), 40.88 (NH-***C***H$_2$), 80.17 ($C_{quat}$, ***C***(CH$_3$)$_3$), 174.97 ($C_{quat}$, ***C***=O), 176.48 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 261 (20) $[M+H]^+$, 521 (40)$[2M+H]^+$, 538 (100) $[2M+H+NH_4]^+$.$C_{11}H_{20}N_2O_5$ (260.29).

**2,2-Dimethyl-4,8,13-trioxo-3-oxa-5,9,12-triazahexadecan-16-oic acid (5.54)**

The title compound was prepared by hydrogenolysis of **5.53** (0.50 g, 1.2 mmol) in 50 mL MeOH in the presence of 10 % Pd/C (50 mg) catalyst yielding **5.54** as white solid (0.39 g, 98 %). $^{1}$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.42 (s, 9H, C(C***H***$_3$)$_3$), 2.35 (t, 3J = 6.7 Hz, CO-C***H***$_2$-CH$_2$-NH) 2.45 (t, $^{3}J$ = 6.9 Hz, 2H, CO-C***H***$_2$), 2.5 (t, $^{3}J$ = 6.8 Hz, 2H, CO-C***H***$_2$), 3.21 – 3.37 (m, overlap with solvent, 10H, 5x NH-C***H***$_2$),; $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.79 (+, C(***C***H$_3$)$_3$), 30.90 (-, CO-***C***H$_2$), 31.98 (-, CO-***C***H$_2$), 37.49 (-, CO-***C***H$_2$-CH$_2$-NH), 38.07 (-, CO-CH$_2$-***C***H$_2$-NH), 39.95 (NH-***C***H$_2$), 40.06 (NH-***C***H$_2$), 80.17 ($C_{quat}$, ***C***(CH$_3$)$_3$), 174.32 ($C_{quat}$, ***C***=O), 175.24 ($C_{quat}$, ***C***=O), 177.04 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 332 (100) $[M+H]^+$, 649 (50) $[M+NH_4]^+$, 663 (70) $[2M+H]^+$, 680 (90) $[2M+NH_4]^+$. $C_{14}H_{25}N_3O_6$ (331.36).

**2,2-Dimethyl-4,9,12,17-tetraoxo-3-oxa-5,8,13,16-tetraazaicosan-20-oic acid (5.56)**

The title compound was prepared by hydrogenolysis of **5.55** (0.30 g, 0.61 mmol) in 100 mL MeOH in the presence of 10 % Pd/C (30 mg) catalyst yielding **5.56** as white solid (0.24 g, 98 %). $^{1}$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 2.39 – 2.48 (m, 6H, CO-C***H***$_2$-C***H***$_2$-CO + CO-C***H***$_2$), 2.39 – 2.56 (m, 2H, CO-C***H***$_2$), 3.09 – 3.18 (m, 2H, NH-C***H***$_2$), 3.19 – 3.29 (m, 6H, NH-C***H***$_2$-C***H***$_2$-N + NH-C***H***$_2$); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.80 (+, C(***C***H$_3$)$_3$), 32.40 (-, CO-***C***H$_2$), 32.45 (-, CO-***C***H$_2$), 32.84 (-, CO-***C***H$_2$), 33.05 (-, CO-***C***H$_2$), 39.89 (-, NH-***C***H$_2$), 40.13 (-, NH-***C***H$_2$), 40.61 (-, NH-***C***H$_2$), 40.88 (-, NH-***C***H$_2$), 80.15 ($C_{quat}$, ***C***(CH$_3$)$_3$), 175.07 ($C_{quat}$, ***C***=O), 175.10 ($C_{quat}$, 2x ***C***=O), 175.89 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1% FA) *m/z* (%): 403 (100) $[M+H]^+$. $C_{17}H_{30}N_4O_7$ (402.44).

**2,2-Dimethyl-4,8,13,16,21-pentaoxo-3-oxa-5,9,12,17,20-pentaazatetracosan-24-oic acid (5.59)**

The title compound was prepared by hydrogenolysis of **5.58** (0.35 g, 0.62 mmol) in 150 mL MeOH in the presence of 10 % Pd/C (35 mg) catalyst yielding **5.59** as white solid (0.29 g, 99 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.36 (s, 9H, C(C***H***$_3$)$_3$), 2.20 (t, $^3J$ = 7.2 Hz, CO-C***H***$_2$-CH$_2$-NH) 2.24 – 2.32 (m, 6H, CO-C***H***$_2$-C***H***$_2$-O + CO-C***H***$_2$), 2.39 (t, $^3J$ = 6.6 Hz, 2H, CO-C***H***$_2$), 2.98 – 3.16 (m, 10H, 2x NH-C***H***$_2$-C***H***$_2$-NH + C***H***$_2$-NHBoc), 6.74 (t, $^3J$ = 5.4 Hz, 1H, N***H***), 7.80 – 8.00 (m, 4 H, 4x N***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 28.11 (+, C(***C***H$_3$)$_3$), 29.52 (-, CO-***C***H$_2$), 30.26 (-, CO-***C***H$_2$), 30.79 (-, 2x CO-***C***H$_2$), 35.71 (-, CO-***C***H$_2$-CH$_2$-NH), 36.58 (-, CO-CH$_2$-***C***H$_2$-NH), 38.19 (-, 2x NH-***C***H$_2$-***C***H$_2$-NH), 77.49 (C$_{quat}$, ***C***(CH$_3$)$_3$), 170.42 (C$_{quat}$, ***C***=O), 171.32 (C$_{quat}$, ***C***=O), 171.50 (C$_{quat}$, ***C***=O), 171.53 (C$_{quat}$, ***C***=O), 174.05 (C$_{quat}$, ***C***=O). MS (ES, MeCN/ 0.1 % FA) *m/z* (%): 474 (100) $[M+H]^+$. $C_{20}H_{35}N_5O_8$ (473.52).

**2,2-Dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazaoctadecan-18-oic acid (5.64)** [29]

The title compound was prepared by hydrogenolysis of **5.62** (0.40 g, 0.91 mmol) in 50 mL MeOH in the presence of 10 % Pd/C (40 mg) catalyst yielding **5.64** as colourless oil (0.31 g, 98 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 2.47 (t, $^3J$ = 6.4 Hz, 2H, CO-C***H***$_2$), 2.56 (t, $^3J$ = 6.6 Hz, 2H, CO-C***H***$_2$), 3.22 (t, $^3J$ = 6.8 Hz, 2H, NH-C***H***$_2$), 3.31 – 3.39 (m, overlap with solvent, 2H, NH-C***H***$_2$), 3.46 – 3.56 (m, 4H, O-C***H***$_2$-C***H***$_2$-O), 3.57 – 3.64 (m, 4H, 2x O-C***H***$_2$-CH$_2$-CH$_2$-NH); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.80 (+, C(***C***H$_3$)$_3$), 30.93 (-, CO-***C***H$_2$), 31.90 (-, CO-***C***H$_2$), 40.42 (-, NH-***C***H$_2$), 31.90 (-, NH-***C***H$_2$), 70.66 (-, O-***C***H$_2$-CH$_2$-CH$_2$-NH), 71.13 (-, O-***C***H$_2$-CH$_2$-CH$_2$-NH), 71.33 (-, O-***C***H$_2$), 71.35 (O-***C***H$_2$), 80.14 (C$_{quat}$, ***C***(CH$_3$)$_3$), 174.88 (C$_{quat}$, ***C***=O), 176.84 (C$_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 349 (70) $[M+H]^+$, 697 (50) $[2M+H]^+$, 714 (100) $[2M+NH_4]^+$. $C_{15}H_{28}N_2O_7$ (348.39).

**2,2-Dimethyl-4,20-dioxo-3,9,12,15-tetraoxa-5,19-diazatricosan-23-oic acid (5.65)** [30]

The title compound was prepared by hydrogenolysis of **5.63** (0.45 g, 0.91 mmol) in 50 mL MeOH in the presence of 10 % Pd/C (45 mg) catalyst yielding **5.65** as a colourless oil (0.38 g, 99 %). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.65 – 1.83 (m, 4H, 2x O-CH$_2$-C***H***$_2$-CH$_2$-NH), 2.44 (t, $^3J$ = 6.5 Hz, 2H, CO-C***H***$_2$), 2.5 (t, $^3J$ = 6.4 Hz, 2H, CO-C***H***$_2$), 3.12 (t, $^3J$ = 6.8 Hz, 2H, NH-C***H***$_2$), 3.25 (t, $^3J$ = 6.8 Hz, 2H, NH-C***H***$_2$), 3.46 – 3.55 (m, 4H, 2x O-C***H***$_2$-CH$_2$-CH$_2$-NH), 3.55 – 3.67 (m, 8H, 2x O-C***H***$_2$-C***H***$_2$-O); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 28.84 (+, C(***C***H$_3$)$_3$), 30.42 (-, O-CH$_2$-***C***H$_2$-CH$_2$-NH), 30.96 (-, O-CH$_2$-***C***H$_2$-CH$_2$-NH), 31.35 (-, CO-***C***H$_2$), 32.23 (-, CO-***C***H$_2$), 37.83 (-, NH-***C***H$_2$), 38.73 (-, NH-***C***H$_2$), 69.88 (-, O-***C***H$_2$-CH$_2$-CH$_2$-NH), 69.91 (-, O-***C***H$_2$-CH$_2$-CH$_2$-NH), 71.27 (-, O-***C***H$_2$-***C***H$_2$-O), 71.58 (O-***C***H$_2$-***C***H$_2$-O), 79.91 (C$_{quat}$, ***C***(CH$_3$)$_3$), 174.82 (C$_{quat}$, ***C***=O), 177.18 (C$_{quat}$, ***C***=O). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 421 (100) $[M+H]^+$, 438 (95) $[M+NH_4]^+$, 841 (45) $[2M+H]^+$, 859 (60) $[2M+NH_4]^+$. $C_{19}H_{36}N_2O_8$ (420.50).

### 5.7.2.3 Preparation of (*S*)- and (*R*)-Configured Monovalent Ligands 5.66 – 5.71

**General procedure for the synthesis of monovalent argininamide-type compounds 5.66 – 5.71**

The spacers **5.52**, **5.54**, **5.56**, **5.59**, **5.64** or **5.65** (1 eq), TBTU (1.2 eq), HOBt (1.2 eq) and DIEA (2.0 eq) were dissolved in $DMF_{abs}$ under argon atmosphere and stirred for 20 min at room temperature. To this mixture a solution of the building block **(*S*)-5.44** or **(*R*)-5.44** (1 eq) in $DMF_{abs}$ was added and stirred overnight. After removal of the solvent under reduced pressure, the crude product was dissolved in EtOAc and washed with water. The organic phase was then dried over $MgSO_4$ and concentrated *in vacuo*. Subsequently, the Boc protection group was removed with 50 % TFA. Therefore the residue was dissolved in DCM. TFA (50% final concentration) was added and the reaction mixture was stirred until the deprotection was complete (3 – 5 h, TLC control). After removal of the solvent *in vacuo*, the crude product was purified by preparative RP-HPLC (column: Gemini-NX C18, 250 × 21 mm, 5 µm, AXIA Packed; flow: 18 mL/min; gradient: A/B 10/90 to 60/40 in 30 min). Acetonitrile was removed under reduced pressure and the residue was subjected to lyophilisation.

**(*S*)-$N^1$-{9-Amino-4-(2,2-diphenylacetamido)-3,11-dioxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12-tetraazatetradec-9-en-14-yl}-$N^4$-(2-aminoethyl)succinamide ((*S*)-5.66)**

The title compound was prepared from **5.52** (47 mg, 0.179 mmol), TBTU (69 mg, 0.214 mmol), HOBt (29 mg, 0.214 mmol), DIEA (93 µL, 0.436 mmol) in 5 mL $DMF_{abs}$ and **(*S*)-5.44** (110 mg, 0.179 mmol) in 32 mL $DMF_{abs}$ according to the general procedure. Deprotection was carried out in 3 mL DCM and 3 mL TFA. Purification by RP-HPLC afforded the product as a white fluffy solid (42 mg, 24 %). $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.48 – 1.62 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.67 – 1.77 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.80 – 1.89 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.44 (t, $^3J$ = 6.5 Hz, 2H, CO-(C***H***$_2$)$_2$-CO), 2.51 (t, $^3J$ = 6.6 Hz, CO-(C***H***$_2$)$_2$-CO ), 3.02 (t, $^3J$ = 5.7 Hz, 2H, NH-$CH_2$-C***H***$_2$-$NH_2$), 3.19 – 3.26 (m, 2H, CH-($CH_2$)$_2$-C***H***$_2$-NH), 3.27 – 3.31 (m, overlap with solvent, 4H, NH-CO-NH-(C***H***$_2$)$_2$-NH), 3.42 (t, $^3J$ = 5.8 Hz, 2H, NH-C***H***$_2$-$CH_2$-$NH_2$), 4.26 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.07 (s, 1H, (Ph)$_2$-C***H***), 7.16 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC) 25.70 (CH-$CH_2$-***C***$H_2$), 30.25 (CH-***C***$H_2$-$CH_2$), 31.75 (CO-(***C***$H_2$)$_2$-CO), 31.93 (CO-(***C***$H_2$)$_2$-CO), 37.95 (NH-$CH_2$-***C***$H_2$-$NH_2$), 40.02 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 40.19 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 41.20 (NH-***C***$H_2$-$CH_2$-$NH_2$), 41.73 (($CH_2$)$_2$-***C***$H_2$-NH-), 43.80 (NH-***C***$H_2$-Ar), 44.32 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.35 (***C***H-$CH_2$-$CH_2$), 58.68 ((Ph)$_2$-***C***H), 128.14 (diPh-***C***-4), 128.22 (diPh-***C***-4), 128.34 (2 Ph-***C***), 128.64 (2 Ph-***C***), 129.51 (2 diPh-***C***), 129.55 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.90 (2 diPh-***C***), 138.40 (Ph-***C***-1), 140.85 (Ph-***C***-4), 140.96 (2 diPh-***C***-1), 155.71 (NH-***C***$NH_2$-NH), 155.81 (NH-***C***O-NH), 162.13 (Ar-$CH_2$-NH-***C***O-$NH_2$), 173.56 (***C***O-NH-$CH_2$-Ar), 174.84 ((Ph)$_2$-CH-***C***O), 175.21 (***C***O-($CH_2$)$_2$-CO)), 176.31 (***C***O-NH-$CH_2$-$CH_2$-$NH_2$); anal. RP-HPLC: 100 % ($t_R$ = 12.60 min, *k*' = 3.13); HRMS (ESI): *m/z* calcd. for $[C_{38}H_{51}N_{11}O_6+H]^+$ 758.4097, found 758.4103. $C_{38}H_{51}N_{11}O_6 \cdot 2TFA$ (985.92).

**(*R*)-$N^1$-{9-Amino-4-(2,2-diphenylacetamido)-3,11-dioxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12-tetraazatetradec-9-en-14-yl}-$N^4$-(2-aminoethyl)succinamide ((*R*)-5.66)**

The title compound was prepared from **5.52** (34 mg, 0.130 mmol), TBTU (50 mg, 0.156 mmol), HOBt (21 mg, 0.156 mmol), DIEA (68 µL, 0.390 mmol) in 5 mL $DMF_{abs}$ and **(*R*)-5.44** (80 mg, 0.130 mmol) in 2 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white fluffy solid (28 mg, 22 %). $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.47 – 1.63 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.67 – 1.77 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.79 – 1.89 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.44 (t, $^3J$ = 6.8 Hz, 2H, CO-(C***H***$_2$)$_2$-CO), 2.51 (t, $^3J$ = 6.7 Hz, CO-(C***H***$_2$)$_2$-CO ), 3.02 (t, $^3J$ = 5.6 Hz, 2H, NH-$CH_2$-C***H***$_2$-$NH_2$), 3.17 – 3.26 (m, 2H, CH-($CH_2$)$_2$-C***H***$_2$-NH), 3.27 – 3.31 (m, overlap with solvent, 4H, NH-CO-NH-(C***H***$_2$)$_2$-NH), 3.42 (t, $^3J$ = 5.8 Hz, 2H, NH-C***H***$_2$-$CH_2$-$NH_2$), 4.26 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.07 (s, 1H, (Ph)$_2$-C***H***), 7.16 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC) 25.70 (CH-$CH_2$-***C***$H_2$), 30.25 (CH-***C***$H_2$-$CH_2$), 31.75 (CO-(***C***$H_2$)$_2$-CO), 31.93 (CO-(***C***$H_2$)$_2$-CO), 37.95 (NH-$CH_2$-***C***$H_2$-$NH_2$), 40.02 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 40.20 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 41.20 (NH-***C***$H_2$-$CH_2$-$NH_2$), 41.73 (($CH_2$)$_2$-***C***$H_2$-NH-), 43.80 (NH-***C***$H_2$-Ar), 44.32 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.35 (***C***H-$CH_2$-$CH_2$), 58.68 ((Ph)$_2$-***C***H), 128.14 (diPh-***C***-4), 128.22 (diPh-***C***-4), 128.34 (2 Ph-***C***), 128.65 (2 Ph-***C***), 129.51 (2 diPh-***C***), 129.55 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.90 (2 diPh-***C***), 138.41 (Ph-***C***-1), 140.85 (Ph-***C***-4), 140.96 (2 diPh-***C***-1), 155.72 (NH-***C***$NH_2$-NH), 155.79 (NH-***C***O-NH), 162.14 (Ar-$CH_2$-NH-***C***O-$NH_2$), 173.55 (***C***O-NH-$CH_2$-Ar), 174.83 ((Ph)$_2$-CH-***C***O), 175.21 (***C***O-($CH_2$)$_2$-CO)), 176.31 (***C***O-NH-$CH_2$-$CH_2$-$NH_2$); anal. RP-HPLC: 100 % ($t_R$ = 12.44 min, *k'* = 3.08); HRMS (ESI): *m/z* calcd. for $[C_{38}H_{51}N_{11}O_6+H]^+$ 758.4097, found 758.4095. $C_{38}H_{51}N_{11}O_6 \cdot 2TFA$ (985.92).

**(*S*)-$N^1$-{9-Amino-4-(2,2-diphenylacetamido)-3,11-dioxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12-tetraazatetradec-9-en-14-yl}-$N^4$-[2-(3-aminopropanamido)ethyl]succinamide ((*S*)-5.67)**

The title compound was prepared from **5.54** (59 mg, 0.179 mmol), TBTU (69 mg, 0.214 mmol), HOBt (29 mg, 0.214 mmol), DIEA (93 µL, 0.436 mmol) in 5 mL $DMF_{abs}$ and **(*S*)-5.44** (110 mg, 0.179 mmol) in 3 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and purification by RP-HPLC afforded the product as a white solid (45 mg, 24 %). $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.47 – 1.63 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.67 – 1.76 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.80 – 1.89 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.41 – 2.50 (m, 4H, CO-C***H***$_2$-C***H***$_2$-CO ), 2.55 (t, $^3J$ = 6.3 Hz, 2H, CO-C***H***$_2$-$CH_2$-$NH_2$), 3.15 (t, $^3J$ = 6.3 Hz, 2H, CO-$CH_2$-C***H***$_2$-$NH_2$), 3.19 – 3.26 (m, 2H, CH-($CH_2$)$_2$-C***H***$_2$-NH), 3.25 – 3.31 (m, overlap with solvent, 8H, 2x NH-(C***H***$_2$)$_2$-NH), 4.26 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.08 (s, 1H, (Ph)$_2$-C***H***), 7.16 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC) 25.72 (CH-$CH_2$-***C***$H_2$), 30.27 (CH-***C***$H_2$-$CH_2$), 31.75 (-CO-(***C***$H_2$)$_2$-CO), 31.93 (CO-(***C***$H_2$)$_2$-CO), 32.89 (CO-***C***$H_2$-$CH_2$-$NH_2$), 37.11 (CO-$CH_2$-***C***$H_2$-$NH_2$),

39.79 (NH-(***C***$H_2$)$_2$-NH), 39.92 (NH-(***C***$H_2$)$_2$-NH), 40.18 (NH-(***C***$H_2$)$_2$-$NH_2$), 40.38 (NH-(***C***$H_2$)$_2$-$NH_2$), 41.74 (($CH_2$)$_2$-***C***$H_2$-NH-), 43.80 (NH-***C***$H_2$-Ar), 44.34 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.36 (***C***H-$CH_2$-$CH_2$), 58.67 (($Ph)_2$-***C***H), 128.14 (diPh-***C***-4), 128.23 (diPh-***C***-4), 128.34 (2 Ph-***C***), 128.66 (2 Ph-***C***), 129.51 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.90 (2 diPh-***C***), 138.41 (Ph-***C***-1), 140.85 (Ph-***C***-4), 140.96 (2 diPh-***C***-1), 155.70 (NH-***C***$NH_2$-NH), 155.75 (NH-***C***O-NH), 162.14 (Ar-$CH_2$-NH-***C***O-$NH_2$), 172.51 (***C***O-$CH_2$-$CH_2$-$NH_2$), 173.55 (***C***O-NH-$CH_2$-Ar), 174.84 (($Ph)_2$-CH-***C***O), 175.16 (***C***O-($CH_2$)$_2$), 175.23 (***C***O-($CH_2$)$_2$); anal. RP-HPLC: 99 % ($t_R$ = 12.45 min, *k*' = 3.08); HRMS (ESI): *m/z* calcd. for $[C_{41}H_{56}N_{12}O_7+H]^+$ 829.4468 , found 829.4473. $C_{41}H_{56}N_{12}O_7$ · 2TFA (1057.00).

**(*R*)-*N*$^1$-{9-Amino-4-(2,2-diphenylacetamido)-3,11-dioxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12-tetraazatetradec-9-en-14-yl}-*N*$^4$-[2-(3-aminopropanamido)ethyl]succinamide ((*R*)-5.67)**

The title compound was prepared from **5.54** (43 mg, 0.130 mmol), TBTU (50 mg, 0.156 mmol), HOBt (21 mg, 0.156 mmol), DIEA (68 µL, 0.390 mmol) in 5 mL $DMF_{abs}$ and **(*R*)-5.44** (110 mg, 0.179 mmol) in 3 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white fluffy solid (32 mg, 23 %)**.** $^1$H-NMR (600 MHz, $CD_3OD$): δ (ppm) 1.47 – 1.63 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.67 – 1.77 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.79 – 1.89 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.41 – 2.49 (m, 4H, CO-C***H***$_2$-C***H***$_2$-CO ), 2.55 (t, $^3J$ = 6.3 Hz, 2H, CO-C***H***$_2$-$CH_2$-$NH_2$), 3.15 (t, $^3J$ = 6.3 Hz, 2H, CO-$CH_2$-C***H***$_2$-$NH_2$), 3.19 – 3.25 (m, 2H, CH-($CH_2$)$_2$-C***H***$_2$-NH), 3.25 – 3.31 (m, overlap with solvent, 8H, 2x NH-(C***H***$_2$)$_2$-NH), 4.27 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.08 (s, 1H, $(Ph)_2$-C***H***), 7.16 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$) 25.73 (CH-$CH_2$-***C***$H_2$), 30.27 (CH-***C***$H_2$-$CH_2$), 32.00 (-CO-(***C***$H_2$)$_2$-CO), 32.11 (CO-(***C***$H_2$)$_2$-CO), 32.88 (CO-***C***$H_2$-$CH_2$-$NH_2$), 37.11 (CO-$CH_2$-***C***$H_2$-$NH_2$), 39.80 (NH-(***C***$H_2$)$_2$-NH), 39.92 (NH-(***C***$H_2$)$_2$-NH), 40.17 (NH-(***C***$H_2$)$_2$-$NH_2$), 40.41 (NH-(***C***$H_2$)$_2$-$NH_2$), 41.75 (($CH_2$)$_2$-***C***$H_2$-NH-), 43.80 (NH-***C***$H_2$-Ar), 44.34 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.34 (***C***H-$CH_2$-$CH_2$), 58.68 (($Ph)_2$-***C***H), 128.15 (diPh-***C***-4), 128.23 (diPh-***C***-4), 128.33 (2 Ph-***C***), 128.67 (2 Ph-***C***), 129.51 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.90 (2 diPh-***C***), 138.41 (Ph-***C***-1), 140.85 (Ph-***C***-4), 140.96 (2 diPh-***C***-1), 155.69 (NH-***C***$NH_2$-NH), 155.77 (NH-***C***O-NH), 162.14 (Ar-$CH_2$-NH-***C***O-$NH_2$), 172.50 (***C***O-$CH_2$-$CH_2$-$NH_2$), 173.52 (***C***O-NH-$CH_2$-Ar), 174.83 (($Ph)_2$-CH-***C***O), 175.16 (***C***O-($CH_2$)$_2$), 175.23 (***C***O-($CH_2$)$_2$); anal. RP-HPLC: 99 % ($t_R$ = 12.32 min, *k*' = 3.04); HRMS (ESI): *m/z* calcd. for $[C_{41}H_{56}N_{12}O_7+H]^+$ 829.4468 , found 829.4469. $C_{41}H_{56}N_{12}O_7$ · 2TFA (1057.00).

**(*S*)-*N*$^1$-{9-Amino-4-(2,2-diphenylacetamido)-3,11,16,19-tetraoxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12,15,20-hexaazadocos-9-en-22-yl}-*N*$^4$-(2-aminoethyl)succinamide ((*S*)-5.68)**

The title compound was prepared from **5.56** (65 mg, 0.179 mmol), TBTU (69 mg, 0.214 mmol), HOBt (29 mg, 0.214 mmol), DIEA (93 µL, 0.436 mmol) in 5 mL $DMF_{abs}$ and **(*S*)-5.44** (110 mg, 0.179 mmol) in

3 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white fluffy solid (53 mg, 29 %). $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.47 – 1.63 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.67 – 1.75 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.80 – 1.88 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.42 – 2.52 (m, 8H, 2x CO-(C***H***$_2$)$_2$-CO), 3.03 (t, $^3J$ = 5.7 Hz, 2H, NH-$CH_2$-C***H***$_2$-$NH_2$), 3.19 – 3.26 (m, 6H, CH-$(CH_2)_2$-C***H***$_2$-NH + NH-(C***H***$_2$)$_2$-NH), 3.27 – 3.31 (m, overlap with solvent, NH-(C***H***$_2$)$_2$-NH), 3.44 (t, $^3J$ = 5.7 Hz, 2H, NH-C***H***$_2$-$CH_2$-$NH_2$), 4.26 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.08 (s, 1H, $(Ph)_2$-C***H***), 7.16 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC): δ (ppm) 25.73 (CH-$CH_2$-***C***$H_2$), 30.27 (CH-***C***$H_2$-$CH_2$), 31.91 (CO-(***C***$H_2$)$_2$-CO), 32.02 (CO-(***C***$H_2$)$_2$-CO), 32.09 (CO-(***C***$H_2$)$_2$-CO), 32.13 (CO-(***C***$H_2$)$_2$-CO), 37.96 (NH-$CH_2$-***C***$H_2$-$NH_2$), 39.85 (NH-(***C***$H_2$)$_2$-NH), 39.92 (NH-(***C***$H_2$)$_2$-NH), 40.00 (NH-(***C***$H_2$)$_2$-NH), 40.40 (NH-(***C***$H_2$)$_2$-NH), 41.18 (NH-***C***$H_2$-$CH_2$-$NH_2$), 41.74 ($(CH_2)_2$-***C***$H_2$-NH-), 43.80 (NH-***C***$H_2$-Ar), 44.33 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.37 (***C***H-$CH_2$-$CH_2$), 58.67 ($(Ph)_2$-***C***H), 128.14 (diPh-***C***-4), 128.23 (diPh-***C***-4), 128.33 (2 Ph-***C***), 128.66 (2 Ph-***C***), 129.51 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.90 (2 diPh-***C***), 138.41 (Ph-***C***-1), 140.86 (Ph-***C***-4), 140.96 (2 diPh-***C***-1), 155.70 (NH-***C***$NH_2$-NH), 155.75 (NH-***C***O-NH), 162.14 (Ar-$CH_2$-NH-***C***O-$NH_2$), 173.56 (***C***O-NH-$CH_2$-Ar), 174.84 ($(Ph)_2$-CH-***C***O), 175.10 (***C***O-$(CH_2)_2$-CO), 175.14 (***C***O-NH-$CH_2$-$CH_2$), 175.20 (***C***O-$(CH_2)_2$-CO), 176.28 (***C***O-NH-$CH_2$-$CH_2$-$NH_2$); anal. RP-HPLC: 100 % ($t_R$ = 12.37 min, *k*' = 3.06); HRMS (ESI): *m/z* calcd. for $[C_{44}H_{61}N_{13}O_8+H]^+$ 900.4839 , found 900.4845. $C_{44}H_{61}N_{13}O_8 \cdot 2TFA$ (1128.08).

**(*R*)-*N*$^1$-{9-amino-4-(2,2-diphenylacetamido)-3,11,16,19-tetraoxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12,15,20-hexaazadocos-9-en-22-yl}-*N*$^4$-(2-aminoethyl)succinamide ((*R*)-5.68)**

The title compound was prepared from **5.56** (65 mg, 0.179 mmol), TBTU (69 mg, 0.214 mmol), HOBt (29 mg, 0.214 mmol), DIEA (93 µL, 0.436 mmol) in 5 mL $DMF_{abs}$ and **(*R*)-5.44** (80 mg, 0.130 mmol) in 3 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white fluffy solid (18 mg, 12 %). $^1$H-NMR (600 MHz, $CD_3OD$): δ (ppm) 1.48 – 1.63 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.67 – 1.75 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.79 – 1.88 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.42 – 2.52 (m, 8H, 2x CO-(C***H***$_2$)$_2$-CO), 3.03 (t, $^3J$ = 5.7 Hz, 2H, NH-$CH_2$-C***H***$_2$-$NH_2$), 3.19 – 3.26 (m, 6H, CH-$(CH_2)_2$-C***H***$_2$-NH + NH-(C***H***$_2$)$_2$-NH), 3.27 – 3.31 (m, overlap with solvent, NH-(C***H***$_2$)$_2$-NH), 3.44 (t, $^3J$ = 5.6 Hz, 2H, NH-C***H***$_2$-$CH_2$-$NH_2$), 4.27 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.41 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.07 (s, 1H, $(Ph)_2$-C***H***), 7.16 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$): δ (ppm) 25.73 (CH-$CH_2$-***C***$H_2$), 30.29 (CH-***C***$H_2$-$CH_2$), 31.88 (CO-(***C***$H_2$)$_2$-CO), 32.00 (CO-(***C***$H_2$)$_2$-CO), 32.08 (CO-(***C***$H_2$)$_2$-CO), 32.11 (CO-(***C***$H_2$)$_2$-CO), 37.96 (NH-$CH_2$-***C***$H_2$-$NH_2$), 39.86 (NH-(***C***$H_2$)$_2$-NH), 39.91 (NH-(***C***$H_2$)$_2$-NH), 40.01 (NH-(***C***$H_2$)$_2$-NH), 40.45 (NH-(***C***$H_2$)$_2$-NH), 41.20 (NH-***C***$H_2$-$CH_2$-$NH_2$), 41.76 ($(CH_2)_2$-***C***$H_2$-NH-), 43.81 (NH-***C***$H_2$-Ar), 44.31 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.33 (***C***H-$CH_2$-$CH_2$), 58.68 ($(Ph)_2$-***C***H), 128.16 (diPh-***C***-4), 128.24

(diPh-***C***-4), 128.33 (2 Ph-***C***), 128.68 (2 Ph-***C***), 129.52 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.91 (2 diPh-***C***), 138.41 (Ph-***C***-1), 140.86 (Ph-***C***-4), 140.97 (2 diPh-***C***-1), 155.68 (NH-***C***$NH_2$-NH), 155.70 (NH-***C***O-NH), 162.15 (Ar-$CH_2$-NH-***C***O-$NH_2$), 173.51 (***C***O-NH-$CH_2$-Ar), 174.83 ($(Ph)_2$-CH-***C***O), 175.10 (***C***O-$(CH_2)_2$-CO), 175.14 (***C***O-NH-$CH_2$-$CH_2$), 175.21 (***C***O-$(CH_2)_2$-CO), 176.30 (***C***O-NH-$CH_2$-$CH_2$-$NH_2$); anal. RP-HPLC: 100 % ($t_R$ = 12.19 min, *k*' = 3.00); HRMS (ESI): *m/z* calcd. for $[C_{44}H_{61}N_{13}O_8+H]^+$ 900.4839 , found 900.4842. $C_{44}H_{61}N_{13}O_8 \cdot 2TFA$ (1128.08).

**(*S*)-*N*$^1$-{9-Amino-4-(2,2-diphenylacetamido)-3,11,16,19-tetraoxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12,15,20-hexaazadocos-9-en-22-yl}-*N*$^4$-[2-(3-aminopropanamido)ethyl]succinamide ((*S*)-5.69)**

The title compound was prepared from **5.59** (62 mg, 0.130 mmol), TBTU (50 mg, 0.156 mmol), HOBt (21 mg, 0.156 mmol), DIEA (68 µL, 0.390 mmol) in $DMF_{abs}$ and **(*S*)-5.44** (80 mg, 0.130 mmol) in $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white solid (27 mg, 17 %)**.** $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.48 – 1.64 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.67 – 1.75 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.79 – 1.89 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.39 – 2.51 (m, 8H, CO-C***H***$_2$-C***H***$_2$-CO ), 2.55 (t, $^3J$ = 6.4 Hz, 2H, CO-C***H***$_2$-$CH_2$-$NH_2$), 3.16 (t, $^3J$ = 6.3 Hz, 2H, CO-$CH_2$-C***H***$_2$-$NH_2$), 3.19 – 3.29 (m, 10H, CH-$(CH_2)_2$-C***H***$_2$-NH + 2 NH-(C***H***$_2$)$_2$-NH), 3.29 – 3.32 (m, overlap with solvent, 2H, 2x NH-(C***H***$_2$)$_2$-NH), 4.27 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.41 – 4.46 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.08 (s, 1H, $(Ph)_2$-C***H***), 7.16 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC): δ (ppm) 25.72 (CH-$CH_2$-***C***$H_2$), 30.29 (CH-***C***$H_2$-$CH_2$), 32.04 (-CO-***C***$H_2$), 32.15 (CO-***C***$H_2$), 32.16 (CO-***C***$H_2$), 32.17 (CO-***C***$H_2$), 32.94 (CO-***C***$H_2$-$CH_2$-$NH_2$), 37.12 (CO-$CH_2$-***C***$H_2$-$NH_2$), 39.79 (NH-(***C***$H_2$)$_2$-NH), 39.90 (2 NH-(***C***$H_2$)$_2$-NH), 39.97 (NH-(***C***$H_2$)$_2$-NH), 40.19 (NH-(***C***$H_2$)$_2$-$NH_2$), 40.46 (NH-(***C***$H_2$)$_2$-$NH_2$), 41.75 ($(CH_2)_2$-***C***$H_2$-NH-), 43.80 (NH-***C***$H_2$-Ar), 44.31 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.36 (***C***H-$CH_2$-$CH_2$), 58.67 ($(Ph)_2$-***C***H), 128.14 (diPh-***C***-4), 128.23 (diPh-***C***-4), 128.33 (2 Ph-***C***), 128.66 (2 Ph-***C***), 129.51 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.90 (2 diPh-***C***), 138.41 (Ph-***C***-1), 140.86 (Ph-***C***-4), 140.97 (2 diPh-***C***-1), 155.68 (NH-***C***$NH_2$-NH), 155.73 (NH-***C***O-NH), 162.14 (Ar-$CH_2$-NH-***C***O-$NH_2$), 172.49 (***C***O-$CH_2$-$CH_2$-$NH_2$), 173.54 (***C***O-NH-$CH_2$-Ar), 174.83 ($(Ph)_2$-CH-***C***O), 175.10 (***C***=O), 175.14 (***C***=O), 175.20 (***C***=O), 175.16 (***C***=O), 175.23 (***C***=O); anal. RP-HPLC: 99 % ($t_R$ = 12.28 min, *k*' = 3.06); HRMS (ESI): *m/z* calcd. for $[C_{47}H_{66}N_{14}O_9+H]^+$ 971.5210 , found 971.5213. $C_{47}H_{66}N_{14}O_9 \cdot 2TFA$ (1199.16).

**(R)-$N^1$-{9-amino-4-(2,2-diphenylacetamido)-3,11,16,19-tetraoxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12,15,20-hexaazadocos-9-en-22-yl}-$N^4$-[2-(3-aminopropanamido)ethyl]succinamide ((*R*)-5.69)**

The title compound was prepared from **5.59** (62 mg, 0.130 mmol), TBTU (50 mg, 0.156 mmol), HOBt (21 mg, 0.156 mmol), DIEA (68 µL, 0.390 mmol) in 5 mL $DMF_{abs}$ and **(*R*)-5.44** (80 mg, 0.130 mmol) in 3 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white solid (22 mg, 14 %). $^1$H-NMR (600 MHz, $CD_3OD$): δ (ppm) 1.49 – 1.62 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.69 – 1.78 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.83 – 1.92 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.43 – 2.53 (m, 8H, CO-C***H***$_2$-C***H***$_2$-CO ), 2.55 (t, $^3J$ = 6.4 Hz, 2H, CO-C***H***$_2$-$CH_2$-$NH_2$), 3.20 (t, $^3J$ = 6.4 Hz, 2H, CO-$CH_2$-C***H***$_2$-$NH_2$), 3.23 – 3.29 (m, 10H, CH-$(CH_2)_2$-C***H***$_2$-NH + 2 NH-(C***H***$_2$)$_2$-NH), 3.29 – 3.32 (m, overlap with solvent, 2H, 2x NH-(C***H***$_2$)$_2$-NH), 4.30 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.31 – 4.43 (m, 2H, Ar-C***H***$_2$-NH), 4.44 – 4.49 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.11 (s, 1H, $(Ph)_2$-C***H***), 7.20 – 7.25 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.26 – 7.36 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$): δ (ppm) 25.73 (CH-$CH_2$-***C***$H_2$), 30.30 (CH-***C***$H_2$-$CH_2$), 32.03 (-CO-(***C***$H_2$)$_2$-CO), 32.14 (CO-***C***$H_2$), 32.16 (2 CO-***C***$H_2$), 32.94 (CO-***C***$H_2$-$CH_2$-$NH_2$), 37.14 (CO-$CH_2$-***C***$H_2$-$NH_2$), 39.80 (NH-(***C***$H_2$)$_2$-NH), 39.90 (2 NH-(***C***$H_2$)$_2$-NH), 39.98 (NH-(***C***$H_2$)$_2$-NH), 40.19 (NH-(***C***$H_2$)$_2$-$NH_2$), 40.48 (NH-(***C***$H_2$)$_2$-$NH_2$), 41.77 ($(CH_2)_2$-***C***$H_2$-NH-), 43.80 (NH-***C***$H_2$-Ar), 44.34 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.35 (***C***H-$CH_2$-$CH_2$), 58.68 ($(Ph)_2$-***C***H), 128.15 (diPh-***C***-4), 128.23 (diPh-***C***-4), 128.33 (2 Ph-***C***), 128.68 (2 Ph-***C***), 129.52 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.91 (2 diPh-***C***), 138.42 (Ph-***C***-1), 140.87 (Ph-***C***-4), 140.97 (2 diPh-***C***-1), 155.66 (NH-***C***$NH_2$-NH), 155.71 (NH-***C***O-NH), 162.14 (Ar-$CH_2$-NH-***C***O-$NH_2$), 172.49 (***C***O-$CH_2$-$CH_2$-$NH_2$), 173.52 (***C***O-NH-$CH_2$-Ar), 174.82 ($(Ph)_2$-CH-***C***O), 175.10 (2x ***C***=O), 175.21 (***C***=O), 175.26 (***C***=O); anal. RP-HPLC: 100 % ($t_R$ = 12.09 min, *k*' = 2.96); HRMS (ESI): *m/z* calcd. for $[C_{47}H_{66}N_{14}O_9+H]^+$ 971.5210 , found 971.5208. $C_{47}H_{66}N_{14}O_9 \cdot 2TFA$ (1199.16).

**(*S*)-$N^1$-{9-amino-4-(2,2-diphenylacetamido)-3,11-dioxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12-tetra-azatetradec-9-en-14-yl}-$N^4$-{2-[2-(2-aminoethoxy)ethoxy]ethyl}succinamide ((*S*)-5.70)**

The title compound was prepared from **5.64** (62 mg, 0.179 mmol), TBTU (69 mg, 0.214 mmol), HOBt (29 mg, 0.214 mmol), DIEA (93 µL, 0.436 mmol) in 5 mL $DMF_{abs}$ and **(*S*)-5.44** (110 mg, 0.179 mmol) in 3 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white fluffy solid (19 mg, 10 %). $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.47 – 1.62 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.66 – 1.77 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.79 – 1.89 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.43 – 2.49 (m, 4H, CO-(C***H***$_2$)$_2$-CO), 3.10 (t, $^3J$ = 5.0 Hz, 2H, O-$CH_2$-C***H***$_2$-$NH_2$), 3.16 – 3.26 (m, 2H, CH-$(CH_2)_2$-C***H***$_2$-NH), 3.27 – 3.31 (m, overlap with solvent, 4H, NH-CO-NH-(C***H***$_2$)$_2$-NH), 3.34 (t, $^3J$ = 5.7 Hz, 2H, NH-CO-C***H***$_2$-$CH_2$-O), 3.53 (t, $^3J$ = 5.7 Hz, 2H, NH-CO-$CH_2$-C***H***$_2$-O), 3.61 – 3.66 (m, 4H, O-C***H***$_2$-C***H***$_2$-O), 3.68 (t, $^3J$ = 5.0 Hz, 2H, O-C***H***$_2$-$CH_2$-$NH_2$) 4.27 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.07 (s, 1H, $(Ph)_2$-C***H***),

7.17 – 7.22 (m, 4H, $CH_2$-$C_6\boldsymbol{H}_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC): δ (ppm) 25.71 (CH-$CH_2$-***C***$H_2$), 30.29 (CH-***C***$H_2$-$CH_2$), 31.96 (CO-(***C***$H_2$)$_2$-CO), 32.05 (CO-(***C***$H_2$)$_2$-CO), 39.95 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 40.27 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 40.43 (CO-NH-***C***$H_2$-$CH_2$-O), 40.69 (O-$CH_2$-***C***$H_2$-$NH_2$), 41.76 ((CH$_2$)$_2$-***C***$H_2$-NH-), 43.81 (NH-***C***$H_2$-Ar), 44.33 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.31 (***C***H-$CH_2$-$CH_2$), 58.69 ((Ph)$_2$-***C***H), 67.89 (O-***C***$H_2$-$CH_2$-$NH_2$), 70.63 (CO-NH-$CH_2$-***C***$H_2$-O), 71.32 (O-***C***$H_2$), 71.36 (O-***C***$H_2$), 128.15 (diPh-***C***-4), 128.23 (diPh-***C***-4), 128.34 (2 Ph-***C***), 128.69 (2 Ph-***C***), 129.51 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.91 (2 diPh-***C***), 138.42 (Ph-***C***-1), 140.86 (Ph-***C***-4), 140.97 (2 diPh-***C***-1), 155.64 (NH-***C***$NH_2$-NH), 155.70 (NH-***C***O-NH), 162.04 (Ar-$CH_2$-NH-***C***O-$NH_2$), 173.50 (***C***O-NH-$CH_2$-Ar), 174.85 ((Ph)$_2$-CH-***C***O), 174.86 (***C***=O), 175.14 (***C***=O); anal. RP-HPLC: 99 % ($t_R$ = 12.60 min, *k*' = 3.13); HRMS (ESI): *m/z* calcd. for $[C_{42}H_{59}N_{11}O_8+H]^+$ 846.4621, found: 846.4617. $C_{42}H_{59}N_{11}O_8 \cdot 2TFA$ (1074.03).

**(*R*)-*N*$^1$-{9-Amino-4-(2,2-diphenylacetamido)-3,11-dioxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12-tetraazatetradec-9-en-14-yl}-*N*$^4$-{2-[2-(2-aminoethoxy)ethoxy]ethyl}succinamide ((*R*)-5.70)**

The title compound was prepared from **5.64** (45 mg, 0.130 mmol), TBTU (50 mg, 0.156 mmol), HOBt (21 mg, 0.156 mmol), DIEA (68 µL, 0.390 mmol) in 5 mL $DMF_{abs}$ and **(*R*)-5.44** (80 mg, 0.130 mmol) in 3 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white fluffy solid (10 mg, 7 %)**.** $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.47 – 1.63 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.66 – 1.76 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.80 – 1.89 (m, 1H, CH-C***H***$_2$-$CH_2$), 2.42 – 2.51 (m, 4H, CO-(C***H***$_2$)$_2$-CO), 3.10 (t, $^3J$ = 5.0 Hz, 2H, O-$CH_2$-C***H***$_2$-$NH_2$), 3.18 – 3.26 (m, 2H, CH-(CH$_2$)$_2$-C***H***$_2$-NH), 3.27 – 3.31 (m, overlap with solvent, 4H, NH-CO-NH-(C***H***$_2$)$_2$-NH), 3.34 (t, $^3J$ = 5.7 Hz, 2H, NH-CO-C***H***$_2$-$CH_2$-O), 3.53 (t, $^3J$ = 5.7 Hz, 2H, NH-CO-$CH_2$-C***H***$_2$-O), 3.61 – 3.66 (m, 4H, O-C***H***$_2$-C***H***$_2$-O), 3.68 (t, $^3J$ = 5.0 Hz, 2H, O-C***H***$_2$-$CH_2$-$NH_2$) 4.27 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.07 (s, 1H, (Ph)$_2$-C***H***), 7.17 – 7.22 (m, 4H, $CH_2$-$C_6\boldsymbol{H}_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC): δ (ppm) 25.71 (CH-$CH_2$-***C***$H_2$), 30.29 (CH-***C***$H_2$-$CH_2$), 31.96 (CO-(***C***$H_2$)$_2$-CO), 32.05 (CO-(***C***$H_2$)$_2$-CO), 39.95 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 40.27 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 40.43 (CO-NH-***C***$H_2$-$CH_2$-O), 40.69 (O-$CH_2$-***C***$H_2$-$NH_2$), 41.76 ((CH$_2$)$_2$-***C***$H_2$-NH-), 43.81 (NH-***C***$H_2$-Ar), 44.32 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.31 (***C***H-$CH_2$-$CH_2$), 58.69 ((Ph)$_2$-***C***H), 67.89 (O-***C***$H_2$-$CH_2$-$NH_2$), 70.63 (CO-NH-$CH_2$-***C***$H_2$-O), 71.32 (O-***C***$H_2$), 71.36 (O-***C***$H_2$), 128.15 (diPh-***C***-4), 128.24 (diPh-***C***-4), 128.34 (2 Ph-***C***), 128.69 (2 Ph-***C***), 129.52 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.91 (2 diPh-***C***), 138.42 (Ph-***C***-1), 140.86 (Ph-***C***-4), 140.97 (2 diPh-***C***-1), 155.69 (NH-***C***$NH_2$-NH), 155.73 (NH-***C***O-NH), 162.14 (Ar-$CH_2$-NH-***C***O-$NH_2$), 173.50 (***C***O-NH-$CH_2$-Ar), 174.83 ((Ph)$_2$-CH-***C***O), 174.85 (***C***=O), 175.14 (***C***=O); anal. RP-HPLC: 98 % ($t_R$ = 12.37 min, *k*' = 3.06); HRMS (ESI): *m/z* calcd. for $[C_{42}H_{59}N_{11}O_8+H]^+$ 846.4621, found: 846.4623. $C_{42}H_{59}N_{11}O_8 \cdot 2TFA$ (1074.03).

**(*S*)-*N*$^1$-{9-amino-4-(2,2-diphenylacetamido)-3,11-dioxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12-tetra-azatetradec-9-en-14-yl}-*N*$^4$-(3-{2-[2-(3-aminopropoxy)ethoxy]ethoxy}propyl)succinamide ((*S*)-5.71)**

The title compound was prepared from **5.65** (75 mg, 0.179 mmol), TBTU (69 mg, 0.214 mmol), HOBt (29 mg, 0.214 mmol), DIEA (93 µL, 0.436 mmol) in 5 mL $DMF_{abs}$ and **(*S*)-5.44** (110 mg, 0.179 mmol) in 3 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white fluffy solid (17 mg, 8 %)**.** $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.47 – 1.62 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.66 – 1.77 (m, 3H, CH-C***H***$_2$-$CH_2$ + O-$CH_2$-C***H***$_2$-$CH_2$-NH), 1.80 – 1.87 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.87 – 1.93 (m, 2H, O-$CH_2$-C***H***$_2$-$CH_2$-$NH_2$), 2.42 – 2.49 (m, 4H, CO-(C***H***$_2$)$_2$-CO), 3.07 (t, $^3J$ = 6.3 Hz, 2H, O-$CH_2$-$CH_2$-C***H***$_2$-$NH_2$), 3.16 – 3.26 (m, 4H, CH-$(CH_2)_2$-C***H***$_2$-NH + O-$(CH_2)_2$-C***H***$_2$-NH-CO), 3.27 – 3.31 (m, overlap with solvent, 4H, NH-CO-NH-(C***H***$_2$)$_2$-NH), 3.49 (t, $^3J$ = 6.1 Hz, 2H, CO-NH-$CH_2$-$CH_2$-C***H***$_2$-O), 3.55 – 3.58 (m, 2H, O-C***H***$_2$), 3.59 – 3.67 (m, 8H, 3x O-C***H***$_2$ + O-C***H***$_2$-$CH_2$-$CH_2$-$NH_2$) 4.26 (s, 2H, Ar-C***H***$_2$-NH-CO-$NH_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-$CH_2$-$CH_2$), 5.07 (s, 1H, $(Ph)_2$-C***H***), 7.17 – 7.22 (m, 4H, $CH_2$-$C_6$***H***$_4$-$CH_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, $CD_3OD$, HSQC): δ (ppm) 25.72 (CH-$CH_2$-***C***$H_2$), 28.04 (O-$CH_2$-***C***$H_2$-$CH_2$-$NH_2$), 30.27 (CH-***C***$H_2$-$CH_2$), 30.45 (O-$CH_2$-***C***$H_2$-$CH_2$-NH), 32.11 (CO-***C***$H_2$), 32.17 (CO-***C***$H_2$), 37.73 (CO-NH-***C***$H_2$-$CH_2$-$CH_2$-O) 39.94 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 40.14 (NH-CO-NH-(***C***$H_2$)$_2$-NH), 40.42 (O-$CH_2$-$CH_2$-***C***$H_2$-$NH_2$), 41.73 $(CH_2)_2$-***C***$H_2$-NH-), 43.79 (NH-***C***$H_2$-Ar), 44.35 (Ar-***C***$H_2$-NH-CO-$NH_2$), 54.34 (***C***H-$CH_2$-$CH_2$), 58.68 ($(Ph)_2$-***C***H), 69.65 (CO-NH-$CH_2$-$CH_2$-***C***$H_2$-O), 70.37 (O-***C***$H_2$-$CH_2$-$CH_2$-$NH_2$), 71.00 (O-(***C***$H_2$)$_2$-O), 71.02 (O-(***C***$H_2$)$_2$-O), 71.08 (O-(***C***$H_2$)$_2$-O), 71.38 (O-(***C***$H_2$)$_2$-O), 128.15 (diPh-***C***-4), 128.23 (diPh-***C***-4), 128.34 (2 Ph-***C***), 128.69 (2 Ph-***C***), 129.51 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.91 (2 diPh-***C***), 138.42 (Ph-***C***-1), 140.86 (Ph-***C***-4), 140.97 (2 diPh-***C***-1), 155.64 (NH-***C***$NH_2$-NH), 155.70 (NH-***C***O-NH), 162.04 (Ar-$CH_2$-NH-***C***O-$NH_2$), 173.50 (***C***O-NH-$CH_2$-Ar), 174.66 (***C***=O), 174.82 ($(Ph)_2$-CH-***C***O), 175.12 (***C***=O); anal. RP-HPLC: 90 % ($t_R$ = 12.92 min, *k*' = 3.24); HRMS (ESI): *m/z* calcd. for $[C_{46}H_{67}N_{11}O_9+H]^+$ 918.5196, found: 918.5199. $C_{46}H_{67}N_{11}O_9 \cdot 2TFA$ (1146.13).

**(*R*)-*N*$^1$-{9-amino-4-(2,2-diphenylacetamido)-3,11-dioxo-1-[4-(ureidomethyl)phenyl]-2,8,10,12-tetra-azatetradec-9-en-14-yl}-*N*$^4$-(3-{2-[2-(3-aminopropoxy)ethoxy]ethoxy}propyl)succinamide ((*R*)-5.71)**

The title compound was prepared from **5.65** (55 mg, 0.130 mmol), TBTU (50 mg, 0.156 mmol), HOBt (21 mg, 0.156 mmol), DIEA (68 µL, 0.390 mmol) in 5 mL $DMF_{abs}$ and **(*R*)-5.44** (80 mg, 0.130 mmol) in 3 mL $DMF_{abs}$ according to the general procedure. Deprotection in 3 mL DCM and 3 mL TFA and subsequent purification by RP-HPLC afforded the product as a white fluffy solid (16 mg, 11 %)**.** $^1$H-NMR (600 MHz, $CD_3OD$, COSY, HSQC): δ (ppm) 1.48 – 1.62 (m, 2H, CH-$CH_2$-C***H***$_2$), 1.67 – 1.76 (m, 3H, CH-C***H***$_2$-$CH_2$ + O-$CH_2$-C***H***$_2$-$CH_2$-NH), 1.80 – 1.87 (m, 1H, CH-C***H***$_2$-$CH_2$), 1.87 – 1.93 (m, 2H, O-$CH_2$-C***H***$_2$-$CH_2$-$NH_2$), 2.42 – 2.49 (m, 4H, CO-(C***H***$_2$)$_2$-CO), 3.07 (t, $^3J$ = 6.4 Hz, 2H, O-$CH_2$-$CH_2$-C***H***$_2$-$NH_2$), 3.17 – 3.26 (m, 4H, CH-$(CH_2)_2$-C***H***$_2$-NH + O-$(CH_2)_2$-C***H***$_2$-NH-CO), 3.27 – 3.31 (m, overlap with solvent, 4H, NH-CO-

NH-(C***H***$_2$)$_2$-NH), 3.49 (t, $^3J$ = 6.1 Hz, 2H, CO-NH-CH$_2$-CH$_2$-C***H***$_2$-O), 3.54 – 3.59 (m, 2H, O-C***H***$_2$), 3.59 – 3.68 (m, 8H, 3x O-C***H***$_2$ + O-C***H***$_2$-CH$_2$-CH$_2$-NH$_2$) 4.27 (s, 2H, Ar-C***H***$_2$-NH-CO-NH$_2$), 4.27 – 4.39 (m, 2H, Ar-C***H***$_2$-NH), 4.40 – 4.45 (m, 1H, C***H***-CH$_2$-CH$_2$), 5.08 (s, 1H, (Ph)$_2$-C***H***), 7.17 – 7.22 (m, 4H, CH$_2$-C$_6$***H***$_4$-CH$_2$), 7.23 – 7.33 (m, 10 H, Ph-***H***); $^{13}$C-NMR (150 MHz, CD$_3$OD, HSQC): δ (ppm) 25.71 (CH-CH$_2$-***C***H$_2$), 28.04 (O-CH$_2$-***C***H$_2$-CH$_2$-NH$_2$), 30.27 (CH-***C***H$_2$-CH$_2$), 30.45 (O-CH$_2$-***C***H$_2$-CH$_2$-NH), 32.11 (CO-***C***H$_2$), 32.17 (CO-***C***H$_2$), 37.73 (CO-NH-***C***H$_2$-CH$_2$-CH$_2$-O) 39.94 (NH-CO-NH-(***C***H$_2$)$_2$-NH), 40.14 (NH-CO-NH-(***C***H$_2$)$_2$-NH), 40.42 (O-CH$_2$-CH$_2$-***C***H$_2$-NH$_2$), 41.73 (CH$_2$)$_2$-***C***H$_2$-NH-), 43.79 (NH-***C***H$_2$-Ar), 44.37 (Ar-***C***H$_2$-NH-CO-NH$_2$), 54.34 (***C***H-CH$_2$-CH$_2$), 58.67 ((Ph)$_2$-***C***H), 69.65 (CO-NH-CH$_2$-CH$_2$-***C***H$_2$-O), 70.37 (O-***C***H$_2$-CH$_2$-CH$_2$-NH$_2$), 71.00 (O-(***C***H$_2$)$_2$-O), 71.02 (O-(***C***H$_2$)$_2$-O), 71.08 (O-(***C***H$_2$)$_2$-O), 71.38 (O-(***C***H$_2$)$_2$-O), 128.13 (diPh-***C***-4), 128.22 (diPh-***C***-4), 128.35 (2 Ph-***C***), 128.66 (2 Ph-***C***), 129.50 (2 diPh-***C***), 129.56 (2 diPh-***C***), 129.86 (2 diPh-***C***), 129.90 (2 diPh-***C***), 138.41 (Ph-***C***-1), 140.86 (Ph-***C***-4), 140.97 (2 diPh-***C***-1), 155.71 (NH-***C***NH$_2$-NH), 155.76 (NH-***C***O-NH), 162.12 (Ar-CH$_2$-NH-***C***O-NH$_2$), 173.54 (***C***O-NH-CH$_2$-Ar), 174.66 (***C***=O), 174.83 ((Ph)$_2$-CH-***C***O), 175.12 (***C***=O); anal. RP-HPLC: 90 % ($t_R$ = 12.69 min, *k*' = 3.16); HRMS (ESI): m/z calcd. for [C$_{46}$H$_{67}$N$_{11}$O$_9$+H]$^+$ 918.5196, found: 918.5194. C$_{46}$H$_{67}$N$_{11}$O$_9$ · 2TFA (1146.13).

## 5.7.3 Pharmacological Methods

### 5.7.3.1 Materials and Cell Culture

Porcine NPY (pNPY), human pancreatic polypeptide (hPP), [K$^4$]-hPP and GW1229 [31] (also designated GR231118 or 1229U91) were a gift of Prof. Cabrele (University of Bochum, Germany). The cyanine labeled fluorescent peptides cy5-pNPY and Cy5-[K$^4$]hPP were prepared in our laboratory as described previously. [32,15]

Unless otherwise indicated, chemicals, buffers and reagents were purchased from Merck (Darmstadt, Germany). Powder for the preparation of media was obtained from Sigma Aldrich (Germany, München). Millipore water was used throughout. HEPES and bovine serum albumin (BSA) were purchased from Serva (Heidelberg, Germany). FCS was obtained from Biochrom (Berlin, Germany), cyanine dye cy5-succinimidyl ester from Amersham Biosciences (Little Chalfont, UK) and coelenterazine h from Biotrend (Cologne, Germany).

*Cell culture:*

Except for HEL (human erythroleukemia) cells, SK-N-MC cells and MCF-7-Y1 cells, expressing the NPY Y$_1$R, all other cells – HEC-1-B-Y$_5$ [33] cells and CHO-hY$_2$/-hY$_4$ - were genetically engineered to stably express the receptor of interest as described elsewhere. [15, 34]

HEL cells (suspension) were passaged in RPMI with FCS (5%). HEC-1-B-Y$_5$ cells (Y$_5$R) were cultured in EMEM with FCS (10 %) and G418 (400 µg/mL) as described previously.[33] CHO cells, expressing Y$_2$R or Y$_4$R were grown in Ham's F12 medium supplemented with FCS (10 %) and G418 (400 mg/mL),

hygromycin (400 mg/mL) and zeocin (200 mg/mL). [15, 35] MCF-7-Y1 cells were maintained in MEM supplemented with 5 % FCS (this cell line originated from MCF-7 cells in the 157$^{th}$ passage and shows a higher $hY_1$ expression (factor 2 – 3) over the original MCF-7 cells).

### 5.7.3.2 Aequorin Assay

See section 3.3.2.1

### 5.7.3.3 Flow Cytometric Binding Assay

See section 3.3.2.2

### 5.7.3.4 Radioligand Binding Assay

The radioligand binding assay was essentially performed as described previously using [$^3$H]UR-MK114[16] instead of [$^3$H]propionyl-pNPY.[36] Binding buffer was prepared by addition of BSA (1 %) and bacitracin (50 µg · $L^{-1}$) to a buffer (pH 7.4) consisting of HEPES (10 mM), NaCl (150 mM), KCl (5 mM), $CaCl_2 \cdot 2H_2O$ (2.5 mm), $KH_2PO_4$ (1.2 mM), $Mg_2SO_4 \cdot 7\ H_2O$ (1.2 mM), and $NaHCO_3$ (25 mM). HEPES buffer without additives was used to wash the cells prior to and after radioligand binding.

Two or three days prior to the experiment, cells were seeded in 24-well plates reaching 70 % confluency at the day of testing. MCF-7 cells were seeded in culture medium with 1 nM β-estradiol in order to up-regulate the $Y_1$ receptor. In a first step, the culture medium was removed, the cells were washed with buffer (500 µL) and covered with binding buffer (200 µL for SK-N-MC, 400 µL for µL for µL for MCF-7 cells). Binding buffer (25/50 µL) and binding buffer (25/50 µL) with radioligand (10-fold concentrated) were added for total binding. For determination of non-specific binding and competition of the radioligand with the test compounds, binding buffer (25/50 µL) with the competing agent (NPY or test compound, 10-fold concentrated) and binding buffer (25/50 µL) with radioligand (10-fold concentrated) was added. During incubation at room temperature for 20 (SK-N-MC) or 30 min (MCF-7-$Y_1$) the plates were gently shaken. Subsequently, the binding buffer was removed, the cells were washed twice with buffer (500 µL, 4 °C, ≤ 30s) and covered with lysis solution (200 µL) consisting of urea (8 M), acetic acid (3 M) and Triton-X-100 (1 5) in water. The plates were shaken for 30 min. The lysis solution was transferred into 6-mL scintillation vials filled with scintillator (3 mL, Rothiszint$^{TM}$ eco plus) and the dishes were washed once with lysis solution (100 µL). Samples were counted with a Beckman LS 6500 beta-counter. Assays were performed in duplicate or triplicate.

## 5.8 References


1. Keller, M.; Teng, S.; Bernhardt, G.; Buschauer, A. Bivalent argininamide-type neuropeptide y y(1) antagonists do not support the hypothesis of receptor dimerisation. *ChemMedChem* **2009,** 4, 1733-1745.
2. Bard, J. A.; Walker, M. W.; Branchek, T. A.; Weinshank, R. L. Cloning and functional expression of a human Y4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. *J. Biol. Chem.* **1995,** 270, 26762-26765.
3. Lundell, I.; Blomqvist, A. G.; Berglund, M. M.; Schober, D. A.; Johnson, D.; Statnick, M. A.; Gadski, R. A.; Gehlert, D. R.; Larhammar, D. Cloning of a human receptor of the NPY receptor family with high affinity for pancreatic polypeptide and peptide YY. *J. Biol. Chem.* **1995,** 270, 29123-29128.
4. Gregor, P.; Millham, M. L.; Feng, Y.; DeCarr, L. B.; McCaleb, M. L.; Cornfield, L. J. Cloning and characterization of a novel receptor to pancreatic polypeptide, a member of the neuropeptide Y receptor family. *FEBS Lett.* **1996,** 381, 58-62.
5. Larhammar, D.; Wraith, A.; Berglund, M. M.; Holmberg, S. K.; Lundell, I. Origins of the many NPY-family receptors in mammals. *Peptides* **2001,** 22, 295-307.
6. Fuhlendorff, J.; Gether, U.; Aakerlund, L.; Langeland-Johansen, N.; Thogersen, H.; Melberg, S. G.; Olsen, U. B.; Thastrup, O.; Schwartz, T. W. [Leu31, Pro34]neuropeptide Y: a specific Y1 receptor agonist. *Proc. Natl. Acad. Sci. USA* **1990,** 87, 182-186.
7. Daniels, A. J.; Matthews, J. E.; Slepetis, R. J.; Jansen, M.; Viveros, O. H.; Tadepalli, A.; Harrington, W.; Heyer, D.; Landavazo, A.; Leban, J. J.; Spaltenstein, A. High-affinity neuropeptide Y receptor antagonists. *Proc. Natl. Acad. Sci. USA* **1995,** 92, 9067-9071.
8. Douglas A. Schober, A. M. v. A., David L. Smiley, Robert F. Bruns, Donald R. Gehlert. The Neuropeptide Y Y1 Antagonist, 1229U91, A Potent Agonist for the Human Pancreatic Polypeptide-Preferring (NPY Y4) Receptor. *Peptides* **1998,** 19, 537-542.
9. Schober, D. A.; Van Abbema, A. M.; Smiley, D. L.; Bruns, R. F.; Gehlert, D. R. The neuropeptide Y Y1 antagonist, 1229U91, a potent agonist for the human pancreatic polypeptide-preferring (NPY Y4) receptor. *Peptides* **1998,** 19, 537-542.
10. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; Koenig, B. NG-Acyl-argininamides as NPY Y1 receptor antagonists: Influence of structurally diverse acyl substituents on stability and affinity. *Bioorg. Med. Chem.* **2010,** 18, 6292-6304.
11. Keller, M. Guanidine-Acylguanidine Bioisosteric Approach to Address Peptidergic Receptors: Pharmacological and Diagnostic Tools for the NPY Y1 Receptor and Versatile Building Block on Arginine Substitutes. Doctoral Thesis, Regensburg, **2008**.

12. Keller, M.; Erdmann, D.; Pop, N.; Pluym, N.; Teng, S.; Bernhardt, G.; Buschauer, A. Red-fluorescent argininamide-type NPY Y1 receptor antagonists as pharmacological tools. *Bioorg. Med. Chem.* **2011,** 19, 2859-2878.

13. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; Koenig, B. Modular synthesis of non-peptidic bivalent NPY Y1 receptor antagonists. *Bioorg. Med. Chem.* **2008,** 16, 9858-9866.

14. Yung-Chi Cheng, W. H. P. Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzymatic reaction. *Biochem. Pharm.* **1973,** 22, 3099-3108.

15. Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Determination of Affinity and Activity of Ligands at the Human Neuropeptide Y Y4 Receptor by Flow Cytometry and Aequorin Luminescence. *J. Recept. Signal. Transduct. Res.* **2007,** 27, 217 - 233.

16. Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Guanidine-acylguanidine bioisosteric approach in the design of radioligands: synthesis of a tritium-labeled $N^G$-propionylargininamide ([$^3$H]-UR-MK114) as a highly potent and selective neuropeptide Y $Y_1$ receptor antagonist. *J. Med. Chem.* **2008,** 51, 8168-8172.

17. Brennauer, A., Dove, S., Buschauer, A. Structure-Activity Relationships of Nonpeptide Neuropeptide Y Receptor Antagonists. *Handb. Exp. Pharm.*; **2004**; 162, 506-537.

18. Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Becksickinger, A. G.; Doods, H. N. The First Highly Potent and Selective Nonpeptide Neuropeptide-Y Y-1-Receptor Antagonist - BIBP3226. *Eur. J. Pharm.* **1994,** 271, R11-R13.

19. Boon, J. M.; Lambert, T. N.; Smith, B. D.; Beatty, A. M.; Ugrinova, V.; Brown, S. N. Structure/activity study of tris(2-aminoethyl)amine-derived translocases for phosphatidylcholine. *J. Org. Chem.* **2002,** 67, 2168-2174.

20. Duschinsky, R.; Dolan, L. A. Imidazolone series. Synthesis of a lower and a higher homolog of desthiobiotin and of related substances. *J. Am. Chem. Soc.* **1946,** 68, 2350-2355.

21. Eschalier, A.; Dureng, G.; Duchene-Marullaz, P.; Berecoechea, J.; Anatol, J. Acylguanidines and substituted ureas derivated from aryloxypropanolamines: β-blocking properties. *Eur. J. Med. Chem.* **1983,** 18, 139-145.

22. Kirmse, W.; Buschhoff, M. Reactions of b-alkoxyalkyl carbenes. *Chem. Berichte* **1967,** 100, 1491-1506.

23. Marx, J.; Marx-Moll, L. The synthesis of pyrazole from *N*-nitroso-*N*-allylurea. *Chem. Berichte* **1954,** 87, 1499-1500.

24. Remenar, J. F.; Blumberg, L. C. Diaryldiazepine prodrugs and their preparation and use for the treatment of neurological and psychological disorders. US20110166128A1, **2011**.

25. Akagi, A.; Soejima, Y.; Koga, K.; Yoshinaga, S.-i.; Izumiya, N. Abnormal circular dichroism of a-acyl-L-ornithines and facile syntheses of d-acyl-L-ornithines. *Kyushu Sangyo Daigaku Kogakubu Kenkyu Hokoku* **1993,** 30, 133-138.

26. Bauer, J.; Martin, R. O.; Rodriguez, S. Preparation of novel acyl dipeptide-like compounds bearing an accessory functional side chain spacer for use in pharmaceutical compositions. US20050192232A1, **2005**.

27. Engel, W.; Eberlein, W.; Rudolf, K.; Doods, H.; Wieland, H.-A.; Willim, K.-D.; Entzeroth, M.; Wienen, W. Preparation of amino acid derivatives as neuropeptide Y antagonists. DE19544687A1, **1997**.

28. Callahan, J. F.; Ashton-Shue, D.; Bryan, H. G.; Bryan, W. M.; Heckman, G. D.; Kinter, L. B.; McDonald, J. E.; Moore, M. L.; Schmidt, D. B.; et, a. Structure-activity relationships of novel vasopressin antagonists containing C-terminal diaminoalkanes and (aminoalkyl)guanidines. *J. Med. Chem.* **1989,** 32, 391-396.

29. Szent-Gyorgyi, C.; Schmidt, B. A.; Creeger, Y.; Fisher, G. W.; Zakel, K. L.; Adler, S.; Fitzpatrick, J. A. J.; Woolford, C. A.; Yan, Q.; Vasilev, K. V.; Berget, P. B.; Bruchez, M. P.; Jarvik, J. W.; Waggoner, A. Fluorogen-activating single-chain antibodies for imaging cell surface proteins. *Nat. Biotechnol.* **2008,** 26, 235-240.

30. Garibay, P. W.; Hoeg-Jensen, T.; Jonassen, I.; Havelund, S.; Madsen, P.; Jakobsen, P. Preparation of insulin derivatives. WO2006082205A1, **2006**.

31. Balasubramaniam, A.; Mullins, D. E.; Lin, S.; Zhai, W.; Tao, Z.; Dhawan, V. C.; Guzzi, M.; Knittel, J. J.; Slack, K.; Herzog, H.; Parker, E. M. Neuropeptide Y (NPY) $Y_4$ receptor selective agonists based on NPY(32-36): development of an anorectic $Y_4$ receptor selective agonist with picomolar affinity. *J. Med. Chem.* **2006,** 49, 2661-2665.

32. Schneider, E.; Keller, M.; Brennauer, A.; Hoefelschweiger, B. K.; Gross, D.; Wolfbeis, O. S.; Bernhardt, G.; Buschauer, A. Synthesis and characterization of the first fluorescent nonpeptide NPY Y1 receptor antagonist. *ChemBioChem* **2007,** 8, 1981-1988.

33. Moser, C.; Bernhardt, G.; Michel, J.; Schwarz, H.; Buschauer, A. Cloning and functional expression of the hNPY $Y_5$ receptor in human endometrial cancer (HEC-1B) cells. *Can. J. Physiol. Pharmacol.* **2000,** 78, 134-142.

34. Ziemek, R. Development of binding and functional assays for the neuropeptide Y Y 2 and Y 4 receptors. Doctoral Thesis, Regensburg, **2006**.

35. Ziemek, R.; Brennauer, A.; Schneider, E.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Fluorescence- and luminescence-based methods for the determination of affinity and activity of neuropeptide Y2 receptor ligands. *Eur. J. Pharm.* **2006,** 551, 10-18.

36. Aiglstorfer, I.; Uffrecht, A.; Gessele, K.; Moser, C.; Schuster, A.; Merz, S.; Malawska, B.; Bernhardt, G.; Dove, S.; Buschauer, A. NPY Y1 antagonists: structure-activity relationships of arginine derivatives and hybrid compounds with arpromidine-like partial structures. *Regul. Pept.* **1998,** 75-76, 9-21.

# CHAPTER 6

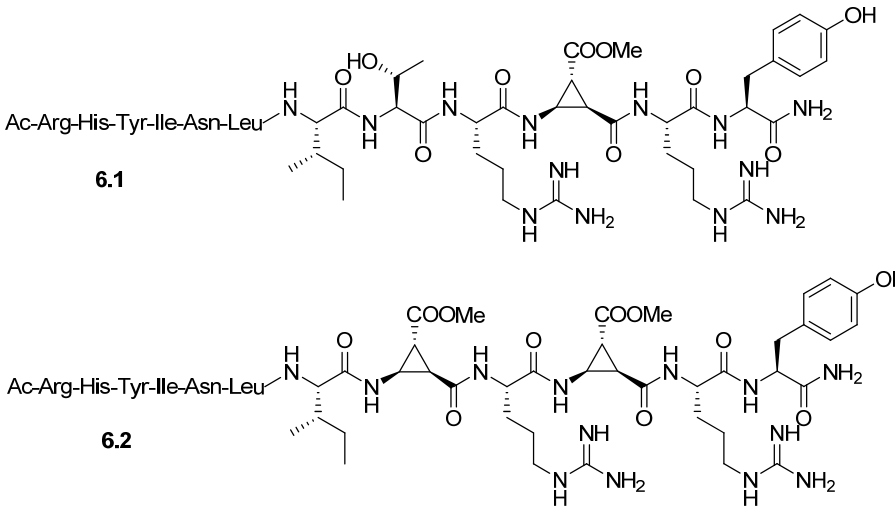
# Derivatives of Truncated pNPY and hPP as Potent and Selective NPY $Y_4R$ Ligands Containing β- or γ-Amino Acids

# 6.1 Introduction

The study of the NPY $Y_4R$ is not only hampered by a lack of appropriate antagonists. Even the availability of suitable $Y_4R$-selective small peptidic agonists is limited. Besides a peptide dimer with picomolar affinity to the NYP $Y_4R$ published by Balasubramaniam et al.[1], no truncated peptide analogs with selectivity for this receptor subtype are known so far. Thus, there is a need for selective molecules as pharmacological tools to identify and investigate NPY receptor subtype mediated processes and to explore the therapeutic potential.[2]

The project presented in this chapter was subject of a cooperation with Prof. Dr. Oliver Reiser (University of Regensburg, Institute of Organic Chemistry), Dr. Lukasz Berlicki (Wroclaw University of Technology, Department of Bioorganic Chemistry), a postdoctoral visiting scientist, and Raquel Gutiérrez Abad (exchange student from the University of Barcelona, Department of Chemistry), both working in Prof. Reiser's laboratory on the synthesis of $Y_4R$ selective peptides.

For the design of the presented compounds, we focused on the C-terminal part of pNPY and hPP because, based on an alanine scan of pNPY, the C-terminal amino acids, in particular the arginine residues $Arg^{33}$ and $Arg^{35}$ as well as $Tyr^{36}$, are suggested to be strongly involved in binding at all NPY receptor subtypes.[3] The replacement of natural amino acids by β-amino acids can be used to induce conformational changes and to influence the selectivity profiles of receptor ligands. Previously, the introduction of β-aminocyclopropanecarboxylic acids in position 34 (and 32) of pNPY(25-36) resulted in $Y_1R$ subtype selective peptides (**Figure 6.1**).[4] This strategy should be extended to conformationally contrained β- and γ-amino acids of different ring size and stereochemical properties to explore the structure-selectivity relationships of peptidic NPY receptor ligands in more detail within this project.




| No. | $Y_1R$ | $Y_2R$ | $Y_5R$ |
|---|---|---|---|
| | $K_i$ [nM] | | |
| **6.1** | 37 | > 1000 | 724 |
| **6.2** | 50 | > 1000 | 118 |

**Figure 6.1**: Structures and $K_i$ values of the β-aminocylopropanecarboxylic acid containing peptide analogs (**6.1** and **6.2**) selective for the $Y_1R$, published by Koglin et al.[4]

Obviously, the introduction of constrained amino acid residues in particular in position 34 – in close vicinity to the most important amino acids $Arg^{33}$, $Arg^{35}$ and $Tyr^{36}$ – resides in a rigidity of the peptide backbone stabilizing a distinct secondary structure preferring one of the NPY receptor subtypes.[4] A similar effect was previously reported for the replacement of $Gln^{34}$ by the constrained natural amino acid Pro in pNPY, resulting in a loss of affinity towards the NPY $Y_2$R while keeping $Y_1$ and $Y_5$R affinity.[5] With regard to the $Pro^{34}$ replacement in pNPY, we decided to introduce the structurally similar (*1R,2S*)-aminopentanecarboxylic acid in position 34 of pNPY(25-36) and hPP(25-36). Surprisingly, the resulting truncated pNPY and hPP analogs proved to be very potent and more or less subtype-selective NPY $Y_4$R ligands. This unexpected success prompted to us to design a second set of compounds replacing the cis-pentacin moiety by another unnatural β-amino acid, (*1R,2S*)-aminocyclobutanecarboxylic acid and a corresponding γ-amino acid to further explore the influence of these conformational constraints on the NPY receptor subtype selectivity, especially with respect to the $Y_4$R.

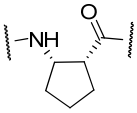
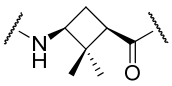
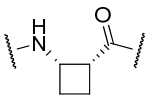
The pharmacological results for all synthesized peptides will be presented in detail in this chapter. Besides flow cytometric binding studies to obtain $Y_4$R affinity as well as selectivity towards the $Y_1$R, $Y_2$R and $Y_5$R, the (unexpected) challenges in analyzing the functional pharmacological activities of these new compounds using different assay systems will be discussed.

## 6.2 Design and Preparation of the Peptides

The structures of the investigated peptides, which were synthesized by Dr. Lukasz Berlicki (Wroclaw University of Technology, Department of Bioorganic Chemistry) and Raquel Gutiérrez Abad (University of Barcelona, Department of Chemistry), are outlined in **Table 6.1**.

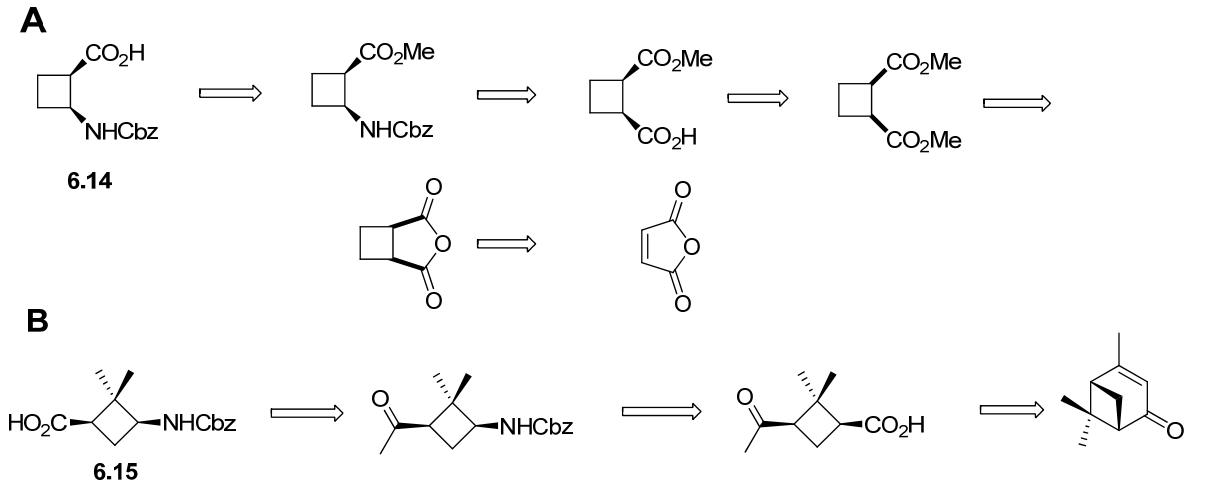
**Table 6.1**: Amino acid sequences of the synthesized pNPY(25-36) and hPP(25-36) analogs **6.3** – **6.13**.

| No. | Sequence | MW (Da) |
|---|---|---|
| **pNPY** | YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-$NH_2$ | 4253.74 |
| **hPP** | APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-$NH_2$ | 4181.80 |
| **pNPY(25-36)** | Ac-RHRILITRQRY-$NH_2$ | 1673.92 |
| **(6.3)** | | |
| **hPP(25-36)*** | Ac-RRYIN-Nle-LTRPRY-$NH_2$ | 1661.95 |
| **(6.4)** | | |
| **[Leu$^{34}$]pNPY(25-36)** | Ac-RHYILITRLRY-$NH_2$ | 1658.94 |
| **(6.5)** | | |
| **6.6** | Ac-RHYINLITR-(*1R*,*2S*)**cpen**-RY-$NH_2$ | 1656.93 |
| **6.7** | Ac-RHYINLI-(*1R*,*2S*)**cpen**-R-(*1R*,*2S*)**cpen**-RY-$NH_2$ | 1666.96 |
| **6.8** | Ac-RRYIN-Nle-LTR-(*1R*,*2S*)**cpen**-RY-$NH_2$ | 1675.98 |
| **6.9** | Ac-RHYINLITR■RY-$NH_2$ | 1641.92 |
| **6.10** | Ac-RHYINLR■RRY-$NH_2$ | 1680.96 |
| **6.11** | Ac-RRYIN-Nle-LTR■RY-$NH_2$ | 1661.00 |
| **6.12** | Ac-RHYINLITR♦RY-$NH_2$ | 1669.95 |
| **6.13** | Ac-RHYINLI♦R♦RY-$NH_2$ | 1694.00 |

**cpen** =  ♦ =  ■ = 

* Met$^{30}$ was replaced bei Nle to avoid instability against oxygen.

The stereoselective synthesis of (*1R*,*2S*)-cis-pentacin was performed by Dr. Lukasz Berlicki according to a protocol reported by Davies et al.[6] and will be described in detail in chapter 7. The (*1R*,*2S*)-aminocyclobutanecarboxylic acid (**6.14**) and the corresponding γ-amino acid (**6.15**) were prepared by Raquel Gutiérrez Abad according to a procedure established in the workgroup of Rosa M. Ortuno (University of Barcelona, Department of Chemistry of Barcelona),[7-10] outlined in **Scheme 6.1** as retro-synthetic scheme.




**Scheme 6.1:** Schematic presentation of the retro-synthesis of (*1R,2S*)-aminocyclobutancarboxylic acid **6.14** (**A**) and the corresponding γ-amino acid **6.15** (B).

The cis-pentacin containing peptides were obtained by automated solid phase peptide synthesis (SPPS), whereas the analogs containing aminocyclobutanecarboxylic acids were synthesized by manually performed solid phase peptide synthesis following an Fmoc-strategy. This part of the work was supervised by Prof. Dr. Chiara Cabrele (Ruhr-University of Bochum, Department of Organic Chemistry). As the β- and γ-cyclobutane amino acid fragments were considered too unstable for direct coupling in SPPS, they were attached to an Fmoc-protected arginine ($Arg^{33}$) prior to use as a building block for peptide synthesis.

The (*1R*,*2S*)-cis-pentacin containing, truncated pNPY(25-36) and hPP(25-36) analogs (**6.6** and **6.8**) were prepared and analyzed first. In addition, the truncated analogs (AS-sequence: 25-36) of the endogenous peptides pNPY as well as hPP, composed of the natural amino acids without any replacement, were included as reference compounds (**6.3** and **6.4**). To avoid instability against oxygen, which could possibly influence functional test results and binding properties, $Met^{30}$ present in hPP was exchanged by norleucine (Nle) in the truncated analog (hPP(25-36)); this approach has often been applied as a bioisosteric replacement.[11, 12] Furthermore, Leu was introduced in position 34 instead of a Gln in the truncated pNPY(25-36) yielding compound **6.5** ([$Leu^{34}$]pNPY(25-36)). This peptide analog was included in the pharmacological investigations as the replacement of $Gln^{34}$ by Leu was demonstrated by Cabrele et al.,[13] to increase $Y_4$R affinity of the endogenous ligand pNPY (18-fold). Due to promising pharmacological results for the cis-pentacin containing peptides, the introduction of (*1R,2S*)-aminocyclobutancarboxylic acid was considered quite interesting as it may result in a constrained C-terminal part, either, but probably with different torsion angles and conformations, which could differ in terms of affinity and/or receptor subtype selectivity.

## 6.3 Circular Dichroism of the Foldamers

For CD measurements depicted in **Figure 6.2,** the compounds were dissolved in phosphate buffer or a mixture of buffer with trifluorethanol (TFE), respectively. TFE is often reported in literature as structure-inducing co-solvent. Even smaller peptide analogs, which are mostly unstructured in aqueous solution, can be forced in a stable conformation in the presence of TFE, preferably inducing helical structures. However, opinions concerning the stabilizing effect of TFE in native protein-like structures are quite controversial as little is known and understood concerning the interaction of this co-solvent with secondary structural elements.[14]

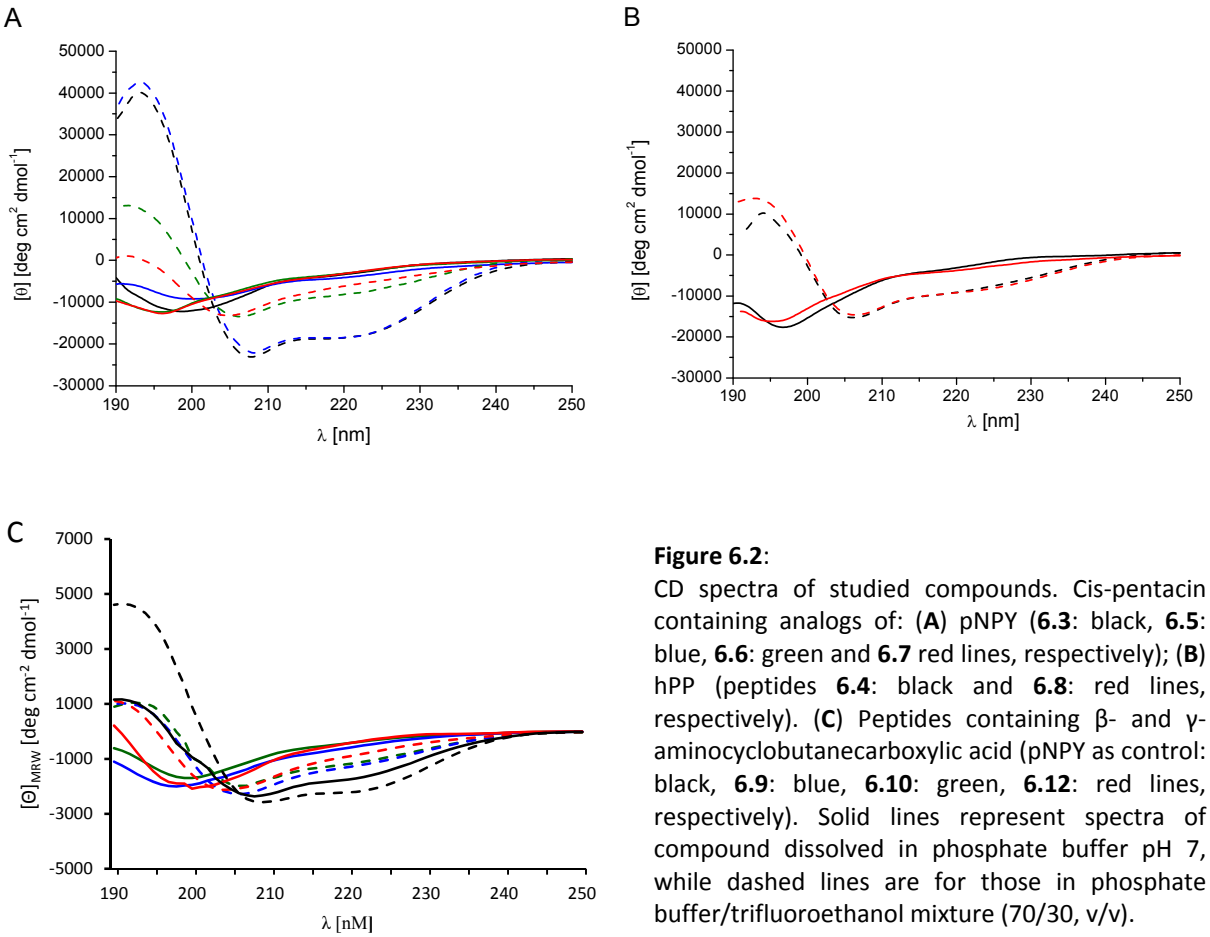



**Figure 6.2**:
CD spectra of studied compounds. Cis-pentacin containing analogs of: (**A**) pNPY (**6.3**: black, **6.5**: blue, **6.6**: green and **6.7** red lines, respectively); (**B**) hPP (peptides **6.4**: black and **6.8**: red lines, respectively). (**C**) Peptides containing β- and γ-aminocyclobutanecarboxylic acid (pNPY as control: black, **6.9**: blue, **6.10**: green, **6.12**: red lines, respectively). Solid lines represent spectra of compound dissolved in phosphate buffer pH 7, while dashed lines are for those in phosphate buffer/trifluoroethanol mixture (70/30, v/v).

CD measurements of the synthesized truncated peptides (**6.3** – **6.10** and **6.2**, **Figure 6.2**) revealed that all pNPY(25-36) and hPP(25-36) analogs, substituted by β-and γ-amino acids as well as unsubstituted, are completely unstructered in aqueous solution (**Figure 6.2,** A,B, C: solid lines). Only the endogenous ligand pNPY (**Figure 6.2**, C: solid, black line) showed helical conformation as expected. After addition of TFE, the unmodified pNPY(25-36) analog **6.3** (A: dashed, black) as well as [Leu$^{34}$]pNPY(25-36) (A: dashed, blue) clearly showed stabilization in a helical conformation. By contrast, the unmodified hPP(25-36) analog (**6.4**, B: black line) as well as the cis-pentacin containing

hPP(25-36) analog (**6.8,** B: red line) revealed only rudimental helicity. Obviously, the replacement of $Pro^{34}$ by cis-pentacin does not have a great impact on the hPP(25-36) conformation. A CD pattern similar to hPP(25-36) **6.4** was observed for all cis-pentacin and aminocyclobutanecarboxylic acid containing analogs (**6.6** – **6.10**, **6.12**). Therefore, one may speculate that peptides **6.6** – **6.10** and **6.12**, having CD spectra comparable to **6.4** (hPP(25-36)), also show an almost identical overall conformation resembling the C-terminal part of hPP and favoring binding to the $Y_4$R. Interestingly, the γ-amino acid substituted analog **6.12** (C: red lines) does not differ from the β-amino acid containing derivatives with regard to conformational properties. This was unexpected as, in contrast to the high affinity β-amino acid containing analogs, the introduction of the cyclic γ-amino acid led to a complete loss of affinity towards the $Y_4$R.

Indeed, the introduction of cyclic β-amino acids led to conformational properties comparable to those of the C-terminal part in hPP and a more unstructured conformation compared to the C-terminal part in pNPY as observed in the prescence of TFE as co-solvent. Nevertheless, it remains a matter of discussion if this structural difference is of relevance with respect to pharmacological assays performed in aqueous solution.

## 6.4 Pharmacological Results

### 6.4.1 Affinity, Subtype Selectivity, Potency and Efficacy

The truncated pNPY and hPP analogs were analyzed in flow cytometric binding studies on CHO-h$Y_4$-qi5-mtAEQ cells by displacement of Cy5-[$K^4$]hPP (c = 3 nM, $K_D$ = 5.62). Results are given as $K_i$ values calculated according to the Cheng-Prusoff equation.[15, 16] Additionally, all compounds were investigated for functional activity at the NPY $Y_4$R in a steady state GTPase assay using Sf9 membranes co-expressing h$Y_4$R plus $G\alpha_{i2}$ plus $G\beta_1\gamma_2$ and the fusion protein RGS4.[17] In the following, agonistic potencies are expressed as $EC_{50}$ values. Intrinsic activities (α, efficacy) refer to the maximal response induced by the standard agonist hPP (10 µM). Binding to the other NPY receptor subtypes was determined by flow cytometry using Cy5-pNPY (c = 5 nM, $K_D$ = 5.2).[18]; results are given as $K_i$ (**Table 6.2**).

The results for pNPY(25-36) **6.3** were in good agreement with data published by Koglin et al. [4] in terms of $Y_2$R subtype selectivity. However, the high affinity of 21 nM, determined on SMS-KAN cells was not confirmed in flow cytometric binding studies on CHO-h$Y_2$ cells; here the affinity was 12-fold lower (**6.3**, $K_i$ = 279 nM). In accordance with the exchange of $Gln^{34}$ by Leu in pNPY, reported by Cabrele et al.,[13] this single amino acid replacement had also a great impact on the pNPY(25-36) fragment (**6.5**), resulting in a complete shift in subtype selectivity from the $Y_2$R to the $Y_4$R with an

affinity in the nanomolar range ($K_i$ = 157 nM). As expected, the truncated hPP(25-36) analog **6.4** showed preference for the $Y_4R$ with an affinity in the two-digit nanomolar range ($K_i$ = 75 nM). Interestingly, the introduction of (*1R*,*2S*)-cis-pentacin in position 34 of hPP(25-36) (**6.8,** $K_i$ = 14 nM) led to a 6-fold increase in affinity compared to unsubstituted truncated hPP analog **6.4** ($K_i$ = 75 nM)**.** However, a substantial loss of subtype selectivity with regard to the $Y_1$ and $Y_2$ receptor became obvious ($Y_1R$: $K_i$ = 281 nM, $Y_2R$: $K_i$ = 217 nM). Remarkably, cis-pentacin does not share such a high degree of similarity with the natural amino acid proline as expected at the beginning. Apparently, the loss of subtype selectivity has to be attributed to a different conformation of the C-terminus of the foldamer compared to that of native hPP.

The introduction of (*1R*,*2S*)-cis-pentacin in position 34 of pNPY(25-36) (**6.6,** $K_i$ = 10 nM) led to a further increase in $Y_4R$ affinity compared to [$Leu^{34}$]pNPY(25-36) **6.5** ($K_i$ = 157 nM). Additionally, no significant decrease in subtype selectivity, as in case of [$cpen^{34}$]hPP(25-36), was observed. Only the $Y_5R$ showed a reduced selectivity for [$cpen^{34}$]pNPY(25-36) **6.6** by a factor of 50, whereas unsubstituted pNPY(25-36) **6.5** appears to be completely inactive at this receptor subtype. This is in good agreement with the data published for the $Y_1R$ selective aminocyclopropanecarboxylic acid containing analogs **6.1** and **6.2**, which show $Y_1R$ binding in the nanomolar range ($K_i$ = 37 and 50 nM) and moderate affinity to the $Y_5R$. Unfortunately, the affinity of these analogs has not been determined at the $Y_4R$ due to lack of substance, although the data would be very interesting to discuss the structure-activity relationships in context of the results for the cis-pentacin containing analogs.

In summary, compound **6.6** ([$cpen^{34}$]pNPY(25-36)) with a $K_i$ value in the low nanomolar range ($K_i$ = 10 nM) revealed the highest $Y_4R$ affinity combined with an excellent subtype selective profile within this series of synthesized peptides.

The incorporation of (*1R*,*2S*)-aminocyclobutanecarboxylic acid into hPP(25-36) (**6.9**) and pNPY(25-36) (**6.11**) led to almost the same affinities at the NPY $Y_4R$ as determined for the cis-pentacin derivatives, previously. Indeed, the (*1R*,*2S*)-aminocyclobutanecarboxylic acid bearing analogs (**6.9**: $K_i$ = 72 nM and **6.11**: $K_i$ = 93 nM) show a 7-fold lower $Y_4R$ affinity compared to the corresponding cis-pentacin derivatives (**6.6**, $K_i$ = 10 nM and **6.8**, $K_i$ = 14 nM), but improved subtype selectivity. Even the cylcobutane containing hPP(25-36) analog **6.11** shows only moderate affinity to the $Y_2R$ subtype ($K_i$ = 709 nM), whereas the $Y_4R$ selectivity of the cis-pentacin containing analog **6.8** was markedly lower, in particular, compared to the $Y_2R$ ($K_i$ = 279 nM) and $Y_1R$ ($K_i$ = 281 nM). Thus, compared to the cis-pentacin modified peptides, changes in torsion angles and conformations of the ring-contracted analogs appear to account for slightly reduced $Y_4R$ affinity but improved subtype selectivity, especially in case of the truncated hPP(25-36)analog.

The introduction of two β-amino acids in position 32 and 34 of pNPY(25-36) (compounds **6.7** and **6.10**) led to a slight decrease in $Y_4$R binding (3-fold) in case of the cis-pentacin and a minor increase in affinity (2-fold) for the (*1R*,*2S*)-aminocyclobutancarboxylic acid modified peptides compared to the corresponding mono-substituted analogs. Obviously, the conformational constraints – due to the second cyclic β-amino acid – do not substantially affect the interaction of the peptide with the $Y_4$R. Additionally, the threonine in position 32 of pNPY doesn't contribute to receptor binding to a high extent, as demonstrated by alanine scan, so that replacement of $Thr^{32}$ seems to be tolerated.[3]

Interestingly, the pNPY(25-36) analogs having a cyclic y-amino acid substructure in position 32 and/or position 34 (**6.12** and **6.13**) were completely devoid of affinity at the $Y_4$R.

Investigations in a steady state GTPase assay revealed $Y_4$R partial agonism for all tested compounds, with efficacies in the range of 0.5 to 0.75 referred to the maximal response elicited by hPP. Only compound **6.5** with a Leu replacement in position 34 of pNPY(25-36) turned out to be a full agonist. Overall, the $EC_{50}$ values were comparable to the binding data.

**Table 6.2**: Potencies, efficacies and affinities of synthesized hPP(25-36) and pNPY(25-36) analogs at the $Y_4R$ and binding data at the $Y_1R$, $Y_2R$ and $Y_5R$.

| No. | $Y_4$ | | | $Y_1$ | $Y_2$ | $Y_5$ |
|---|---|---|---|---|---|---|
| | $EC_{50}$ [nM][a] | α | $K_i$ [nM]/($pK_i$)[b] | $K_i$ [nM][c] | $K_i$ [nM][c] | $K_i$ [nM][c] |
| **6.1** | - | - | - | 37 | > 1000 | 724 |
| **6.2** | - | - | - | 50 | > 1000 | 118 |
| **6.3** | 530 ± 160 | 0.56 | > 1000 | > 1000 | 279 ± 64 | > 1000 |
| **6.4** | 53 ± 8.0 | 0.70 | 75 ± 2.2 | > 1000 | > 1000 | > 1000 |
| **6.5** | 100 ± 24 | 1.00 | 157 ± 47 | > 1000 | > 1000 | > 1000 |
| **6.6** | 38 ± 5.5 | 0.72 | 10 ± 1.8 | > 1000 | > 1000 | > 500 |
| **6.7** | 48 ± 29 | 0.49 | 37 ± 7.0 | > 1000 | > 1000 | > 500 |
| **6.8** | 51 ± 18 | 0.70 | 14 ± 7.2 | 281 ± 89 | 217 ± 28 | > 500 |
| **6.9** | 122 ± 28 | 0.67 | 72 ± 7.2 | > 1000 | >1000 | > 1000 |
| **6.10** | 66 ± 26 | 0.76 | 35 ± 1.9 | > 1000 | >1000 | > 1000 |
| **6.11** | 284 ± 46 | 0.74 | 93 ± 4.3 | > 1000 | 709 ± 90 | > 1000 |
| **6.12** | - | - | > 1000 | > 1000 | >1000 | > 1000 |
| **6.13** | - | - | > 1000 | > 1000 | >1000 | > 1000 |
| **pNPY** | 417 ± 42[17] | 1.00 | (6.55 ± 0.06)[16] | 0.5 ± 0.2[18] | 0.8 ± 0.2[19] | 2.2 ± 0.8[18] |
| **hPP** | 11 ± 3.6[17] | 1.00 | (9.62 ± 0.07)[16] | - | - | - |

[a] Functional [γ-$^{33}$P]GTPase assay with membrane preparations of Sf9 cells expressing the $hY_4R$ + $hY_4R$ $G\alpha_{i2}$ + $G\beta_1\gamma_2$ +RGS4; the intrinsic activity (α) of hPP was set to 1.0 and α-values (efficacies) of other compounds were referred to this value; mean ± SEM from 2 or 3 independent experiments performed in duplicate. [b] Flow cytometric binding assay using 3 nM Cy5-[K$^4$]hPP (c = 3 nM, $K_D$ = 5.62 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [c] Flow cytometric binding assay using 5 nM Cy5-pNPY (c = 5 nM, $K_D$ = 5.2 nM) in CHO-$hY_2$ cells, HEL ($Y_1$) and HEC-1B-$Y_5$ cells, respectively.

## 6.4.2 Behavior in Functional Assays with Different Read-Outs: $[Ca^{2+}]_i$ Mobilization vs. GTPase Activity

Flow cytometric binding studies revealed high $Y_4R$ affinities of the synthesized peptides with $K_i$ values in the low nanomolar range (**Table 6.2** and **Figure 6.3**). Whereas the determination of the binding data was very well reproducible under standard conditions, the quantification of the functional activities of these compounds turned out to be more difficult than expected. Numerous experiments had to be performed to identify the best suited method to determine the agonistic or antagonistic properties of these special peptides, although the applied established test systems were validated and used for the characterization of various classes of compounds, previously. In the following

paragraphs different approaches to a functional characterization of the synthesized peptides will be presented.

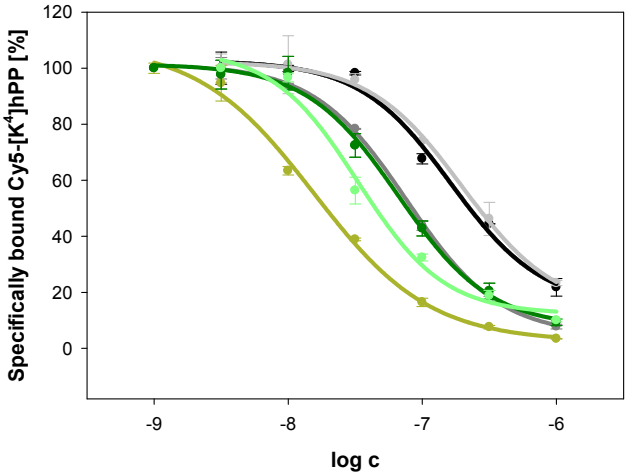



**Figure 6.3**:
Flow cytometric competition binding experiments performed with compounds **6.6** (●), **6.7** (●), **6.8** (●), **6.9** (●), **6.10** (●) and **6.11** (●) using Cy5-[$K^4$]hPP (c = 3 nM, $K_D$ = 5.62 nM) as fluorescent ligand. Unspecific binding was determined in the presence of GW1129 (c = 1 µM)

### 6.4.2.1 Results of the Aequorin Assay

Taking into account that most of the peptides and peptide analogs for the NPY $Y_4$R published in literature are agonists, the synthesized compounds were first tested in the agonist mode in a $Ca^{2+}$-based luminescence assay (aequorin assay) at CHO-h$Y_4$-qi5-mtAEQ cells. These stably transfected cells express mitochondrially targeted apoaequorin, which forms a holoenzyme with the chromophore coelenterazine h, entering the cells during a 5 h preincubation period. The docking of agonists to the $Y_4$R induces the interaction of the receptor with the chimeric G-protein $G\alpha_{qi5}$, resulting in activation of the $G_q$-coupled signaling, i.e. release of intracellular calcium which interacts with the aequorin complex to enable the oxidation of coelenterazine under emission of light.[16, 20] Unfortunately, as shown in **Figure 6.4**, in contrast to the positive control hPP, none of the tested peptides triggered a luminescence signal, indicating agonistic activity.

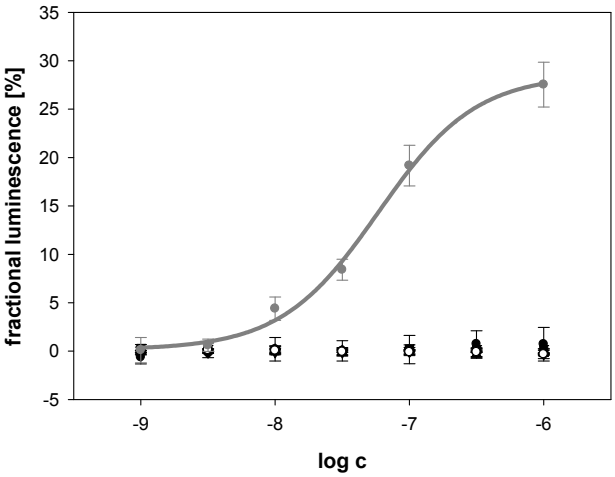



**Figure 6.4**:
Investigations on agonistic activity in the aequorin assay using CHO-h$Y_4$-qi5-mtAEQ cells. Concentration-response curves of peptidic ligands **6.2**(▲), **6.3** (■), **6.4** (▼), **6.5** (●), **6.6** (○), **6.7** (♦) and hPP (●) as positive control.

Although, a closer look to the kinetic measurements (**Figure 6.5**), performed in agonist mode, revealed the emission luminescence of very low intensity (**A**), this phenomenon was also observed when analyzing blanks (**B**). Probably, this luminescence resulted from the injection at high speed leading to the release of aequorin by individual cells as a consequence of the disruption of cell membranes.

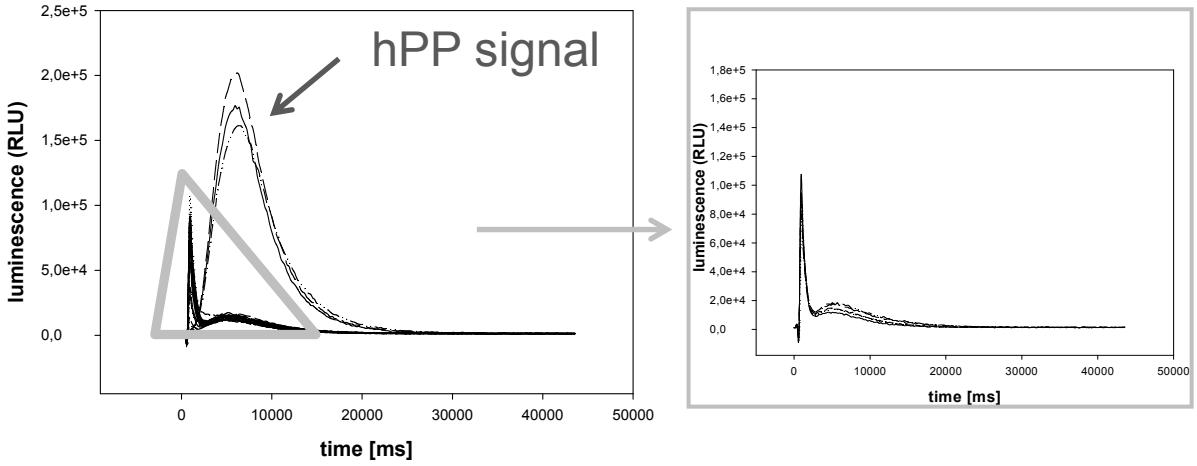



**Figure 6.5**: Aequorin assay performed with CHO-h$Y_4$-qi5-mtAEQcells. 162 µL of cell suspension were injected into 18 µL of hPP or ligand solution. (A) Luminescence responses of compound **6.6** at different concentrations (1 nM – 1000 nM) compared to hPP. (B) Blank signal as control (luminescence signal probably only due to rupture of the cells during injection by the Tecan GENios Pro).

Subsequently, the peptides were analyzed in the aequorin assay in the antagonist mode (**Figure 6.6**). Thereby, the luminescence signal elicited by hPP (100 nM) should be decreased by the tested compounds in case of antagonistic activity. Surprisingly, the truncated hPP and pNPY analogs **6.5** – **6.8** were able to suppress the hPP induced luminescence at higher concentrations. Only **6.3** and **6.4** were devoid of activity. Strikingly, the obtained $IC_{50}$ values did not correlate well with the measured $K_i$ values determined by flow cytometry. The measured antagonistic activities were lower than expected from the high $Y_4R$ affinities (**Table 6.2**, **Figure 6.3**).

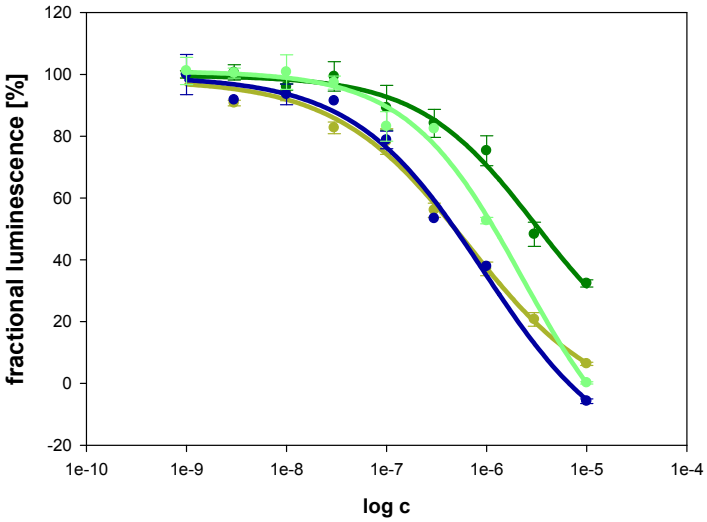



**Figure 6.6:**
Peptidic ligands **6.5** (●), **6.6** (●), **6.7** (●), **6.8** (●) when tested in the aequorin assay inhibiting the luminescence signal elicited with 100 nM hPP using CHO-h$Y_4$-qi5-K8-mtAEQ cells.

### 6.4.2.2 Results from the Steady State GTPase Assay

To verify the results from the aequorin assay, the peptides were investigated in the steady state GTPase assay using Sf9 membranes, co-expressing the h$Y_4R$, G$\alpha_{i2}$, G$\beta_1\gamma_2$ and the fusion protein RGS4. Antagonistic activity was determined from the concentration-dependent inhibition of the $Y_4R$-mediated hPP-induced stimulation of GTPase activity, determined by measuring the cleavage of [$\gamma$-$^{33}$P]GTP. The results were contradictory to those from the aequorin assay as presented in **Figure 6.7**. Interestingly, the investigated compounds showed no $Y_4R$ antagonism in the GTPase assay. In fact, a slight amplification of the hPP signal could be detected at increasing concentrations of the test compounds.

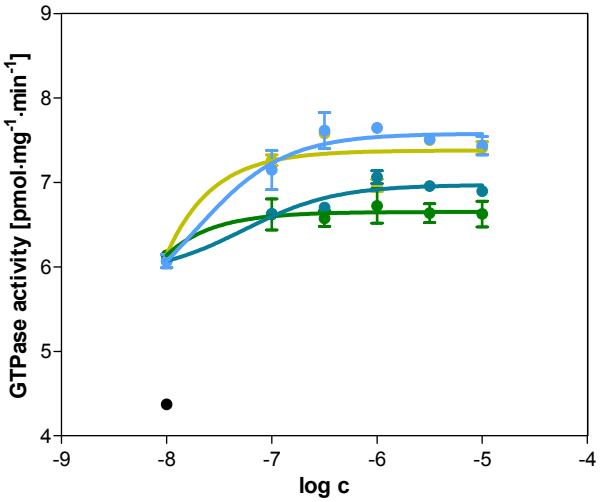



**Figure 6.7**
Investigations in the GTPase assay for $Y_4R$ agonistic activity of compounds **6.3** (●), **6.4** (●), **6.6** (●), **6.7** (●) at Sf9 membranes expressing h$Y_4R$, G$\alpha_{i2}$ and G$\beta_1\gamma_2$. Basal activity: ●. Data are means ± SEM from representative experiments performed in duplicate

This raised the question, if the observed stimulation could be caused by direct G-Protein activation.[21, 22] Moreover, such a receptor-independent effect could also explain the apparent antagonistic activity

in the aequorin assay. As the test compounds were incubated with the CHO-h$Y_4$-qi5-mtAEQ cells for 15 minutes prior to the addition of hPP, direct G-protein activation, eliciting luminescence prior to the measurement and reducing/depleting $Ca^{2+}$ and apoaequorin reservoirs, could result in weak luminescence signal after stimulation with hPP, pretending an antagonistic effect. Therefore, the compounds should be investigated for direct G-protein activation in the GTPase assay system.

Receptor independent activation has been observed for various compounds, for instance, amphiphilic histamine $H_1$-receptor antagonists such as diphenhydramine and chlorpheniramine as well as for mastoparan, an amphiphilic peptide from wasp venom.[21, 23, 24] Hence, experiments using Sf9 membranes expressing the G protein subunits $G\alpha_{i2}$ and $G\beta_1\gamma_2$ but no NPY receptor were performed using mastoparan as a positive control. The results, depicted in **Figure 6.8**, did not confirm our assumption. Mastoparan acted as a G-protein activator at concentrations higher than 1 µM, whereas the investigated peptidic ligands were completely inactive.

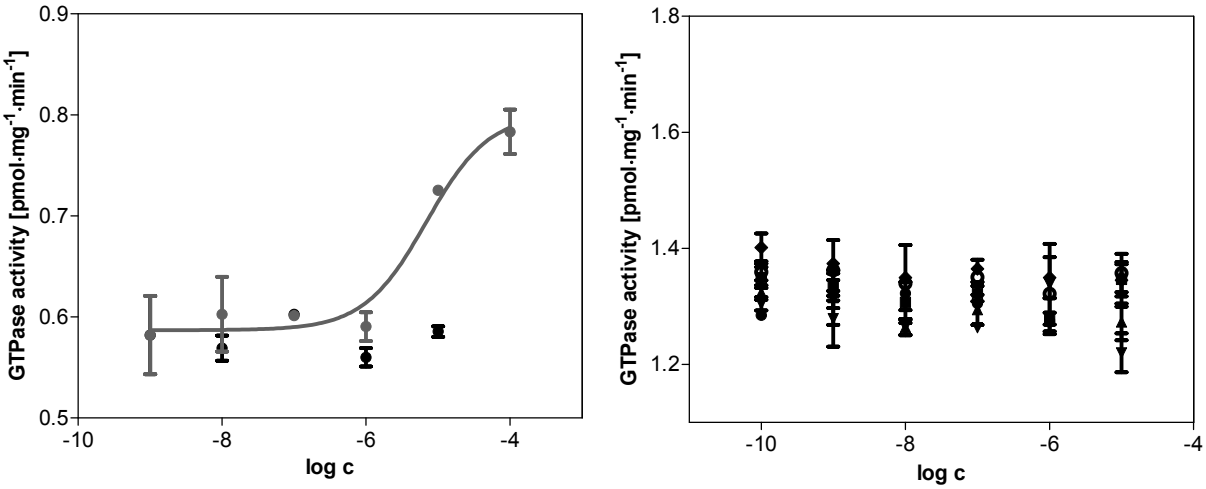



**Figure 6.8:** Concentration response curve of mastoparan (●) (left) and effects of the peptidic ligands **6.2**(▲), **6.3** (■), **6.4** (▼), **6.5** (●), **6.6** (○), **6.7** (♦) (right) investigated for direct G-protein activation in the GTPase assay at Sf9 membranes expressing only $G\alpha_{i2}$ and $G\beta_1\gamma 2$. Data are means ± SEM from representative experiments performed in duplicate.

Finally, the peptides where analyzed in the steady state GTPase assay on Sf9 membranes for agonism to exclude potential errors overlooked before (**Figure 6.9**). Actually, the synthesized compounds turned out to be (partial) agonists. The determined $EC_{50}$ values were in good agreement with $Y_4R$ binding data. Obviously, when the compounds were studied in the antagonist mode in the aequorin assay, the minor amplification of the hPP signal, initially considered as receptor independent activation, was due to real agonistic stimulation.

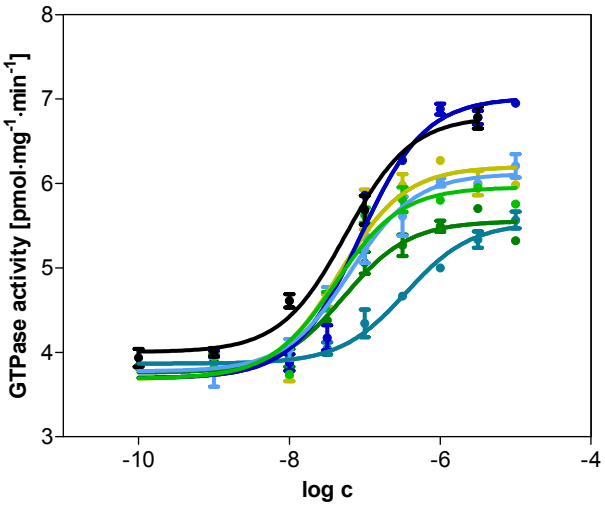



**Figure 6.9**:
Concentration response curve for peptidic ligands **6.2** (●), **6.3** (●), **6.4** (●), **6.5** (●), **6.6** (●), **6.7** (●), and hPP(●) as a positive control at Sf9 membranes expressing $hY_4R$, $G\alpha_{i2}$ and $G\beta_1\gamma 2$. Data are means ± SEM from representative experiments performed in duplicate

### 6.4.2.3 Results from the Fura-2 Assay

The contradictory results from aequorin and GTPase assay prompted us to investigate several compounds in an additional functional assay, the classical Fura-2 based calcium assay. It should be stressed that the confusing data obtained in the aequorin assay may results from the peculiarities of this protocol. As the assay is performed in the microplate format, the required injection of the cells into the wells containing hPP appears to be the critical issue, because the CHO-$hY_4$-qi5-mtAEQ cells proved to be very sensitive against shear stress. This is avoided in the Fura-2 assay performed in cuvettes. However, the results of these investigations were again different from those obtained in the GTPase assay, as becomes obvious from **Figure 6.10**. In particular, compound **6.5,** which turned out to be a full agonist in the GTPase assay, only showed quite weak agonistic effects reaching only 50 % of the luminescence signal compared to hPP in the Fura-2 assay. In addition, the potency of **6.5** was 2-fold lower than in the GTPase assay. The discrepancy between results from different assays becomes even more obvious from the results for compound **6.6**. This peptide was a partial agonist in the GTPase assay, but agonistic activity was hardly detectable in the Fura-2 assay, the $EC_{50}$ value being 102 nM compared to 37 nM in the GTPase assay.

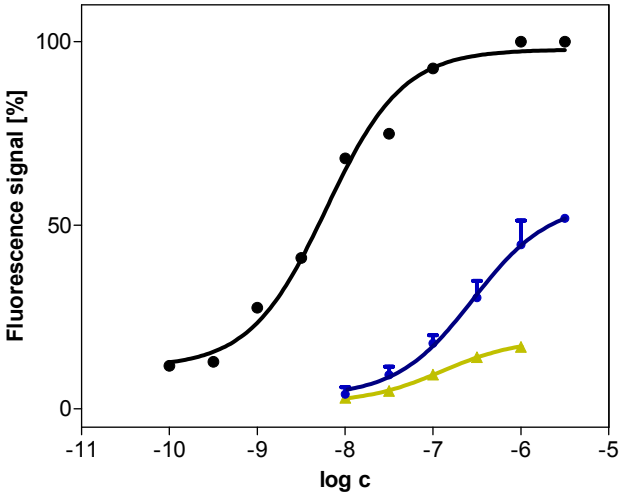



**Figure 6.10**:
$Ca^{2+}$-mobilization assay (Fura-2, agonist mode) with CHO-hY4-qi5-mtAEQ cells. Dose-response curves of peptidic ligands **6.5** (●), **6.6** (●) and hPP (●) as positive control. The fluorescence signal is calculated relatively to hPP (1 µM).

### 6.4.3 Discussion of the Discrepancies Observed in Different Functional Assays

Different possible explanations for the contradictory results in various assay systems were discussed. Probably, the observed antagonism is in fact due to pre-incubation of the cells in the presence of the test compounds prior to the aequorin assay. A luminescence signal immediately after addition of the compounds could not be detected under these conditions, while active aequorin would be consumed. Consequently, only a decreased luminescence signal would be elicited after addition of the agonist, thus pretending an antagonistic effect of the compound. But then the question arises: why was it impossible to analyse the agonistic activity of these peptides in the aequorin assay? Possibly, this can be explained as outlined below.

In contrast to the determination of agonsim in the aequorin assay, the compounds were preincubated with the CHO-$hY_4$-qi5-mtAEQ cells for 15 min prior to the measurements in the antagonist mode. A similar incubation time is applied in the GTPase assay, either. Probably, the peptides are unable to stimulate the receptor within the limited time frame of about 44 s set for the agonist mode of the aequorin assay, as the binding kinetics of these peptides is very slow. Association experiments performed for [$^{125}$I]-pNPY at SK-N-MC cells revealed that equilibrium was reached not before 90 to 120 min using standard conditions. Even after increasing the temperature to 37 °C this process lasts 30 to 40 min.[25] Hence, with a measurement period of less than one minute for the determination of agonistic potency, the compounds are far from equilibrium state. By contrast, in the GTPase assay, with an incubation time of 20 min (25 – 27 °C), the conditions are probably more suitable for the investigation of peptides.

Additionally, regarding the difference (65-fold) between the $K_i$ value of hPP determined by flow cytometry ($K_i$ = 0.24 nM) and the relatively high $EC_{50}$ (15.5 nM) value obtained in the aequorin assay, such assay-dependent discrepancies may also add to the problems. Supposed that a factor of 65 between functional and binding studies also holds for the investigated peptides, $EC_{50}$ values in the range of 650 nM to 10 µM would be expected. Taking into account that most of the truncated pNPY and hPP analogs were only tested up to concentrations as high as 1 µM, the detection of agonistic activity seems almost impossible under these conditions. As the synthetic peptides are partial agonists, the reduced maximal response compared to the full agonist hPP will additionally hamper the detection. These speculations are supported by studies with compound **6.5**, the only full agonist identified within this series of pNPY analogs, but a compound which has only moderate $Y_4$R affinity ($K_i$ = 157 nM). Peptide **6.5** was capable of eliciting 95 % of the luminescence signal induced by hPP, but concentrations as high as 10 µM were required (**Figure 6.11**).

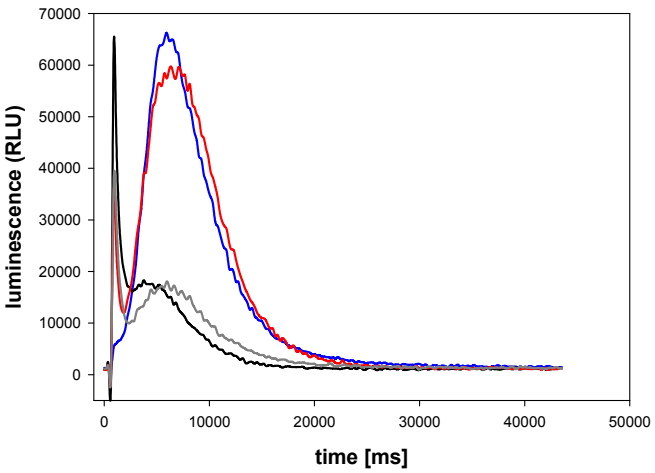



**Figure 6.11**:
Luminescence signal elicited by **6.5** at 10 µM (blue) and 1 µM (grey), respectively, with hPP (red) as a positive and the solvent (black) as negative control.

Furthermore, the discrepancies between different test systems might reflect functional selectivity of the receptor ligands.[26, 27] It is now widely accepted that GPCR can adopt different active or inactive conformations depending on the ligands used. Ligand-specific conformations have the potential to interact with different cellular proteins resulting in distinct cellular responses. For example, it is possible that G-protein independent signalling occurs via arrestins, or that a G-protein activation different from the typical $G_q$ coupled pathway, which is the basis for the determination of calcium in the aequorin assay with the genetically modified CHO-h$Y_4$-qi5-mtAEQ cells, is preferred. In addition, receptor desensitization due to receptor phosphorylation, followed by binding of β-arrestin, receptor internalization and sequestration can occur, as previously described for NPY receptors by Berglund et al.[28, 29]

Finally, instability of the used cell line has to be taken into consideration. Ralf Ziemek who established the stably transfected CHO-h$Y_4$-qi5-mtAEQ cell system already observed two different cell populations varying in terms of receptor expression and response upon agonist stimulation (**Figure 6.12**, a). Therefore, he isolated the clone with the preferred properties revealing higher receptor expression as well as high and homogenous binding of Cy5-[$K^4$]hPP.[20]

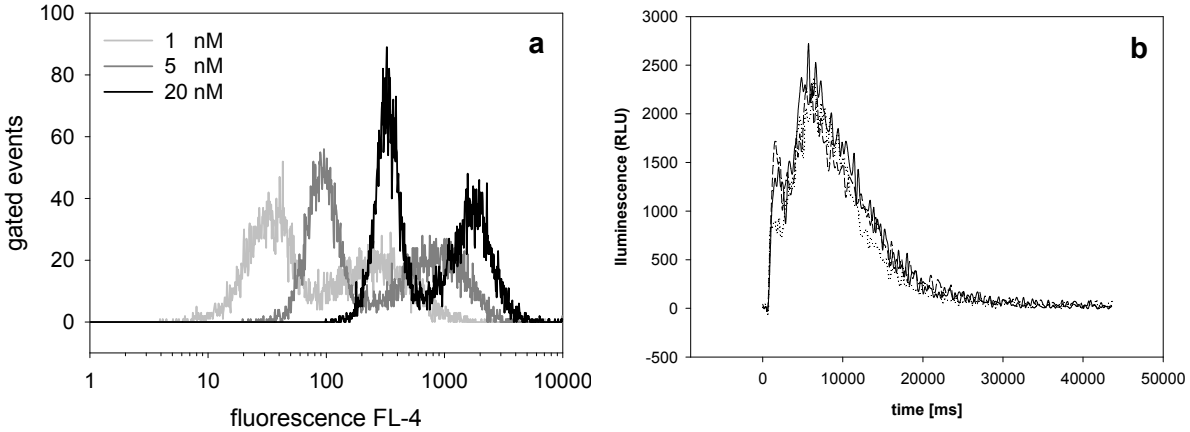



**Figure 6.12**: a) Inhomogenous binding of Cy5-[$K^4$]-hPP to two subpopulations of CHO-h$Y_4$-qi5-mtAEQ. b) Extremely reduced luminescence signal of 100 nM hPP.

Unfortunately, we could also observe two subpopulations of CHO-h$Y_4$-qi5-mtAEQ cells after a short time period of culturing (passage 7 - 10) the freshly thawed cells. Obviously, the clone carrying the "worse properties" couldn't be separated completely. In addition, it seems to be more efficient to eliminate the cell population with the unfavorable properties successively. Interestingly, these cells could be further used in flow cytomtetric binding studies without any limitations but show a clearly reduced signal in the aequorin assay as depticted in **Figure 6.11** (b). This might be due to reduced receptor expression or a loss of genetic information affecting $G_q$ coupling or mitochondrial aequorin.

Taken together, the available cell-based $Ca^{2+}$-mobilization assays used in this doctoral thesis turned out to be inappropriate for the determination of agonistic activities, especially partial agonism, of peptides at the NPY $Y_4$ receptor. Presumably, the determination of $EC_{50}$ values for the peptides of the NPY family and synthetic peptides with similarly high affinity to the $Y_4$R is only possible because of the great excess applied in the aequorin assay. Peptides showing a lower affinity compared to hPP or NPY could not be functionally characterized under the assay conditions, as equilibrium of ligand binding is by far not reached due to very slow kinetics.

## 6.5 Summary and Conclusion

The investigations on (*1R*,*2S*)-cis-pentacin as well as (1*R*,2*S*)-aminocyclobutanecarboxylic acid containing pNPY(25-36) and hPP(25-36) analogs led to surprising but also promising results with respect to the search of new NPY $Y_4$R ligands.

Obviously, it is possible to change subtype selectivity by a single amino acid replacement in position 34 of pNPY(25-36) using different cyclic β-amino acids. While the aminocyclopropanecarboxylic acid containing compounds (**6.1** and **6.2**), published by Koglin et al.[4], revealed $Y_1$R selectivity (the affinity towards the $Y_4$R has never been analyzed), the cis-pentacin comprising analogs (**6.6** – **6.8**) presented within this cooperation project showed preference for the NPY $Y_4$R with affinities in the low nanomolar range. Only the $Y_5$R was able to bind the compounds with moderate affinity but the preference for the $Y_4$R was higher by a factor of 20 and 50, respectively. Compounds bearing an aminocylobutanecarboxylic acid moiety in position 34 (**6.9** – **6.11**) had a slightly lower affinity to the $Y_4$R but increased subtype selectivity, in particular compared to the $Y_5$R. In contrast, the introduction of cyclic γ-amino acid into pNPY(25-36) (**6.12** and **6.13**) obviously led to unfavorable conformations in terms of binding to all NPY receptor subtypes; receptor affinity was completely lost.

In summary, we identified compound **6.6** ($K_i$ = 10 nM) as the most potent and selective $Y_4$R agonist among a series of β-amino acid modified peptides, superior to the endogenous ligands NPY and hPP and their truncated analogs. Compound **6.6** will be useful as a pharmacological tool with improved properties compared to hPP and was therefore subjected to fluorescence labeling as described in Chapter 8. Moreover, the results are considered a promising starting point for the design of new $Y_4$R selective ligands – optimized truncated β-amino acid modified peptides and possibly non-peptidic ligands.

## 6.6 Experimental Section

### 6.6.1 CD-Measurements

All spectra were recorded with a JASCO model J-710/720 (1; for compounds **6.3** – **6.9**) or a JASCO J-815 (2; for compounds **6.9**, **6.10**, **6.12**) instrument, respectively. Measurements were performed at room temperature scanning a wavelength range from 260 to 190 (1) or 280 to 190 nm (2). The band width was 1.0 nm, the sensitivity 20 mdeg (1) or 100 mdeg (2), and the scan speed 20 nm/min (1) or 50 nm/min (2). Quartz cuvettes of 1.0 mm path length were used. The background spectrum (phosphate buffer or a phosphate buffer/TFE mixture) was subtracted from each spectrum. The compounds were dissolved in phosphate buffer (100 mM (1) or 10 mM (2)) and a phosphate buffer/TFE mixture (70/30 v/v) yielding a concentration of 100 µM (**6.3** – **6.9** and pNPY) and 200 µM

(**6.9**, **6.10**, **6.12**). The difference in absorbance is given as molar ellipticity (1) or mean residue ellipticity (2) calculated from degrees of ellipticity according to the following equation reported by Schmid[30]:

(1) Molar ellipticity:

$$[\Theta] = \frac{\Theta \cdot MW \cdot 100}{c \cdot d}$$

(2) Mean residue ellipticity:

$$[\Theta]_{MRW} = \frac{\Theta \cdot MW \cdot 100}{c \cdot d \cdot N_A}$$

**Equation 6.1**: Calculation of molar [Θ] and residue ellipticity $[\Theta]_{MRW}$ with Θ: degrees of ellipticity [deg], MW: molecular weight [g/mol], $N_A$: number of amino acids, d: optical path length of cuvette [cm], c: concentration of peptide solution [mg/mL].

The absorbances of the peptide solutions for the calculation of the concentrations in case of (2) were determined on a Jasco V-650 spectrometer (bandwidth 2.0, scan range: 340 – 220, wavelength interval: 1.0 nM, path length of quartz cuvette: 1.0 cm). The concentrations were calculated according to Beer's law with $\varepsilon = n_{Tyr} * \varepsilon_{Tyr}$ ($\varepsilon_{Tyr}$(280 nM) = 1490 $M^{-1}cm^{-1}$).

## 6.6.2 Pharmacological Methods

### 6.6.2.1 Materials and Cell Culture

See section 3.3.2.1

Mastoparan was purchased from Bachem (Bubendorf, Switzerland). Guanosine 5′-[γ-$^{33}$P]-triphosphate ([γ-$^{33}$P]GTP) was prepared in our laboratory using guanosine 5′-diphosphate (GDP) and [$^{33}$P]-orthophosphoric acid (150 mCi/mL, obtained from Hartmann Analytics GmbH (Braunschweig, Germany) according to a previously described enzymatic labeling procedure.[31] $MgCl_2$ was purchased from Merck (Darmstadt, Germany) and Tris base was obtained from USB (Staufen, Germany). 3-Phosphoglycerate kinase and L-α-glycerol phosphate were from Sigma-Aldrich Chemie GmbH (München, Germany). All unlabeled nucleotides, glycerol-3-phosphate dehydrogenase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase were from Roche (Mannheim, Germany). Radioactive samples were counted in a Beckman LF6500 liquid scintillation analyzer.

### 6.6.2.2 Aequorin Assay

See section 3.3.2.1

### 6.6.2.3 Flow Cytometric Binding Assay

See section 3.3.2.2

### 6.6.2.4 Steady State GTPase Assay

The steady state GTPase activity assay was performed as described previously. [17, 32, 33]

$Y_4R$ assays: Sf9 insect cell membranes, co-expressing the $hY_4R$, the mammalian $G\alpha_{i2}$ and $G\beta_1\gamma_2$ as well as the RGA4 fusion protein, were employed. $Y_2R$ assay: Sf9 insect cell membranes co-expressing the $hY_2R$, the mammalian $G\alpha_{i2}$ and $G\beta_1\gamma_2$ were used.

The membranes were thawed and sedimented by a 10 min centrifugation step at 4 °C and 13 000 *g*. Subsequently, they were re-suspended in 10 mM Tris/HCl (pH 7.4). Each assay tube contained Sf9 membranes, co-expressing the respective YR subtype (10 µg of protein/tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1mM ATP, 100 nM GTP, 0.1 mM adenylyl imidodiphosphate, 1.2 mM creatine phosphate, 1 µg of creatine kinase, and 0.2% (w/v) bovine serum albumin in 50 mM Tris-HCl, pH 7.4, and the investigated ligands at various concentrations (10 µL)*. For experiments run in the antagonist mode, the reaction mixture additionally contained 100 nM hPP ($Y_4R$) or 100 nM pNPY ($Y_2R$). After incubation of the reaction mixtures (80 µL) at 25 °C for 2 min, 20 µL of [$\gamma$-$^{33}$P]-GTP (0.1 µCi/tube) were added. All stock and work solutions of [$\gamma$-$^{33}$P]-GTP were prepared in 20 mM Tris-HCl (pH 7.4). Reactions were terminated by addition of a slurry (900 µL), consisting of 5 % (w/v) activated charcoal and 50 mM $NaH_2PO_4$ (pH 2.0). Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 13 000 *g*. 600 µL of the supernatant of the reaction mixtures were removed, and $^{33}P_i$ was determined by liquid scintillation counting using Optiphase Supermix® (Perkin Elmer, Rodgau, Germany). Enzyme activities were corrected for spontaneous hydrolysis of [$\gamma$-$^{33}$P]GTP, which was determined in tubes containing all of the above-described components plus a very high concentration of unlabeled GTP (1 mM). In the presence of Sf9 membranes this high excess of unlabeled GTP prevents enzymatic hydrolysis of [$\gamma$-$^{33}$P]GTP. Spontaneous hydrolysis of [$\gamma$-$^{33}$P]GTP amounted to < 1 % of the total amount of radioactivity added.[17, 32] All experimental data were analyzed by non-linear regression with the Prism 5 program (GraphPad Software, San Diego, CA).

* for the investigations on direct G-protein activation, 3 µM GDP per assay tube were added following a protocol of Burde et al.[21]

### 6.6.2.5 $Ca^{2+}$-Assay (Fura-2)

The spectrofluorimetric calcium assay using the ratiometric $Ca^{2+}$-indicator Fura-2 was performed by analogy with a procedure described for HEL cells by Müller et al. and Gessele et al. [34, 35].

CHO-$hY_4$-qi5 cells (stably transfected, Ziemek et al. 2006) were grown for three days to 80-90 % confluency, trypsinized and detached with Ham's F12 supplemented with 10 % FCS. Cells were counted, centrifuged at 300 *g* for 5 min and resuspended at $1.3 \cdot 10^6$ cells/mL in loading buffer (120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM HEPES, 10 mM Glucose). Subsequently, 0.75 mL cell suspension were added to 0.25 mL of loading suspension, which consists of 20 mg of BSA, 5 µL of pluronic F-127 (20 % in DMSO) and 4 µL of fura-2/AM (Molecular Probes; 1 mM in anhydrous DMSO) per 1 mL of loading buffer (final concentrations: $1 \cdot 10^6$ cell/mL, 1 µM fura-2/AM, 0.2 % DMSO and 0.025 %, pluronic F-127). After 30 min of incubation in the dark, the cells were centrifuged and re-suspended in the same volume of loading buffer. In order to achieve complete intracellular cleavage of the AM-ester, the cells were incubated for additional 30 min in the dark, washed twice with loading buffer and re-suspended at a density of $1 \cdot 10^6$ cells/mL. For the measurements on a Perkin Elmer LS 50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany) 1 mL of cell suspension was added to 1 mL of loading buffer in disposable cuvettes. The baseline was recorded 30 s before the agonist was added (instrument settings and conditions: 25 °C, stirring: low, $\lambda_{ex}$: 340 and 380 (slit 10 nm), $\lambda_{em}$: 510 nm (slit 10 nm)). For the calculation of the calcium concentration the Grynkiewicz equation[36] was used.

## 6.7 References


1. Balasubramaniam, A.; Mullins, D. E.; Lin, S.; Zhai, W.; Tao, Z.; Dhawan, V. C.; Guzzi, M.; Knittel, J. J.; Slack, K.; Herzog, H.; Parker, E. M. Neuropeptide Y (NPY) Y4 receptor selective agonists based on NPY(32-36): development of an anorectic Y4 receptor selective agonist with picomolar affinity. *J. Med. Chem.* **2006,** 49, 2661-2665.
2. Brothers, S. P.; Wahlestedt, C. Therapeutic potential of neuropeptide Y (NPY) receptor ligands. *EMBO Mol. Med.* **2010,** 2, 429-439.
3. Beck-Sickinger, A. G.; Wieland, H. A.; Wittneben, H.; Willim, K. D.; Rudolf, K.; Jung, G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* **1994,** 225, 947-958.
4. Koglin, N.; Zorn, C.; Beumer, R.; Cabrele, C.; Bubert, C.; Sewald, N.; Reiser, O.; Beck-Sickinger, A. G. Analogues of neuropeptide Y containing beta-aminocyclopropane carboxylic acids are the shortest linear peptides that are selective for the Y1 receptor. *Angew. Chem. Int. Ed. Engl.* **2003,** 42, 202-205.
5. Fuhlendorff, J.; Gether, U.; Aakerlund, L.; Langeland-Johansen, N.; Thogersen, H.; Melberg, S. G.; Olsen, U. B.; Thastrup, O.; Schwartz, T. W. [Leu31, Pro34]neuropeptide Y: a specific Y1 receptor agonist. *Proc. Natl. Acad. Sci. USA* **1990,** 87, 182-186.

6. Davies, S. G.; Ichihara, O.; Lenoir, I.; Walters, I. A. S. Asymmetric synthesis of (-)-(1R,2S)-cispentacin and related cis- and trans-2-amino cyclopentane- and cyclohexane-1-carboxylic acids. *J. Chem.Soc., Perkin Transactions 1* **1994**, 1411-1415.

7. Moglioni, A. G.; Garcia-Exposito, E.; Aguado, G. P.; Parella, T.; Branchadell, V.; Moltrasio, G. Y.; Ortuno, R. M. Divergent routes to chiral cyclobutane synthons from (-)-alpha-pinene and their use in the stereoselective synthesis of dehydro amino acids. *J. Org. Chem.* **2000,** 65, 3934-3940.

8. Izquierdo, S.; Rua, F.; Sbai, A.; Parella, T.; Alvarez-Larena, A.; Branchadell, V.; Ortuno, R. M. (+)- and (-)-2-aminocyclobutane-1-carboxylic acids and their incorporation into highly rigid beta-peptides: stereoselective synthesis and a structural study. *J. Org. Chem.* **2005,** 70, 7963-7971.

9. Fernandez, D.; Torres, E.; Aviles, F. X.; Ortuno, R. M.; Vendrell, J. Cyclobutane-containing peptides: evaluation as novel metallocarboxypeptidase inhibitors and modelling of their mode of action. *Bioorg Med Chem* **2009,** 17, 3824-3828.

10. Gutierrez-Abad, R.; Illa, O.; Ortuno, R. M. Synthesis of chiral cyclobutane containing C3-symmetric peptide dendrimers. *Org. Lett.* **2010,** 12, 3148-3151.

11. Mammi, S.; Mammi, N. J.; Peggion, E. Conformational studies of human Des-Trp1,Nle12 minigastrin in water-trifluoroethanol mixtures by proton NMR and circular dichroism. *Biochemistry* **1988,** 27, 1374-1379.

12. Moroder, L. Isosteric replacement of sulfur with other chalcogens in peptides and proteins. *J. Pept. Sci.* **2005,** 11, 187-214.

13. Cabrele, C.; Beck-Sickinger, A. G. Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J. Pept. Sci.* **2000,** 6, 97-122.

14. Sonnichsen, F. D.; Van Eyk, J. E.; Hodges, R. S.; Sykes, B. D. Effect of trifluoroethanol on protein secondary structure: an NMR and CD study using a synthetic actin peptide. *Biochemistry* **1992,** 31, 8790-8798.

15. Yung-Chi Cheng, W. H. P. Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzymatic reaction. *Biochem. Pharm.* **1973,** 22, 3099-3108.

16. Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Determination of Affinity and Activity of Ligands at the Human Neuropeptide Y Y4 Receptor by Flow Cytometry and Aequorin Luminescence. *J. Recept. Signal. Transduct. Res.* **2007,** 27, 217 - 233.

17. Pop, N.; Igel, P.; Brennauer, A.; Cabrele, C.; Bernhardt, G. N.; Seifert, R.; Buschauer, A. Functional reconstitution of human neuropeptide Y (NPY) Y(2) and Y(4) receptors in Sf9 insect cells. *J. Recept. Signal Transduct. Res.* **2011,** 31, 271-285.

18. Schneider, E.; Mayer, M.; Ziemek, R.; Li, L.; Hutzler, C.; Bernhardt, G.; Buschauer, A. A simple and powerful flow cytometric method for the simultaneous determination of multiple parameters at G protein-coupled receptor subtypes. *ChemBioChem* **2006,** 7, 1400-1409.

19. Ziemek, R.; Brennauer, A.; Schneider, E.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Fluorescence- and luminescence-based methods for the determination of affinity and activity of neuropeptide Y2 receptor ligands. *Eur. J. Pharmacol.* **2006,** 551, 10-18.

20. Ziemek, R. Development of binding and functional assays for the neuropeptide Y Y 2 and Y 4 receptors. Doctoral Thesis, Regensburg, **2006**.

21. Burde, R.; Dippel, E.; Seifert, R. Receptor-independent G protein activation may account for the stimulatory effects of first-generation H1-receptor antagonists in HL-60 cells, basophils, and mast cells. *Biochem. Pharm.* **1996,** 51, 125-131.

22. Klinker, J. F.; Hagelueken, A.; Gruenbaum, L.; Seifert, R. Direct and indirect receptor-independent G-protein activation by cationic-amphiphilic substances. Studies with mast cells, HL-60 human leukemic cells and purified G-proteins. *Exp. Dermatol.* **1995,** 4, 231-239.

23. Higashijima, T.; Burnier, J.; Ross, E. M. Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. Mechanism and structural determinants of activity. *J. Biol. Chem.* **1990,** 265, 14176-14186.

24. Mousli, M.; Bueb, J. L.; Rouot, B.; Landry, Y.; Bronner, C. G-proteins as targets for non-immunological histamine releasers. *Inflamm. Res.* **1991,** 33, 81-83.

25. Lobaugh, L. A.; Blackshear, P. J. Neuropeptide Y binding and inhibition of cAMP accumulation in human neuroepithelioma cells. *Am. J. Physiol. - Cell Physiology* **1990,** 258, C913-C922.

26. Kenakin, T. Functional selectivity and biased receptor signaling. *J. Pharmacol. Exp. Ther.* **2011,** 336, 296-302.

27. Kenakin, T.; Miller, L. J. Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacol. Rev.* **2010,** 62, 265-304.

28. Berglund, M. M.; Hipskind, P. A.; Gehlert, D. R. Function, distribution and molecular pharmacology of NPY-family receptors. The NPY Family of Peptides in Immune Disorders, Inflammation, Angiogenesis and Cancer, *Prog. Inflamm. Res.*; **2005**; 29-62.

29. Berglund, M. M.; Schober, D. A.; Statnick, M. A.; McDonald, P. H.; Gehlert, D. R. The use of bioluminescence resonance energy transfer 2 to study neuropeptide Y receptor agonist-induced beta-arrestin 2 interaction. *J. Pharmacol. Exp. Ther.* **2003,** 306, 147-156.

30. Schmid, F. X. Spectral methods of characterizinig protein conformation and conformational changes. Potein Structure, *A Practical Approach*; **1989**; 251-285.

31. Walseth, T. F.; Johnson, R. A. The enzymatic preparation of [alpha-(32)P]nucleoside triphosphates, cyclic [32P] AMP, and cyclic [32P] GMP. *Biochim. Biophys. Acta.* **1979,** 562, 11-31.

32. Preuss, H.; Ghorai, P.; Kraus, A.; Dove, S.; Buschauer, A.; Seifert, R. Constitutive activity and ligand selectivity of human, guinea pig, rat, and canine histamine $H_2$ receptors. *J. Pharmacol. Exp. Ther.* **2007,** 321, 983-995.

33. Seifert, R.; Lee, T. W.; Lam, V. T.; Kobilka, B. K. Reconstitution of $\beta_2$-adrenoceptor-GTP-binding-protein interaction in Sf9 cells-high coupling efficiency in a $\beta_2$-adrenoceptor-$G_s\alpha$ fusion protein. *Eur. J. Biochem.* **1998,** 255, 369-382.

34. Gessele, K. Zelluläre Testsysteme zur pharmakologischen Charakterisierung neuer Neuropeptid Y-Rezeptorantagonisten. Doctoral Thesis, **1998**.

35. Muller, M.; Knieps, S.; Gessele, K.; Dove, S.; Bernhardt, G.; Buschauer, A. Synthesis and neuropeptide Y Y1 receptor antagonistic activity of N,N-disubstituted omega-guanidino- and omega-aminoalkanoic acid amides. *Arch. Pharm. (Weinheim)* **1997,** 330, 333-342.

36. Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **1985,** 260, 3440-3450.

# CHAPTER 7

# Cis-Pentacin Containing $Y_4R$ Selective Agonists

# 7.1 Introduction

The identification of the truncated [cpen[34]]NPY(25-36) analog **6.6** (**Figure 7.1**) as a new potent and selective NPY $Y_4R$ partial agonist was considered an excellent starting point for the synthesis of additional truncated and structurally modified peptides. Thereby, the cis-pentacin moiety should play a central role as it was found to be a very useful building block for the synthesis of $Y_4R$ selective peptides (see Chapter 6).

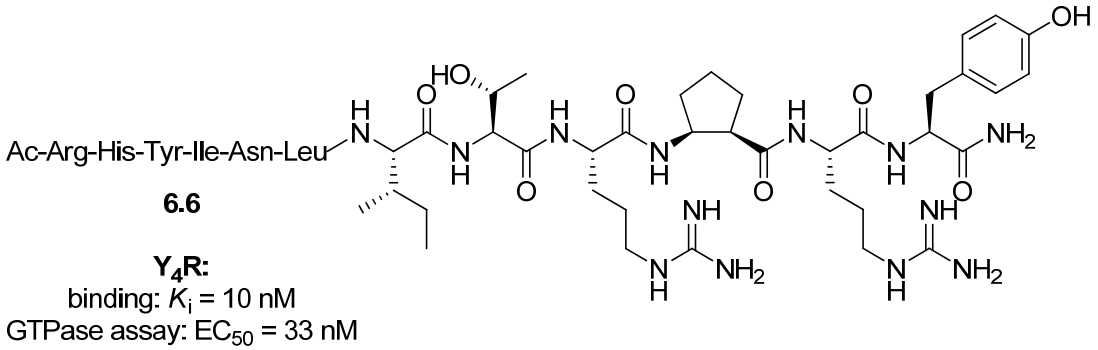
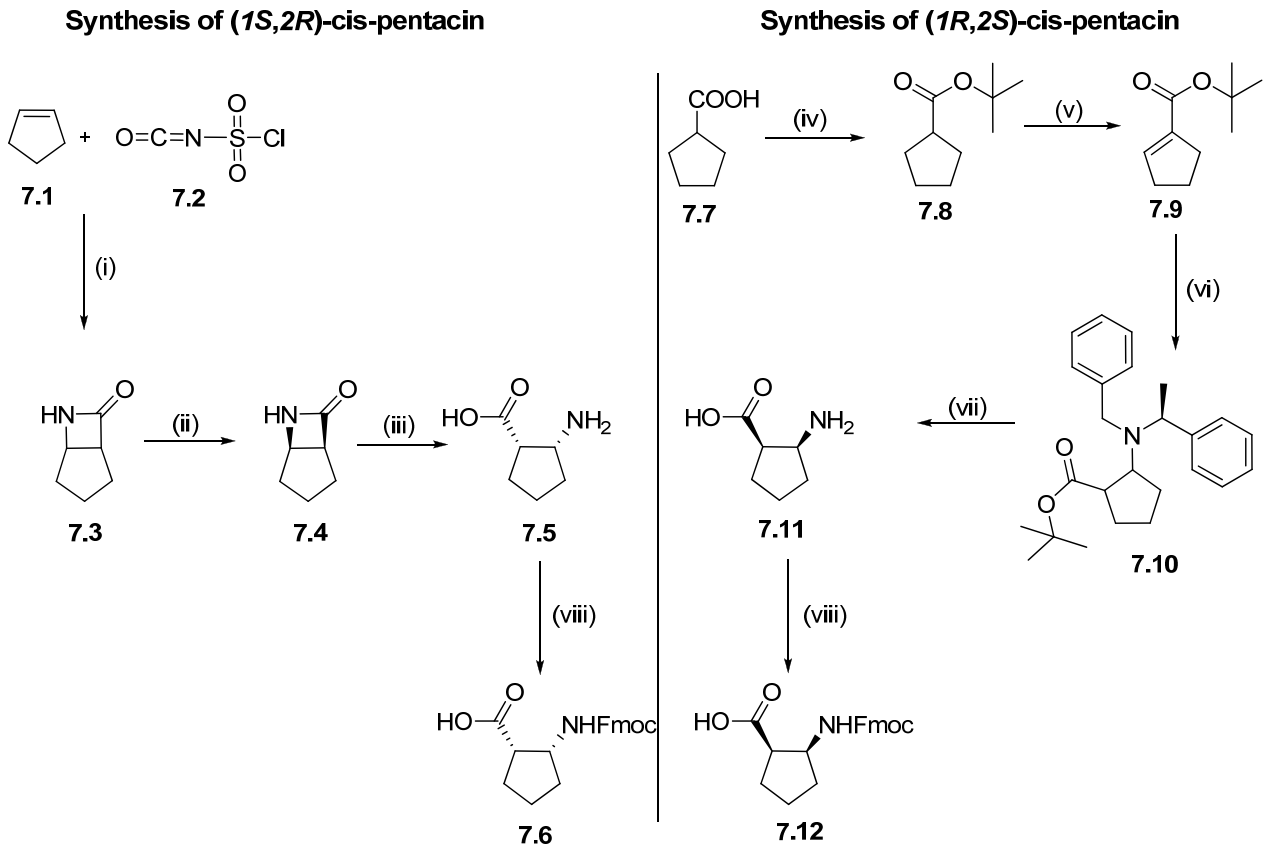



**Figure 7.1**: $Y_4R$ selective pNPY (25-36) agonist **6.6** containing a cis-pentacin residue.

The introduction of cyclic β-amino acids like cis-pentacin is one of the techniques often used in the field of peptidomimetic research.[1, 2] Peptidomimetics are small molecules mimicking the structure of biologically active peptides. Compared to natural polyamino acids, such molecules show higher proteolytic stability, making them less prone to biotransformation.[3] This can be advantageous for the application of such peptide mimics in *in vivo* studies or with regard to drug design. The latter aspect may regain importance in the NPY field considering recent publications on the influence of peptidic $Y_4R$ agonists on food intake and their possible role in the treatment of obesity.[4-8] Furthermore, the introduction of constrained, unnatural and β-amino acids can lead to enhanced affinity and/or selectivity towards a biological target as observed in case of the cis-pentacin insert in pNPY(25-36) (**6.6**). This peptidomimetic approach could also support the design of non-peptidic $Y_4R$ ligands. Hence, the cis-pentacin containing analog **6.6** was used as a basis for the design of additional truncated and cis-pentacin modified peptides presented in this chapter. Besides the synthesis and pharmacological characterization of pentapeptides derived from the C-terminus of the endogenous ligands pNPY and hPP, the influence of *D*-amino acids, in particular *D*-Pro, was investigated. Additionally, experiments to link the C- and N-terminal part of pNPY via a lysine side chain will be discussed. Finally, a stereochemical approach to the exchange of (*1R*,*2S*)-cis-pentacin by its enantiomer will be presented.

## 7.2 Chemistry

The stereoselective synthesis of (*1R*,*2S*)-cis-pentacin was performed according to a protocol published by Davies et al.[9] whereas the (*1S*,*2R*)-enantiomer was obtained via an enzymatic approach established by Forro et al.[10] as outlined in **Scheme 7.1**.



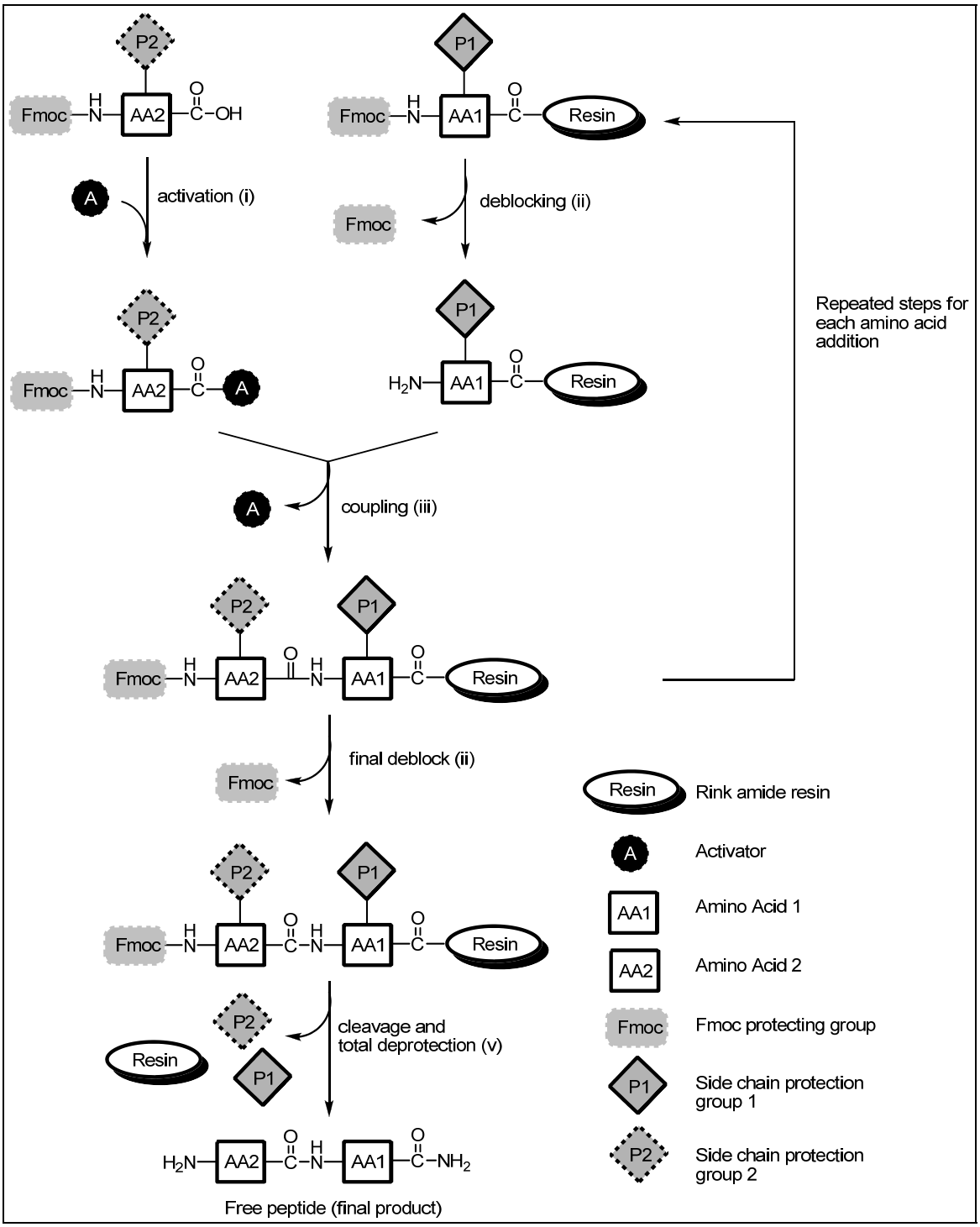

**Scheme 7.1**: Synthetic routes for the preparation of (*1S*,*2R*)- and (*1R*,*2S*)-cis-pentacin **7.6** and **7.12**: (i) a) -78 °C to rt, overnight, b) aq. sodium sulfite (25 %), conc. KOH; (ii) resin-bound Lipase B, $H_2O$, diisopropyl ether, 60 °C, shaking, 13 days; (iii) conc. HCl, $H_2O$, -5 °C to rt, overnight;(iv) isobutene (7.0 eq), $H_2SO_4$ (0.2 eq), DCM, -78 °C to rt, overnight; (v) a) LDA (1.5 eq), $I_2$ (1.7 eq), $THF_{abs}$, 0 °C to rt, 30 min; b) DBU (1.6 eq), $THF_{abs}$, 0 °C to rt, 48 h; (vi) a) (*S*)-*N*-benzyl-α-methylbenzylamine (1.6 eq), nBuLi (1.5 eq), $THF_{abs}$, 15 min; b) addition of **7.9** to a), toluene, -78 °C, 2h; c) 2,6-di-*tert*-butylphenol (3 eq), THFabs, -95 °C to rt, 30 min; (vii) a) 10 % Pd/C, $H_2$, acetic acid, 5 bar, 60 °C, overnight; b) TFA, overnight; (viii) Fmoc-OSu, aq. $Na_2C0_3$ (9 %), dioxane, 0 °C to rt, 30 min.

The synthesis of the (*1S*,*2R*)-cis-pentacin started from cyclopentene and *N*-chlorosulfonylisocyanate via a [2+2]-cycloaddition yielding a *N*-chlorosulfonyl-β-lactam. This thermally unstable intermediate could not be isolated. Instead, it was directly reduced in situ using a 25 % sodium sulfite solution affording an unstable *N*-sulfinic acid which spontaneously forms the desired product **7.3** under release of sulfur dioxide. The reduction is carried out under slightly basic conditions (pH 7-8) to avoid acid catalyzed ring opening in aqueous solution. Subsequently, resin-bound lipase B selectively catalyzed the hydrolysis of one enantiomer of the racemic β-lactam (**7.3**) leaving **7.4** in high enantiomeric purity and acceptable yield. The (*1R*,*2S*)-enantiomer of cis-pentacin, which precipitates

during the enzymatic process, was separated by filtration together with the resin after a 13 days reaction time. Cleavage of the β-lactam **7.4** with hydrochloric acid afforded (*1S*,*2R*)-cis-pentacin (**7.5**), which was converted to the Fmoc protected building block **7.6**.

The (*1R*,*2S*)-cis-configured pentacin (**7.11**) was obtained by a four-step stereoselective synthesis starting with a *tert*-butyl protection of cyclopentanecarboxylic acid **7.7** followed by a iodination and elimination of hydrogen iodide from the resultant α-iodo ester which afforded the a,β-unsaturated compound **7.9**. After conversion of the (*S*)-*N*-benzyl-α-methylbenzylamine to the corresponding lithium amide, **7.9** was added slowly to the lithium amide conjugate yielding the (*1R*,*2S*,α*S*) adduct **7.10**. Finally, the benzyl groups were cleaved off by hydrogenation over Pd/C catalyst affording the desired (*1R*,*2S*)-cis-pentacin **7.11**. Finally, **7.11** was Fmoc-protected according to a standard procedure yielding entantiomerically pure **7.12**.[11]

The cis-pentacin enantiomers were used as building blocks in standard solid phase peptide synthesis (SPPS) following the Fmoc-strategy as outlined in **Scheme 7.2**. As all peptides of the neuropeptide Y family are amidated at the C-terminus, Rink amide resin was used for SPPS to obtain the C-terminal amides after final cleavage. Prior to the first coupling step, the resin was swollen in a mixture of DMF/NMP (80/20 v/v). Every amino acid addition was performed following a double coupling strategy (2x 40 min) using standard activation reagents (HBTU and HOBt). The Fmoc protection group could be easily removed under basic conditions with a 20 % piperidine solution. The final cleavage from the resin combined with the removal of the acid labile side chain protection groups was carried out under strongly acidic conditions using TFA in the presence of anisol, thioanisole and ethanedithiol as scavengers. This cleavage cocktail was superior to the standard method (TFA, triisosilylpropylsilane (TIS) and water) as in particular the Pbf-protection groups of the numerous Arg residues couldn't be removed thoroughly under standard conditions, even after three hours reaction time. After precipitating the peptides in diethyl ether and several washing steps, the crude products were subjected to RP-HPLC yielding highly pure compounds.




**Scheme 7.2**: Solid phase peptide synthesis following the Fmoc-standard protocol for peptide synthesis (scheme adopted from Sigma Aldrich). Reagents and conditions: (i) + (iii) HBTU (4 eq), HOBt, (4eq), DIEA (8 eq), DMF/NMP (80/20 v/v), rt, shaking, 2x 40 min; (ii) 20 % piperidine solution (in DMF/NMP 80/20 v/v), shaking, 2 x 10 min, rt; (v) TFA/thioanisol/EDT/anisol (90/5/3/2 v/v), rt, shaking, 2.5 h. Step (ii) and (iii) always follows a washing step with DMF (5x).

# 7.3 Replacement of the Amino Acid in Position 34 by Cis-Pentacin or Leucine

## 7.3.1 Structural Overview of the Synthesized Peptides

One part of structural modifications of the cis-pentacin and aminocyclobutane containing pNPY(25-36) and hPP(25-36) analogs (**Table 7.1**) focused on the exploration of the stereodiscrimination of the peptides with regard to the cis-pentacin moieties (**7.13** and **7.14**) and on the extension of the peptide sequences of both the high affinity $Y_4R$ agonist **6.6** (**7.15** and **7.16**) and the corresponding (*1R*,*2S*)-aminocyclobutanecarboxylic acid analog **6.9** (**7.17**, **Table 7.1**), which are presented in Chapter 6.

To further explore structural variations of the recently identified cis-pentacin containing pNPY(25-36) analog **6.6**, the compounds presented in **Table 7.1** were designed and synthesized. As small subtype-selective molecules are urgently needed as pharmacological tools to study the biological role of NPY receptors, the pentapeptides **7.18**, **7.20**, **7.22** and **7.24** could also serve as a basis for the design of non-peptidic $Y_4R$ ligands. Besides the introduction of cis-pentacin (**7.18** and **7.20**), pentapeptides containing a leucine in postion 34 (**7.22** and **7.24**) were synthesized for reasons of comparison. The positive effect of $Leu^{34}$ on $Y_4R$ affinity of pNPY was previously reported by Cabrele et al.[12] Additionally, the impact of the N-terminal part of hPP on $Y_4R$ binding should be analyzed by linking the C-terminal cis-pentacin or leucine containing pentapeptides with a 5 amino acid sequence of the N-terminus of hPP via a lysine side chain (**7.19**, **7.21**, **7.23** and **7.25**).
Inspired by studies by Balasubramaniam et al.,[13] who developed high affinity peptide dimers for the $Y_4R$, the effect of an Thr-Tyr exchange at position 32, which includes similar functionality but higher lipophilicity, was taken into consideration and should be combined with $Leu^{34}$ and cis-pentacin modifications of the pentapeptides (**7.20** and **7.22)**.

A further approach aimed at expanding peptidomimetic substructures by introduction of *D*-Pro. The use of *D*-amino acids was taken into consideration due to their ability to form and stabilize reverse β-turns – hairpin-like structures – which led to increased affinities in many cases, indicating that such a β-turn or constrained conformation is important for biological activity.[14] Such a hairpin-like conformation is also reported for the endogenous ligands hPP and pNPY.[15] Therefore, the synthesis of compounds **7.27** and **7.29** should combine the natural or cis-pentacin containing C-terminal part with the N-terminal pentapeptide of hPP linked by a *D*-Pro sequence imitating a structure comparable to that of the endogenous ligands.

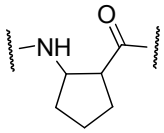
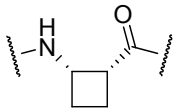
Furthermore, *D*-Pro was used to replace $Leu^{31}$ and $Met^{30}$ in hPP(25-36) (**7.26** and **7.28**), as this could be helpful to induce a certain conformation or further constrain the peptide backbone in this region. The incorporation of such local constraints by replacing specified amino acids, is apart from the truncation of natural peptides a widely applied strategy for the design of peptidomimetics.[14] *D*-Pro as an unnatural amino acid with a conformationally constrained structure seems highly suitable in this case.

Finally, the compatibility of the cis-pentacin approach with high affinity of already published $Y_4R$ ligands and the impact of this modification on affinity and selectivity should be analyzed. Therefore, the truncated hPP analog $[Tyr^{5-20}]$hPP (**7.30**) described by Eckard et al.[16] as potent $Y_4R$ ligand with only minor $Y_1R$ affinity was selected as a reference compound.

The synthesis of peptides **7.18** – **7.31** presented in **Table 7.3** was carried out with a cis-pentacin racemic mixture.

**Table 7.1**: Amino acid sequences of the synthesized peptides **7.13** – **7.31**.

| No. | Sequence | MW [Da] |
|---|---|---|
| **pNPY(25-36)** | Ac-RHRILITRQRY-$NH_2$ | 1673.92 |
| **(6.3)** | | |
| **hPP(25-36)**[*] | Ac-RRYIN-Nle-LTRPRY-$NH_2$ | 1661.95 |
| **(6.4)** | | |
| **[Leu$^{34}$]pNPY(25-36)** | Ac-RHYILITRLRY-$NH_2$ | 1658.94 |
| **(6.5)** | | |
| **7.13** | Ac-RHYINLITR-(*1S*,*2R*)**cpen**-RY-$NH_2$ | 1656.93 |
| **7.14** | Ac-RRYIN-Nle-LTR-(*1S*,*2R*)**cpen**-RY-$NH_2$ | 1675.98 |
| **7.15** | Ac-ALRHYINLITR-(*1R*,*2S*)**cpen**-RY-$NH_2$ | 1841.47 |
| **7.16** | Ac-YYSALRHYINLITR-(*1R*,*2S*)**cpen**-RY-$NH_2$ | 2254.59 |
| **7.17** | Ac-YYSALRHYINLITR■RY-$NH_2$ | 2240.21 |
| **7.18** | Ac-TR-**cpen**-RY-$NH_2$ | 746.86 |
| **7.19** | Ac-APLEP- $N^{\omega}$Lys -TR-**cpen**-RY-$NH_2$ | 1340.57 |
| **7.20** | Ac-YR-**cpen**-RY-$NH_2$ | 808.43 |
| **7.21** | Ac-APLEP-$N^{\omega}$Lys-YR-**cpen**-RY-$NH_2$ | 1402.64 |
| **7.22** | Ac-YRLRY-$NH_2$ | 810.94 |
| **7.23** | Ac-APLEP- $N^{\omega}$Lys -YRLRY-$NH_2$ | 1404.65 |
| **7.24** | Ac-TRLRY-$NH_2$ | 748.87 |
| **7.25** | Ac-APLEP- $N^{\omega}$Lys -TRLRY-$NH_2$ | 1342.59 |
| **7.26** | RRYINppTRPRT-$NH_2$ | 1424.66 |
| **7.27** | APLEPppTRPRT-$NH_2$ | 1392.60 |
| **7.28** | RRYINppTR-**cpen**-RT-$NH_2$ | 1438.68 |
| **7.29** | APLEPppTR-**cpen**-RT-$NH_2$ | 1406.63 |
| **7.30** | APLEYAADLRRTINMLITRPRY-$NH_2$ | 2695.15 |
| **7.31** | APLEYAADLRRTINMLITR-**cpen**-RY-$NH_2$ | 2709.18 |

**cpen** =  ■ =  (bACBC)

[*] Met$^{30}$ was replaced bei Nle to avoid instability against oxygen.

## 7.3.2 Pharmacological Results and Discussion

The truncated pNPY and hPP analogs were analyzed for $Y_4R$ binding by flow cytometry using CHO-h$Y_4$-qi5-mtAEQ cells. Affinity was determined by displacing Cy5-[$K^4$]hPP (c = 3 nM, $K_D$ = 5.62 nM).[17] Results are given as $K_i$ calculated according to the Cheng-Prusoff equation.[18] Additionally, all compounds were investigated for functional activity at the NPY $Y_4R$ in a steady state GTPase assay using Sf9 membranes co-expressing h$Y_4R$ plus $G\alpha_{i2}$ plus $G\beta_1\gamma_2$ and the fusion protein RGS4. In the following, agonistic potencies are expressed as $EC_{50}$ values. Intrinsic activities (α, efficacy) refer to the maximal response induced by the standard agonist hPP (10 µM). [19] Binding to the other NPY receptor subtypes was determined by flow cytometry using Cy5-pNPY (c = 5 nM, $K_D$ = 5.2);[20] results are given as $K_i$ values (**Table 7.2**).

The (*1S*,*2R*)-cis-pentacin containing peptides **7.13** and **7.14** did not show a significant difference in $Y_4R$ binding compared to the corresponding (*1R*,*2S*)-cis-pentacin containing analogs **6.6** and **6.8**. Obviously, in this case the enantomeric replacement does not have relevant impact on structure and $Y_4R$ binding of the ligand. By contrast, the $Y_5R$ binding of the (*1S*,*2R*)-cis-pentacin modified peptides pNPY(25-36) (**7.13**) and hPP(25-36) (**7.14**) was lower than that of the corresponding (*1R*,*2S*)-cis-pentacin containing diastereomers **6.6** and **6.8**.

Considering the elongation of **6.6** and the corresponding (*1R*,*2S*)-aminocyclobutanecarboxylic acid modified analog **6.9**, quite different results were obtained (cf. **7.16** and **7.17**). Whereas **7.16** shows only a decrease in affinity towards the $Y_4R$ by a factor of 9 compared to **6.6**, compound **7.17** was devoid of affinity to all NPY receptor subtypes. Surprisingly, the extension of the amino acid sequence in case of **7.16** led to a reduced $Y_4R$ subtype selectivity compared to the $Y_2R$ (**7.15** and **7.16**, **Table 7.2**).

The investigated pentapeptides mimicking the C-terminal part of pNPY (**7.18**, **7.20**, **7.22** and **7.24**) still show acceptable $Y_4R$ affinities and remarkable subtype selectivity. Compounds containing a Tyr in position 32 (**7.20**, $K_i$ = 105 nM and **7.22**, $K_i$ **=** 50 nM) showed 5- and 10-fold higher $Y_4R$ affinity than the corresponding $Thr^{32}$ parent peptides (**7.18**, $K_i$ = 509 and **7.24**, $K_i$ = 490). In comparison with [$cpen^{34}$]pNPY(25-36) **6.6** ($K_i$ = 10 nM) the cis-pentacin containing pentapeptides reveal a 50-fold lower affinity in case of the $Thr^{32}$-containing sequence (**7.18**), but only a 10- fold decrease in affinity regarding the $Tyr^{32}$-including analog (**7.20**). In contrast, the introduction of leucine to pNPY(32-36) (**7.24**, $K_i$ = 490 nM) only resulted in a 3-fold decreased affinity, whereas the [$Tyr^{32}$,$cpen^{34}$]pNPY(32-36) (**7.22**, $K_i$ = 50 nM) showed a 3-fold higher $Y_4R$ binding with regard to the corresponding extended [$Leu^{34}$]pNPY[25-36) analog **6.5** ($K_i$ = 157). Obviously, pentapeptides **7.20** ($cpen^{34}$) and **7.22** ($Leu^{34}$) with a Thr-Tyr-exchange in position 32 show only slightly reduced $Y_4R$ affinities (5 – 10-fold) compared to

[cpen$^{34}$]pNPY(25-36) **6.6** and are a promising structural basis for the development of peptidomimetic and non-peptidic $Y_4R$ ligands. The cis-pentacin containing analog (**7.20**) with its peptidomimetic-like character and its partial agonistic activity might be of advantage as a model compound with regard to the development of (non-peptidic) antagonists.
The linkage of pentapeptides of the C- and N-terminal part of hPP via a lysine side chain (**7.19**, **7.21**, **7.23** and **7.25**) didn't lead to improved $Y_4R$ binding, instead a slight decrease in affinity compared to the corresponding C-terminal pentapeptides (**7.18**, **7.20**, **7.22** and **7.24**) could be observed. This suggests that the N-terminus is of less importance in case of truncated hPP analogs.

The insert of two *D*-Pro residues into hPP(25-36) replacing Leu$^{31}$ and Met$^{30}$ revealed surprising and promising results in particular in terms of $Y_4R$ selectivity. Compared to the natural amino acid sequence of hPP(25-36) **6.8** ($K_i$ = 75 nM) the *D*-Pro modification of hPP(25-36) (**7.26,** $K_i$ = 940 nM) led to a pronounced decrease in $Y_4R$ affinity by a factor of 12. By contrast, compound **7.28,** a truncated hPP(25-36) analog which contains *D*-Pro as well as a cis-pentacin residue ([p$^{30}$,p$^{31}$,cpen$^{34}$]hPP(25-36)), showed nearly identical $Y_4R$ affinity as compound **6.8** ([cpen$^{34}$]hPP(25-36), $K_i$ = 75 nM) with a cis-pentacin replacement only. Compared to **7.26** ($K_i$ = 940 nM) the additional cpen$^{34}$ substitution leads to a 10-fold improved $Y_4R$ binding (**7.28**, $K_i$ = 90 nM). Interestingly, [p$^{30}$,p$^{31}$,cpen$^{34}$]hPP(25-36) **7.28** also showed a clearly improved subtype selectivity profile ($Y_1R$, $Y_2R$, $Y_5R$: $K_i$ > 1000 nM) compared to the [cpen$^{34}$]hPP(25-36) analog **6.8** ($Y_1R$: $K_i$ = 281 nM, $Y_2R$: $K_i$ = 217 nM, $Y_5R$: $K_i$ > 500), which is only cis-pentacin modified in position 34. Obviously, compared to **6.8** the introduction of *D*-amino acids, especially Pro which already includes a conformationally constrained structure, leads to a constrained peptide backbone, which seems to be advantageous to induce or stabilize conformations responsible for improved subtype selectivity. In contrast, the combination of pentapeptides of the C- and N-terminal part of hPP via a *D*-Pro sequence(**7.27** and **7.19**), which was assumed to be capable of inducing a hairpin-like structure as present in the endogenous ligand hPP, led to a complete loss of affinity.

The last approach – introducing cis-pentacin to [Tyr$^{5-20}$]hPP – led to contradictory results. $Y_4R$ affinity of the unsubstituted sequence **7.30** could not be detected. This is in contrast to the literature (**Table 7.4**, **7.30** obtained data + results from literature) where a $Y_4R$ affinity of 27 nM is reported.[16] Interestingly, modification of the peptide by introduction of cis-pentacin resulted in $Y_4R$ affinity in the nanomolar range (**7.31**, $K_i$ = 88 nM). However, this means - compared to the data published in literature - a decrease in $Y_4R$ affinity. Most striking after cis-pentacin incorporation is the complete loss of $Y_4R$ selectivity regarding $Y_2R$ and $Y_5R$. This phenomenon was already observed in a similar manner for [cpen$^{34}$]hPP(25-36) which shows a reduced selectivity towards the $Y_1R$ and $Y_2R$ after cis-

pentacin introduction. Obviously, this is a hPP specific problem. The replacement of $Pro^{34}$ seems to lead to a massive structural change going along with reduced subtype selectivity, although cis-pentacin was considered a more or less isosteric replacement.[21]

The the GTPase assay all cis-pentacin containing peptides showed partial agonism whereas all leucine containing analogs turned out to be full agonists.

**Table 7.2**: Potencies, efficacies and affinities of synthesized peptides **7.18** – **7.31** at the $Y_4R$ and binding data determined at the $Y_1R$, $Y_2R$ and $Y_5R$.

| No. | $Y_4R$ | | | $Y_1R$ | $Y_2R$ | $Y_5R$ |
|---|---|---|---|---|---|---|
| | $EC_{50}$ [nM][a] | α | $K_i$ [nM][b] | $K_i$ [nM][c] | $K_i$ [nM][c] | $K_i$ [nM][c] |
| **pNPY** | 417 ± 42[19] | 1.00 | (6.55 ± 0.06)[17] | 0.5 ± 0.2[20] | 0.8 ± 0.2[22] | 2.2 ± 0.8[20] |
| **hPP** | 11 ± 3.6[19] | 1.00 | (9.62 ± 0.07)[17] | - | - | - |
| **6.3** | 530 ± 160 | 0.56 | > 1000 | > 1000 | 279 ± 64 | > 1000 |
| **6.4** | 53 ± 8.0 | 0.70 | 74.6 ± 2.2 | > 1000 | > 1000 | > 1000 |
| **6.5** | 100 ± 24 | 1.00 | 157 ± 47 | > 1000 | > 1000 | > 1000 |
| **6.6** | 38 ± 5.5 | 0.72 | 10 ± 1.8 | > 1000 | > 1000 | > 500 |
| **6.8** | 51 ± 18 | 0.70 | 14 ± 7.2 | 281 ± 89 | 217 ± 28 | > 500 |
| **7.13** | 52 ± 11 | 0.59 | 29 ± 0.8 | > 1000 | > 1000 | 1747 ± 241 |
| **7.14** | 48 ± 16 | 0.54 | 6.4 ± 0.5 | 242 ± 33 | 380 ± 30 | 1468 ± 390 |
| **7.15** | 255 ± 92 | 0.52 | 33 ± 6.0 | > 1000 | 550 ± 91 | 1660 ± 413 |
| **7.16** | 356 ± 142 | 0.44 | 91 ± 20 | > 1000 | 770 ± 108 | 923 ± 355 |
| **7.17** | - | - | > 1000 | > 1000 | > 1000 | > 1000 |
| **7.18** | 451 ± 241 | 0.59 | 509 ± 160 | > 1000 | > 1000 | > 1000 |
| **7.19** | 605 ± 144 | 0.73 | 1423 ± 350 | > 1000 | > 1000 | > 1000 |
| **7.20** | 94 ± 21 | 0.73 | 105 ± 42 | > 1000 | > 1000 | > 1000 |
| **7.21** | 120 ± 9 | 0.95 | 139 ± 10 | > 1000 | > 1000 | > 1000 |
| **7.22** | 65 ± 9 | 0.97 | 50 ± 17 | > 1000 | > 1000 | > 1000 |
| **7.23** | 112 ± 26 | 0.99 | 109 ± 40 | > 1000 | > 1000 | > 1000 |
| **7.24** | 291 ± 48 | 0.97 | 490 ± 90 | > 1000 | > 1000 | > 1000 |
| **7.25** | 264 ± 58 | 1.00 | 691 ± 61 | > 1000 | > 1000 | > 1000 |
| **7.26** | 141 ± 37 | 0.71 | 940 ± 166 | > 1000 | > 1000 | > 1000 |
| **7.27** | n.d. | - | > 1000 | > 1000 | > 1000 | > 1000 |
| **7.28** | 32 ± 3 | 0.84 | 90 ± 17 | > 1000 | > 1000 | > 1000 |
| **7.29** | n.d. | - | > 1000 | > 1000 | > 1000 | > 1000 |
| **7.30** | n.d. | - | > 1000 | > 1000 | > 1000 | > 1000 |
| | n.d. | - | 27[16] | > 500[16] | > 1000[16] | > 5000[16] |
| **7.31** | 311 ± 139 | 0.34 | 88 ± 21 | > 1000 | 13 ± 5 | 40 ± 5 |

[a] Functional [γ-$^{33}$P]GTPase assay with membrane preparations of Sf9 cells expressing the $hY_4R$ + $hY_4R$ $G\alpha_{i2}$ + $G\beta_1\gamma_2$ +RGS4; the intrinsic activity (α) of hPP was set to 1.0 and α-values of other compounds were referred to this value; mean ± SEM from 3 independent experiments performed in duplicate. [b] Flow cytometric binding assay using 3 nM Cy5-[K$^4$]hPP (c = 3 nM, $K_D$ = 5.62 nM) in CHO-$hY_4$-$qi_5$-mtAEQ cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [c] Flow cytometric binding assay using 5 nM Cy5-pNPY (c = 5 nM, $K_D$ = 5.2 nM) in CHO-$hY_2$ cells, HEL($Y_1$) and HEC-1B-$Y_5$ cells, respectively.

## 7.4 Summary, Conclusion and Outlook

Cis-Pentacin and leucine (positon 34) containing analogs of pNPY(32-36) proved to be more potent $Y_4R$ agonists, in case of an additional exchange of $Thr^{32}$ by Tyr ($K_i$ values ≈ 50 – 100 nM). These short peptides could serve as a structural basis for the development of (non)peptidc $Y_4R$ ligands. Thereby, cis-pentacin containing analogs are considered more promising due to their peptidomimetic-like structure. Additionally, the cis-pentacin induced partial agonism may be interpreted a hint to the development of antagonists by structural modifications. As described in literature, the identification of peptide antagonists was often based on truncated and modified peptides with partial agonistic activity.[14] The introduction of cis-pentacin in hPP-derived amino acid sequences (hPP(25-36) and [$Tyr^{5-20}$]hPP) always led to high affinity $Y_4R$ ligands, but also to a tremendous decrease in $Y_4R$ selectivity. This is surprising, since the parent peptide hPP, is preferably bound by the $Y_4R$ and has considerably lower affinity towards the other NPY receptor subtypes. Obviously, cis-pentacin causes a dramatic change in conformation although it is considered a bioisosteric replacement for proline.[21] Surprisingly, the introduction of two *D*-Pro residues in position 30 and 31 of [$cpen^{34}$]hPP(25-36) led to a tremendous increase in subtype selectivity. Probably, the ability of *D*-amino acids to stabilize a certain structure and to conformationally constrain the peptide backbone had a positive effect on $Y_4R$ selectivity. Additionally, the use of *D*-amino acids and cis-pentacin can be considered a step towards peptidomimetic structures.

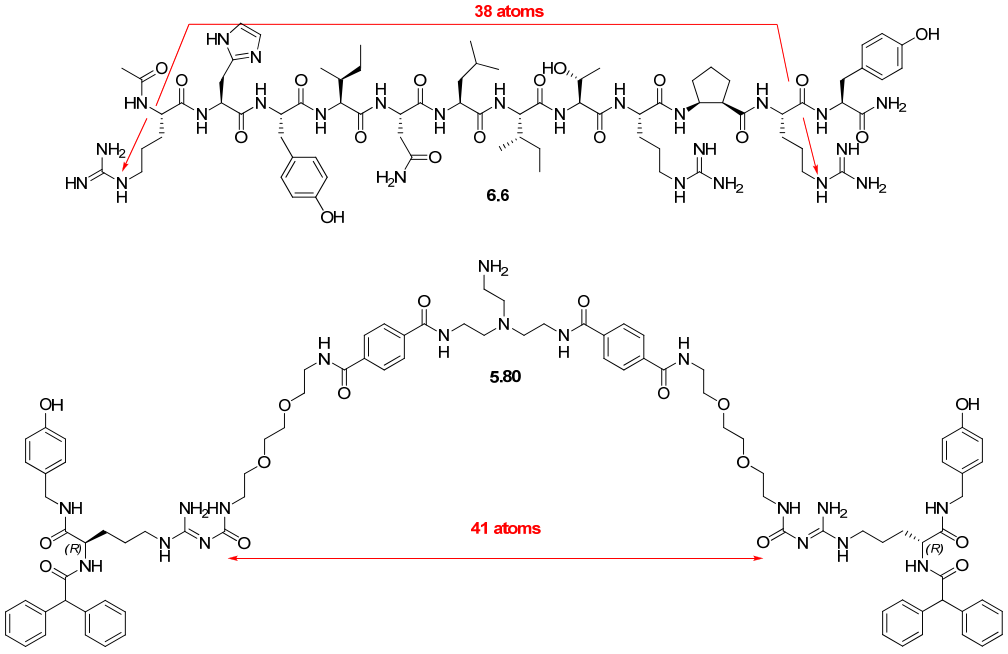



**Figure 7.2**: Comparison of the cis-pentacin containing pNPY(25-36) analog **6.6** with the structure of the NPY $Y_4R$ antagonist **5.80**.

The knowledge obtained within these studies of different peptidic structures should also be taken into consideration concerning the synthesis of new non-peptidic argininamide-type $Y_4R$ ligands based on the recently discovered bivalent structure **5.80** (see Chapter 5). As these BIBP 3226 derivatives, originally designed as $Y_1R$ antagonists are known to imitate the C-terminus of NPY, the identification of other important amino acids including their functional groups within the analog **6.6** could be helpful with respect to variations and improvements concerning linker structure and functional groups of the building block in **5.80** (**Figure 7.2**). Therefore, an alanine scan of this 12-amino acid analog could be useful. Additionally, variations of the presented pentapeptides should be considered concerning peptide backbone replacements by non-peptidic structures or by introduction of additional unnatural and D-configured amino acids. Also, modifications of side chains in terms of steric demand, basic or acidic properties can performed to identify the preferred structural elements and to suggest non-peptidic replacements. Such could also stimulate the variation of twin compounds based on **5.80**, aiming at low(er) molecular weight $Y_4R$ ligands.

# 7.5 Experimental Section

## 7.5.1 Chemistry

### 7.5.1.1 General Conditions

See section 3.3.1.1

Lipase B (from *Candida antarctica*) was from Sigma-Aldrich Chemie GmbH (Munich, Germany). Optical rotations were measured on a Perkin Elmer 141 polarimeter in the specified solvent. Concentrations are indicated in [g/100 mL].

### 7.5.1.2 Preparation of (*1S*,*2R*)-2-Aminocyclopentanecarboxylic acid

**cis-(±)-6-Azabicyclo[3.2.0]heptan-7-one (7.3)**[10]

Chlorosulfonylisocyanate **7.2** was added dropwise to cyclopentene **7.1** at – 78 °C under argon atmosphere within 45 min. The clear, pale yellow solution was slowly allowed to warm up to room temperature upon stirring overnight. Subsequently, the reaction mixture was slowly poured into an aqueous solution of sodium sulfite (25 %) keeping a pH of 8 by simultaneously adding an aqueous solution of conc. KOH at 0 °C. When addition was complete, the aqueous phase was extracted four times with 70 mL EtOAc. The combined organic phase was dried over $MgSO_4$ and the solvent was evaporated yielding a pale yellow oil that slowly crystallized to a pale yellow solid (8.54 g, 67 %).

Optical rotation (c 3.22, $CHCl_3$): $[\alpha]^{25}$ (nm): -0.20 (589), -0.2 (564), -0.7 (365). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.20 – 1.42 (m, 2H Cyclopentyl-***H***), 1.47 – 1.65 (m, 1H, Cyclopentyl-***H***), 1.66 – 1.82 (m, 3H, Cyclopentyl-***H***), 3.30 – 3.38(m, 1H, C***H***-NH), 3.89 (t, $^3J$ = 4.0 Hz , 1H, C***H***-CO), 7.54 (bs, 1H, N***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 22.08 (-, Cyclopentyl-***C***), 24.65 (-, Cylclopentyl-***C***), 29.45 (-, Cyclopentyl-***C***), 52.17 (+, ***C***H-NH), 54.93 (+, ***C***H-CO), 169.41 ($C_{quat}$, ***C***=O). MS (CI, $NH_3$) *m/z* (%): 129 (100) $[M+NH_4]^+$. $C_6H_9NO$ (111.14).

**(*1S*,*5R*)-6-Azabicyclo[3.2.0]heptan-7-one (7.4)[10]**

To a solution of **7.3** (5.0 g, 45.0 mmol, 1.0 eq) in 100 mL diisopropyl ether was added 320 µL of water and the enzyme Lipase B (on acryl resin, 5.0 g). The mixture was shaken 13 days at 60 °C in an incubator. Subsequently, the reaction mixture was filtrated over Celite and AloxB and washed with 50 mL diisopropyl ether after each filtration step. After concentrating the filtrate under reduced pressure, the residue was taken up in 150 mL DCM and extracted with aqueous $NaHCO_3$ (sat., 3x). Drying of the organic phase over $MgSO_4$ and evaporation of the solvent afforded the product as pale yellow oil (1.95 g, 39 %). Optical rotation (c 1.33, $CHCl_3$): $[\alpha]^{25}$ (nm) : +19.8 (589), +23.3 (564), +57.7 (365); Ref.: $[\alpha]^{25}$: +35.9 (589 nm, c 0.5, $CHCl_3$). $^1$H-NMR (300 MHz, $CD_3OD$): δ (ppm) 1.32 – 1.53 (m, 2H, Cyclopentyl-***H***), 1.63 – 1.77 (m, 1H, Cylclopentyl-***H***), 1.78 – 1.97 (m, 3H, Cyclopentyl-***H***), 3.41 – 3.47 (m, 1H, C***H***-NH), 4.03 (t, $^3J$ = 4.0 Hz , 1H, C***H***-CO); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 23.37 (-, Cyclopentyl-***C***), 25.91 (-, Cylclopentyl-***C***), 30.62 (-, Cyclopentyl-***C***), 55.01 (+, ***C***H-NH), 56.32 (+, ***C***H-CO), 173.67 ($C_{quat}$, ***C***=O). (CI, $NH_3$) *m/z* (%): 129 (100) $[M+NH_4]^+$. $C_6H_9NO$ (111.14).

**(*1S*,*2R*)-2-Aminocyclopentanecarboxylic acid (7.5)[10]**

Compound **7.4** (1.95 g, 17.6 mmol, 1 eq) was dissolved in 5 mL water and cooled to -5 °C. Subsequently, 5 mL of conc. HCl were added dropwise under vigorous stirring over a period of 45 min. The reaction mixture was allowed to warm to ambient temperature and was stirred overnight. After evaporation of the solvent, the colorless solid residue was triturated with MeCN and the white product was sucked off. Compound **7.5** was obtained as hydrochloride (2.45 g, 85 %). Optical rotation (c 1.61, $H_2O$): $[\alpha]^{25}$ (nm) : +10.7 (589), +15.2 (546), +16.0 (365); Ref.[10]: +5.2 $[\alpha]^{25}$: +.1 (589 nm, c 1.0, $H_2O$). $^1$H-NMR (300 MHz, $D_2O$): δ (ppm) 1.56 – 1.95 (m, 4H, Cyclopentyl-***H***), 1.97 – 2.10 (m, 2H, Cyclopentyl-***H***), 2.99 – 3.09 (m, 1H, C***H***-COOH), 3.70 – 3.80 (m, 1H, C***H***-$NH_2$); $^{13}$C-NMR (75 MHz, $D_2O$): δ (ppm) 21.23 (-, Cyclopentyl-***C***), 27.23 (-, Cyclopentyl-***C***), 29.73 (-, Cyclopentyl-***C***), 45.50 (+, C***H***-NH), 52.72 (+, C***H***-CO), 176.55 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 130 (40) $[M+H]^+$, 171 (100) $[M+MeCN+H]^+$, 259 (60) $[2M+H]^+$. $C_6H_{11}NO_2 \cdot HCl$ (165.62).

### 7.5.1.3 Preparation of (*1R*, *2S*)-2-Aminocyclopentanecarboxylic acid

**_tert_-Butyl cyclopentanecarboxylate (7.8)[9]**

Cyclopentanecarboxylic acid **7.7** (12.3 g, 90.0 mmol, 1 eq) was dissolved in DCM (200 mL) and cooled to -78 °C in a nitrogen flask. Concentrated sulfuric acid (1 mL, 19.3 mmol, 0.2 eq) was added and the reaction mixture was vigorously stirred during the condensation of isobutene (58 mL, 630 mmol, 7.0 eq). The colorless solution was then allowed to warm to ambient temperature within 20 h. After extraction with diethyl ether (3 x 100 mL) from aqueous $NaHCO_3$ (sat.) the organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (PE/diethyl ether 10/1 v/v) yielding a colorless oil (14.71 g, 96 %); $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.59 – 1.46 (m, 2H, cyclopentyl-***H***), 1.59 – 1.92 (m, 6H, cyclopentyl-***H***), 2.56 – 2.68 (m, 1H, C***H***-CO-O-*t*-Bu). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ (ppm) 25.81 (cyclopentyl-***C***), 28.10 (C-(***C***H$_3$)$_3$), 29.96 (cyclopentyl-***C***), 44.90 (***C***H-CO-O-t-Bu), 79.63 ($C_{quat.}$, ***C***-(CH$_3$)$_3$), 176.27 ($C_{quat.}$, ***C***O-O-t-Bu). $C_{10}H_{18}O_2$ (170.25).

**_tert_-Butyl Cyclopent- 1 -ene- 1 –carboxylate (7.9)[9]**

A solution of **7.8** (14.5 g, 85 mmol, 1 eq) in cooled $THF_{abs}$ (40 mL) was added to a mixture of LDA (16.86 mL, 128 mmol, 1.5 eq) in 200 mL $THF_{abs}$ by cannula at -78 °C within 5 min. After stirring for 1 h the generated enolate was transferred to a solution of iodine (36.7 g, 145 mmol, 1.7 eq) in $THF_{abs}$ (200 mL) and stirred for further 30 min at -78 °C prior to addition of conc. HCl (30 mL). The brown reaction mixture was then allowed to warm to room temperature and was extracted with diethyl ether (2x 100 mL) in presence of aq. sodium thiosulfate. Drying of the organic phase over $MgSO_4$ and evaporation of the solvent yielded an orange-brown oil (15.1 g). The obtained product was immediately dissolved in $THF_{abs}$ (100 mL). To this solution a mixture of DBU (12.1 mL, 80 mmol, 1.6 eq) dissolved in 20 mL $THF_{abs}$ was added dropwise by cannula at 0 °C. After 1 h the reaction mixture was allowed to warm to room temperature and was stirred for further 48 h. Subsequently, the solvent was evaporated and the obtained crude product was partitioned between water (100 mL) and diethyl ether (50 mL). The organic phase was dried over $MgSO_4$ and concentrated *in vacuo*. Flash chromatography with PE/diethyl ether (100/1 v/v) afforded the desired compound as pale yellow oil (5.05 g, 60 %) contaminated with 25 % **7.8**; $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.49 (s, 9H, C(C***H***$_3$)$_3$), 1.87 – 1.99 and 2.42 – 2.56 (m, 6H, (C***H***$_2$)$_3$), 6.64 – 6.68 (m, 1H, C***H***-C-CO-O-t-Bu); $^{13}$C-NMR (75 MHz, $CDCl_3$): δ (ppm) 23.17 (-, (***C***H$_2$)$_3$), 28.18 (+, C-(***C***H$_3$)$_3$), 31.39 (-, (***C***H$_2$)$_3$), 33.24 (-, (***C***H$_2$)$_3$), 79.94 ($C_{quat.}$, ***C***-(CH$_3$)$_3$), 138.35 ($C_{quat.}$, CH-***C***-CO-O-t-Bu ), 142.43 (+, ***C***H-C-CO-O-t-Bu), 164.99 ($C_{quat.}$, ***C***O-O-t-Bu). MS (CI, $NH_3$) *m/z* (%): 169 (40) $[M+H]^+$, 186 (100) $[M+NH_4]^+$. $C_{10}H_{16}O_2$ (168.23).

**(*1R,2S,*α*S*)-*tert*-Butyl 2-(*N*-Benzyl-α-methylbenzylamino)cyclopentane carboxylate (7.10)[9]**

To a solution of (*S*)-*N*-benzyl-α-methylbenzylamine (10.1 g, 48.0 mmol, 1.6 eq) in 250 mL $THF_{abs}$ n-butyllithium (in hexane, 2.5 M, 45.2 mL, 45.0 mmol, 1.5 eq) was added slowly by syringe at 0 °C under an argon atmosphere. The resultant pink solution was stirred for 15 min before cooling to -95 °C. Subsequently **7.9**, dissolved in 50 mL toluene, were added dropwise. After complete addition the red reaction mixture was stirred for 2 h, gradually warming to -78 °C. Quenching of the reaction mixture with 2,6-di-*tert*-butylphenol (18.6 g, 90.0 mmol, 3.0 eq) in 50 mL $THF_{abs}$ at -95 °C yielded a clear green solution which was allowed to warm to rt within 30 min. The solvent was removed under reduced pressure and the obtained residue was partitioned between diethyl ether and brine. After drying over $MgSO_4$ and evaporation of the solvent, the crude product was subjected to flash chromatography (PE/diethyl ether 20/1 v/v) affording a colorless, oily product (6.56 g, 57 %). Optical rotation (c 1.61, $CHCl_3$): $[\alpha]^{25}$ (nm) : -76.6 (589), -92.0 (546), -118.1(365) ; Ref.[9]: $[\alpha]^{25}$: - 69.1 (589 nm, c 1.14, $CHCl_3$). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.38 (d, $^3J$ = 15.5 Hz, N-CH-C***H***$_3$), 1.51 (s, 9H, C(C***H***$_3$)$_3$), 1.35-1.87 (m, 6H, (C***H***$_2$)$_3$), 2.87 – 2.95 and 3.05 – 3.17 (m, 2H, CH2-C***H***-N and C***H***-CO-O-t-Bu), 3.54 and 4.03 (2H, AB system, $J_{AB}$ = 15.5 Hz, Ph-C***H***$_2$-N), 4.32 (q, $^3J$ = 6.9 Hz, 1H, N-C***H***-$CH_3$), 7.12 – 7.47 (m, 10H, Ph-***H***); $^{13}$C-NMR (75 MHz, $CDCl_3$): δ (ppm) 17.42 (+, CH-***C***$H_3$), 22.09, 27.45 and 29.01 (-, (***C***$H_2$)$_3$), 28.26 (+, C-(***C***$H_3$)$_3$), 48.42 (+, ***C***H-CO-O-t-Bu), 51.74 (-, N-***C***$H_2$-Ph), 57.90 and 66.01 (+, ***C***H-N), 79.96 ($C_{quat.}$, ***C***-$(CH_3)_3$), 126.20 (+, Ph-***C***), 126.74 (+, Ph-***C***), 127.89 (+, Ph-***C***), 127.91 (+, Ph-***C***), 128.00 (+,), 128.08 (+, Ph-***C***), 142.52 ($C_{quat.}$, Ph-***C***-1), 142.97 ($C_{quat.}$, Ph-***C***-1) 175.14 ($C_{quat.}$, ***C***O-O-t-Bu). MS (ES, MeCN/0.1 % FA) *m/z* (%): 380 (100) $[M+H]^+$. $C_{25}H_{33}NO_2$ (379.54).

**(*1R*,*2S*)-2-Aminocyclopentanecarboxylic acid (7.10)[9]**

Compound **7.9** (6.45 g, 17.0 mmol, 1.0 eq) was dissolved in 85 mL acetic acid and a 10 % Pd/C catalyst (1.9 g) was added. The reaction mixture was vigorously stirred under a hydrogen atmosphere (5 bar) overnight. Subsequently, the Pd/C catalyst was removed by filtration over Celite and washed with MeOH. After removal of the solvent an oily residue was obtained, which was partitioned between aqueous $NaHCO_3$ (sat., 400 mL) and DCM (400 mL). The organic phase was dried over $MgSO_4$ and the solvent was concentrated *in vacuo*. Purification by flash chromatography (DCM/MeOH 90/10 – 80/20 v/v) afforded product **7.10** as a colorless oil (3.32 g, 89%). Subsequently, the intermediate (2.96 g, 16.0 mmol, 1.0 eq) was dissolved in 25 mL trifluoroacetic acid and stirred at room temperature overnight. The solvent was evaporated and the oily residue was taken up in 30 mL methanol and sat. HCl in diethyl ether (30 mL). Subsequently, the diethylether was removed under reduced pressure. The obtained pale brown solid was washed with ether yielding a white solid (1.95 g, 74 %). Optical rotation (c 1.41, $H_2O$): $[\alpha]^{25}$ (nm): -3.8 (589), -35.2 (546), -34.3 (365) ; Ref.: $[\alpha]^{25}$: - 8.8 (589 nm, c 1.0, $H_2O$). $^1$H-NMR (300 MHz, $D_2O$): δ (ppm) 1.50 – 1.94 (m, 4H, Cyclopentyl-***H***), 1.96 – 2.10 (m, 2H, Cyclopentyl-***H***), 2.99 – 3.09 (m, 1H, C***H***-COOH), 3.70 – 3.80 (m, 1H, C***H***-$NH_2$); $^{13}$C-NMR (75

MHz, $D_2O$): δ (ppm) 21.23 (-, Cyclopentyl-***C***), 27.22 (-, Cyclopentyl-***C***), 29.73 (-, Cyclopentyl-***C***), 45.50 (+, C***H***-NH), 52.72 (+, C***H***-CO), 176.54 ($C_{quat}$, ***C***=O) . MS (ES, MeCN/0.1 % FA) *m/z* (%): 130 (100) $[M+H]^+$, 171 (70) $[M+MeCN+H]^+$, 259 (60) $[2M+H]^+$. $C_6H_{11}NO_2 \cdot HCl$ (165.62).

### 7.5.1.4 Preparation of Fmoc-Protected cis-2-Aminopentanecarboxylic acid

**General procedure for the synthesis of Fmoc-amino acids**

**7.5** or **7.11** (1.0 eq) was dissolved in a 9 % sodium carbonate solution and cooled to 0 °C prior to addition of a solution of succinimidyl 9-fluorenylmethyl carbonate (1.0 eq) in dioxane. After 30 min stirring at room temperature, the reaction mixture was diluted with water and extracted with ether (1x) and EtOAc (2x). The remaining aqueous phase was cooled and acidified with conc. hydrochloric acid to pH 2 prior to extraction of the precipitated product (oil) in the aqueous phase with EtOAc (4x 100 mL). The combined organic phase was treated with brine (2x 60 mL) and water (2x 60 mL). After drying over $MgSO_4$, the solvent was evaporated and the remaining half-solid product was recrystallized from petroleum ether affording a white solid.

**(*1R*,*2S*)-*N*-Fmoc-2-aminocyclopentanecarboxylic acid (7.12)**

The title compound was prepared from **7.11** (1.93 g, 11.7 mmol) in sodium carbonate solution (2.47 g in 14 mL water) and succinimidyl 9-fluorenylmethyl carbonate (3.93 g, 11.7 mmol) in 30 mL dioxane according to the general procedure affording **7.12** as a white solid (1.70 g, 42 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.36 – 2.03 (m, 6H, Cyclopentyl-***H***), 2.76 – 2.92 (m, 1H, C***H***-CO), 4.08 – 4.32 (m, 4H, C***H***-C***H***$_2$ + C***H***-NH), 7.26 – 7.46 (m, 4H, Ph-***H***), 7.65 – 7.78 (m, 2H, Ph-***H***), 7.89 (d, $^3J$ = 7.4 Hz, 2H, Ph-***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 21.55 (-, Cyclopentyl-***C***), 26.25 (-, Cyclopentyl-***C***), 31.24 (-, Cyclopentyl-***C***), 46.59 (+, ***C***H-$CH_2$-O), 47.35 (+, ***C***H-CO), 53.91 (+, ***C***H-NH), 65.44 (-, CH-***C***$H_2$-O), 119.96 (+, 2 Ph-***C***), 126.97 (+, 2 Ph-***C***), 127.02 (+, 2 Ph-***C***), 127.50 (+, 2 Ph-***C***), 140.58 ($C_{quat}$, Ph-***C***), 143.94 ($C_{quat}$, Ph-***C***), 155.47 ($C_{quat}$, O-***C***O-NH), 174.20 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 352 (40) $[M+H]^+$, 369 (45) $[M+NH_4]^+$, 393 (50) $[M+MeCN+H]^+$, 703 (100) $[2M+H]^+$. $C_{21}H_{21}NO_4$ (351.40).

**(*1S*,*2R*)-*N*-Fmoc-2-aminocyclopentanecarboxylic acid (7.6)**

The title compound was prepared from **7.5** (2.40 g, 14.5 mmol) in sodium carbonate solution (3.07 g in 18 mL water) and succinimidyl 9-fluorenylmethyl carbonate (4.89 g, 14.5 mmol) in 40 mL dioxane according to the general procedure affording **7.6** as a white solid (3.60 g, 60 %). $^1$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.37 – 2.05 (m, 6H, Cyclopentyl-***H***), 2.76 – 2.92 (m, 1H, C***H***-CO), 4.07 – 4.34 (m, 4H, C***H***-C***H***$_2$ + C***H***-NH), 7.25 – 7.46 (m, 4H, Ph-***H***), 7.65 – 7.78 (m, 2H, Ph-***H***), 7.89 (d, $^3J$ = 7.3 Hz, 2H, Ph-***H***); $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 21.55 (-, Cyclopentyl-***C***), 26.25 (-, Cyclopentyl-***C***), 31.25 (-, Cyclopentyl-***C***), 46.59 (+, ***C***H-$CH_2$-O), 47.36 (+, ***C***H-CO), 53.91 (+, ***C***H-NH), 65.44 (-, CH-***C***$H_2$-O), 119.97

(+, 2 Ph-***C***), 126.97 (+, 2 Ph-***C***), 127.02 (+, 2 Ph-***C***), 127.51 (+, 2 Ph-***C***), 140.58 ($C_{quat}$, Ph-***C***), 143.94 ($C_{quat}$, Ph-***C***), 155.47 ($C_{quat}$, O-***C***O-NH), 174.22 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 352 (40) $[M+H]^+$, 369 (40) $[M+NH_4]^+$, 393 (80) $[M+MeCN+H]^+$, 703 (100) $[2M+H]^+$. $C_{21}H_{21}NO_4$ (351.40).

## 7.5.2 Peptide Synthesis According to a Standard Fmoc-Protocol

### 7.5.2.1 General Conditions

Rink-amide resin (loading 0.45 mmol/g), Fmoc-protected natural D- and L-amino acids as well as HBTU, HOBt, DIEA and NMP were purchased from IRIS Biotech (Marktredwitz, Germany). Piperidine, thioanisole, and ethane-1,2-dithiol were from Fluka (now Sigma Aldrich, Munich, Germany). DMF, DCM and diethylether were obtained from Acros Organics (Geel, Belgium). (±)-Cis-Pentacin was obtained from PolyPeptide Group (Hillerod, Denmark). The Fmoc- amino acids comprised the following side chain protection groups: Asp (*tert*-Butyl), Glu (*tert*-Butyl), His (Trt), Lys (Boc), Asn (Trt), Gln (Trt), Arg (Pbf), Ser (*tert*-Butyl), Thr (*tert*-Butyl), Tyr (*tert*-Butyl). Lysine had a Fmoc- side chain protection and $N^{\alpha}$-Boc-protection, instead.

Mass spectra (MS) were recorded on a Finnigan ThermoQuest TSQ 7000 (ES-MS). For high resolution mass spectrometry an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) equipped with an ESI source was used. The peak intensity in % relative to the strongest signal is indicated in parenthesis. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Nucleodur 100-5 C18ec, 250 × 21 mm, 5 µm, Knauer, Berlin, Germany) at a flow rate 18 mL/min. Mixtures of acetonitrile and 0.1 % aq. TFA were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure prior to lyophilisation. Analytical HPLC analysis was performed with a Jupiter column (250 x 4.6 mm, 5 µm, 300 Å, Phenomenex, Aschaffenburg, Germany) on a Merck Hitachi systems composed of a L-6200-A pump, a AS-2000A autosampler, a L-4000A UV-VIS detector and a F-1050 fluorescence spectrophotometer (flow rate: 0.8 mL/min; UV detection 220 nm). Acetonitrile (A) and 0.05 % TFA (B) was used as mobile phase. Helium degassing was used throughout.

### 7.5.2.2 General Procedure for SPPS (Fmoc-Protocol)

The synthesis of the peptides was performed by manual solid-phase peptide synthesis starting from the Fmoc-deprotected Rink Amide resin (loading 0.45 mmol/g) using a standard Fmoc-strategy. Prior to the first coupling step, the resin was swollen in a mixture of DMF/NMP (80/20 v/v) under shaking

for 30 min. To improve the complete conversion of every amino acid, chain elongation was performed by double coupling protocol (2x 45 min per amino acid) using a 4-fold excess of the Fmoc-protected amino acid, HBTU (0.45 M) and HOBt (0.45 M) in DMF/NMP (80/20 v/v) and a 8-fold excess of DIEA (1.0 M) in NMP for every coupling step. The Fmoc-deprotection was accomplished by a solution of 20 % piperidine in DMF/NMP (80/20 v/v, 2x 10 min). Acetylation of the N-terminus was achieved by treating the peptide with acetic anhydride (10 eq) in presence of DIEA (10 eq) in DMF. After every double coupling step, as well as every Fmoc-deprotection step the reaction mixture was sucked off and the resin was washed with DMF (5x). For the removal of the side chains and the total cleavage from the resin a mixture of TFA/thioanisole/EDT/anisole (90/5/3/2 v/v) was used. The completely deprotected peptides were precipitated by ice-cold diethyl ether or *tert*-butyl ether (5 – 10 mL) and collected by centrifugation for 5 – 10 min at 4 °C (4500 rpm) followed by two washing steps with ice-cold ether. All synthesized peptides were purified with preparative RP-HPLC and characterized by analytical RP-HPLC (gradient: MeCN/ 0.05 % TFA: 10/90 – 90/10 in 30 min) and HRMS.

### 7.5.2.3 Synthesis of NPY and hPP Analogs

**Ac-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-(*1S*,*2R*)cpen-Arg-Tyr-$NH_2$ (7.13)**

The title compound was prepared from 100 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 15 – 35 % in 30 min) yielded **7.13** as a white fluffy solid (14 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 425 (10) $[M+4H]^{4+}$, 553 (100) $[M+3H]^{3+}$, 829 (5) $[M+2H]^{2+}$; anal. RP-HPLC: 100 % ($t_R$ = 15.57 min, *k*' = 2.75); HRMS (ESI): *m/z* calcd. for $[C_{76}H_{121}N_{25}O_{178}+ 2H]^{2+}$ 828.9759, found: 828.9771. $C_{76}H_{121}N_{25}O_{17} \cdot 4TFA$ (2113.01).

**Ac-Arg-Arg-Tyr-Ile-Asn-Nle-Leu-Thr-Arg-(*1S*,*2R*)cpen-Arg-Tyr-$NH_2$ (7.14)**

The title compound was prepared from 100 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 15 – 35 % in 30 min) yielded **7.14** as a white fluffy solid (16 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 575 (50) $[M+3H]^{3+}$, 586 (100) $[M+HCOOH+3H]^{3+}$, 902 (10) $[M+2H]^{2+}$; anal. RP-HPLC: 98 % ($t_R$ = 16.85 min, *k*' = 3.06); HRMS (ESI): *m/z* calcd. for $[C_{76}H_{126}N_{26}O_{17}+ 3H]^{3+}$ 559.3338, found: 559.3355. $C_{76}H_{126}N_{26}O_{17} \cdot 4TFA$ (2132.06).

**Ac-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-(*1R*,*2S*)cpen-Arg-Tyr-$NH_2$ (7.15)**

The title compound was prepared from 100 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 15 – 35 % in 30 min) yielded **7.15** as a white fluffy solid (18 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 461 (10) $[M+4H]^{4+}$, 615 (100) $[M+3H]^{3+}$, 921 (5)

$[M+2H]^{2+}$; anal. RP-HPLC: 99 % ($t_R$ = 17.87 min, *k'* = 3.31); HRMS (ESI): *m/z* calcd. for $[C_{106}H_{160}N_{30}O_{25}+3H]^{3+}$ 614.3601, found: 614.3607. $C_{85}H_{137}N_{27}O_{19} \cdot 3TFA$ (2297.55).

**Ac-Tyr-Tyr-Ser-Ala-Leu-Arg-Hs-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-(*1R*,*2S*)cpen-Arg-Tyr-$NH_2$ (7.16)**

The title compound was prepared from 100 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 15 – 35 % in 30 min) yielded **7.16** as a white fluffy solid (22 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 565 (50) $[M+4H]^{4+}$, 753 (100) $[M+3H]^{3+}$, 1151 (5) $[M+2H]^{2+}$; anal. RP-HPLC: 92 % ($t_R$ = 19.87 min, *k'* = 3.79); HRMS (ESI): *m/z* calcd. for $[C_{106}H_{160}N_{30}O_{25}+3H]^{3+}$ 752.0796, found: 752.0814. $C_{106}H_{160}N_{30}O_{25} \cdot 4TFA$ (2710.67).

**Ac-Thr-Arg-(*1R*,*2S*)cpen-Arg-Tyr-$NH_2$ (7.18)**

The title compound was prepared from 80 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 40 % in 30 min) yielded **7.18** as a white fluffy solid (5 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 374 (100) $[M+2H]^{2+}$, 395 (25) $[M+MeCN+2H]^{2+}$, 793 (15) $[M+HCOOH+H]^{+}$; anal. RP-HPLC: 93 % ($t_R$ = 11.22 min, *k'* = 1.70); HRMS (ESI): *m/z* calcd. for $[C_{33}H_{54}N_{12}O_8 + H]^{+}$ 747.4260, found: 747.42629. $C_{33}H_{54}N_{12}O_8 \cdot 2TFA$ (974.90).

**Ac-Ala-Pro-Leu-Gln-Pro-($N^{\omega}$)Lys-Thr-Arg-(*1R*,*2S*)cpen-Arg-Tyr-$NH_2$ (7.19)**

The title compound was prepared from 80 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 60 % in 30 min) yielded **7.19** as a white fluffy solid (12 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 356 (25) $[M+2MeCN+4H]^{4+}$, 448 (100) $[M+3H]^{3+}$, 671 (30) $[M+2H]^{2+}$; anal. RP-HPLC: 97 % ($t_R$ = 21.22 min, *k'* = 4.11); HRMS (ESI): *m/z* calcd. for $[C_{61}H_{101}N_{19}O_{15} + 2H]^{2+}$ 670.8935, found: 670.8949. $C_{61}H_{101}N_{19}O_{15} \cdot 4TFA$ (1796.65).

**Ac-Tyr-Arg-(*1R*,*2S*)cpen-Arg-Tyr-$NH_2$ (7.20)**

The title compound was prepared from 80 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 5 - 35 % in 30 min) yielded **7.20** as a white fluffy solid (3 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 405 (100) $[M+2H]^{2+}$, 809 (5) $[M+H]^{+}$; anal. RP-HPLC: 96 % ($t_R$ = 12.70 min, *k'* = 2.06); HRMS (ESI): *m/z* calcd. for $[C_{38}H_{56}N_{12}O_8 + H]^{+}$ 809.4417, found: 809.4422. $C_{38}H_{56}N_{12}O_8 \cdot 2TFA$ (1036.47).

**Ac-Ala-Pro-Leu-Gln-Pro-($N^{\omega}$)Lys-Tyr-Arg-(*1R*,*2S*)cpen-Arg-Tyr-$NH_2$ (7.21)**

The title compound was prepared from 80 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 5 – 35 % in 30 min) yielded **7.21** as a white fluffy solid (10 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 469 (100) $[M+3H]^{3+}$, 702 (25) $[M+2H]^{2+}$; anal.

RP-HPLC: 96% ($t_R$ = 12.81 min, $k'$ = 2.09); HRMS (ESI): *m/z* calcd. for $[C_{66}H_{103}N_{19}O_{15} + H]^+$ 1402.7954, found: 1402.7955. $C_{66}H_{103}N_{19}O_{15} \cdot 4TFA$ (1858.72).

**Ac-Tyr-Arg-Leu-Arg-Tyr-$NH_2$ (7.22)**

The title compound was prepared from 80 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 40 % in 30 min) yielded **7.22** as a white fluffy solid (4 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 406 (100) $[M+2H]^{2+}$, 857 (5) $[M+HCOOH+H]^+$; anal. RP-HPLC: 94 % ($t_R$ = 13.91 min, $k'$ = 2.53); HRMS (ESI): *m/z* calcd. for $[C_{38}H_{58}N_{12}O_8 + H]^+$ 811.4573, found: 811.4579. $C_{38}H_{58}N_{12}O_8 \cdot 2TFA$ (1038.98).

**Ac-Ala-Pro-Leu-Gln-Pro-($N^{\omega}$)Lys –Tyr-Arg-Leu-Arg-Tyr-$NH_2$ (7.23)**

The title compound was prepared from 80 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 50 % in 30 min) yielded **7.23** as a white fluffy solid (16 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 372 (25) $[M+2MeCN+4H]^{4+}$, 469 $[M+3H]^{3+}$, 703 (40)$[M+2H]^{2+}$; anal. RP-HPLC: 97 % ($t_R$ = 13.91 min, $k'$ = 2.35); HRMS (ESI): *m/z* calcd. for $[C_{66}H_{105}N_{19}O_{15} + H]^+$ 1404.8110, found: 1404.8107. $C_{66}H_{105}N_{19}O_{15} \cdot 4TFA$ (1860.73).

**Ac-Thr-Arg-Leu-Arg-Tyr-$NH_2$ (7.24)**

The title compound was prepared from 80 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 40 % in 30 min) yielded **7.24** as a white fluffy solid (5 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 375 (100) $[M+2H]^{2+}$, 795 (10) $[M+HCOOH+H]^+$; anal. RP-HPLC: 97 % ($t_R$ = 12.33 min, $k'$ = 1.97); HRMS (ESI): *m/z* calcd. for $[C_{33}H_{56}N_{12}O_8 + H]^+$ 749.4417 , found: 749.4417. $C_{33}H_{56}N_{12}O_8 \cdot 2TFA$ (976.91).

**Ac-Ala-Pro-Leu-Gln-Pro-($N^{\omega}$)Lys-Thr-Arg-Leu-Arg-Tyr-$NH_2$ (7.25)**

The title compound was prepared from 80 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 50 % in 30 min) yielded **7.25** as a white fluffy solid (13 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 522 (100) $[M+3H]^{3+}$, 783 (50) $[M+2H]^{2+}$; anal. RP-HPLC: 99 % ($t_R$ = 21.55 min, $k'$ = 4.19); HRMS (ESI): *m/z* calcd. for $[C_{61}H_{103}N_{19}O_5 + 2H]^{2+}$, found: 782.9370. $C_{61}H_{103}N_{19}O_5 \cdot 4TFA$ (1798.67).

**Arg-Arg-Tyr-Ile-Asn-*D*-Pro-*D*-Pro-Thr-Arg-Pro-Arg-Tyr-$NH_2$ (7.26)**

The title compound was prepared from 150 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 60 % in 30 min) yielded **7.26** as a white fluffy solid (24 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 398 (25) $[M+4H]^{4+}$, 545 (100)

$[M+HCOOH+3H]^{3+}$, 841 (20) $[M+2HCOOH+2H]^{2+}$; anal. RP-HPLC: 97 % ($t_R$ = 12.07 min, $k'$ = 1.91); HRMS (ESI): *m/z* calcd. for $[C_{71}H_{114}N_{26}O_{16} + 3H]^{3+}$ 529.9708, found: 529.9708. $C_{71}H_{114}N_{26}O_{16} \cdot 5TFA$ (1994.76).

**Ala-Pro-Leu-Gln-Pro-*D*-Pro-*D*-Pro-Thr-Arg-Pro-Arg-Tyr-$NH_2$ (7.27)**

The title compound was prepared from 150 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 60 % in 30 min) yielded **7.27** as a white fluffy solid (36 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 465 (100) $[M+3H]^{3+}$, 479 (100) $[M+MeCN+3H]^{3+}$, 697 (65) $[M+2H]^{2+}$ ; anal. RP-HPLC: 100 % ($t_R$ = 13.15 min, $k'$ = 2.17); HRMS (ESI): *m/z* calcd. for $[C_{64}H_{101}N_{19}O_{16} + H]^+$ 1392.7746, found: 1392.7746. $C_{64}H_{101}N_{19}O_{16} \cdot 3TFA$ (1734.66).

**Arg-Arg-Tyr-Ile-Asn-*D*-Pro-*D*-Pro-Thr-Arg-cpen-Arg-Tyr-$NH_2$ (7.28)**

The title compound was prepared from 150 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 60 % in 30 min) yielded **7.28** as a white fluffy solid (26 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 401 (15) $[M+4H]^{4+}$; anal. RP-HPLC: 95 % ($t_R$ = 12.72 min, $k'$ = 2.07); HRMS (ESI): *m/z* calcd. for $[C_{65}H_{103}N_{19}O_{16}^{+} 3H]^{3+}$ 534.6427, found: 534.6440. $C_{72}H_{116}N_{26}O_{16} \cdot 5TFA$ (2008.78).

**Ala-Pro-Leu-Gln-Pro-*D*-Pro-*D*-Pro-Thr-Arg-cpen-Arg-Tyr-$NH_2$ (7.29)**

The title compound was prepared from 150 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 60 % in 30 min) yielded **7.29** as a white fluffy solid (36 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 470 (100) $[M+3H]^{3+}$, 704 (75) $[M+2H]^{2+}$, 1407 (5) $[M+H]^+$; anal. RP-HPLC: 99 % ($t_R$ = 13.92 min, $k'$ = 2.35); HRMS (ESI): *m/z* calcd. for $[C_{65}H_{103}N_{19}O_{16}^{+} H]^+$ 1406.7903, found: 1406.7896. $C_{65}H_{103}N_{19}O_{16} \cdot 3TFA$ (1748.69).

**Ala-Pro-Leu-Gln-Tyr-Ala-Ala-Asp-Leu-Arg-Arg-Thr-Ile-Asn-Met-Leu-Ile-Thr-Arg-Pro-Arg-Tyr-$NH_2$ (7.30)**

The title compound was prepared from 150 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 60 % in 30 min) yielded **7.30** as a white fluffy solid (39 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 675 (100) $[M+4H]^{4+}$, 900 (25) $[M+3H]^{3+}$; anal. RP-HPLC: 99 % ($t_R$ = 19.53, $k'$ = 3.71); HRMS (ESI): *m/z* calcd. for $[C_{122}H_{196}N_{36}O_{31}S + 3H]^{3+}$ 898.8269, found: 898.8282. $C_{122}H_{196}N_{36}O_{31}S \cdot 5TFA$ (3265.25).

**Ala-Pro-Leu-Gln-Tyr-Ala-Ala-Asp-Leu-Arg-Arg-Thr-Ile-Asn-Met-Leu-Ile-Thr-Arg-cpen-Arg-Tyr-$NH_2$ (7.31)**

The title compound was prepared from 150 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 – 60 % in 30 min) yielded **7.31** as a white fluffy solid (23 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 678 (100) $[M+4H]^{4+}$, 904 (20) $[M+3H]^{3+}$; anal. RP-HPLC: 99 % ($t_R$ = 19.96 min, *k*' = 3.81); HRMS (ESI): *m/z* calcd. for $[C_{123}H_{198}N_{36}O_{31}S + 3H]^{3+}$ 903.4988, found: 903.5005. $C_{123}H_{198}N_{36}O_{31}S \cdot 5TFA$ (3279.28).

### 7.5.3 Pharmacology Methods

#### 7.5.3.1 Flow Cytometric Binding Assay

See section 3.3.2.3

#### 7.5.3.2 Steady State GTPase Assay

See section 6.7.2.4

## 7.6 References

1. Aguilar, M.-I.; Purcell, A. W.; Devi, R.; Lew, R.; Rossjohn, J.; Smith, A. I.; Perlmutter, P. β-Amino acid-containing hybrid peptides-new opportunities in peptidomimetics. *Org. Biomol. Chem.* **2007,** 5, 2884-2890.
2. Seebach, D.; Gardiner, J. β-Peptidic Peptidomimetics. *Acc. Chem. Res.* **2008,** 41, 1366-1375.
3. Vagner, J.; Qu, H.; Hruby, V. J. Peptidomimetics, a synthetic tool of drug discovery. *Curr. Opin. Chem. Biol.* **2008,** 12, 292-296.
4. Brothers, S. P.; Wahlestedt, C. Therapeutic potential of neuropeptide Y (NPY) receptor ligands. *EMBO Mol. Med.* **2010,** 2, 429-439.
5. Kamiji, M. M.; Inui, A. Neuropeptide Y Receptor Selective Ligands in the Treatment of Obesity. *Endocr. Rev.* **2007,** 28, 664-684.
6. M.M. Kamiji, A. I. NPY Y2 and Y4 Receptors Selective Ligands: Promising Anti-Obesity Drugs? *Curr. Top. Med. Chem.* **2007,** 7, 1734-1742.
7. Walther, C.; Mörl, K.; Beck-Sickinger, A. G. Neuropeptide Y receptors: ligand binding and trafficking suggest novel approaches in drug development. *J. Pept. Sci.* **2011,** 17, 233-246.
8. Lumb, K.; Decarr, L. Preparation of Neuropeptide y4 receptor agonists as in vivo or in vitro agents to reduce food intake. WO2006091506A2, **2006**.

9. Davies, S. G.; Ichihara, O.; Lenoir, I.; Walters, I. A. S. Asymmetric synthesis of (-)-(1R,2S)-cispentacin and related cis- and trans-2-amino cyclopentane- and cyclohexane-1-carboxylic acids. *J. Chem.Soc., Perkin Transactions 1* **1994**, 1411-1415.

10. Forro, E.; Fülöp, F. Lipase-catalyzed enantioselective ring opening of unactivated alicyclic-fused beta-lactams in an organic solvent. *Org. Lett.* **2003,** 5, 1209-1212.

11. Lapatsanis, L.; Milias, G.; Froussios, K.; Kolovos, M. Synthesis of N-2,2,2-(Trichloroethoxycarbonyl)-L-Amino Acids and N-(9-Fluorenylmethoxycarbonyl)-L-Amino Acids Involving Succinimidoxy Anion as a Leaving Group in Amino-Acid Protection. *Synthesis* **1983**, 671-673.

12. Cabrele, C.; Beck-Sickinger, A. G. Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J. Pept. Sci.* **2000,** 6, 97-122.

13. Balasubramaniam, A.; Mullins, D. E.; Lin, S.; Zhai, W.; Tao, Z.; Dhawan, V. C.; Guzzi, M.; Knittel, J. J.; Slack, K.; Herzog, H.; Parker, E. M. Neuropeptide Y (NPY) Y4 receptor selective agonists based on NPY(32-36): development of an anorectic Y4 receptor selective agonist with picomolar affinity. *J. Med. Chem.* **2006,** 49, 2661-2665.

14. Hruby, V. J. Designing peptide receptor agonists and antagonists. *Nat. Rev. Drug. Discov.* **2002,** 1, 847-858.

15. Allen, J.; Novotny, J.; Martin, J.; Heinrich, G. Molecular structure of mammalian neuropeptide Y: analysis by molecular cloning and computer-aided comparison with crystal structure of avian homologue. *Proc. Natl. Acad. Sci. USA* **1987,** 84, 2532-2536.

16. Eckard, C.; Cabrele, C.; Wieland, H.; Beck-Sickinger, A. Characterisation of Neuropeptide Y Receptor Subtypes by Synthetic NPY Analogues and by Anti-receptor Antibodies. *Molecules* **2001,** 6, 448-467.

17. Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Determination of Affinity and Activity of Ligands at the Human Neuropeptide Y Y4 Receptor by Flow Cytometry and Aequorin Luminescence. *J. Recept. Signal. Transduct. Res.* **2007,** 27, 217 - 233.

18. Yung-Chi Cheng, W. H. P. Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzymatic reaction. *Biochem. Pharm.* **1973,** 22, 3099-3108.

19. Pop, N.; Igel, P.; Brennauer, A.; Cabrele, C.; Bernhardt, G. N.; Seifert, R.; Buschauer, A. Functional reconstitution of human neuropeptide Y (NPY) Y(2) and Y(4) receptors in Sf9 insect cells. *J. Recept. Signal Transduct. Res.* **2011,** 31, 271-285.

20. Schneider, E.; Mayer, M.; Ziemek, R.; Li, L.; Hutzler, C.; Bernhardt, G.; Buschauer, A. A simple and powerful flow cytometric method for the simultaneous determination of multiple parameters at G protein-coupled receptor subtypes. *ChemBioChem* **2006,** 7, 1400-1409.

21. Noteberg, D.; Branalt, J.; Kvarnstrom, I.; Classon, B.; Samuelsson, B.; Nillroth, U.; Danielson, U. H.; Karlen, A.; Hallberg, A. Synthesis of enantiomerically pure cis and trans 2-aminocyclopentanecarboxylic acids. Use of proline replacements in potential HIV-protease inhibitors. *Tetrahedron* **1997,** 53, 7975-7984.

22. Ziemek, R.; Brennauer, A.; Schneider, E.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Fluorescence- and luminescence-based methods for the determination of affinity and activity of neuropeptide Y2 receptor ligands. *Eur. J. Pharmacol.* **2006,** 551, 10-18.

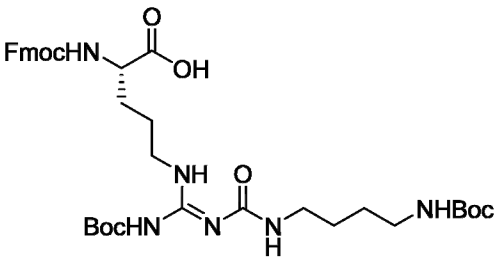
# CHAPTER 8

# Green- and Red-Fluorescent Subtype-Selective Peptides for the NPY $Y_2$, $Y_4$ and $Y_5$ Receptor

## 8.1 Introduction

Fluorescence-labeled ligands have become valuable pharmacological tools for investigations of ligand-receptor interactions over the past two decades. Compared to radiotracers, they can be considered superior in terms of safety precautions and waste disposal. Additionally, fluorescence-labeled compounds provide the possibility to be applied to fluorescence microscopy and flow cytometry, powerful techniques becoming daily routine in many laboratories.[1]

A widely distributed and successfully applied approach in the field of peptidergic G-protein coupled receptors is the preparation and use of the corresponding fluorescence-labeled endogenous ligands. The attachment of fluorescent dyes is often tolerated without a distinct decrease in affinity, as the properties and the ligand-receptor interactions of peptides are much less affected by the introduction of a bulky compared to low molecular weight GPCR ligands. Numerous fluorescence-labeled peptides have been reported to date in literature also for NPY receptors.[2-4] Besides the application as standard ligands in fluorescence-based binding studies,[5-7] such fluorescent NPY analogs can be used e.g. to investigate receptor internalization[8] or to perform colocalisation[9] experiments by means of confocal microscopy. The major disadvantage of endogenous ligands and their fluorescence-labeled derivatives is the lack of discrimination between the different receptor subtypes. The need of selective high-affinity fluorescent ligands e.g. for the identification and investigation of receptor subtypes on cells and in tissues prompted us to prepare green and red fluorescently-labeled peptide agonists for the $Y_1R$, $Y_2R$, $Y_4R$ and $Y_5R$. Hence, subtype-selective, preferably truncated peptide agonists derived from the endogenous ligand NPY were selected from literature. At first, special attention was paid to peptide derivatives containing a lysine residue which enables the introduction of dyes via the amino functionality of the side chain. For example, [Ala$^{31}$,Aib$^{32}$]pNPY, a selective NPY $Y_5R$ ligand contains a Lys at position 4.[10] However, due to the limited number of available Lys-containing subtype-selective NPY derivatives a different strategy was required. Recently, the design of a functionalized arginine-building block was developed by our research group.[11] The bioisosteric replacement of guanidines by acyl- or the more stable carbamoylguanidines was reported to strongly decrease the basicity of guanidine but to retain a sufficient degree of protonation at physiological pH to enable interactions with key amino acids of the receptors of interest. For instance, incorporating $N^G$-acylated arginine building blocks into non-peptidic $Y_1R$ and $Y_2R$ antagonists derived from BIBP3226 and BIIE0246, respectively, led to valuable pharmacological tools.[12-16] The latter results prompted us to explore the




**8.1**

**Figure 8.1**: Functionalized arginine building block Arg($N^{\omega}$-Ahx)

broader applicability of this approach, based on $N^G$-functionalized arginine building blocks (**Figure 8.1**), which can be used in solid phase peptide synthesis (SPPS). A suitable functional group in the $N^G$-substituent should allow for the attachment of fluorescent dyes. The search for appropriate subtype-selective NPY analogs was therefore extended to peptides comprising an N-terminal Arg-residue. pNPY(18-36) was identified as an appropriate candidate for the design of a subtype selective $Y_2R$ agonist as outlined in this chapter.[17] In addition, the design of the functionalized arginine building block **8.1** made it possible to include the recently developed, selective $Y_4R$ ligand [cpen$^{34}$]pNPY(25-36) (cf. Chapter 6), containing an N-terminal Arg-residue, in fluorescence labeling experiments. This could be of special importance, due to the lack of pharmacological tools for the NPY $Y_4R$.

## 8.2 Chemistry

With respect to potential use in cellular assays a red fluorescent dye with an emission wavelength > 600 nm was selected to reduce background fluorescence and thus to improve the signal-to-noise ratio. As Cy5 (**Figure 8.2**) has already been successfully applied to labeling of pNPY and [K$^4$]hPP,[5, 7] this fluorescent dye was considered as the first-choice for the labeling of subtype-selective and truncated NPY derivatives. The cyanine dye Cy5 is excitable at 635 nm with a red diode laser used as typical light source in commercial flow cytometers. The fluorescence emission can be detected around 660 nm.

Additionally, aiming at higher flexibility for application in confocal microscopy and flow cytometry, green-fluorescent dyes were considered. Ligands emitting at distinct wavelengths, e.g., green and red fluorescent compounds, are of special value in multiparametric assays.[7, 18] Furthermore, the combination of green- and red-fluorescent ligands might be useful for confocal microscopy in colocalization[9], FRET or receptor trafficking experiments.

In a first approach 5(6)-carboxyfluorescein, highlighted in **Figure 8.2** (here with an additional hexanoic acid side chain: 5(6)-SFX), which is one of the most popular and commonly used fluorophores, was chosen as a green-fluorescent dye for labeling of subtype-selective NPY analogs. However, besides the common problem of autofluorescence of cells and tissues upon excitation at 488 nm, fluorescein and its derivatives are disadvantageous with regard to pH sensitivity and photostability. Therefore, a green-fluorescent dye with improved properties, especially higher stability against photobleaching, was desired. A new series of fluorescent dyes, namely Alexa Fluor dyes, bearing negatively charged sulfonate groups in addition to fluorescein, were reported as more hydrophobic and brighter dyes with improved photostability and more favorable pH tolerance.[9, 19] Excitability by the widely used 488 nm laser, a standard component of flow cytometers and confocal microscopes is a common advantage of 5(6)-SFX and Alexa Fluor 488.

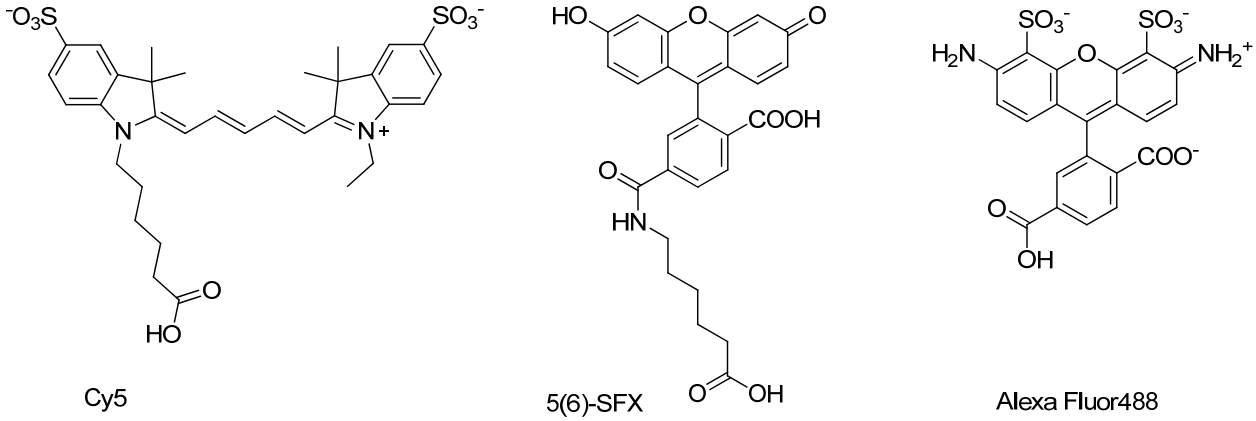
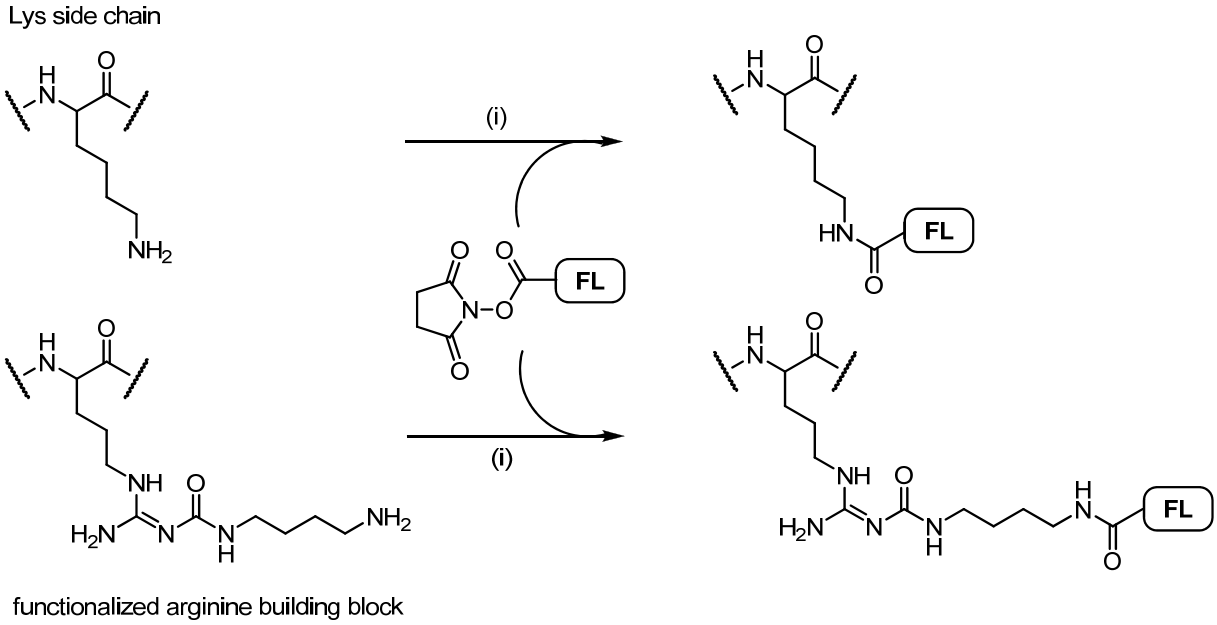



**Figure 8.2**: Structures of the dyes which were used for the preparation of the fluorescent peptide agonists selective for the different NPY receptor subtypes.

All dyes presented in **Figure 8.2** contain a carboxylated side chain (Cy5 and 5(6)-SFX) or a carboxyl group at the phenyl moiety (Alexa Fluor 488) and were available as succinimidyl esters. These active esters allowed for convenient labeling of primary amino groups, i.e. at the Lys side chain or a $N^G$-functionalized arginine building block (**Figure 8.1**), of the selected peptides in a $NaCO_3/NaHCO_3$-buffered solution (10 mM, pH 8.5) as outlined in **Scheme 8.1**. Thereby, the Cy5- and the 5(6)-SFX ester reacted very rapidly, whereas the acylation with the Alexa Fluor dye was hampered and required considerably longer reactions times, probably because of sterical hindrance due to the direct attachment of the carboxyl group to the phenyl moiety. The introduction of Alexa Fluor 488 to the $Lys^4$ side chain in [$Ala^{31}$,$Aib^{32}$]pNPY even completely failed. The yields of all labeled peptides were low, presumably, due the high hydrolytic instability of the NHS-ester in the used buffer system.

Protection of the N-terminal amino group ($N^\alpha$) in the selective $Y_2R$ ligand pNPY(18-36) and the selective $Y_5R$ ligand [$Ala^{31}$,$Aib^{32}$]pNPY was not required. Obviously, the $N^\omega$-amino group of the lysine side chain and the functionalized arginine side chain, respectively, are preferably acylated due to better accessibility. Additionally, the $N^\alpha$-position at the N-terminus shows reduced reactivity due to the neighboring electron-withdrawing carboxamide group. Indeed, besides the desired mono-labeling at the $N^\omega$-amino group only minor amounts of twofold labeled peptides ($N^\alpha$ and $N^\omega$-position) were detected in both cases. The labeling reactions were stopped with 10 % aq. TFA and the crude products were purified by preparative HPLC.




**Scheme 8.1**: Synthesis of the fluorescent ligands **8.2** - **8.4**. Reagents and conditions: (i) $NaHCO_3/Na_2CO_3$-buffer (10 mM, pH 8.5) with 33 % MeCN.

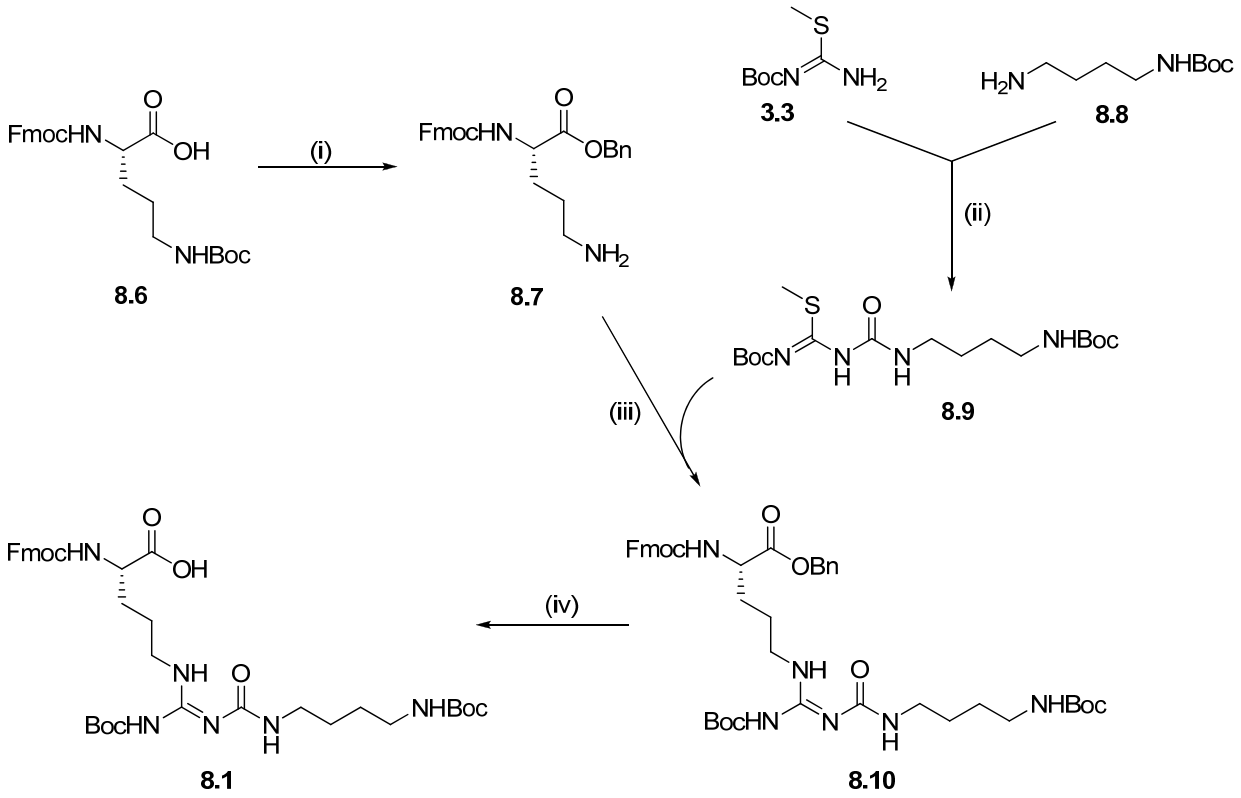
The subtype selective peptides chosen for fluorescence labeling (**Table 8.1**) were synthesized according to a standard protocol for solid phase peptide synthesis (SPPS) following an Fmoc-protection strategy, as described in detail in Chapter 7.

**Table 8.1**: Amino acid sequences of the synthesized peptides **8.2 – 8.5.**

| No. | Sequence | Selective for | MW [Da] |
|---|---|---|---|
| **8.2** | INP-Nle-Bpa-LRY-$NH_2$ | $Y_1R$ | 1294.55 |
| **8.3** | Arg($N^{\omega}$-Ahx)-HYINLITRQRY-$NH_2$ | $Y_2R$ | 2499.87 |
| **8.4** | Ac-Arg($N^{\omega}$-Ahx)-HYINLITR-cpen-RY-$NH_2$ | $Y_4R$ | 1771.08 |
| **8.5** | YPSKPDNPGEDAPAEDLARYYSALRHYINLA-Aib-RQRY-$NH_2$ | $Y_5R$ | 4211.09 |

A procedure for the synthesis of the functionalized arginine building block (Arg($N^{\omega}$-Ahx)) present in peptides **8.3** and **8.4** was previously developed in our work group.[11] First approaches using precursor molecules possessing alkanoylguanidines moiety gave unsatisfactory results in terms of stability and yields. Therefore, the introduction of the more stable carbamoylguanidine substructure was considered superior. The optimized four-step synthesis is outlined in **Scheme 8.2**. Commercially available $N^{\alpha}$-Fmoc-$N^{\omega}$-Boc-ornithine (**8.6**) was treated with benzylalcohol using DCC as activation reagent to protect the free carboxyl group. After Boc-deprotection of the $N^{\omega}$-amino group was allowed to react with the guandinylating reagent **8.9** in the presence of $HgCl_2$ and $NEt_3$ (for reaction details see Chapter 3) affording the carbamoylguanidine **8.10** in good yield. The building block **8.9**

was obtained from Boc-protected S-methylisothiourea **3.3** and Boc-diaminobutane **8.8** in the presence of triphosgene. Finally, compound **8.10** was subjected to hydrogenolytic debenzylation, yielding the functionalized arginine building block **8.1** after purification by flash chromatography. The introduction of **8.1** into the desired peptides was performed under standard SPPS conditions (HBTU, HOBt, $Et_3N$) in a 14 – 16 hours reaction (single coupling).




**Scheme 8.2**: Synthesis of the functionalized arginine building block **8.1**. Reagents and conditions: (i) a) benzyl alcohol (1.3 eq), DCC (1.1 eq), DMAP (0.03 eq), DCM, rt, 20 h; b) TFA, DCM, rt, 16h; (ii) triphosgene (0.6 eq), DIPEA (eq), DCM, 0 °C to rt, 3 h. (iii) $HgCl_2$ (2 eq), DIPEA (2 eq), DCM, rt, 20 h; (iv) $H_2$, Pd/C (10 %), MeOH, rt, 3 h.

## 8.3 NPY Receptor Affinity, Agonism and Selectivity

The synthesized peptides were analyzed for NPY receptor binding by displacing Cy5-[$K^4$]hPP (c = 3 nM, $K_D$ = 5.62 nM, $Y_4R$) or Cy5-pNPY (c = 5 nM, $K_D$ = 5.2 nM, $Y_1R$, $Y_2R$, $Y_5R$).[5] Results are given as $K_i$ calculated according to the Cheng-Prusoff equation.[20] Additionally, some compounds were investigated for functional activity at the NPY $Y_4R$ or NPY $Y_2R$ in a steady state GTPase assay using Sf9 membranes co-expressing $hY_4R$ plus $G\alpha_{i2}$ and $G\beta_1\gamma_2$ plus RGS4 and $hY_2R$ plus $G\alpha_{i2}$ and $G\beta_1\gamma_2$, respectively. In the following, agonistic potencies are expressed as $EC_{50}$ values. Intrinsic activities (α, efficacy) refer to the maximal response induced by the standard agonist hPP (10 µM, $hY_4R$) or pNPY (10 µM, $hY_2R$).[21] A determination of NPY receptor affinity in case of the Cy5-labeled analogs was not possible due to the same excitability (635 nm, red diode laser) as Cy5-pNPY and Cy5-[$K^4$]hPP. The results are summarized in **Table 7.2**.

Initially, the design of subtype-selective fluorescent ligands was planned for all human NPY receptor subtypes ($Y_1R$, $Y_2R$, $Y_4R$ and $Y_5R$). Unfortunately, the truncated $Y_1R$ agonist (**8.2**), recently discovered by Zwanziger et al.,[22] didn't show $Y_1R$ selectivity in our test systems. Instead, this compound turned out to be an even more potent $Y_4R$ agonist ($Y_4R$: $K_i$ = 17 nM, $Y_1R$: $K_i$ = 38 nM). Therefore, **8.2** was not included in the fluorescence labeling.

In contrast, the replacement of $Arg^{18}$ in the $Y_2R$ agonist pNPY(18-36) by the functionalized arginine building block resulted in acceptable $Y_2R$ affinity (**8.3**: $K_i$ = 21 nM) despite of considerably lower $Y_2R$ binding compared to the results published for unmodified pNPY(18-36) ($K_i$ = 0.25 nM).[17] In addition, the selectivity profile of **8.3** was retained. After introduction of the fluorescent dyes (**8.3-SFX**, **8.3-alexa** and **8.3-Cy5**) the $Y_2R$ affinity, potency and selectivity remained nearly unchanged ($K_i$ (**8.3-SFX**) = 15 nM, $K_i$ (**8.3-alexa**) = 20 nM and $EC_{50}$ (**8.3-Cy5**) = 10 nM) compared to the unlabeled peptide **8.3** ($K_i$ = 21 nM).

The replacement of $Arg^{25}$ in the $Y_4R$ partial agonist [$cpen^{34}$]pNPY(25-36) by the functionalized arginine building block resulted in similar $Y_4R$ affinity in the low nanomolar range (**8.4**: $K_i$ = 6.8 nM vs. $K_i$ = 10.1 nM) but a reduced subtype selectivity with regard to the $Y_1R$ ($K_i$ = 509 nM) and the $Y_2R$ ($K_i$ = 534 nM), compared to the corresponding parent compound [$cpen^{34}$]pNPY(25-36) ($Y_1R$ > 1000 nM, $Y_2R$ >1000 nM). Fluorescence labeling of compound **8.4** was well tolerated and resulted only in slight decrease (2-fold) in $Y_4R$ affinity and potency ($K_i$ (**8.4-SFX**) = 25 nM, $K_i$ (**8.4-alexa**) = 21 nM and $EC_{50}$ (**8.4-Cy5**) = 66 nM) compared to **8.4** ($K_i$ = 10 nM, $EC_{50}$ = 28 nM). Furthermore, the introduction of the fluorescent dyes led to an increase in $Y_4R$ selectivity ($K_i$ at $Y_1R$ and $Y_2R$ > 1000 nM) compared to **8.4**.

The $Y_5R$ selective peptide [$Ala^{31}$,$Aib^{32}$]pNPY **8.5**, previously reported by Cabrele et al.[10], revealed a 3-fold lower affinity to the $Y_5R$ in our test systems ($K_i$ = 23 nM), using HEC-1B-$Y_5$ cells[23] as described in literature ($K_i$ = 5.9 nM). As expected, the introduction of a fluorophore into this peptide of considerably high molecular weight was tolerated without remarkable decrease in $Y_5R$ affinity.

**Table 8.2**: Potencies, efficacies and affinities of synthesized peptides **8.2** – **8.5** and the corresponding fluorescently-labeled analogs at the different NPY receptor subtypes.

| No. | $Y_1R$ | $Y_2R$ | | | $Y_4R$ | | | $Y_5R$ |
|---|---|---|---|---|---|---|---|---|
| | $K_i$ [nM][a] | $K_i$ [nM][a] | $EC_{50}$ [nM][b] | α | $K_i$ [nM][a] | $EC_{50}$ [nM][b] | α | $K_i$ [nM][a] |
| **pNPY (18-36)** | 2700[17] | 0.25[17] | - | - | - | - | - | - |
| **[cpen[34]] NPY(25-36)** | > 1000 | > 1000 | - | - | 10 ± 2 | 38 ± 6 | 0.72 | > 500 |
| **8.2** | 38 ± 13 | > 1000 | n.d. | n.d. | 17 ± 5 | 48 ± 20 | 0.98 | > 1000 |
| (Lit.)[22] | 29.7 ± 6.8 | > 1000 | - | - | > 1000 | - | - | > 1000 |
| **8.3** | > 1000 | 21 ± 11 | 43 ± 7 | 1.10 | > 1000 | n.d. | n.d. | > 1000 |
| **8.4** | 509 ± 164 | 534 ± 118 | n.d. | n.d. | 6.8 ± 2.8 | 38 ± 8.8 | 0.67 | 466 ± 36 |
| **8.5** | > 1000 | > 1000 | n.d. | n.d. | > 1000 | n.d. | n.d. | 23 ± 8 |
| (Lit.)[10] | > 1000 | 760 | - | - | > 1000 | - | - | 5.9 |
| **8.3-SFX** | > 1000 | 9 ± 5 | 36 ± 9 | 1.00 | > 1000 | n.d. | n.d. | > 1000 |
| **8.3-alexa** | > 1000 | 20 ± 5 | 30 ± 1 | 1.02 | > 1000 | n.d. | n.d. | > 1000 |
| **8.3-cy5** | n.d. | n.d. | 10 ± 2 | 0.92 | n.d. | n.d. | n.d. | n.d. |
| **8.4-SFX** | > 1000 | > 1000 | n.d. | n.d. | 25 ± 8 | 117 ± 12 | 0.68 | 391 ± 63 |
| **8.4-alexa** | > 1000 | > 1000 | n.d. | n.d. | 21 ± 7 | 34 ± 7 | 0.65 | 325 ± 42 |
| **8.4-cy5** | n.d. | n.d. | n.d. | n.d. | n.d. | 66 ± 2 | 0.56 | n.d. |
| **8.5-SFX** | > 1000 | > 1000 | n.d. | n.d. | > 1000 | n.d. | n.d. | 36 ± 8 |

[a] Flow cytometric binding assay using Cy5-[$K^4$]-hPP (c = 3 nM, $K_D$ = 5.62 nM) in CHO-h$Y_4$-q$i_5$-mtAEQ cells or Cy5-pNPY (c = 5 nM, $K_D$ = 5.2 nM) in HEL ($Y_1$R), CHO-h$Y_2$-qi5-mtAEQ and HEC-1-B-Y5 cells; mean ± SEM from 2 or 3 independent experiments performed in triplicate. [c] Flow cytometric binding assay using 5 nM Cy5-pNPY (c = 5 nM, $K_D$ = 5.2 nM) in CHO-h$Y_2$ cells, HEL ($Y_1$) and HEC-1B-$Y_5$ cells, respectively. [b] Functional [γ-$^{33}$P]GTPase assay using membrane preparations of Sf9 cells expressing the h$Y_4$R + h$Y_4$R G$\alpha_{i2}$ + G$\beta_1\gamma_2$ +RGS4 or h$Y_2$R + G$\alpha_{i2}$ + G$\beta_1\gamma_2$; the intrinsic activity (α) of hPP ($Y_4$R) or pNPY ($Y_2$R) was set to 1.0 and α-values of other compounds were referred to this value; mean ± SEM from 2 independent experiments performed in duplicate.

## 8.4 Fluorescence Properties of the Labeled Peptides

The fluorescence properties of the labeled peptides are summarized in **Table 8.4**. The fluorescence quantum yields were determined (reference: cresyl violet perchlorate (for Cy5) and quinine sulfate

(for 5(6)-SFX and Alexa Fluor 488)) in phosphate buffered saline (PBS) at pH 7.0 and in PBS with 1 % bovine serum albumin (BSA) to simulate assay conditions and to study the influence of proteins on the fluorescence properties.

**Table 8.3**: Spectroscopic properties of the fluorescent labeled peptides **8.3** and **8.4**: Influence of protein (BSA) on the quantum yield **Φ** (reference: cresyl violet perchlorate or quinine sulfate) as well as excitation/emission maxima.

| No. | Dye | PBS | | PBS + 1 % BSA | |
|---|---|---|---|---|---|
| | | $\lambda_{ex}/\lambda_{em}$ [nm] | Φ [%] | $\lambda_{ex}/\lambda_{em}$ [nm] | Φ [%] |
| **8.3-cy5** | Cy5 | 651/667 | 6.7 ± 0.5 | 654/669 | 31.5 ± 3.0 |
| **8.3-alexa** | Alexa Fluor 488 | 498/520 | 15.7 ± 2.4 | 498/520 | 12.7 ± 1.9 |
| **8.3-SFX** | 5(6)-SFX | 500/528 | 17.1 ± 2.6 | 501/526 | 13.5 ± 2.4 |
| **8.4-cy5** | Cy5 | 650/666 | 5.2 ± 0.2 | 652/668 | 7.3 ± 0.1 |
| **8.4-alexa** | Alexa Fluor 488 | 496/526 | 16.6 ± 2.3 | 499/525 | 15.9 ± 3.5 |
| **8.4-SFX** | 5(6)-SFX | 498/518 | 13.7 ± 2.0 | 499/520 | 15.1 ± 3.0 |

The Cy-5 labeled peptide **8.3** revealed the highest quantum yield (QY) in PBS with 1 % BSA (31.5 %) and a very low quantum yield in pure PBS (7 %). Possible reasons for this increase after BSA addition are intermolecular interactions, particularly hydrophobic and electrostatic interactions with the protein. Furthermore, binding of the fluorescent ligands to proteins can lead to a rigidization, which generally results in an increase in fluorescence intensity and therefore in quantum yield. Thus, when BSA-free buffer is used as medium to perform flow cytometric binding assays with **8.3-Cy5**, the increase in fluorescence intensity may reflect the increase in the receptor bound state. However, an increase in fluorescence intensity can also be related to non-specific interactions of the ligand with other proteins or with the cell membrane. Nevertheless, the Stokes shift seems not to be influenced by BSA-addition. Surprisingly, the addition of BSA doesn't have a great impact on fluorescence quantum yield in case of the Cy5-labeled peptide **8.4** (PBS: 5 %, PBS + 1 % BSA: 7 %). The Alexa Fluor and fluorescein labeled peptides **8.3** and **8.4** revealed similar quantum yields between 15 and 17 % in PBS as well as in PBS + 1 % BSA. Obviously, in these cases the spectroscopic properties are less sensitive to rigidization and protein binding.

The absorption and corrected emission spectra of the fluorescence labeled peptides **8.3** and **8.4** in PBS containing 1 % of BSA are depicted in **Figure 8.3**. The Cy5-labeled peptides show only low Stokes shifts (≈ 15 - 20 nm) and are excitable at 635 nm with the red diode laser, which is commonly used in flow cytometry. The Alexa Fluor 488 and 5(6)-SFX-labeled peptides show low Stokes shifts of 20 to 30 nm, either, and can be excited by the argon laser at 488 nm.

In conclusion, due to their affinities and fluorescence properties all synthesized fluorescent ligands should be suitable for application in flow cytometric binding studies at equilibrium and confocal microscopy. Thereby, it is noticeable that, in principle, due to different excitation wavelengths, the Alexa Fluor 488 as well as the 5(6)-SFX labeled subtype selective ligands can be investigated in the presence of the cyanine-labeled fluorescent compounds in multi-parametric assays or in colocalisation experiments.

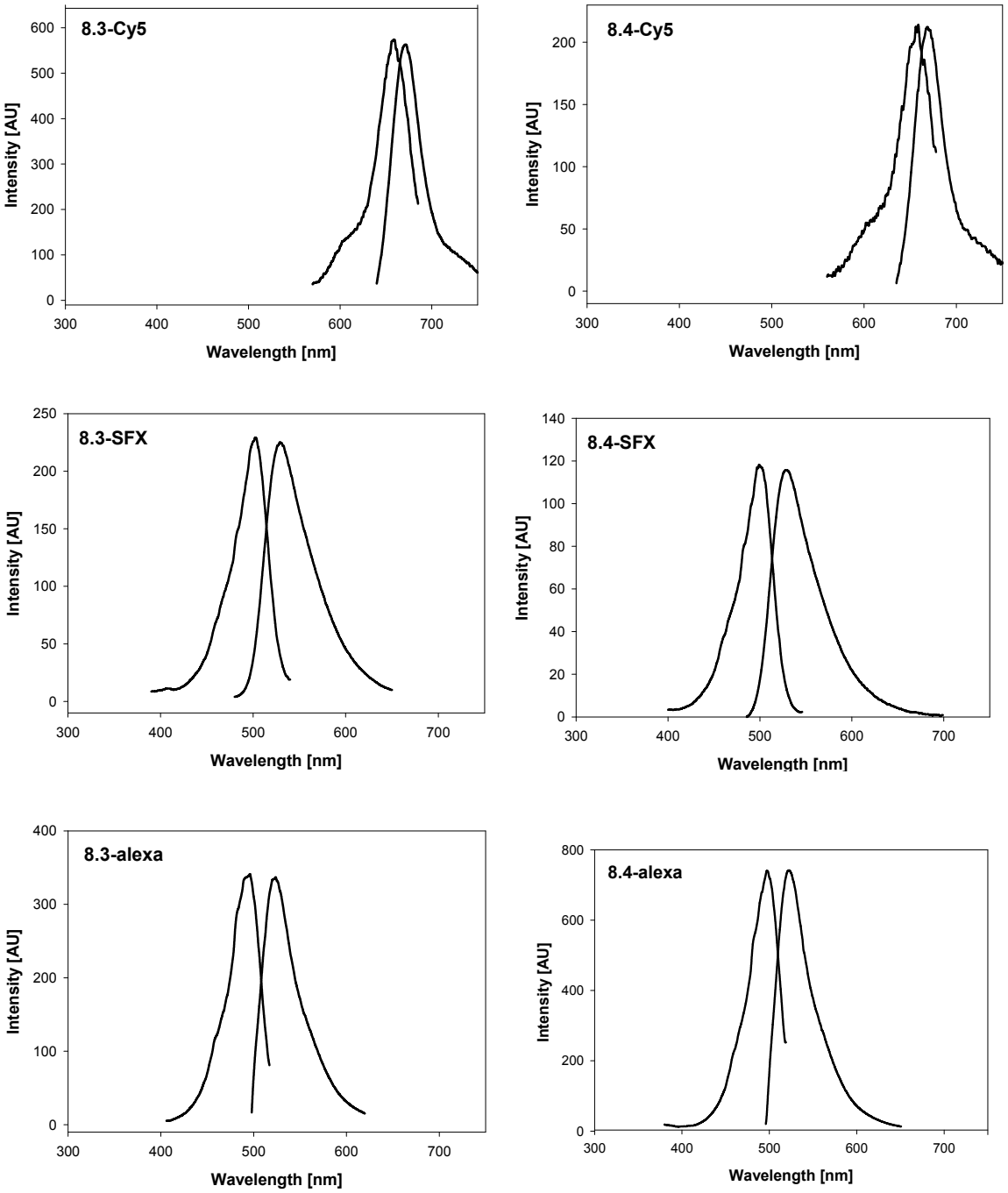



**Figure 8.3**: Absorption and corrected emission spectra in PBS + 1 % BSA exemplified for 5(6)-SFX, Alexa Fluor 488 and Cy5-labeled peptides **8.3** and **8.4** (recorded at 22 °C).

### 8.4.1 Application of Subtype-Selective Fluorescently-Labeled Peptides to Confocal Laser Scanning Microscopy

All Cy-5 and Alexa Fluor labeled peptides (**8.3-Cy5**, **8.4-Cy5**, **8.5-Cy5**, **8.3-alexa**, **8.4-alexa**) as well as **8.5-SFX** were screened for their applicability in confocal laser scanning microscopy on CHO cells expressing the $hY_2R$ or $hY_4R$ or on HEC-1-B-$Y_5$ cells. The non-specific binding was determined in the presence of the corresponding non-fluorescent ligands, namely BIIE 0246 ($Y_2R$), GW1229 ($Y_4R$) and pNPY ($Y_5R$) in 50 to 100-fold excess. For total as well as for non-specific binding, concentrations around the $K_D$ value (mostly 2x $K_D$) were used.

As shown in (**Figure 8.4**) specific binding to the cell membrane was detected by confocal microscopy. However, the extent of specifically bound Cy5-labeled ligands (**Figure 8.4**) differed widely between moderate (**8.4-Cy5**, **Figure 8.4**, panel B) to high (**8.3-Cy5** and **8.5-Cy5**, **Figure 8.4**, panel A and C).
In case of the $Y_5R$ ligand **8.5-Cy5** (**Figure 8.4**, panel C), bearing the closest similarity with the endogenous ligand pNPY, fluorescence could also be detected inside the cells (cytoplasma). All investigated Cy5-labeled peptides can be completely displaced by non-fluorescent competitors, i.e. the non-specific binding is very low.

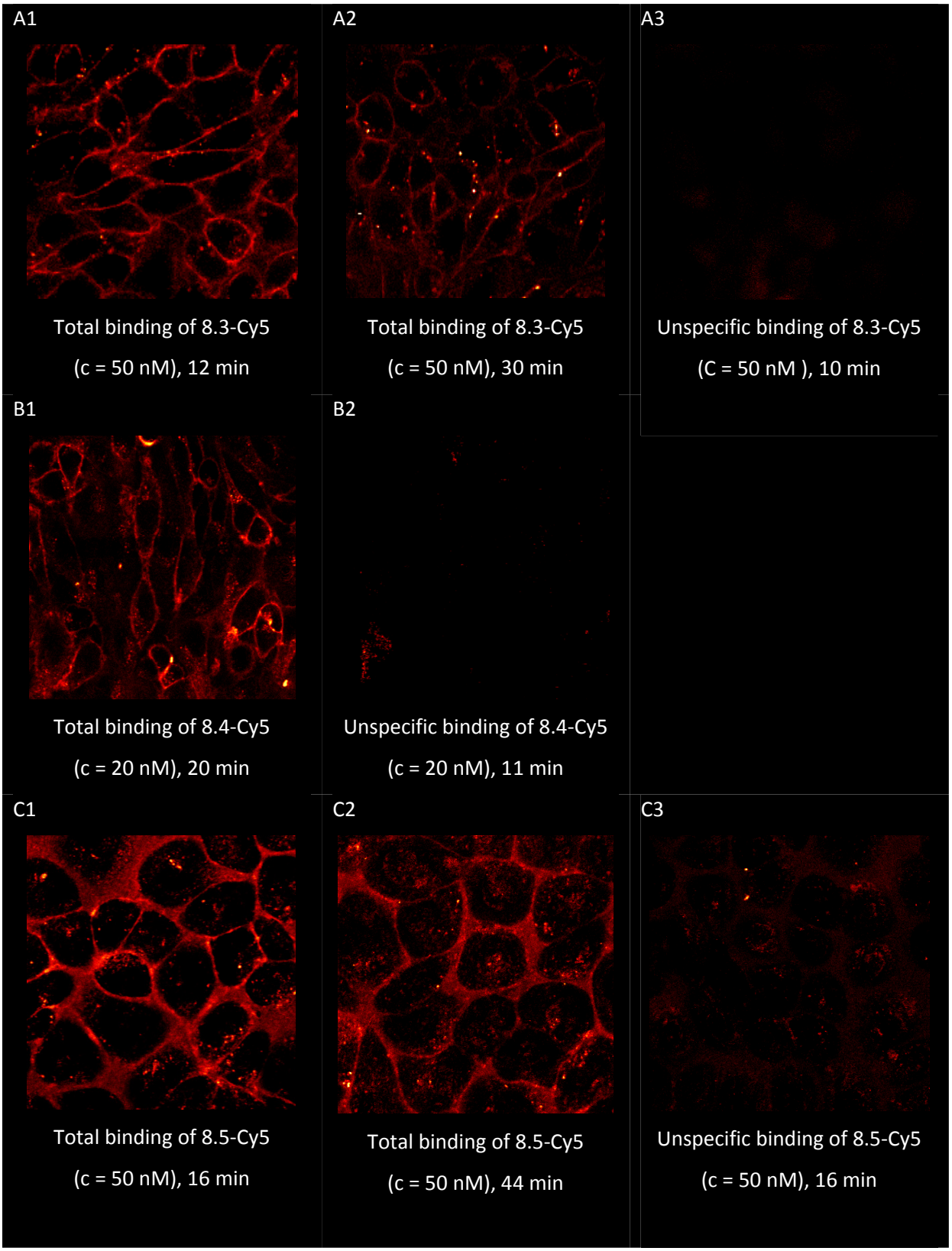



**Figure 8.4**: Binding of the fluorescent ligands **8.3-Cy5** (panel A), 8.4-Cy5 (panel B), **8.5-Cy5** (panel C) to $Y_2R$, $Y_4R$ and $Y_5R$ expressed in CHO-h$Y_2R$, CHO-h$Y_4$ and HEC-1-B-$Y_5$ cells, visualized by confocal microscopy. Non-specific binding was determined in the presence of BIIE 0246 ($Y_2R$, 100-fold excess), GW1229 ($Y_4R$, 50-fold-excess) and pNPY ($Y_5R$, 50-fold excess). Cells were incubated with the fluorescent ligands at rt in Leibowitz L15 culture medium. All images were acquired with a Zeiss Axiovert 200 M microscope.

Fluorescence at the cell membrane could also be observed for the green-fluorescent-labeled peptides at low concentrations (1-2x $K_D$). Nevertheless, the determination of total and non-specific binding of Alexa Fluor 488 and 5(6)-SFX labeled peptides (**8.3-alexa**, **8.4-alexa** and **8.5-SFX**, **Figure 8.5**) is quite challenging due to the high background fluorescence caused by the autofluorescence of the cells at an excitation wavelength of 488 nm. Using a "small pinhole" (124 µm) as in case of **8.3-alexa** (**Figure 8.5**, panel A), revealed only a weak fluorescence signal at the cell membranes. Only with the aid of an overlay of the fluorescence image with a light transmission picture of the cells, it could be proven that **8.3-alexa** indeed binds to the cell membranes (**Figure 8.5**, A1) and shows a difference in total and non-specific binding. Expanding the pinhole (212 µm) as in case of **8.4-alexa** (**Figure 8.5**, panel B), led to enhanced fluorescence intensity, but also to an increase in background fluorescence. Nevertheless, a distinct binding of **8.4-alexa** to the cell membranes could be observed. Additionally, the fluorescent labeled peptide could be completely removed by GW1229 (**Figure 8.5**, B2). Compound **8.5-SFX**, labeled with a fluorescein derivative comprised a reduced fluorescent intentsity compared to Alexa Fluor 488 labeled peptide **8.3** under identical measurement conditions (same pinhole, same laser transmission). Obviously, **8.5-SFX** is more sensitive against photobleaching leading to a reduced fluorescence intensity and brightness even after shorter incubation periods compared to the Alexa Fluor-labeled compound. Hence, the Alexa Fluor 488 fluorophore seems to be superior to fluorescein with regard to confocal microscopy as already reported in literature.[19]

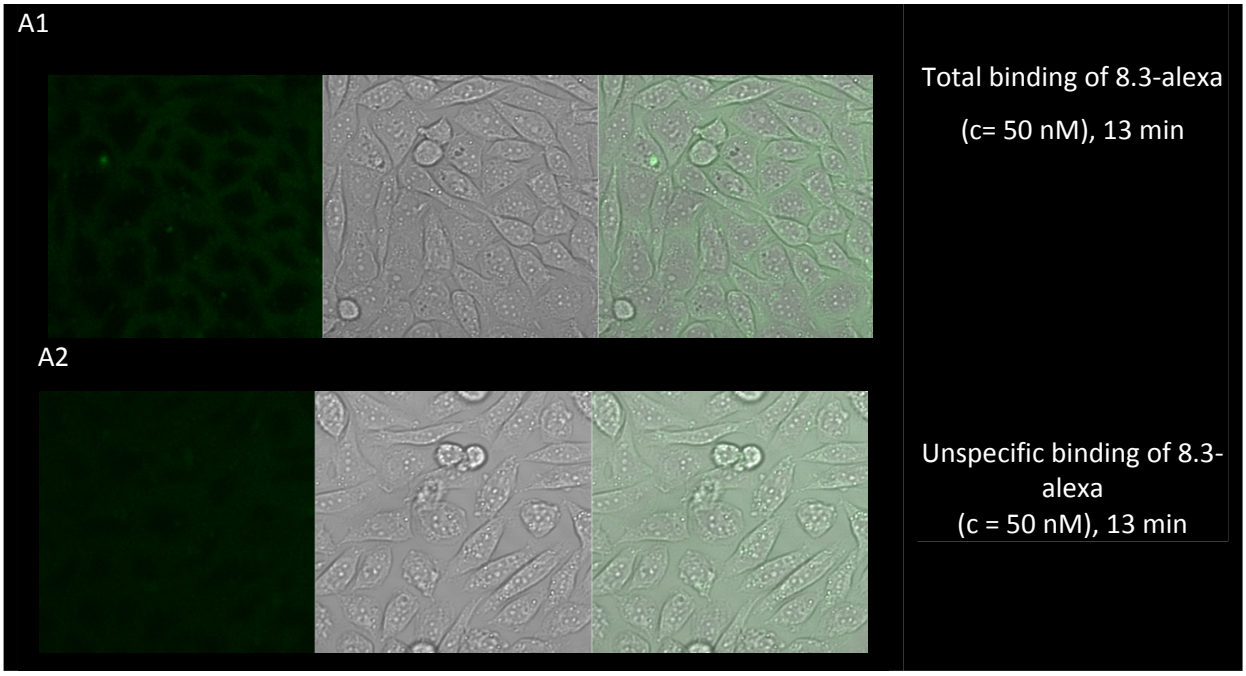



**Figure 8.5** continued on next page

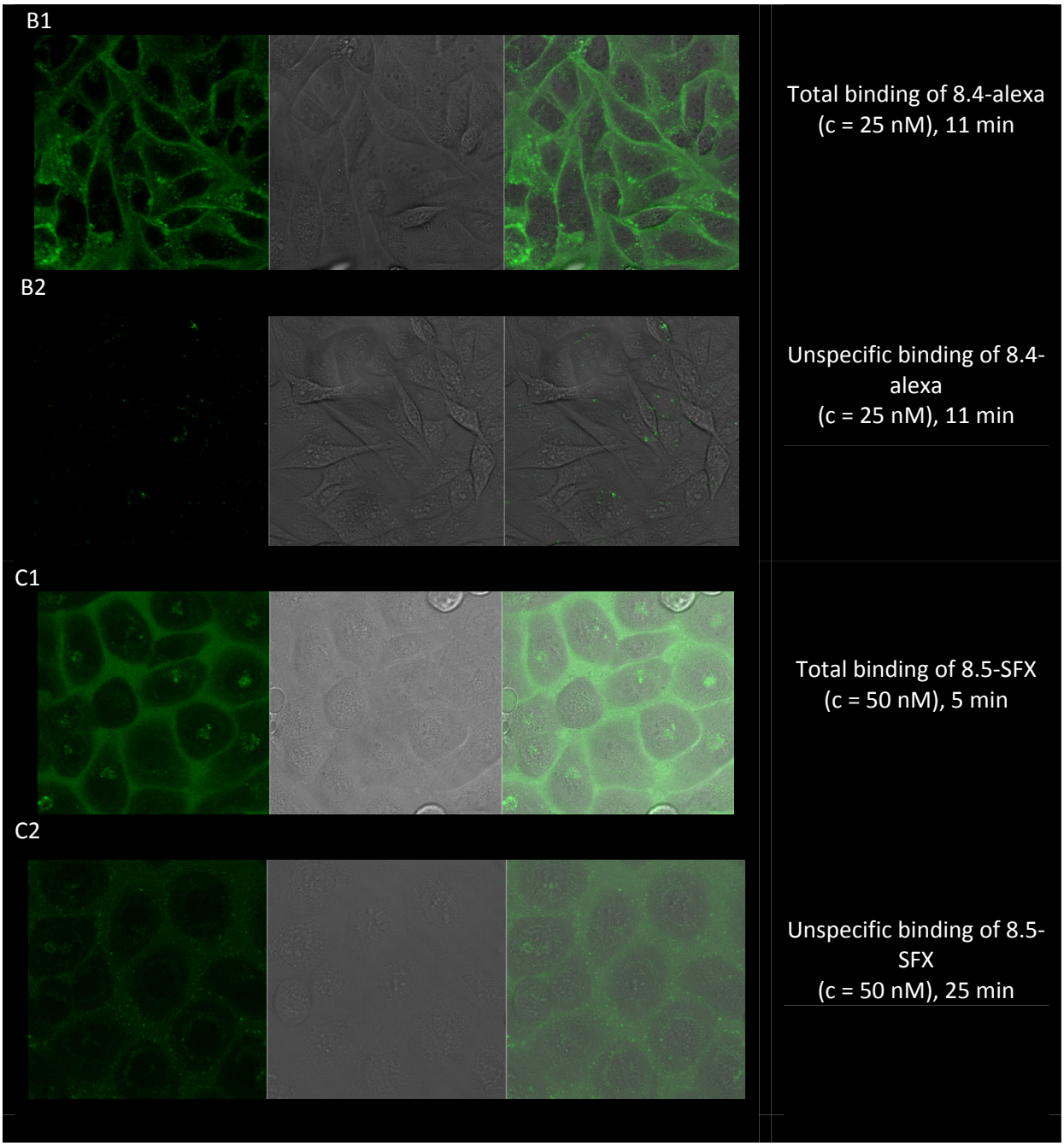



**Figure 8.5**: Binding of the fluorescent ligands **8.3-alexa** (panel A: fluorescence, transmission light picture and overlay), **8.4-alexa** (panel B, fluorescence, transmission light picture and overlay), **8.5-SFX** (panel C, fluorescence, transmission light picture and overlay) to $Y_2R$, $Y_4R$ and $Y_5R$ expressed in CHO-h$Y_2R$, CHO-h$Y_4$ and HEC-1-B-$Y_5$ cells, visualized by confocal microscopy. Non-specific binding was determined in the presence of BIIE 0246 ($Y_2R$, 100-fold excess), GW1229 ($Y_4R$, 50-fold-excess) and pNPY ($Y_5R$, 50-fold excess). Cells were incubated with the fluorescent ligands at rt in Leibowitz L15 culture medium. All images were acquired with a Zeiss Axiovert 200 M microscope.

## 8.4.2 Application of Fluorescence-Labeled Peptides in Flow Cytometry: Saturation, Kinetics and Competition Binding Experiments

The flow cytometric binding studies were performed with the same NPY receptor expressing cell lines as described above for confocal microscopy (section 8.4.1).

All Cy5-labeled peptides (**8.3-Cy5**, **8.4-Cy5** and **8.5-Cy5**) were investigated in saturation binding experiments (cf. **Figure 8.6**). Generally speaking, the Cy5-labeled peptides (**8.3-Cy5**, **8.4-Cy5** and **8.5-Cy-5**) showed low non-specific binding when the fluorescence was recorded in channel Fl-4 (661 ± 8 nm). The obtained $K_D$ values (**8.3-Cy5**: 31.7 ± 4.3 nM, **8.4-Cy5**: 7.1 ± 0.7 nM and **8.5-Cy5**: 19.5 ± 4.2 nM; mean values ± SEM; 2 – 3 independent experiments performed in duplicate) were in good accordance with the potencies determined in the GTPase assay.

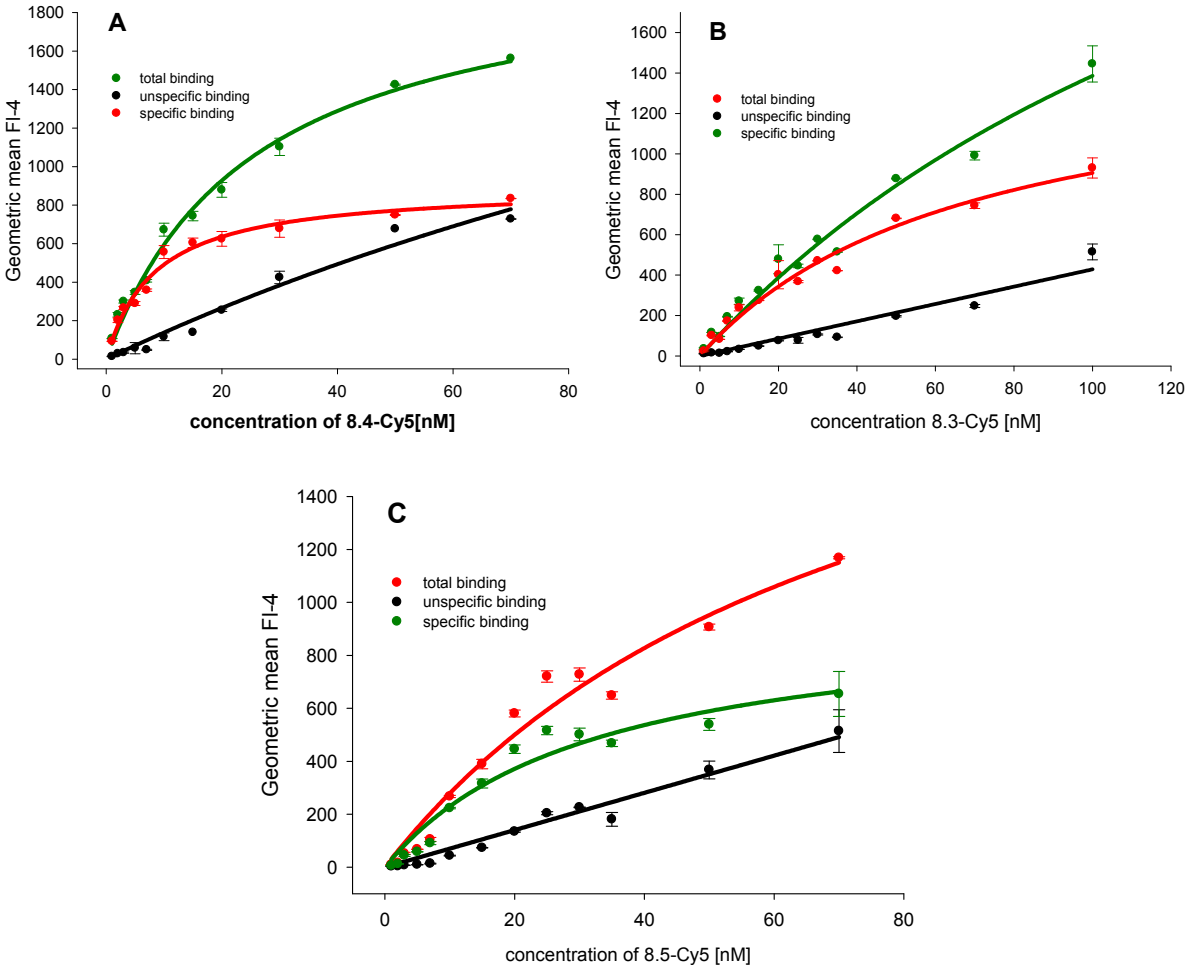



**Figure 8.6**: Flow cytometric saturation binding experiment with fluorescent ligand: (**A**) **8.4-Cy5** at CHO-h$Y_4$-qi5-mtAEQ cells. Unspecific binding was determined in the presence of GW1229 (100-fold excess). Determined $K_D$ value: 7.1 ± 0.7 nM (mean value ± SEM; three independent experiments performed in duplicate); (**B**) **8.3-Cy5** at CHO-h$Y_2$-qi5-mtAEQ cells. Unspecific binding was determined in the presence of BIIE 0246 (100-fold excess). Determined $K_D$ value: 31.7 ± 4.3 nM (mean value ± SEM; two independent experiments performed in duplicate); (**C**) **8.5-Cy5** at HEC-1B-$Y_5$ cells. Unspecific binding was determined in presence of GW1129 (100-fold excess). Determined $K_D$ value: 19.5 ± 4.2 nM (mean value ± SEM; two independent experiments performed in duplicate).

In addition, compound **8.4-Cy5** was investigated in dissociation studies to get more detailed information about the binding properties of this $Y_4R$ partial agonist. As outlined in **Figure 8.7** (B) fluorescent-labeled **8.4** showed a quite fast dissociation rate with a half-life of 2.0 min. After 15 min complete dissociation could be observed. By contrast, the dissociation of the endogenous ligand Cy5-[$K^4$]hPP was about 6 times slower, with a half-life of 12.5 min, reaching complete dissociation only after approximately 90 min (**Figure 8.7**: A). Therefore, **8.4-Cy5** can be considered an appropriate candidate for the application in competition binding experiments with improved properties compared to Cy5-[$K^4$]hPP, e.g. a reduced incubation time.

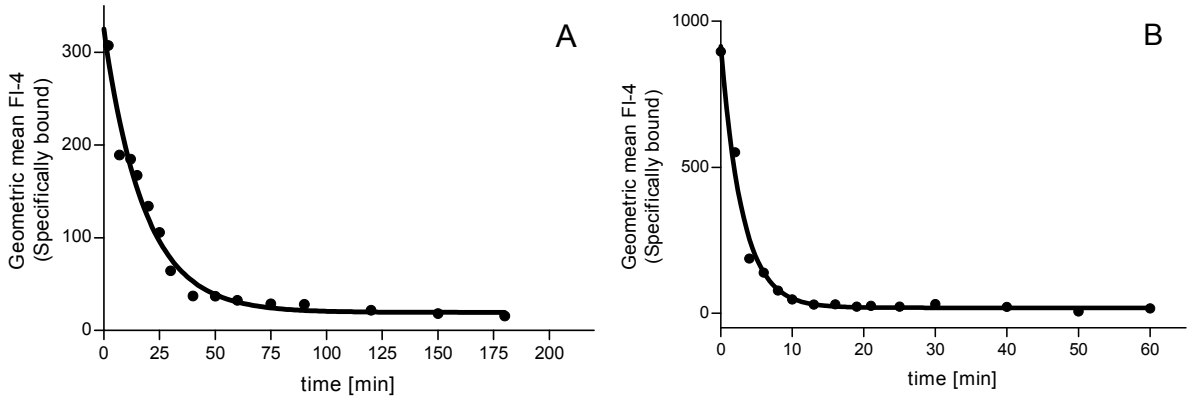



**Figure 8.7**: Dissociation of (**A**) Cy5-[$K^4$]-hPP (c = 3 nM, $t_{1/2}$ = 12.5 min) and (**B**) **8.4-Cy5** (c = 10 nM, $t_{1/2}$ = 2.0 min) from the NPY $Y_4R$ as a function of time in the presence of hPP (100-fold excess), monophasic exponential decay.

Subsequently, the applicability of fluorescent ligands **8.3-Cy5** and **8.4-Cy5** as standard labeled compounds for the determination of binding data was explored in competition binding experiments using several reference ligands, namely hPP and GW1129 ($Y_4R$, **Figure 8.8:** A) as well as BIIE 0246 and pNPY ($Y_2R$, **Figure 8.8:** B). Thereby, the fluorescence ligands were used at concentrations around their $K_D$ values. The $K_i$ values determined by displacement of **8.3-Cy5** from $Y_2R$ by BIIE 0246 and pNPY, and **8.4-Cy5** from $Y_4R$ by hPP and GW1229 (pNPY: 3.9 nM, BIIE 0246: 31.9 nM, hPP: 0.58 nM, GW1229: 0.29 nM, summary cf. **Table 8.4**) were in excellent agreement with results, which were previously obtained by equilibrium competition binding experiments using Cy5-pNPY ($Y_2R$)[7] and Cy5-[$K^4$]hPP ($Y_4R$)[5].

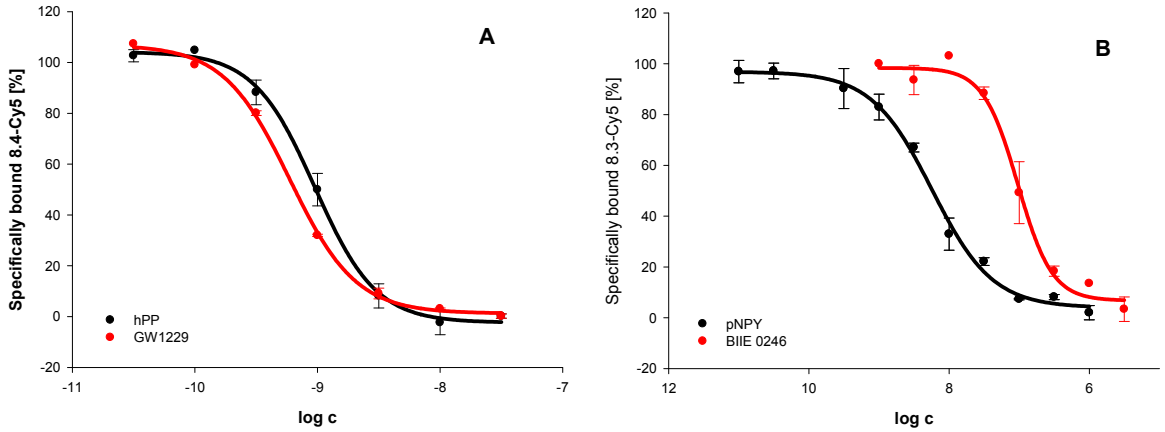



**Figure 8.8**: Flow cytometric $Y_4R$ competition binding assay with (**A**) **8.4-Cy5** (c = 10 nM) in the presence of hPP and GW1229 using CHO-h$Y_4$-qi5-mtAEQ cells. The geometrical mean values of fluorescence intensities (fluorescence channel Fl-4) obtained from the competition binding assay were converted to percentage inhibition values according to the procedure described previously.[7, 18] Samples were incubated for 60 min at room temperature. Calculated $K_i$ values, hPP: 0.58 ± 0.01 nM, GW1229: 0.29 ± 0.11 nM (mean values ± SEM, n = 2-3); (**B**) Flow cytometric $Y_2R$ competition binding assay with **8.3-Cy5** (25 nM) in presence of pNPY using CHO-h$Y_2$-qi5-mtAEQ cells. The geometrical mean values of fluorescence intensities (fluorescence channel Fl-4) obtained from the competition binding assay were converted to percentage inhibition values according to the procedure described previously.[7, 18] Samples were incubated for 90 min at room temperature. Calculated $K_i$ value of pNPY: 3.9 ± 1.5 nM (mean value ± SEM, n = 2).

Furthermore, saturation binding experiments as well as displacement studies using green fluorescent-labeled peptides were performed to test their applicability in flow cytometry when excitated by the argon laser (488 nm) and measuring the fluorescence in channel Fl-1. Unfortunately, the saturation binding experiments with 5(6)-SFX labeled peptides **8.3-SFX** and **8.4 SFX** (**Figure 8.9**: A and B) showed very high non-specific binding and only low fluorescence intensities for total binding, presumably due to insufficient photostability and a high rate of photobleaching.

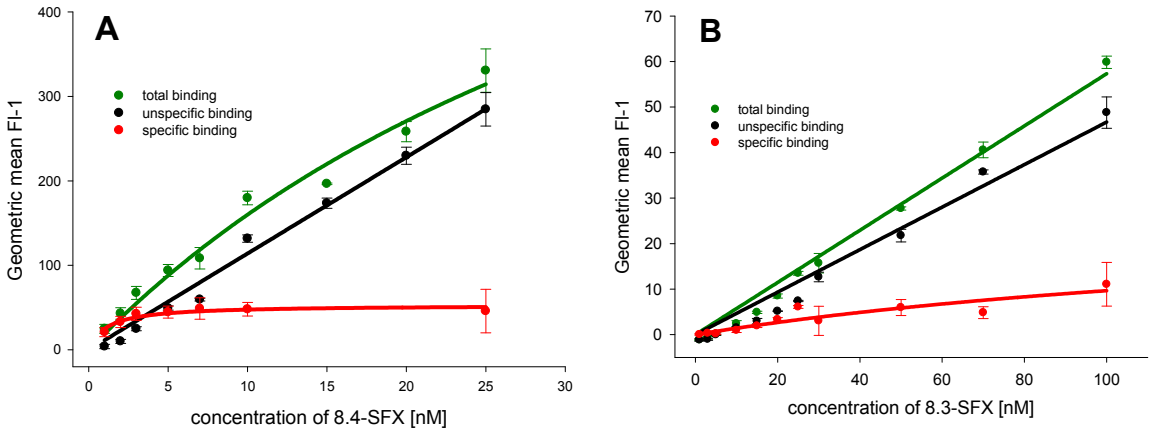



**Figure 8.9**: Flow cytometric saturation binding experiment with fluorescent ligand: (**A**) **8.4-SFX** at CHO-h$Y_4$-qi5-mtAEQ cells. Unspecific binding was determined in the presence of GW1229 (100-fold excess); (**B**) **8.3-SFX** at CHO-h$Y_4$-qi5-mtAEQ cells. Unspecific binding was determined in the presence of BIIE 0246 (100-fold excess).

In contrast, the Alexa Fluor 488 labeled $Y_4$R-selective **8.4-alexa** (**Figure 8.10**) revealed appropriate total and unspecific binding on CHO-h$Y_4$ cells under the same measurement conditions, confirming the advantages in terms of increased brightness, improved photostability and reduced photobleaching compared to the fluorescein derivatives.[19] Additionally, the determined $K_D$ value of 10.4 nM is in good agreement with the binding data determined by the displacement of Cy5-[K$^4$]hPP ($K_i$ = 21 nM). As the difference between specific and non-specific binding seemed to be acceptable in the range of the $K_D$ value, the equilibrium competition binding experiments with standard ligands were expected to reveal reasonable results at an acceptable signal-to-noise ratio. Indeed, the displacement of **8.4-alexa** by hPP and GW1229 ($K_i$ = 0.38 and 0.29 nM) gave highly reproducible results which were in good agreement with affinities determined with the corresponding Cy5-labeled derivative (**8.4-Cy5**) and Cy5-[K$^4$]hPP A(**Table 8.4**). The autofluorescence of the cells – often a problem at an excitation wavelength of 488 nm – was comparably low and was subtracted from the measured intensities.

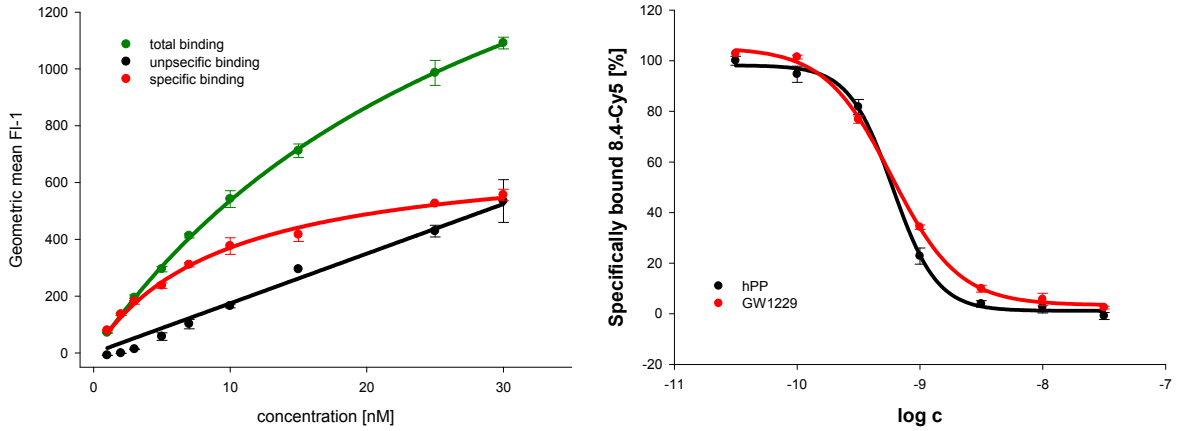



**Figure 8.10**: (A) Flow cytometric saturation binding experiment with fluorescent ligand **8.4-alexa** at CHO-h$Y_4$-qi5-mtAEQ cells. Unspecific binding was determined in the presence of GW1129 (100-fold excess). Determined $K_D$ value: 10.4 nM (mean value ± SEM; two independent experiments performed in duplicate); (B) Flow cytometric $Y_4$R competition binding assay with **8.4-alexa** (10 nM) in the presence of hPP and GW1229 using CHO-h$Y_4$. The geometrical mean values of fluorescence intensities (fluorescence channel Fl-1) obtained from the competition binding assay were converted to percentage inhibition values according to the procedure described previously.[7, 18] Samples were incubated for 60 min at room temperature. Calculated $K_i$ values: hPP = 0.38 ± 0.01 nM, GW1229 = 0.29 ± 0.12 nM (mean values ± SEM, n = 2-3)

**Table 8.4**: $K_i$ values of standard ligands determined in flow cytometric binding assay on CHO-h$Y_2$ and CHO-h$Y_4$ cells displacing different fluorescent ligands

| $Y_xR$ | Compd. | $K_i$ [nM] or ($pK_i$) values determined by flow cytometry through displacement of | | | | |
|---|---|---|---|---|---|---|
| | | **8.3-Cy5**[a] | **8.4-Cy5**[b] | **8.4-alexa**[c] | **Cy5-pNPY**[d] | **Cy5-[K$^4$]hPP**[e] |
| **$Y_2R$** | **pNPY** | 3.9 ± 1.5 | n.d. | n.d. | 0.8 ± 0.2[6] | n.d. |
| | **BIIE 0246** | 31.9 ± 19.2 | n.d. | n.d. | 10.2 ± 1.1[6] | n.d. |
| **$Y_4R$** | **hPP** | n.d. | 0.58 ± 0.05 | 0.38 ± 0.01 | n.d. | (9.62 ± 0.07)[5] |
| | **GW1129** | n.d. | 0.29 ± 0.11 | 0.29 ± 0.12 | n.d. | (9.66 ± 0.09)[5] |

[a] $K_D$ =31.7 nM (Fl-4), c = 25 nM. [b] $K_D$ = 7.1 nM (Fl-4), c = 10 nM. [c] $K_D$ = 10.4 nM (Fl-1), c = 10 nM. [d] $K_D$ = 5.2 nM (Fl-4), c = 5 nM. [e] $K_D$ = 5.62 nM (Fl-4), c = 3 nM.

## 8.5 Summary and Conclusion

The synthesis of fluorescence-labeled, subtype-selective peptides for the different NPY receptor subtypes was successful. The selected peptides showed $Y_xR$ subtype affinities in the low nanomolar range, which remained essentially unchanged after fluorescence labeling. Besides, the replacement of N-terminal arginine by an $N^G$-functionalized arginine building block allowed for an alternative to Lys-side chain labeling. The synthesis of the corresponding building block and its application in SPPS were optimized. The red-fluorescent compounds were superior to the green fluorescent ligands in flow cytometry and confocal microscopy due to excellent signal-to-noise ratios. As expected, the applicability of green-fluorescent compounds proved to be limited due to overlap of ligand-bound fluorescence with high background fluorescence (autofluorescence) of the cells. Comparison of fluorescein, a quite popular green-fluorescent dye commonly used over the last decades, with a new dye, Alexa Fluor 488, revealed significant differences concerning fluorescent properties. Despite of similar quantum yields, an application of the fluorescein derivative 5(6)-SFX in flow cytometry failed presumably because of fast photobleaching. Flow cytometric investigation of the Alexa Fluor labeled peptide instead, gave sound saturation binding curves and allowed to perform displacements studies with standard ligands. Furthermore, improved brightness and reduced susceptibility towards photobleaching was observed for the Alexa Flour labeled peptides when used in confocal microscopy.

In summary, the subtype-selective green- and red-fluorescent ligands represent valuable pharmacological tools for the investigation of NPY receptors. This is of special interest with respect to the $Y_4R$, since high affinity selective ligands and appropriate pharmacological tools are still lacking for this NPY receptor subtype. Despite the general disadvantages of green fluorescence in cellular imaging, Alexa Fluor labeled peptides proved to superior compared to the fluorescein derivatives in

terms of fluorescence properties and could offer alternatives and complementary tools to red fluorescence labeled ligands with regards to multi-parametric assays and colocoalisation experiments.

# 8.6 Experimental Section

## 8.6.1 Chemistry

### 8.6.1.1 General Conditions

See section 3.3.1.1

### 8.6.1.2 Preparation of the Functionalized Arginine Building Block 8.1

**(*S*)-$N^{\alpha}$-(9-Fluorenylmethoxycarbonyl)ornithine benzyl ester (8.7)[24]**

(*S*)-$N^{\omega}$-Boc-$N^{\alpha}$-Fmoc-ornithine (2.5 g, 5.5 mmol, 1.0 eq) was suspended in DCM (200 mL). Benzyl alcohol (0.77 mL, 7.2 mmol, 1.3 eq) was added resulting in a decrease in the amount of white precipitate. After addition of DCC (1.25 g, 6.1 mmol, 1.1 eq) the mixture became completely clear. A catalytic amount of DMAP (20 mg, 0.17 mmol, 0.03 eq) was added and the mixture was stirred at rt for 20 h. The white solid (DCU) was removed by filtration and the solvent was concentrated under reduced pressure. The intermediate was purified by flash chromatography (DCM/MeOH 100/0 – 95/5 v/v) yielding a white solid, which was dissolved in DCM (150 mL). TFA (10 mL) was added and the mixture was stirred at rt for 16 h, Subsequently, the clear solution was concentrated under reduced pressure, and the remaining yellow oil was suspended in water (50 mL). Lyophilisation afforded the product as a white solid (2.03 g, 93 %). $^{1}$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.70 (bs, 3H, CH-C***H***$_2$-C***H***$_2$), 1.89 (bs, 1H, CH-C***H***$_2$-CH$_2$), 2.93 (bs, 2H, CH-(CH$_2$)$_2$-C***H***$_2$-NH), 4.05 – 4.16 (m, 1H, C***H***$^{\alpha}$), 4.20 – 4.45 (m, 3H, C***H***-C***H***$_2$, Fmoc), 5.07 (s, 2H, Ph-C***H***$_2$), 5.70 – 5.82 (m, 1H, N***H***), 7.13 – 7.39 (m, 9H, Ph-***H*** + Fmoc-***H***), 7.46 – 7.57 (m, 2H, Fmoc), 7.70 (d, 2H, $^{3}J$ = 7.5 Hz, Fmoc-***H***), 7.77 (bs, 3H, N***H***$_3^+$); $^{13}$C-NMR (75 MHz, $CDCl_3$): δ (ppm) 23.46 (-, CH-(***C***H$_2$)$_2$), 29.20 (-, CH-(***C***H$_2$)$_2$), 39.34 (-, ***C***H$_2$-NH$_2$), 46.94 (+, ***C***H-CH2, Fmoc), 53.33 (+, ***C***H$^{\alpha}$), 67.35 (-, ***C***H$_2$-O-CO), 67.35 (-, ***C***H$_2$-O-CO), 119.98 (+, 2 Fmoc-***C***), 127.11 (+, 2 Fmoc-***C***), 127.76 (+, 2 Fmoc-***C***), 128.30 (+, 2 Ph-C + Ph-***C***-4), 128.59 (+, Fmoc-***C***), 128.64 (+, 2 Ph-***C***), 134.93 (C$_{quat}$, Ph-***C***-1), 141.24 (C$_{quat}$, 2 Fmoc-***C***), 143.66 (C$_{quat}$, 2 Fmoc-***C*** ), 156.48 (C$_{quat}$, O-***C***O-NH), 171.81 (C$_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 445 (100) [M + H]$^+$. $C_{27}H_{28}N_2O_4$ · TFA (558.54).

***N*-(*tert*-Butoxycarbonyl)butane-1,4-diamine (8.8)[25]**

A solution of $(Boc)_2O$ (5.46 g, 25.0 mmol, 1.0 eq) in 85 mL dioxane was added dropwise to a solution of butane-1,4-diamine (17.6 g, 200 mmol, 8.0 eq) in 85 mL dioxane over a period of 2.5 h. After complete addition, the reaction mixture was stirred for 22 h at rt. The solvent was evaporated and the residue was taken up in water (200 mL). The precipitated di-Boc-protected by-product was removed by filtration. The filtrate was extracted with DCM (3x 100 mL). Drying of the organic phase over $MgSO_4$ and removal of the solvent *in vacuo* afforded the product as pale yellow oil (5.06 g, 70 %). $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.38 (s, 9H, C(C***H***$_3$)$_3$), 1.41 (m, 4H, C***H***$_2$), 2.65 (t, $^3J$ = 6.6 Hz, 2H, C***H***$_2$-$NH_2$), 3.06 (m, 2H, C***H***$_2$-NHBoc); $^{13}$C-NMR (75 MHz, $CDCl_3$): δ (ppm) 27.46 (-, ***C***$H_2$), 28.39 (+, C(***C***$H_3$)$_3$), 30.92 (-, ***C***$H_2$), 40.39 (-, ***C***$H_2$-N), 41.82 (-, ***C***$H_2$-N), 78.90 ($C_{quat}$, ***C***$(CH_3)_3$), 156.01 ($C_{quat}$, ***C***=O). MS (CI, $NH_3$) *m/z* (%): 189 (100) $[M+H]^+$. $C_9H_{20}N_2O_2$ (188.27).

***N*-*tert*-Butoxycarbonyl-*N'*-[*N*-(4-*tert*-butoxycarbonylaminobutyl)aminocarbonyl]-*S*-methylisothiourea (8.9)[11]**

A solution of *N*-(*tert*-butoxycarbonyl)butan-1,4-diamine **8.8** (0.94 g, 5.0 mmol, 1.0 eq) and DIEA (2.61 mL, 15.0 mmol, 3.0 eq) in anhydrous DCM (30 mL) was added dropwise to a solution of triphosgene (0.89 g, 3.0 mmol, 0.6 eq) in 15 mL DCM over a period of 30 min under argon atmosphere and cooling with ice. *N*-*tert*-butoxycarbonyl-*S*-methylisothiourea **3.3** (0.95 g, 5.0 mmol, 1.0 eq) was added and stirring was continued for 2.5 h. After removal of the solvent under reduced pressure, the residue was directly subjected to column chromatography (PE/EE 9:1) yielding **8.9** as white solid (1.75 g, 77%); $^1$H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.43 (s, 9H, C(C***H***$_3$)$_3$), 1.48 (s, 9H, C(C***H***$_3$)$_3$), 1.50 – 1.60 (m, 4H, NH-$CH_2$-(C***H***$_2$)$_2$) 2.32 (s, 3H, S-C***H***$_3$), 3.07 – 3.18 (m, 4H, C***H***$_2$-NH), 3.19 – 3.29 (m, 2H, C***H***$_2$-NH); $^{13}$C-NMR (75 MHz, $CD_3OD$): δ (ppm) 14.38 (+, S-***C***$H_3$), 26.94 (-, NH-$CH_2$-***C***$H_2$), 27.57 (-, NH-$CH_2$-***C***$H_2$), 28.03 (+, C(***C***$H_3$)$_3$), 28.42 (+, C(***C***$H_3$)$_3$), 9.76 (-, ***C***$H_2$-NH), 40.16 (-, ***C***$H_2$-NH), 79.22 ($C_{quat}$, ***C***$(CH_3)_3$), 82.77 ($C_{quat}$, ***C***$(CH_3)_3$), 149.41 ($C_{quat}$,***C***=O), 151.05 ($C_{quat}$, ***C***=O), 156.03 ($C_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) *m/z* (%): 405 (100) $[M+H]^+$. $C_{17}H_{32}N_4O_5S$ (404.52).

**(*S*)-$N^{\omega}$-*tert*-Butoxycarbonyl-$N^{\omega'}$-[4-(*tert*-butoxycarbonylamino)butylaminocarbonyl]-$N^{\alpha}$-(9-fluorenylmethoxycarbonyl)arginine benzyl ester (8.10)[11]**

Compound **8.9** (1.42 g, 3.5 mmol, 1 eq), $HgCl_2$ (1.90 g, 7.0 mmol, 2.0 eq) and **8.7** (1.56 g, 3.5mmol, 1.0 eq) were dissolved in 60 mL $DCM_{abs}$. DIEA (0.98 mL, 7.0 mmol, 2 eq) was slowly added and the reaction mixture was stirred for 16 h at room temperature. After TLC control, another equivalent of DIEA was added and the reaction mixture was stirred for further 4 h to guarantee complete conversion of the starting material. Subsequently, the reaction was filtrated over Celite, the solvent was evaporated and the crude product was directly subjected to column chromatography (PE/EtOAc

50/50 v/v) yielding the product as colorless foam (2.04 g, 73 %). $^{1}$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.36 (bs, 12 H, C(C***H***$_3$)$_3$ + CH-CH$_2$-C***H***$_2$), 1.43 and 1.46 (s, split signal, C(C***H***$_3$)$_3$ + CH-C***H***$_2$), 1.50 – 1.60 (m, 4H, NH-CH$_2$-(C***H***$_2$)$_2$), 2.83 – 2.95 (m, 2H, CH-(CH$_2$)$_2$-C***H***$_2$-NH), 2.96 – 3.10 (m, 2H, C***H***$_2$-NH), 3.23 – 3.34 (m, 2H, C***H***$_2$-NH), 4.07 – 4.16 (m, 1H, C***H***$^{\alpha}$), 4.18 – 4.35 (m, 2H, CH-C***H***$_2$, Fmoc), 5.12 (s, Ph-C***H***$_2$), 7.26 – 7.36 (m, 7H, Ph-***H*** + Fmoc-***H***), 7.37 – 7.45 (m, 2H, Fmoc-***H***), 7.71 (d, $^{3}J$ = 7.4 Hz, 2H, Fmoc-***H***), 7.89 (d, $^{3}J$ = 7.3 Hz, 2H, Fmoc-***H***). MS (ES, MeCN/0.1 % FA) *m/z* (%): 802 (100) [M+H]$^{+}$. $C_{43}H_{56}N_6O_9$ (800.94).

**(*S*)-*N*$^{\omega}$-*tert*-Butoxycarbonyl-*N*$^{\omega'}$-[4-(*tert*-butoxycarbonylamino)butylaminocarbonyl]-*N*$^{\alpha}$-(9-fluorenylmethoxycarbonyl)arginine (8.1)[11]**

Compound **8.10** (2.00 g, 2.5 mmol, 1.0 eq) was dissolved in 150 mL MeOH, a 10 % Pd/C catalyst (160 mg) was added and hydrogen was led through the reaction mixture under vigorous stirring. After 3 h benzyl deprotection was complete (TLC control) and the Pd/C catalyst was removed by filtration over Celite. The solvent was concentrated under reduced pressure and directly subjected to flash chromatography (DCM/MeOH/AcOH 100/0/0 – 90/9.5/0.5). After chromatography, the solvent was evaporated *in vacuo* under mild conditions (30 °C). Subsequently, the product was suspended in 50 mL water. Lyophilisation afforded the product as a white solid (1.63 g, 90 %). $^{1}$H-NMR (300 MHz, DMSO-$d_6$): δ (ppm) 1.36 (bs, 12 H, C(C***H***$_3$)$_3$ + CH-CH$_2$-C***H***$_2$), 1.40 and 1.45 (bs, 10H, split signal, C(C***H***$_3$)$_3$ + CH-C***H***$_2$), 1.49 – 1.80 (m, 4H, NH-CH$_2$-(C***H***$_2$)$_2$), 2.83 – 2.10 (m, 4H, CH-(CH$_2$)$_2$-C***H***$_2$-NH + C***H***$_2$-NH), 3.20 – 3.34 (m, 2H, C***H***$_2$-NH), 3.87 – 4.04 (m, 1H, C***H***$^{\alpha}$), 4.18 – 4.35 (m, 2H, C***H***-C***H***$_2$ , Fmoc), 6.70 – 6.84 (m, 2H, N***H***), 6.86 – 7.02 (m, 1H, N***H***), 7.32 (t, $^{3}J$ = 7.4 Hz, H, Fmoc-***H***), 7.41 (t, $^{3}J$ = 7.4 Hz, 2H, Fmoc-***H***), 7.72 (d, $^{3}J$ = 7.5 Hz, 2H, Fmoc-***H***), 7.89 (d, $^{3}J$ = 7.4 Hz, 2H, Fmoc-***H***), 8.16 (t, $^{3}J$ = 4.6 Hz, 1H, N***H***), 9.17 (t, $^{3}J$ = 5.0 Hz, N***H***) 12.25 (bs, 1H, COO***H***). $^{13}$C-NMR (75 MHz, DMSO-$d_6$): δ (ppm) 25.45 (-, CH-(***C***H$_2$)$_2$), 26.21 and 26.83 (-, ***C***H$_2$-***C***H$_2$-NH), 28.15 (+, 2 x C(***C***H$_3$)$_3$), 30.58 (-, CH-(***C***H$_2$)$_2$), 39.40 (-, ***C***H$_2$-NH$_2$), 39.46 (-, 2 x ***C***H$_2$-NH), 46.55 (+, ***C***H-CH2, Fmoc), 53.33 (+, ***C***H$^{\alpha}$), 67.35 (-, ***C***H$_2$-O-CO), 65.48 (-, ***C***H$_2$-O-CO), 119.99 (+, 2 Fmoc-***C***), 125.18 (+, 2 Fmoc-***C***), 126.95 (+, 2 Fmoc-C), 127.51 (+, Fmoc-***C***), 141.24 (C$_{quat}$, 2 Fmoc-***C***), 143.74 (C$_{quat}$, 2 Fmoc-***C*** ), 155.46 (C$_{quat}$, ***C***-N), 156.48 (C$_{quat}$, ***C***-N), 171.81 (C$_{quat}$, ***C***=O). MS (ES, MeCN/0.1 % FA) m/z (%): 711 (100) [M+H]$^{+}$. $C_{36}H_{50}N_6O_9$ (710.84).

## 8.6.2 Peptides Synthesis According to a Standard Fmoc-Protocol

### 8.6.2.1 General Conditions

See section 7.5.2.1

### 8.6.2.2 General Procedure for the Coupling of the Functionalized Arginine Building Block

The functionalized arginine building block (3 eq) was dissolved in 0.45 M HOBt (3 eq, in DMF/NMP 80/20 v/v) and added to the resin together with HBTU (3 eq, in DMF/NMP 80/20 v/v) as coupling reagent and 1.0 M DIEA (6 eq, in NMP). The mixture was shaken overnight (14 – 16 h). The deprotection step was performed with 20 % piperidine in DMF (2x 10 min).

### 8.6.2.3 General Procedure for SPPS

See section 7.5.2.2

**Ile-Asn-Pro-Nle-Bpa-Arg-Leu-Arg-Tyr-$NH_2$ (8.2)[22]**

The title compound was prepared from 100 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 15 – 50 % in 30 min) yielded **8.2** as a white fluffy solid (17 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 432 (25) $[M+3H]^{3+}$ 446 (40) $[M+MeCN+3H]^{3+}$, 648 (100) $[M+2H]^{2+}$, 1295 (5) $[M+H]^{+}$; anal. RP-HPLC: 98 % ($t_R$ = 18.39 min, *k*' = 3.43); HRMS (ESI): *m/z* calcd. for $[C_{64}H_{95}N_{17}O_{12} + 2H]^{2+}$ 647.8746 , found: 647.8751. $C_{64}H_{95}N_{17}O_{12} \cdot 3TFA$ (1636.61).

**pNPY (18-36): Arg($N^{\omega}$-Ahx)Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-$NH_2$ (8.3)[17]**

The title compound was prepared from 100 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 10 - 50 % in 30 min) yielded **8.3** as a white fluffy solid (16 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 525 (100) $[M+2MeCN+CHOOH+5H]^{5+}$, 636 (80) $[M+HCOOH+4H]^{4+}$, 848 (10) $[M+HCOOH+3H]^{3+}$; anal. RP-HPLC: 99 % ($t_R$ = 14.87 min, *k*' = 2.58); HRMS (ESI): *m/z* calcd. for $[C_{114}H_{179}N_{37}O_{27}+MeCN+4H]^{4+}$ 635.8703, found: 635.8687. $C_{114}H_{179}N_{37}O_{27} \cdot 7TFA$ (3298.01).

**Arg($N^{\omega}$-Ahx)-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-(*1R*,*2S*)cpen-Arg-Tyr-$NH_2$ (8.4)**

The title compound was prepared from 30 mg Rink amide resin according to the standard Fmoc-protocol. Purification by preparative RP-HPLC (MeCN 15 – 45 % in 30 min) yielded **8.4** as a white fluffy solid (9.1 mg); MS (ESI, MeCN/0.1 % FA) *m/z* (%): 444 (10) $[M+4H]^{4+}$, 591 (100) $[M+3H]^{3+}$, 886 (10) $[M+2H]^{2+}$; anal. RP-HPLC: 98 % ($t_R$ = 15.71 min, *k*' = 2.79); HRMS (ESI): *m/z* calcd. for $[C_{81}H_{131}N_{27}O_{18}+ 2H]^{2+}$ 886.0155, found: 886.0164. $C_{81}H_{131}N_{27}O_{18} \cdot 4TFA$ (2227.16).

## 8.6.3 Preparation of Fluorescently-Labeled Peptides

### 8.6.3.1 General Conditions

Chemicals and solvents were purchased from commercial suppliers and used without further purification. Cy5-NHS ester, 5(6)-SFX-NHS ester and Alexa Fluor 488-NHS ester were obtained from Molecular Probes (now Invitrogen; Darmstadt, Germany). [Ala$^{31}$,Aib$^{32}$]pNPY was a gift from Prof. Dr. Chiara Cabrele (Ruhr University Bochum, Germany).

Mass spectra (MS) were recorded on a Finnigan ThermoQuest TSQ 7000 (ES-MS). For high resolution mass spectrometry an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) equipped with an ESI source was used. The peak intensity in % relative to the strongest signal is indicated in parenthesis. Analytical HPLC analysis was performed with a Jupiter column (250 x 4.6 mm, 5 µm, 300 Å, Phenomenex, Aschaffenburg, Germany) on a Merck Hitachi systems composed of a L-6200-A pump, a AS-2000A autosampler, a L-4000A UV-VIS detector and a F-1050 fluorescence spectrophotometer (flow rate: 0.8 mL/min; UV detection 220 nm). Acetonitrile (A) and 0.05 % TFA (B) was used as mobile phase (Gradient: A/B 10/90 to A/B 90/10 in 30 min; A/B 95/5: 31 to 40 min). Helium degassing was used throughout.

### 8.6.3.2 General Procedure for Peptide Labeling

All peptides were fluorescently labeled as previously described.[5, 7]

For the labeling reaction, the peptides (1 eq) were dissolved in 20 µL of DMF (Merck, Darmstadt, Germany). 250 µL of 0.1 M sodium carbonate/bicarbonate buffer (pH 8.5), containing 33 % acetonitrile were added. The fluorescent dye (1.2 eq (Cy5), 1.5 eq (5(6)-SFX and Alexa Fluor)) was dissolved in 20 µL of anhydrous DMF and added to the peptide solution.

Purification was performed by preparative HPLC using a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Nucleodur 100-5 C18ec, 250 × 21 mm, 5 µm, Knauer, Berlin, Germany) at a flow rate of 18 mL/min. Mixtures of acetonitrile and 0.1 % aq. TFA were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure prior to lyophilisation. The labeled peptides were dissolved in water and diluted with $CH_3CN$/0.1 aq. TFA (20/80, v/v, Cy5), PBS (pH 7.0, Alexa Fluor 488) or ammonium carbonate buffer (10 mM, pH 9, 5(6)-SFX). The concentration was determined by UV/Vis spectroscopy at 649 nm (Cy5), 495 nm (Alexa Fluor 488) and 494 nm 5(6)-SFX, respectively, using the molar extinction coefficient for the corresponding dye and solvent mixture (Cy5: $\varepsilon^{26}$ = 313568 L · mol$^{-1}$ · cm$^{-1}$, Alexa fluor 488: 74000 L · mol$^{-1}$ · cm$^{-1}$, (5(6)-SFX: 73000 L · mol$^{-1}$ · cm$^{-1}$). Afterwards, the labeled peptides were aliquoted (1 – 10 nmol) into siliconized microtubes (1.5 mL) and the solvent was evaporated in the vacuum

concentrator at room temperature. The purified products were analyzed by analytical HPLC, MS and HRMS.

**Peptide 8.3 labeled with the fluorescent dye Cy5 (8.3-Cy5)**

The title compound was prepared from **8.3** (0.74 mg) and NHS-Cy5 (0.15 mg) according to the general procedure. Purification by preparative RP-HPLC (MeCN 15 – 40 % in 30 min) afforded **8.3-Cy5** as a blue fluffy solid (0.04 mg, 6 %).MS (ESI, MeCN/0.1 % FA) *m/z* (%): 531 (20) $[M+6H]^{6+}$, 637 (100) $[M+5H]^{5+}$, 796 (50) $[M+4H]^{4+}$, 1061 (20) $[M+3H]^{3+}$; anal. RP-HPLC: 95 % ($t_R$ = 16.21 min, *k*' = 2.91); HRMS (ESI): *m/z* calcd. for $[C_{147}H_{216}N_{39}O_{34}S_2$ +MeCN + $5H]^{5+}$ 636.5310, found: 636.5394. $C_{147}H_{216}N_{39}O_{34}S_2 \cdot 6TFA$ (3822.79).

**Peptide 8.3 labeled with the fluorescent dye (5)6-SFX (8.3-SFX)**

The title compound was prepared from **8.3** (2 mg) and NHS-5(6)-SFX (0.62 mg) according to the general procedure. Purification by preparative RP-HPLC (MeCN 10 – 50 % in 30 min) afforded **8.3-SFX** as an orange fluffy solid (0.26 mg, 14 %).MS (ESI, MeCN/0.1 % FA) *m/z* (%): 603 (75) $[M+5H]5^{+}$, 754 (100) $[M+4H]^{4+}$, 1005 (30) $[M+3H]^{3+}$; anal. RP-HPLC: 98 % ($t_R$ = 19.61 min, double peak (dye: constitution isomers), *k*' = 3.73); HRMS (ESI): *m/z* calcd. for $[C_{141}H_{200}N_{38}O_{34} + 4H]^{4+}$ 754.1455, found: 754.1523. $C_{141}H_{200}N_{38}O_{34} \cdot 6TFA$ (3657.2392).

**Peptide 8.3 labeled with the fluorescent dye Alexa Fluor 488 (8.3-alexa)**

The title compound was prepared from **8.3** (1.2 mg) and NHS-Alexa fluor 488 (0.29 mg) according to the general procedure. Purification by preparative RP-HPLC (MeCN 10 – 50 % in 30 min) afforded **8.3-alexa** as an orange-red fluffy solid (0.17 mg, 22 %). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 612 (100) $[M+MeCN+5H]^{5+}$, 765 (50) $[M+MeCN+4H]^{4+}$, 1020 (20) $[M+MeCN+3H]^{3+}$; anal. RP-HPLC: 98 % *(*$t_R$ = 18.32 min, double peak (dye: constitution isomers), *k*' = 3.41); HRMS (ESI): *m/z* calcd. for $[C_{135}H_{191}N_{39}O_{37}S_2 + 5H]^{5+}$ 612.0874, found: 612.0955. $C_{135}H_{191}N_{39}O_{37}S_2 \cdot 6TFA$ (3700.43).

**Peptide 8.4 labeled with the fluorescent dye Cy5 (8.4-Cy5)**

The title compound was prepared from **8.4** (0.45 mg) and NHS-Cy5 (0.15 mg) according to the general procedure. Purification by preparative RP-HPLC (MeCN 10 – 50 % in 30 min) afforded **8.4-Cy5** as blue fluffy solid (0.10 mg, 16 %). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 805 (100) $[M+3H]^{3+}$, 1206 (55) $[M+2H]^{2+}$; anal. RP-HPLC: 99 % ($t_R$ = 17.35 min, *k*' = 3.18); HRMS (ESI): *m/z* calcd. for $[C_{114}H_{169}N_{29}O_{25}S_2$ + $3H]^{3+}$ 803.7507 , found: 803.7518. $C_{114}H_{169}N_{29}O_{25}S_2 \cdot 4TFA$ (2863.30).

**Peptide 8.4 labeled with the fluorescent dye (5)6-SFX (8.4-SFX)**

The title compound was prepared from **8.4** (2 mg) and NHS-5(6)-SFX (1.0 mg) according to the general procedure. Purification by preparative RP-HPLC (MeCN 15 – 45 % in 30 min) **8.4-SFX** as a yellow fluffy solid (0.89 mg, 29 %).MS (ESI, MeCN/0.1 % FA) *m/z* (%): 561 (100) $[M+4H]^{4+}$, 748 (80) $[M+3H]^{3+}$, 1122 (10) $[M+2H]^{2+}$; anal. RP-HPLC: 95 % ($t_R$ = 18.11 min, *k*' = 3.36); HRMS (ESI): *m/z* calcd. for $[C_{108}H_{152}N_{28}O_{25}+ 4H]^{4+}$ 564.5451, found: 561.5464. $C_{108}H_{152}N_{28}O_{25} \cdot 4TFA$ (2698.62).

**Peptide 8.4 labeled with the fluorescent dye Alexa Fluor 488 (8.4-alexa)**

The title compound was prepared from **8.4** (0.8 mg) and NHS-Alexa Fluor 488 (0.29 mg) according to the general procedure. Purification by preparative RP-HPLC (MeCN 15 – 45 % in 30 min) afforded **8.4-alexa** as an orange-red fluffy solid (0.07 mg, 12 %).MS (ESI, MeCN/0.1 % FA) *m/z* (%): 763 (100) $[M+3H]^{3+}$, 1145 (40) $[M+2H]^{2+}$; anal. RP-HPLC: 99 % ($t_R$ = 16.18 min, *k*' = 2.90); HRMS (ESI): *m/z* calcd. for $[C_{102}H_{143}N_{29}O_{28}S_2 + 3H]^{3+}$ 763.0106, found: 763.0120. $C_{102}H_{143}N_{29}O_{28}S_2 \cdot 4TFA$ (2743.61).

**Peptide 8.5 labeled with the fluorescent dye Cy5 (8.5-Cy5)**

The title compound was prepared from **8.5** (0.76 mg) and NHS-Cy5 (0.15 mg) according to the general procedure. Purification by preparative RP-HPLC (MeCN 15 – 45 % in 30 min) afforded **8.5-Cy5** as a blue fluffy solid (0.05 mg, 4 %).MS (ESI, MeCN/0.1 % FA) *m/z* (%): 968 (100) $[M+5H]^{5+}$, 1209 (30) $[M+4H]^{4+}$; anal. RP-HPLC: 75 % ($t_R$ = 18.90 min, *k*' = 3.55); $C_{219}H_{319}N_{57}O_{64}S_2 \cdot 6TFA$ (5518.19).

**Peptide 8.4 labeled with the fluorescent dye (5)6-SFX (8.5-SFX)**

The title compound was prepared from **8.5** (0.7 mg) and NHS-5(6)-SFX (0.30 mg) according to the general procedure. Purification by preparative RP-HPLC (MeCN 20 – 50 % in 30 min) afforded **8.5**-**SFX** as a yellow fluffy solid (0.14 mg, 9 %). MS (ESI, MeCN/0.1 % FA) *m/z* (%): 668 (40) $[M+7H]^{7+}$, 779 (100) $[M+6H]^{6+}$, 934 (40) $[M+5H]^{5+}$, 1168 (30) $[M+4H]^{4+}$; anal. RP-HPLC: 75 % ($t_R$ = 19.42 min, *k*' = 3.68); HRMS (ESI): *m/z* calcd. for $[C_{214}H_{305}N_{56}O_{64}+6H]^{6+}$ 778.5449, found: 778.5456. $C_{214}H_{305}N_{56}O_{64} \cdot 6TFA$ (5351.86).

## 8.6.4 Determination of Quantum Yields

The determination of quantum yields was performed with a Cary Eclipse spectrofluorimeter and a Cary 100 UV/VIS photometer (Varian Inc., Mulgrave, Victoria, Australia). The photomultiplier voltage of the Cary Eclipse spectrofluorimeter was set to 400 V throughout. The excitation spectra depicted in **Figure 8.3** were recorded with an excitation slit of 5 and an emission slit of 10 nm. For the presented emission spectra (**Figure 8.3**) recording was performed with an excitation slit and emission slit of 10 nm. The quantum yield was determined according to a previously described protocol using

cresyl violet (Acros Organics, Geel, Belgium) as a red fluorescent standard which revealed a quantum yield of 54 % in ethanol as reported in literature.[27] As a green fluorescent standard quinine sulfate (Fluka, now Sigma Aldrich, Munich, Germany) was selected for which a quantum yield of 57.7 % is described in literature.[28] Spectra were recorded in disposable acryl cuvettes (10x10 mm, Ref. 67.755, Sarstedt, Nümbrecht, Germany) providing a less time consuming alternative to glass cuvettes as solutions had not be transferred when recording fluorescence and absorption spectra. Quantum yields seem to be nearly unaffected by the material of the cuvette (glass/acryl). At first the concentrations of the fluorescent ligands for the determination of the quantum yield was determined. For this purpose, an absorption spectrum was recorded in a concentration range of 0.8 – 1.0 µM. For the determination of the quantum yields solutions with absorbances between 0.08 and 0.24 at the excitation wavelength were chosen as close to the absorption maximum (Alexa Fluor 488 labeled compounds) or at a plateau of the absorption spectrum (Cy5 and 5(6)-SFX labeled compounds). It was strictly avoided to excite the fluorescent compounds in flank of the excitation spectrum. Solutions of the fluorescent ligands were freshly prepared from 100 µM or 1 mM stock solutions (water) of the compounds in PBS or PBS + 1 % BSA and immediately protected from light. Fluorescence spectra were recorded at two different slit adjustments (excitation/emission): 10/5 nm and 10/10 nm. For the determination of reference spectra the pure solvents with the same water content but without fluorescent ligand were used. Instrument settings for the determination of the emission spectra were chosen as followed: T = 22 °C; medium scan rate; filter settings: auto (excitation filter), open (emission filter); $\lambda_{em} = \lambda_{ex} + 10 – 15$ nm. From every emission spectrum the corresponding reference spectrum was subtracted, yielding the net spectra, which were multiplied with the corresponding lamp correction spectra. The resulting corrected spectra were integrated up to 850 nm. The absorbance at the excitation wavelength was determined by recording absorption spectra immediately after the recording of the emission spectra (within 30 min after preparation of test solutions). Baselines were stored using reference solutions and subtracted from the raw spectra. The quantum yield was calculated according to the following equation:

$$\Phi_{F(X)} = (A_s/A_x)\,(F_x/F_s)\,(n_x/n_s)^2\,\Phi_{F(S)} \text{ with}$$

A: absorbance of the corrected absorption spectrum at the excitation wavelength;

F: integral of the corrected emission spectrum;

n: refraction index of the solvent;

$\Phi_F$: quantum yield [%];

s: cresyl violet perchlorate standard: $\Phi_{F(S)} = 54$ % or quinine sulfate standard: $\Phi_{F(S)} = 57.7$ %

x: fluorescent ligand (sample)

## 8.6.5 Pharmacological Methods

### 8.6.5.1 Materials and Cell culture

See section 3.3.2.1

### 8.6.5.2 Steady State GTPase Assay

See section 6.7.2.4

### 8.6.5.3 Flow Cytometric Binding Experiments

**Flow cytometric competition binding assay**

See section 3.3.2.2

**Displacement of fluorescent ligands 8.3-Cy5, 8.4-Cy5 and 8.4-alexa**

The cells (CHO-h$Y_4$-qi5-mtAEQ and CHO-h$Y_2$-qi5-mtAEQ) were seeded three days prior to the experiment (90-95 % confluency), trypsinized and resuspended in Ham´s F12 medium, containing 10 % fetal calf serum for the inactivation of trypsin. After centrifugation at 1,000 rpm for 5 min, the cells were suspended in binding buffer with BSA (1 %) to a density of $10^6$ cells/mL. Bacitracin (0.1 g/L) was added in order to prevent protease-mediated degradation of the peptides. The experiments were performed with the respective fluorescent ligand (c (**8.4-Cy5**) = 10 nM, c (**8.4-alexa**) = 10 nM, c (**8.3-Cy5**) = 25 nM) in the presence and absence of various competitors ($Y_4$R: GW1229 and hPP, $Y_2$R: pNPY) at different concentrations ($10^{-10}$ to $10^{-6}$ M). Non-specific binding was determined in the presence of 1 µM GW1229 ($Y_4$R) or 1 µM pNPY ($Y_2$R). The samples were incubated in siliconized reaction vessels for 60 min (**8.4-Cy5** and **8.4-alexa**) or 90 min (**8.3-Cy5**) at room temperature before flow cytometer analysis. Instrument settings for the different fluorescent ligands:

**8.4-alexa**: FSC: E-1, SSC: 280 V, Fl-1: 550 V, Flow: HI;
**8.4-Cy5** and **8.3-Cy5**: FSC: E-1, SSC: 280 V, Fl-4: 800 V, Flow: HI.
The measurement stopped when 15,000 gated events were counted.

**Saturation binding experiments with 8.3-Cy4, 8.4-Cy5, 8.5-Cy5 and 8.4-alexa**

Saturation binding experiments were performed by analogy with the above mentioned competition binding assays with following adaptations. The cells were incubated with the respective fluorescent ligand at increasing concentrations (c ≈ 0.1 × $K_D$ - 10 × $K_D$) for 60 min (**8.4-Cy5**, **8.4-alexa** and **8.4-carboxy**) or 90 min (**8.3-Cy5** and **8.3-carboxy**). Non-specific binding was determined with 100-fold

excess of GW1229 ($Y_4R$) or BIIE 0246 ($Y_2R$). Data from saturation binding were evaluated by one-site saturation fits using the Sigma plot 11 analysis software (Systat Software GmbH, Erkrath, Germany). For the green fluorescent compound **8.4-alexa** the autofluorescence was subtracted from all geometric mean values obtained from flow cytometric measurements prior to data analysis.

**8.4-alexa**: FSC: E-1, SSC: 280 V, Fl-1: 550 V, Flow: HI;
**8.5-Cy5, 8.4-Cy5** and **8.3-Cy5**: FSC: E-1, SSC: 280 V, Fl-4: 700 V, Flow: HI.
The measurement stopped when 15,000 gated events were counted.

**Dissociation kinetics of $Y_4R$ binding of Cy5-[$K^4$]-hPP and 8.4-Cy5**

The cells were treated as already described and pre-incubated with a fix concentration (c (**8.4-Cy5**) = 15 nM, c (Cy5-[$K^4$]hPP) = 3 nM) of the labeled ligand for 60 min (**8.4-Cy5**) or 90 min (Cy5-[$K^4$]hPP) in binding buffer with BSA (1 %), bacitracin, except for Cy5-pNPY (buffer + 1 % BSA and 0.1 g/L bacitracin). For non-specific binding, pre-incubation was performed in presence of 100-fold excess of hPP. Afterwards, cells were washed with cold binding buffer and re-suspended in binding buffer consisting of hPP (100-fold excess). Samples were taken at different time periods and measured. (For instrument settings see the respective competition binding protocols). Data was fitted using Graph Pad Prism 5 software (San Diego, CA) using monophasic exponential decay.

### 8.6.5.4 Confocal Microscopy

Two days prior to the experiment CHO-h$Y_4$-qi5-mtAEQ cells, CHO-h$Y_2$-qi5-mtAEQ cells and HEC-1-B-Y5 cells were trypsinized and seeded in Nunc LabTekTM II chambered coverglasses with 8 chambers (Nunc, Wiesbaden, Germany) in Hams F12 containing 10 % FCS (CHO cells) or EMEM containing 10 % FCS (HEC cells). On the day of the experiment confluency of the cells was 70 – 90 %. The culture medium was removed, the cells were washed twice with Leibowitz L15 culture medium (200 µL) and covered with L15 medium (150 µL). L15 medium (50 µL) and L15 medium (50 µL) with the fluorescent probe (5-fold concentrated) was added for total binding. For non-specific binding L15 medium (50 µL) with the competing agent GW1229 ($Y_4R$), BIIE 0246 ($Y_2R$) and pNPY ($Y_5R$) (5-fold concentrated) and L15 medium (50 µL) with the fluorescent probe (5-fold concentrated)
Confocal microscopy was performed with a Zeiss Axiovert 200 M microscope, equipped with the LSM 510 laser scanner. The objective was a Plan-Apochromat 63x/1.4 with oil or water immersion. The most important settings for the detection of the investigated fluorescent ligands are outlined in **Table 8.5**.

**Table 8.5**: Conditions for the detection of the fluorescent ligands **8.3-Cy5**, **8.3-alexa**, **8.4-Cy5**, **8.4-alexa**, **8.5-Cy5** and **8.5-SFX** with the Zeiss Axiovert 200 M microscope

| No. | Excitation (laser transmission) | Filter | Pinhole [µm] |
|---|---|---|---|
| **8.3-Cy5** | 633 nm (10 %) | LP650 | 122 |
| **8.3-alexa** | 488 nm (5 %) | LP505 | 124 |
| **8.4-cy5** | 633 nm (10 %) | LP650 | 124 |
| **8.4-alexa** | 488 nm (5 %) | LP505 | 212 |
| **8.5-Cy5** | 633 nm (10 %) | LP650 | 164 |
| **8.5-carboxy** | 488 nm (5 %) | LP505 | 212 |

## 8.7 References


1. Middleton, R. J.; Kellam, B. Fluorophore-tagged GPCR ligands. *Curr. Opin. Chem. Biol.* **2005,** 9, 517-525.
2. Ingenhoven, N.; Beck-Sickinger, A. G. Fluorescent Labelled Analogues of Neuropeptide Y for the Characterization of Cells Expressing NPY Receptor Subtypes. *J. Recept. Signal. Transduct. Res.* **1997,** 17, 407-418.
3. Fabry, M.; Cabrele, C.; Höcker, H.; Beck-Sickinger, A. G. Differently labeled peptide ligands for rapid investigation of receptor expression on a new human glioblastoma cell line. *Peptides* **2000,** 21, 1885-1893.
4. Dumont, Y.; Gaudreau, P.; Mazzuferi, M.; Langlois, D.; Chabot, J. G.; Fournier, A.; Simonato, M.; Quirion, R. BODIPY-conjugated neuropeptide Y ligands: new fluorescent tools to tag Y1, Y2, Y4 and Y5 receptor subtypes. *Br. J. Pharmacol.* **2005,** 146, 1069-1081.
5. Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Determination of Affinity and Activity of Ligands at the Human Neuropeptide Y Y4 Receptor by Flow Cytometry and Aequorin Luminescence. *J. Recept. Signal. Transduct. Res.* **2007,** 27, 217 - 233.
6. Ziemek, R.; Brennauer, A.; Schneider, E.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Fluorescence- and luminescence-based methods for the determination of affinity and activity of neuropeptide Y2 receptor ligands. *Eur. J. Pharmacol.* **2006,** 551, 10-18.
7. Schneider, E.; Mayer, M.; Ziemek, R.; Li, L.; Hutzler, C.; Bernhardt, G.; Buschauer, A. A simple and powerful flow cytometric method for the simultaneous determination of multiple parameters at G protein-coupled receptor subtypes. *ChemBioChem* **2006,** 7, 1400-1409.

8. Fabry, M.; Langer, M.; Rothen-Rutishauser, B.; Wunderli-Allenspach, H.; Hocker, H.; Beck-Sickinger, A. G. Monitoring of the internalization of neuropeptide Y on neuroblastoma cell line SK-N-MC. *Eur. J. Biochem.* **2000,** 267, 5631-5637.

9. French, A. P.; Mills, S.; Swarup, R.; Bennett, M. J.; Pridmore, T. P. Colocalization of fluorescent markers in confocal microscope images of plant cells. *Nat. Protocols* **2008,** 3, 619-628.

10. Cabrele, C.; Wieland, H. A.; Koglin, N.; Stidsen, C.; Beck-Sickinger, A. G. Ala31-Aib32: identification of the key motif for high affinity and selectivity of neuropeptide Y at the Y5-receptor. *Biochemistry* **2002,** 41, 8043-8049.

11. Keller, M. Guanidine-Acylguanidine Bioisosteric Approach to Address Peptidergic Receptors: Pharmacological and Diagnostic Tools for the NPY Y1 Receptor and Versatile Building Block on Arginine Substitutes. Doctoral Thesis, Regensburg, **2008**.

12. Kraus, A.; Ghorai, P.; Birnkammer, T.; Schnell, D.; Elz, S.; Seifert, R.; Dove, S.; Bernhardt, G.; Buschauer, A. N(G)-acylated aminothiazolylpropylguanidines as potent and selective histamine H(2) receptor agonists. *ChemMedChem* **2009,** 4, 232-240.

13. Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. Guanidine-acylguanidine bioisosteric approach in the design of radioligands: synthesis of a tritium-labeled $N^G$-propionylargininamide ([$^3$H]-UR-MK114) as a highly potent and selective neuropeptide Y $Y_1$ receptor antagonist. *J. Med. Chem.* **2008,** 51, 8168-8172.

14. Brennauer, A., Dove, S., Buschauer, A. Structure-Activity Relationships of Nonpeptide Neuropeptide Y Receptor Antagonists. *Handb. Exp. Pharm.*; **2004**; 162, 506-537.

15. Pluym, N.; Brennauer, A.; Keller, M.; Ziemek, R.; Pop, N.; Bernhardt, G.; Buschauer, A. Application of the Guanidine-Acylguanidine Bioisosteric Approach to Argininamide-Type NPY Y2 Receptor Antagonists. *ChemMedChem* **2011,** 6, 1727-1738.

16. Pluym, N. Application of the Guanidine–Acylguanidine Bioisosteric Approach to NPY Y2 Receptor Antagonists: Bivalent, Radiolabeled and Fluorescent Pharmacological Tools. Doctoral Thesis, **2011**.

17. Cabrele, C.; Beck-Sickinger, A. G. Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J. Pept. Sci.* **2000,** 6, 97-122.

18. Burchiel, S. W.; Edwards, B. S.; Kuckuck, F. W.; Lauer, F. T.; Prossnitz, E. R.; Ransom, J. T.; Sklar, L. A. Analysis of Free Intracellular Calcium by Flow Cytometry: Multiparameter and Pharmacologic Applications. *Methods* **2000,** 21, 221-230.

19. Panchuk-Voloshina, N.; Haugland, R. P.; Bishop-Stewart, J.; Bhalgat, M. K.; Millard, P. J.; Mao, F.; Leung, W.-Y.; Haugland, R. P. Alexa Dyes, a Series of New Fluorescent Dyes that Yield Exceptionally Bright, Photostable Conjugates. *J. Hisotchem. Cytochem.* **1999,** 47, 1179-1188.

20. Yung-Chi Cheng, W. H. P. Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzymatic reaction. *Biochem. Pharm.* **1973,** 22, 3099-3108.

21. Pop, N.; Igel, P.; Brennauer, A.; Cabrele, C.; Bernhardt, G. N.; Seifert, R.; Buschauer, A. Functional reconstitution of human neuropeptide Y (NPY) Y(2) and Y(4) receptors in Sf9 insect cells. *J. Recept. Signal Transduct. Res.* **2011,** 31, 271-285.

22. Zwanziger, D.; Bohme, I.; Lindner, D.; Beck-Sickinger, A. G. First selective agonist of the neuropeptide Y1-receptor with reduced size. *J. Pept. Sci.* **2009,** 15, 856-866.

23. Moser, C.; Bernhardt, G.; Michel, J.; Schwarz, H.; Buschauer, A. Cloning and functional expression of the hNPY Y5 receptor in human endometrial cancer (HEC-1B) cells. *Can. J. Physiol. Pharmacol.* **2000,** 78, 134-142.

24. Masiukiewicz, E.; Wiejak, S.; Rzeszotarska, B. Scalable syntheses of Nα-benzyloxycarbonyl-L-ornithine and of Nα-(9-fluorenylmethoxy)carbonyl-L-ornithine. *Org. Prep. Proced. Int.* **2002,** 34, 521-527.

25. Krapcho, P. K., Christopher,S. Mono-Protected Diamines. N-tert-Butoxycarbonylalkanediamines from Alkanediamines. *Synth. Commun.* **1990,** 20, 2559-2564.

26. Ziemek, R. Development of binding and functional assays for the neuropeptide Y Y 2 and Y 4 receptors. Doctoral Thesis, Regensburg, **2006**.

27. Magde, D.; Brannon, J. H.; Cremers, T. L.; Olmsted, J., III. Absolute luminescence yield of cresyl violet. A standard for the red. *J. Phys. Chem.* **1979,** 83, 696-699.

28. Eastman, J. W. Quantitative spectrofluorometry: the fluorescence quantum yield of quinine sulfate. *Photochem. Photobiol.* **1967,** 6, 55-72.

# CHAPTER 9

# Summary

The neuropeptide Y hormone family consists of three 36-amino acid peptides, namely neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP), involved in the regulation of numerous physiological processes such as food intake, blood pressure, stress, pain and hormone secretion. Among the four NPY receptor subtypes ($Y_1R$, $Y_2R$, $Y_4R$, $Y_5R$) activated by the NPY peptide family in humans, the $Y_4R$ is unique due to the preferential binding of PP over NPY and PYY. The biological role of the $Y_4R$ is far from being understood, not least because of the lack of appropriate pharmacological tools. Although several PP analogs have been reported to inhibit food intake, there is a need for potent and selective peptides with reduced molecular weight and, in particular, for non-peptide $Y_4R$ ligands. Therefore, this work aimed at the development of both, peptidic agonists and non-peptidic antagonists, selective for the $Y_4R$.

The first part of this thesis focused on the structural optimization of UR-AK49 ($K_i$ = 68 µM, $IC_{50}$ = 60.9 µM), a $N^G$-acylated imidazolylpropylguandine, originally designed as $H_2R$ agonist and identified as the first weak $Y_4R$ antagonist. Replacement of the imidazolyl ring by moieties known as "privileged structures" of GPCR ligands, led to the identification of a $N^G$-acylated phenylpiperazinylguanidine with 12-fold higher $Y_4R$ activity compared to UR-AK49. However, variation of the $N^G$-substituent by introducing highly lipophilic residues (e.g. diphenyl, cyclohexyl), which turned out to be superior to polar or basic substituents, did not further improve $Y_4R$ antagonistic potency. Unfortunately, the increase in lipophilicity and amphiphilic character of the acylguanidines was accompanied with hemolysis and cytotoxicity. The latter turned out to be incompatible with whole cell based assays, so that this project was discontinued.

Serendipitously, on the occasion of selectivity studies moderate $Y_4R$ antagonists were identified among a set of bivalent BIBP 3326 analogs, which were originally designed as $Y_1R$ antagonists. Systematic investigation of monovalent and bivalent argininamide-type derivatives led to the discovery of the first non-peptidic antagonist for the NPY $Y_4R$, a bivalent ligand with submicromolar affinity ($K_i$ = 132 nM). As the $Y_1R$ prefers (*R*)-configured compounds such as BIBP 3226, the selectivity ratio $Y_4R/Y_1R$ was considerably improved by incorporation of the (*S*)-configured argininamide building block. Hence, this class of compounds is considered a promising starting point in the search of more potent and selective $Y_4R$ antagonist. Furthermore, the bivalent ligand approach may be useful to obtain pharmacological tools for studying receptor dimerization.

The major part of this work focused on truncated pNPY and hPP analogs comprising unnatural and *D*-amino acids as selective $Y_4R$ agonists. Within a cooperation with Prof. Dr. Oliver Reiser (University of Regensburg) cis-pentacin (cpen) containing pNPY(25-36) was identified as a $Y_4R$ ligand with a $K_i$ value

in the low nanomolar range ($K_i$ = 10 nM) and an excellent selectivity profile. On this basis additional peptide-derived ligands were synthesized and characterized. To reduce the peptidic nature of these compounds, truncated ligands, containing unnatural amino acids, were prepared. The C-terminal pentapeptide [cpen$^{34}$]pNPY(32-36) showed $Y_4$R affinity in the low nanomolar range, in particular when Thr$^{32}$ was exchanged by Tyr. In general, the developed pNPY analogs were superior to the hPP analogs with respect to $Y_4$R selectivity. Interestingly, the incorporation of *D*-Pro (position 30 and 31) into [cpen$^{34}$]hPP(25-36) led to potent $Y_4$R ligands with a highly improved selectivity profile.

Aiming at pharmacological tools for the study of NPY receptors, fluorescence-labeling of subtype selective peptides was performed. [cpen$^{34}$]pNPY(25-36) as well as the $Y_2$R selective pNPY(18-36) were conjugated to green- and red-fluorescent dyes (5(6)-SFX, Alexa Fluor 488, Cy5). With the introduction of an $N^G$-functionalized arginine building block, developed in our workgroup and optimized in terms of stability and synthetic accessibility, convenient fluorescence labeling became possible, leading to $Y_2$R and $Y_4$R selective pharmacological tools with high affinity. Compared to the green-fluorescent compounds, the red-fluorescent peptides were superior due to higher signal-to-noise ratios. Regardless of that, especially the Alexa Fluor 488 labeled compounds proved to be suitable for confocal microscopy and flow cytometry.

In conclusion, the peptides/peptidomimetics and fluorescent pharmacological tools described in this thesis provide a promising basis for the optimization of $Y_4$R ligands. Combining this knowledge with the experience made with the argininamide-type bivalent ligands may pave the way to the design of non-peptidic antagonists of the $Y_4$R.

# CHAPTER 10

# Appendix

## Abbreviations

| | |
|---|---|
| α | intrinsic activity or selectivity factor |
| abs | absolute |
| Ahx | 6-aminohexanoic acid |
| Aib | 2-aminoisobutyric acid |
| Ala | alanine |
| aq. | aqueous |
| Ar | aryl |
| Arg | arginine |
| Asp | asparate |
| Asp | aspartic acid |
| BIIE 0246 | (2S)-N-[2-(3,5-Dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]-Nα-[2-(1-{2-oxo-[4-(6-oxo-6,11-dihydro-5H-dibenzo[b,e]azepin-11-yl)piperazine-1-yl]ethyl}cyclo-pentyl)acetyl]argininamide |
| Bn | benzyl |
| Boc | *tert*-butoxycarbonyl |
| Bpa | p-benzoylphenylalanine |
| bs | broad singulet |
| BSA | bovine serum albumin |
| c | concentration |
| $[Ca^{2+}]_i$ | intracellular calcium ion concentration |
| calcd. | calculated |
| cAMP | cyclic 3', 5' adenosine monophosphate |
| cat. | catalytical amounts |
| Cbz | benyloxycarbonyl |
| CD | circular dichroism |
| $CDCl_3$ | deuterated chloroform |
| CDI | carbonyldiimidazole |
| CHO | chinese hamster ovary |
| CI | chemical ionization |
| CLSM | confocal laser scanning microscopy |
| CNS | central nervous system |
| COSY | correlated spectroscopy |

| | |
|---|---|
| $C_{quat}$ | quarternary carbon atom |
| Cys | cysteine |
| d | doublet |
| δ | chemical shift |
| DCC | N,N'-dicyclohexylcarbodiimide |
| DCM | dichlormethane |
| DEPT | distortionless enhancement by polarization transfer |
| DIAD | diisopropyl azodicarboxylate |
| DIEA | N,N'-diisopropyl-ethylamine |
| DMAP | 4-(dimethylamino)pyridine |
| DMEM | Dulbecco's modified eagle medium |
| DMF | dimethylformamide |
| DMSO | dimethylsulfoxide |
| DMSO-$d_6$ | per-deuterated dimethylsulfoxide |
| $EC_{50}$ | agonist concentration which induces 50 % of maxium response |
| EDAC | N-(3-dimethylaminopropyl)-N'-ethyldicarbodiimide |
| EDT | ethane-1,2-dithiol |
| EI | electron impact ionization |
| EMEM | Eagle's minimal essential medium |
| eq | equivalent(s) |
| ES | electrospray ionization |
| $Et_3N$ | triethylamine |
| EtOAc | ethylacetate |
| EtOH | ethanol |
| FA | formic acid |
| FACS | fluorescence activated cell sorter |
| FCS | fetal calf serum |
| Fmoc | 9-fluorenylmethoxycarbonyl |
| FRET | fluorescence-resonance energy transfer |
| GDP | guanosine diphosphate |
| Gln | glutamine |
| Glu | glutamate |
| Gly | glycine |
| GPCR | G-Protein coupled receptor |
| GTP | guanosine triphosphate |

| | |
|---|---|
| h | hour(s)/human |
| HBTU | 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate |
| HEC-1B | human endometrial carcinoma |
| HEL | human erythroleukemia |
| HEPES | 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethansulfonic acid |
| His | histidine |
| HMBC | heteronuclear multiple bond correlation |
| HOBt | 1-hydroxybenzotriazole |
| HPLC | high performance liquid chromatography |
| HRMS | high resolution mass spectrometry |
| HSQC | heteronuclear multiple bond correlation |
| HT-29 | human colon adenocarcinoma cell line |
| HTS | high throughpout screening |
| $IC_{50}$ | antagonist concentration which suppresses 50 % of an agonist induced effect or discplaces 50 % of a labeled ligands from the binding side |
| Ile | Isoleucine |
| IR | infrared |
| *k'* | retention (capacity) factor |
| $K_B$ | dissociation constant derived from functional assay |
| $K_D$ | dissociation constant derived from saturation experiments or kinetics |
| $K_i$ | dissociation constant derived from a competitioin binding assay |
| Leu | Leucine |
| LSI-MS | liquid secondary ion mass spectrometry |
| Lys | Lysine |
| m | multiplet or milli |
| M | molar (mol/L) |
| MCF-7 cells | human breast adenocarcinoma cells |
| MeCN | acetonitrile |
| MeOD | pre-deuterated methanol |
| MeOH | methanol |
| Met | Methionine |
| min | minute(s) |
| mp | melting point |
| MS | mass spectrometry |
| MW | molecular weight |

| | |
|---|---|
| n | nano |
| $N^G$ | guanidine nitrogen |
| NHS | N-hydroxysuccinimide |
| $^nJ$ | coupling constant |
| Nle | Norleucine |
| NMR | nuclear magnetic resonance |
| NPY | neuropeptide Y |
| PBS | phosphate buffered saline |
| PE | petroleum ether |
| Ph | phenyl |
| Phe | Phenylalanine |
| Phth | phthalimide |
| $P_i$ | inorganic phosphate |
| ppm | pars per million |
| Pro | Proline |
| Py | pyridyl |
| q | quartet |
| QY | quantum yield |
| ref | reference |
| RP | reversed phase |
| rt | room temperature |
| s | singlet/second(s) |
| SAR | structure-activity relationships |
| sat. | saturated |
| SEM | standard error of the mean |
| Ser | Serine |
| Sf9 | *Spodoptera frugiperda* insect cell line |
| SK-N-MC cells | human neuroblastoma cell line established from the supraorbital metastasis of a neuroblastoma of a 14-year old girl in 1971 |
| t | triplet/time |
| $t_0$ | hold-up time |
| TBTU | 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate |
| tBu | tert-butyl |
| TFA | trifluoroacetic acid |
| TFE | trifluoroethanol |

| | |
|---|---|
| Tfl | triflyl |
| THF | tetrahydrofurane |
| THF | tetrahydrofuran |
| Thr | Threonine |
| TLC | thin layer chromatography |
| TM | transmembrane domain |
| TMS | trimethylsilyl |
| $t_R$ | retention time |
| Tris | tris(hydroxymethyl)aminomethane |
| Tyr | Tyrosine |
| UV | ultraviolet |
| $Y_n$ | NPY receptor subtypes, n = 1 ,2, 4, 5, 6 |

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Regensburg, im Mai 2012

(Melanie Kaske)