Guanidine−Acylguanidine Bioisosteric Approach to Address Peptidergic Receptors: Pharmacological and Diagnostic Tools for the NPY Y_1 Receptor and Versatile Building Blocks Based on Arginine Substitutes

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Naturwissenschaftlichen Fakultät IV - Chemie und Pharmazie -
der Universität Regensburg

vorgelegt von
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2008

Die vorliegende Arbeit entstand in der Zeit von März 2005 bis November 2008 unter der Leitung von Herrn Prof. Dr. A. Buschauer am Institut für Pharmazie der Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie- der Universität Regensburg.

Das Promotionsgesuch wurde eingereicht im November 2008

Tag der mündlichen Prüfung: 18. Dezember 2008

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Danksagungen

An dieser Stelle möchte ich mich bedanken bei:

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

Herrn Prof. Dr. Günther Bernhardt für seine wissenschaftlichen Ratschläge, seine intensive Betreuung und für die kritische Durchsicht der Arbeit,

Herrn Prof. Dr. Burkhard König und seinem Mitarbeiter Stefan Weiß für die interessanten Diskussionen, Ideen und Ratschläge,

Herrn Prof. Dr. Otto S. Wolfbeis und seinen Mitarbeitern für die Bereitstellung der Pyryliumfarbstoffe,

Herrn Prof. Dr. Hans-Jürgen Wester für die Möglichkeit der Durchführung zweier PET-Experimente am Nuklearmedizinischen Institut des Klinikums rechts der Isar der Technischen Universität München,

Herrn Dr. Shang-Jung Teng für die Durchführung eines großen Teils der Synthese der in Kapitel 3 beschriebenen bivalenten Y_1-Rezeptor Antagonisten,

Herrn Dr. Thilo Spruß für die Betreuung und Unterstützung bei der Durchführung der Tierversuche sowie für das Anfertigen der Kryoschnitte von Gewebeproben,

Frau Elvira Schreiber für die Durchführung der zahlreichen pharmakologischen Bestimmungen (Calcium-Assay) am Fluorimeter,

Frau Susanne Bollwein und Frau Brigitte Wenzl für die Kultivierung der SK-N-MC und MCF-7 Zellen und ihre Unterstützung bei den Radioligand-Bindungsexperimenten,

Herrn Franz Wiesenmayer und Herrn Oskar Baumann für die Unterstützung bei der Durchführung der Tierexperimente und die freundlichen Gespräche,

meiner Kollegin Nathalie Pop für die Durchführung der durchflusszytometrischen Experimente und die Unterstützung bei der Durchführung von Konfokalmikroskopie-Experimenten,

Herrn Dr. Dietmar Gross und meinem Kollegen Miroslaw Lopuch ebenfalls für die Unterstützung bei der Durchführung von Konfokalmikroskopie-Experimenten,

Herrn Dr. Erich Schneider für die hilfreichen Diskussionen, fachlichen Ratschläge, die Charakterisierung der Verbindung **4.36** (mk22) am Durchflusszytometer (Kapitel 4) und die gute Zusammenarbeit bei der Veröffentlichung eines Teils von Kapitel 4,

Herrn Dr. Albert Brennauer für das Bereitstellen von Synthesevorstufen sowie für die fachlichen Diskussionen und Ratschläge,

Herrn Dr. Norman Koglin für die Organisation, Durchführung und Betreuung der PET-Experimente,

Herrn Dipl.-Chem. Michael Herz für die zweimalige Herstellung von 4-Nitrophenyl-2-[^{18}F]fluorpropanoat,

Frau Dr. Chiara Cabrele und Dr. Jaroslava Svobodová für die Durchführung der Peptidsynthese,

Caroline Ivanica für ihre Hilfe bei Synthese, Aufreinigung und Charakterisierung einer Reihe an Verbindungen,

Frau Petra Pistor für die Anfertigung der histologischen Färbungen,

meinem Kollegen Martin Memminger für die sehr gute, freundschaftliche Zusammenarbeit im Labor und die vielen hilfreichen fachlichen Diskussionen,

meinem Kollegen Patrick Igel für die fachlichen Diskussionen, Ratschläge und die angenehme Kooperation bei der Fertigstellung unserer Arbeiten,

Herrn Peter Richthammer für die zahlreichen guten Gespräche und Diskussionen, für die vielen Tipps und Ratschläge bei Geräte-technischen Dingen und Reparaturen sowie für die schöne gemeinsame Motorradtour,

Frau Silvia Heinrich und Frau Martina Wechler für die freundliche Hilfestellung bei organisatorischen und bürokratischen Angelegenheiten,

Herrn Dr. Thomas Burgemeister für die Unterstützung bei der Auswertung von NMR-Spektren,

Herrn Josef Kiermaier und Herrn Wolfgang Söllner für die Anfertigung zahlreicher MS-Analysen und für die fachlichen Diskussionen,

allen Mitgliedern des Lehrstuhls für ihre Kollegialität und das gute Arbeitsklima,

der Deutschen Forschungsgemeinschaft für die finanzielle Unterstützung und für die Hilfe bei der Verbesserung meiner *soft skills* im Rahmen des Graduiertenkollegs 760,

meiner Frau Stefanie Keller und unseren Kindern Franziska und Jacob für die Unterstützung meiner Arbeit von zu Hause aus und für das geduldige Warten an so manchem langen Arbeitstag.

Publications, Posters, Oral Presentations and Professional Training

Publications (published results prior to the submission of this thesis):

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**. in press (2008), doi: 10.1021/jm801018u

Ghorai, P., Kraus, A., Keller, M., Götte, C., Igel, P., Schneider, E., Schnell, D., Bernhardt, G., Dove, S., Zabel, M., Elz, S., Seifert, R., Buschauer, A., Acylguanidines as bioisosteres of guanidines: N^G-acylated imidazolylpropylguanidines, a new class of histamine H_2 receptor agonists. **J. Med. Chem.** in press (2008), doi: 10.1021/jm800841w

Weiss, S., Keller, M., Bernhardt, G., Buschauer, A., König, B., Modular synthesis of non-peptidic bivalent NPY Y_1 receptor antagonists. **Bioorg. Med. Chem.** (2008), 16, 9858–9866

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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 Y_1 receptor antagonist. **Chembiochem** (2007), 8 (16), 1981-1988.

Poster Presentations:

Memminger, M., Keller, M., Bernhardt, G., Buschauer A., von Angerer, E., Estrogen receptor mediated NPY Y_1 receptor up-regulation in MCF-7 breast cancer cells, **4th International Summer School "Medicinal Chemistry", Regensburg (Germany), September 2008**

Pluym, N., Keller, M., Brennauer, A., Pop, N., Bernhardt, G., Wolfbeis, O.S., Buschauer, A., Synthesis of highly potent, selective neuropeptide Y Y_2 receptor antagonists as fluorescent probes and potential radioligands, **4th International Summer School "Medicinal Chemistry", Regensburg (Germany), September 2008**

Keller, M., Teng, S.J., Wolfbeis, O.S., Bernhardt, G., Buschauer, A., Bivalent neuropeptide Y Y_1 receptor ligands, **Annual Meeting of the GDCh, Fachgruppe Medizinische Chemie, "Frontiers in Medicinal Chemistry", Regensburg (Germany), March 2008**, and **4th International Summer School "Medicinal Chemistry", Regensburg (Germany), September 2008**

Pluym, N., Keller, M., Brennauer, A., Schreiber, E., Wolfbeis, O.S., and Buschauer, A., Towards radio- and fluorescence labeled neuropeptide Y Y_2 receptor antagonists, **Annual Meeting of the GDCh, Fachgruppe Medizinische Chemie, "Frontiers in Medicinal Chemistry", Regensburg (Germany), March 2008**

Weiss, S., Keller, M., Buschauer, A., König, B., General modular synthesis of nonpeptidic bivalent NPY Y_1 receptor antagonists, **Annual Meeting of the GDCh, Fachgruppe Medizinische Chemie, "Frontiers in Medicinal Chemistry", Regensburg (Germany), March 2008**

Memminger, M., Keller, M., Bernhardt, G., Buschauer A., von Angerer, E., Studies on the cross-talk between the NPY Y_1 receptor and the estrogen receptor in MCF-7 breast cancer cells, **Annual Meeting of the GDCh, Fachgruppe Medizinische Chemie, "Frontiers in Medicinal Chemistry", Regensburg (Germany), March 2008**

Keller M., Pop N., Schneider E., Hoefelschweiger B.K., Brennauer A., Gross D., Wolfbeis O.S., Bernhardt G., Dove S. and Buschauer A., Fluorescence labeled NPY Y_1 receptor antagonists, **Conference of the German Pharmaceutical Society (DPhG), Erlangen (Germany), October 2007**, and **Annual Meeting of the GDCh, Fachgruppe Medizinische Chemie, "Frontiers in Medicinal Chemistry", Regensburg (Germany), March 2008**

Kraus, A., Ghorai, P., Preuss, H., Keller, M., Bernhardt, G., Dove, S., Elz, S., Seifert, R., and Buschauer, A., N^G-Acylated hetarylpropylguanidines: Towards centrally active selective histamine H_2R agonists, **Conference of the German Pharmaceutical Society (DPhG), Erlangen (Germany), October 2007**

Keller, M., Koglin, N., Brennauer, A., Freund, M., Spruß, T., Bernhardt, G., Dove, S., Wester, H.J. and Buschauer, A., Characterization and application of radiolabeled neuropeptide Y Y_1 receptor antagonists, **3rd International Summer School "Medicinal Chemistry", Regensburg (Germany), September 2006**

Brennauer, A., Keller, M., Freund, M., Graichen, F., Hutzler, C., Ziemek, R., Bernhardt, G., Dove, S., and Buschauer, A., Guanidine replacement in NPY receptor ligands: Synthesis of N^G-acylargininamides as Y_1 and Y_2 receptor antagonists, **Annual Meeting of the GDCh, Fachgruppe Medizinische Chemie, "Frontiers in Medicinal Chemistry", Frankfurt/Main (Germany), March 2006**

Brennauer, A., Keller, M., Freund, M., Bernhardt, G., Dove, S., and Buschauer, A., Towards the development of neuropeptide Y Y_1 receptor selective tracers, **Annual Meeting of the GDCh, Fachgruppe Medizinische Chemie, "Frontiers in Medicinal Chemistry", Leipzig (Germany), March 2005**

Short Lectures:

NPY Y_1 receptor antagonists: acylated argininamides as specialized pharmacological/diagnostic tools, **Conference of the German Pharmaceutical Society (DPhG), Erlangen (Germany), October 2007**

Characterization and application of radiolabeled neuropeptide Y Y_1 receptor antagonists, **3rd International Summer School "Medicinal Chemistry", Regensburg (Germany), September 2006**

Professional Training:

Since December 2004 associated member of the Research Training Group (Graduiertenkolleg 760) "*Medicinal Chemistry: Molecular Recognition – Ligand Receptor Interactions*"

Contents

Chapter 1

General Introduction

1.1 Neuropeptide Y

Neuropeptide Y (NPY), a 36 amino acid peptide, is one of the most abundant neuropeptides in the central and peripheral nervous system.[1] NPY was first isolated by Tatemoto and co-workers from porcine brain in 1982.[2] Together with the homologous 36 amino acid peptides pancreatic polypeptide (PP) and peptide YY (PYY), NPY belongs to the pancreatic polypeptide (or NPY) family. For all these peptides C-terminal amidation is essential for biological activity.[3] The sequence of NPY is highly conserved in various species.[4]

The three-dimensional structure of NPY has been subject of numerous studies. One of the first models was based on the x-ray structure of avian pancreatic polypeptide (aPP).[5] In the aPP structure, amino acid residues 1 – 8 form a polyproline-like helix, which is linked through a turn (amino acids 9 – 13) with an anti-parallel α-helix (amino acids 14 – 31). The C-terminal pentapeptide is in flexible loop conformation. This hairpin-like structure, the so called PP-fold (cf. Figure 1), which brings N- and C-terminus into close proximity, was also proposed for NPY due to its high sequence homology to PP (50 %).[6]

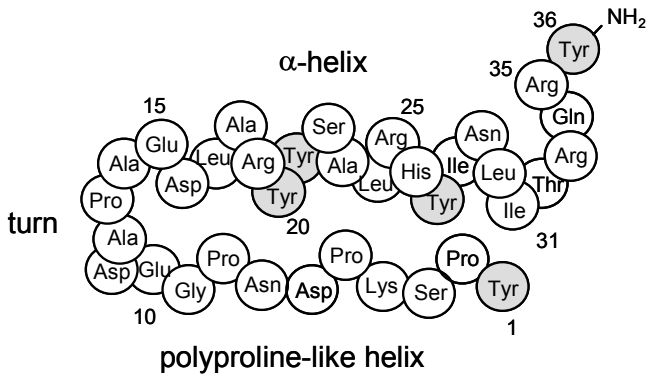

Figure 1. Tertiary structure of porcine NPY (according to Allen, 1987[6]).

The tertiary structure of NPY in solution was intensively investigated over two decades using NMR and CD spectroscopy as well as FRET based approaches. Some of these studies confirmed the PP-fold structure[7, 8], others reported contradictory findings, e.g. dimeric structures through α-helical contacts and conformations with non-helical and flexible N-termini.[9-12] Major drawbacks of NMR studies are physiologically non-relevant concentrations of the peptides and

pH values considerably lower than pH 7.4 (required to increase the solubility of NPY). However, CD spectroscopic measurements revealed that, depending on the concentration of NPY and the pH, different conformations of NPY co-exist in a dynamic equilibrium, and the PP-fold conformation is probably favored under physiological conditions.[13] Also interactions of NPY with the cell membrane, supporting the formation of the active conformation, were discussed.[14, 15]
In the periphery NPY is abundant in sympathetic neurons, where it is co-stored and co-released with noradrenaline, and it was also found in the parasympathetic nervous system[16, 17]. In the central nervous system (CNS) NPY was found in numerous brain regions including basal ganglia, hypothalamus, amygdala, hippoampus, locus coeruleus, nucleus accumbens, and the cerebral cortex.[18-20]

Five mammalian NPY receptors have been cloned so far, the Y_1, Y_2, Y_4, Y_5, and y_6 receptor.[21-28] The y_6 receptor was found to be functional in mice, but is non-functional as a pseudogene in most mammalian species, and in the rat genome it is missing at all.[29] NPY receptors belong to the superfamily of heptahelical G-protein coupled receptors. Their main signal transduction pathway is the coupling to pertussis toxin sensitive $G_{i/o}$ proteins, resulting in an inhibition of forskolin stimulated cAMP accumulation.[30, 31] Besides the inhibition of adenylyl cyclase, elevation of the intracellular calcium concentration after NPY receptor stimulation was reported for subtypes Y_1, Y_2, Y_4 and Y_5,[32-39] but the magnitude of the calcium response depends on the cell type.[30]
NPY binds strongly to the Y_1, Y_2 and Y_5 receptor, but exhibits low affinity for the Y_4 receptor. The latter is nearly exclusively addressed by pancreatic polypeptide.

NPY is involved in the regulation of numerous physiological processes, and was therefore referred to as a “universal soldier”.[40] Important biological functions, which are regulated or co-regulated by NPY, are summarized in Table 1.

Table 1. Overview of biological effects mediated by NPY and its receptors in humans.

Y_1	blood pressure (peripheral effect: ↑, due to vasoconstriction; via central effects: ↓), food intake (↑), anxiolysis, sedation, hormone release, pain sensitivity, depression, angiogenesis, ethanol consumption
Y_2	blood pressure, seizures and food intake (↓), anxiety, pain sensitivity, depression, angiogenesis, gastrointestinal motility, NPY release (↓, presynaptic autoreceptor), circadian rhythms, bone formation, effects on vasculature
Y_4	food intake, gastrointestinal motility
Y_5	food intake (↑), seizure, anxiety, luteinizing hormone release (↓), circadian rhythms

Recently, the Y_1 and Y_2 receptor were found to be expressed in different tumors such as breast cancer[41] ($Y_1 > Y_2$), prostate cancer[42] ($Y_1 \approx Y_2$), adrenal cortical tumors[43] (Y_1), ovarian tumors[44] ($Y_1 \approx Y_2$) and distinct gliomas[45] (Y_2). For some of these malignancies NPY production was detected in the tumor cells (e.g. neuroblastoma, paraganglioma), in some cases NPY was found in nerve fibres contacting the tumor cells (e.g. adrenal cortical tumors, nephroblastoma) and others showed no intratumoral NPY (e.g. mammary carcinomas). Thus, NPY is supposed to exert autocrine and paracrine effects on tumor cell metabolism and tumoral blood supply. The peptide was reported to inhibit tumor growth, and conversely to stimulate tumor growth *in vitro*.[46] Although the role of NPY in tumor biology is unclear *in vivo*, Y_1 and Y_2 receptors have been proposed as potential tumor markers.[46]

1.2 NPY Receptor Ligands

1.2.1 NPY Y_1 Receptor Ligands

Approaches to develop Y_1 receptor (Y_1R) selective agonists by truncation and modification of physiological NPY were more or less successful. In the early 1990th Pro^{34}-substituted analogs of neuropeptide Y and peptide YY were reported to have selectivity for Y_1 over Y_2 receptors. An iodine-125 labeled analog of the most established one, [Leu^{31}, Pro^{34}]peptide YY, was used for numerous autoradiographic binding studies of Y receptor expressing tissues.[47, 48] Later binding studies at the Y_4 receptor revealed that this PYY analog shows also high affinity to NPY Y_4 receptors[49] (cloned in 1995[24]).

Several selective NPY Y_1R agonists were described by Mullins *et al* in 2001.[50] The success was based on the introduction of D-amino acids (e.g. [D-Arg^{25}]NPY) as well as on truncation and cyclization (e.g. Des-AA$^{11-18}$[Cys^{7,21}, D-Lys^{9}(Ac), D-His^{26}, Pro^{34}]NPY). Other NPY analogs with a preference for the Y_1 receptor, namely [Phe^{7}, Pro^{34}]NPY and the cyclic peptide c[D-Cys^{29}-L-Cys^{34}]NPY Ac$^{29-36}$ (YM-42454) were described by Söll *et al* and Takebayashi *et al*, respectively.[51, 52] Also N-terminally shortened peptides, containing β-aminocyclopropane-carboxylic acids (β-ACCs) as unnatural amino acids stabilizing the secondary structure, were reported to be potent and selective Y_1R ligands.[53]

The first non-peptidic Y_1R antagonist described in literature was the arpromidine-type histamine H_2 receptor agonist BU-E-76 (HE 90481)[54] with a pA_2 value of 4.4 in human erythroleukemia cells[55]. In the last two decades a multitude of highly potent and selective non-peptidic Y_1R antagonists with affinities in the nanomolar and subnanomolar range have been developed. A selection is shown in Figure 2 (for a review see Brennauer *et al*[56]).

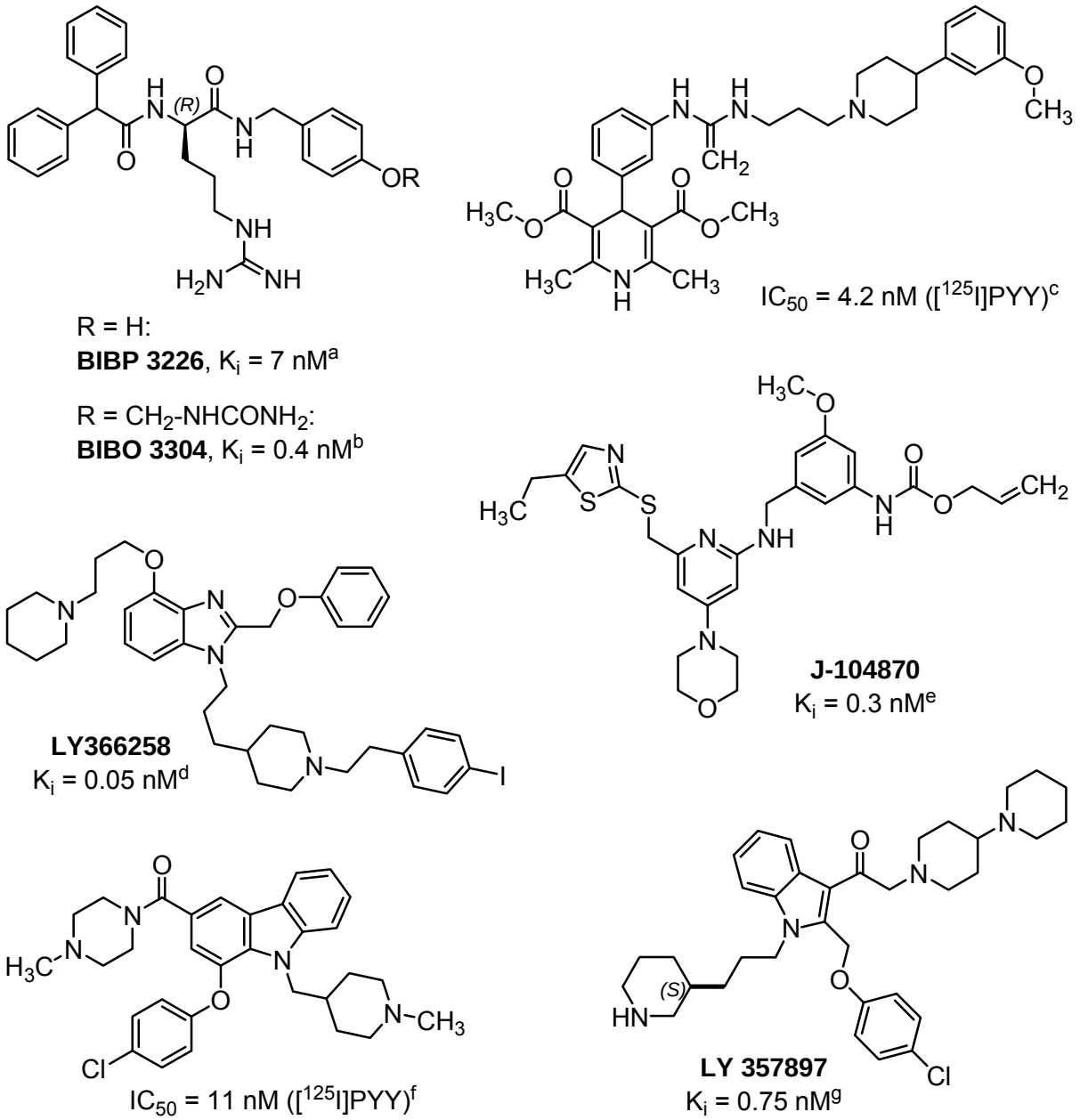

Figure 2. Selection of non-peptide selective Y_1R antagonists. [a]Rudolf *at al* [57], [b]Wieland *et al* [58], [c]Slit *et al* [59], [d]Zarrinmayeh *et al* [60], [e]Kanatani *et al* [61], [f]Leslie *et al* [62], [g]Hipskind *et al* [63].

1.2.2 BIBP 3226

In 1994 the (*R*)-argininamide BIBP 3226 (cf. Figure 2 and 3) was described as the first highly potent and selective Y_1R antagonist.[57] In literature Y_1R binding data of BIBP 3226 ranging from 0.5 to 10 nM are reported.[31, 64] As an alanin scan of NPY revealed that the C-terminal part of the peptide, especially Arg^{35} and Tyr^{36}, are of major importance for the interaction with the Y_1R and the Y_2 receptor (Y_2R),[65] several hundred low molecular weight analogs mimicking the C-terminus of NPY were synthesized and characterized at Boehringer Ingelheim Pharma. Structure optimization with respect to Y_1R affinity including a replacement of L-arginine by the D-enantiomer resulted in the identification of the first highly selective and potent non-peptidic

Y_1R antagonist BIBP 3226 (Scheme 3). The corresponding (*S*)-enantiomer, BIBP 3435, was reported to be at least 1000 times less potent.[57] The replacement of the phenolic OH group by an ureidomethyl entity yielded another selective Y_1R antagonist, BIBO 3304, which is more potent than BIBP 3226 by a factor of about ten (K_i = 0.4 nM, cf. Figure 2).[58, 66]

BIBP 3226 has not been considered as an appropriate drug candidate due to lack oral bioavailability and inability to penetrate across the blood brain barrier, but it has been very useful as a pharmacological tool for the characterization of NPY Y_1 receptors located in the periphery as well as in the CNS.

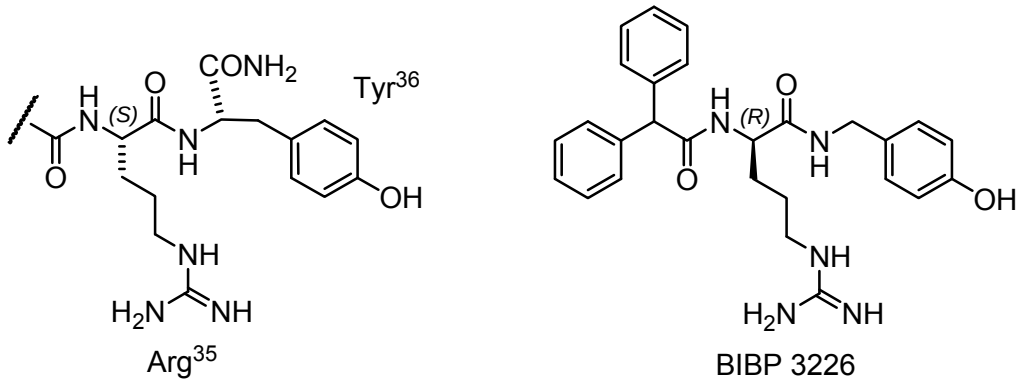

Figure 3. Structural similarity between the C-terminus of NPY and the selective Y_1R antagonist BIBP 3226.

Based on results using [^{125}I]NPY and [^{3}H]BIBP 3226 and several hY_1R mutants Sautel *et al* proposed a model for the binding of BIBP 3226 and NPY to the human Y_1R, disclosing an overlapping binding site of the two ligands.[67, 68] Recently, a new model of BIBP 3226 binding to the Y_1R was generated on the basis of the crystal structure of bovine rhodopsin, confirming the binding mode derived from the formerly reported receptor mutants (Sautel *et al*[68]). This topic was discussed in detail by Brennauer *et al*[56].

In these models the strongly basic guanidine group interacts with an aspartate residue (Asp^{287}) at the top of transmembrane domain six. Therefore, it has been assumed that substituents attached to the N^{ω} guanidine nitrogen, and, upon binding to the receptor, pointing to the extracellular loop region, could be tolerated in terms of Y_1R affinity. A recently prepared series of N^{ω}–substituted BIBP 3226 derivatives revealed that especially electron-withdrawing substituents such as acyl, alkoxycarbonyl and carbamoyl are indeed tolerated, and therefore, a bioisosterism of guanidines and acylguanidines was suggested for this class of compounds.[56, 69, 70]

1.2.3 Y_2, Y_4 and Y_5 Receptor Antagonists

In 1997, a peptidic Y_2R antagonist, $T_4[NPY(33-36)]_4$, consisting of four C-terminal NPY fragments attached to a cyclic template molecule, was reported to bind selectively to the Y_2 receptor with considerable affinity[71, 72]. To date, only the (*S*)-argininamide BIIE 0246 and some N^{ω}-substituted derivatives are known as selective non-peptidic Y_2R antagonists with affinities in the low nanomolar range (Figure 4).[70, 73, 74] Other Y_2R antagonists with affinities around and higher than 100 nM were described, e.g. the Johnson & Johnson compound JNJ-5207787 (Figure 4).[75]

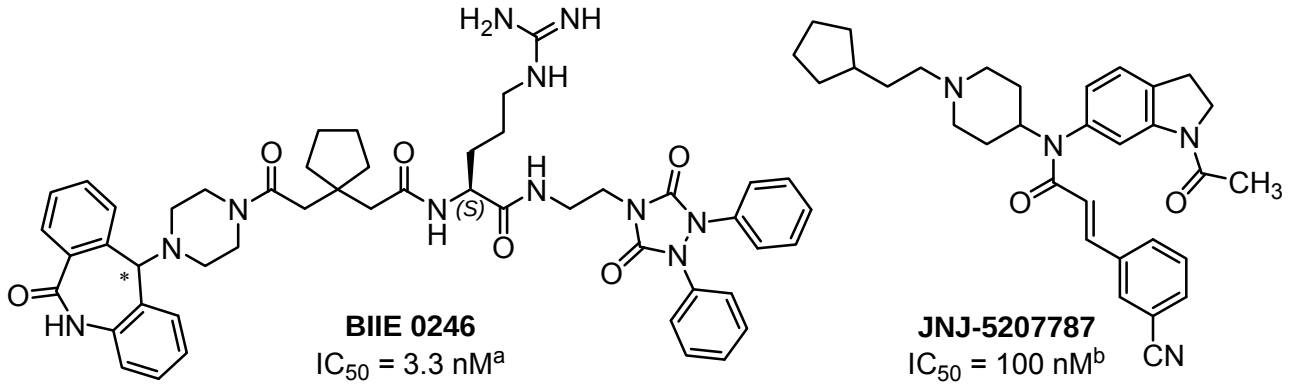

Figure 4. Structures of the most potent non-peptide Y_2R antagonists described in literature to date. [a]Doods *at al* [73], [b]Bonaventure *et al* [75].

Recently, an analog of the C-terminus of NPY, the peptide VD-11, which is structurally closely related to the peptidic Y_4R agonist and Y_1R antagonist GW1229 (1229U91)[76, 77], was shown to have Y_4R antagonistic characteristics.[78] High affinity non-peptide Y_4R ligands are not known so far. However, very recently some acylguanidine derivatives synthesized in the laboratory of Prof. Dr. A. Buschauer (University of Regensburg) as histamine receptor ligands proved to be weak Y_4R antagonists (most potent compound: $IC_{50} \approx 5$ µM) in a functional assay performed with genetically engineered CHO cells, expressing the hY_4R, the chimeric G protein G_{qi5} and mitochondrially targeted apoaequorin[79]. The structure of one of these compounds is shown in Figure 5.

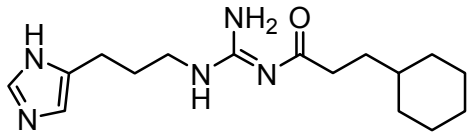

Figure 5. Example of an acylguanidine derivative with moderate Y_4R antagonistic activity. [a]Inhibition of PP induced luminescence in CHO cells expressing the hY_4R, the chimeric G protein G_{qi5} and mitochondrially targeted apoaequorin[79].

In case of the Y_5 receptor the situation is similar as for the Y_1 receptor: the search for new anti-obesity drugs led to numerous highly potent and selective non-peptidic antagonists with broad structural diversity. The Novartis compound CGP 71683A[37, 80], a naphthylsulfonamide, linked through cyclohexane with a 2,4-diaminoquinazoline moiety, was the first selective Y_5R antagonist with nanomolar affinity (IC_{50} = 2.9 nM) (Figure 6).

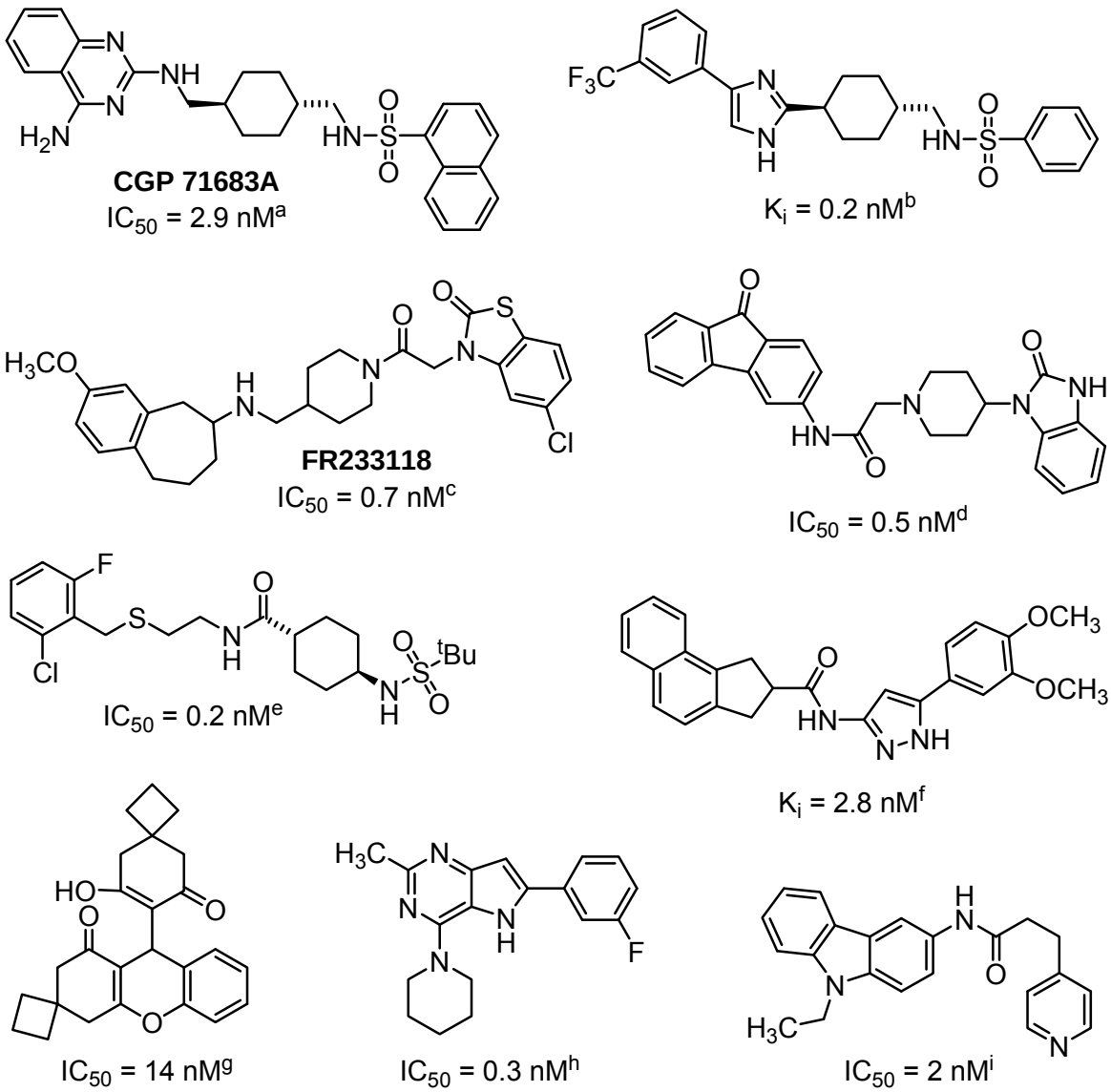

Figure 6. Exemplary structures of selective non-peptide Y_5R antagonists. [a]Criscione *at al* [37], [b]Blum *et al* [81], [c]Itani *et al* [82], [d]Connell *et al* [83], [e]Kawanishi *et al* [84], [f]Sato *et al* [85], [g]Fukami *et al* [86], [h]Norman *et al* [87], [i]Block *et al* [88].

In the last decade numerous arylsulfonamides were reported to be highly potent Y_5R antagonists, and many other Y_5R ligands comprising a broad variety of heteroaryl cycles were described. A selection of Y_5R antagonists with affinities in the low nanomolar range is given in Figure 6. A more detailed overview on Y_5R ligands is included in a review by A. Brennauer *et al*.[56]

1.3 Scope of the Thesis

Fluorescent and radiolabeled peptides such as Cy5-pNPY and [^{125}I]PYY are the preferred tools for the pharmacological evaluation of NPY receptor subtypes and their ligands since labeled small molecules are not available for routine use up to date. As an exception [^{3}H]BIBP 3226 was formerly commercially available, but at present this compound can only be obtained on special orders at high costs. The use of peptide tracers is often compromised, especially in studies on living cells due to intrinsic properties of these compounds such as limited selectivity and stability (enzymatic degradation). In addition, their applicability as pharmacological tools may be hampered by long incubation periods accompanied by receptor desensitization due to the slow binding kinetics of peptides acting as agonists. Thus, there is a demand for fluorescence and radiolabeled NPY receptor subtype selective low molecular weight antagonists.

The attachment of acyl, alkoxycarbonyl and carbamoyl substituents to the guanidine group of the highly potent, Y_1R-antagonistic argininamide BIBP 3226 resulted in analogs with moderately decreased, retained or even increased affinity.[56, 69, 70], suggesting a strategy for the synthesis of potent radio- and fluorescence labeled Y_1R selective antagonists. The aim of this thesis was the development of such functionalized non-peptidic ligands for the Y_1R. The design strategy is based on the hypothesis that a fluorescent or radioactive label can be attached by an acyl linker to the guanidine group in argininamide-type Y_1R antagonists such as BIBP 3226 or BIBO 3304. ω-Amino acyl linkers were considered most promising, since the majority of fluorophores and radionuclide containing entities is usually provided with electrophilic groups (e.g. activated carboxylic acids, isothiocyanates, etc.) requiring nucleophilic labeling precursors.

Very recently, first attempts by A. Brennauer[70, 89] and E. Schneider[90] to prepare N^{ω}–(ω-amino-alkanoyl)argininamides were affected by the extreme instability of the primarily chosen amino-precursor N^{ω}–5-aminopentanoyl-BIBP 3226. At pH > 8 this compound is very rapidly cleaved to BIBP 3226 and valerolactame.[70, 89] Nevertheless, the outlined strategy is considered promising. Therefore, the present work was focused on the identification and optimization of more stable amino-functionalized acyl linkers to pave the way for Y_1R selective fluorescent and radioactive tracers.

With respect to the preparation of radioligands for routine use, tritiated antagonists are preferred in our laboratory, and the synthetic pathway should take into account the commercial availability of appropriate tritiated reagents, in particular [^{3}H]-propionic acid succinimidyl ester. Such a Y_1R selective radioligand is anticipated to serve as a pharmacological tool for the detection of Y_1 receptors on cells and tissues, as well as for autoradiographic studies and for the determination of binding constants at native receptors or receptor mutants.

Beside tritiated compounds the synthesis of fluorine-18 labeled Y_1R antagonists as PET ligands was planned for the *in vivo* imaging of Y_1 receptors. Y_1R PET ligands are of potential value as

diagnostic radiopharmaceuticals, since the Y_1R was recently reported to be expressed in several tumors.[46, 91] In order to explore the structure-activity relationships of potential PET ligands, a series of "cold" fluorinated compounds had to be synthesized and characterized. Thereafter, the most promising candidates had to be radioactively labeled to explore the suitability of argininamide-type Y_1R antagonists as PET ligands *in vivo* using human tumor xenograft models in nude mice.

By analogy with this approach, fluorescent Y_1R antagonists had to be designed, synthesized and characterized, since fluorescence labeled compounds, compared to radioligands, offer advantages with respect to safety precautions, waste disposal and applicability to the powerful techniques flow cytometry and fluorescence microscopy. A major challenge in this field was the preservation of the receptor affinity which is usually considerably affected when large fluorophoric entities are bound to small receptor ligands.

As ligands containing two pharmacophoric entities are discussed to be of potential value as tools for the investigation of GPCR homodimerization,[92] the feasibility of the bivalent ligand approach to argininamide-type Y_1R ligands was intended to be evaluated by linking of two BIBP 3226 moieties through dicarboxylic acids of different structure and length.

Finally, with respect to a broader application of the guanidine-acylguanidine bioisosteric approach in combination with labeling strategies, the preparation and the synthetic use of N^{α}-Fmoc protected arginine building blocks, containing amino-functionalized N^{ω}-acyl substituents, was planned to explore. The replacement of arginine with such building blocks harbors the potential for the preparation of versatile imaging probes derived from biologically relevant oligopeptides, in particular, if the N^{ω}-acylated arginine building blocks are suitable for solid phase peptide synthesis.

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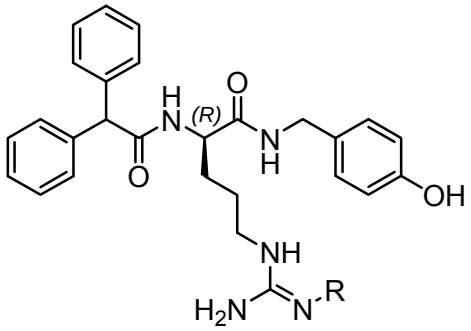
Chapter 2

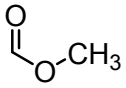
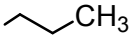
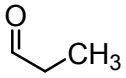
Guanidine - Acylguanidine Bioisosteric Approach in the Design of Radioligands: Synthesis of a Tritium-Labeled N^{ω}-Propionylargininamide ([^{3}H]-UR-MK114) as a Highly Potent and Selective Neuropeptide Y Y_1 Receptor Antagonist

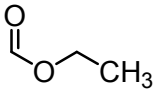
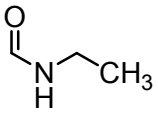
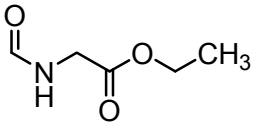
2.1 Introduction

The (*R*)-configured argininamide BIBP 3226 (**2.14**, Chart 1), the first highly potent and selective nonpeptide Y_1 receptor antagonist,[1] has been commonly used as a pharmacological tool for studying the physiological role of the Y_1 receptor (Y_1R). The compound is considered a mimic of the C-terminus, i. e. Arg^{35} and Tyr^{36}, in NPY.[2] On one hand the guanidino group in **2.14** has been considered important for the biological activity due to interaction with Asp^{287} of the human Y_1R, on the other hand the strongly basic group is a major drawback with respect to oral availability and brain penetration. Recently, we prepared a series of N^{ω}-substituted derivatives of **2.14**, which revealed a preference for electron-withdrawing substituents in terms of retaining or even increasing the Y_1R affinity regardless of the by 4-5 orders of magnitude reduced basicity[3] (selection shown in Chart 1). Especially the introduction of carbamoyl residues into the N^{ω}-position yielded Y_1R antagonists with considerably increased affinity (Chart 1),[3, 4] These findings support the concept that the acylguanidines are bioisosteres of guanidines. The high affinities and selectivities achieved with this class of compounds prompted us to develop a Y_1R selective antagonistic radioligand.

Chart 1. Structures and Y_1R affinities of BIBP 3226 (**2.14**) and selected N^{ω}-substituted derivatives, arranged according to increasing size of the substituent "R".



no.	R	K_i [nM]
2.14	H	2[a]
2.15		12[a]
2.16		33[a]
2.17		27[a]
2.8a		1.0[b]

no.	R	K_i [nM]
2.18		4.5[a]
2.19		0.1[a]
2.20		0.1[a]

[a]Determined on SK-N-MC cells with [^{3}H]-propionyl-pNPY as radioligand (1 nM).[3] [b]Determined on SK-N-MC cells with [^{3}H]-UR-MK114 (**2.8b**) as radioligand (1.5 nM). The subnanomolar affinities of the carbamoylated argininamides **2.19** and **2.20** were confirmed using **2.8b** (1.5 nM) as radioligand.

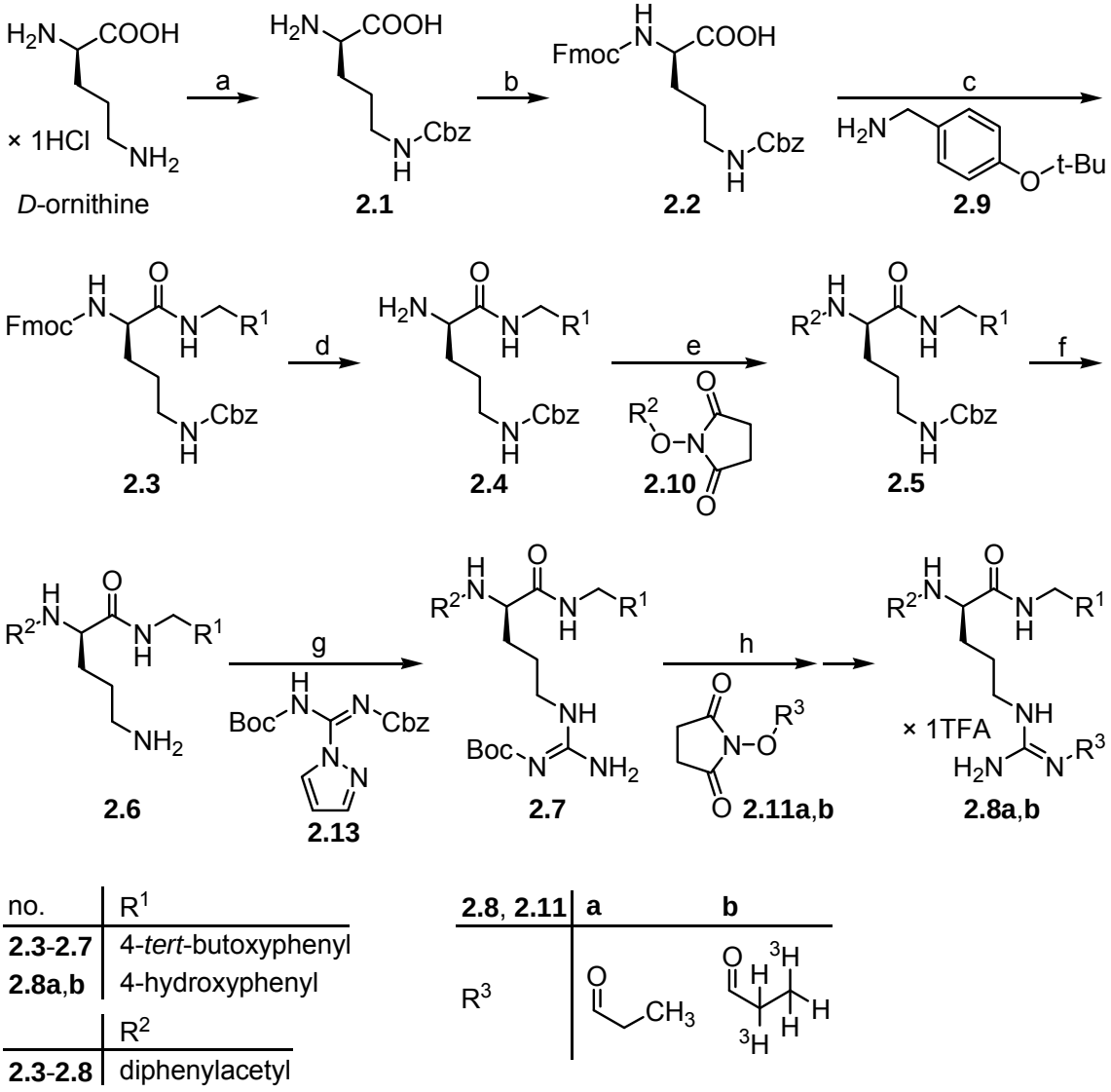
2.2 Results and Discussion

N^{ω}-acetyl (**2.15**), N^{ω}-methoxycarbonyl (**2.16**) and N^{ω}-propyl (**2.17**) substituted argininamides (Chart 1) exhibit lower Y_1R affinities (two-digit nM-range) than the parent compound BIBP 3226 (**2.14**), whereas the N^{ω}-propionyl derivative **2.8a** turned out to be a highly potent Y_1 antagonist with a K_B value of 0.8 nM (cf. Figure 2). Obviously, the extension of the acyl substituent by one methylene group enables an additional hydrophobic interaction, which compensates for the moderate acylation-induced decrease in affinity observed for the lower homolog **2.15**. This was also true for the methoxycarbonyl derivative **2.16** (K_i = 33 nM) compared to the ethoxycarbonyl homolog **2.18** (K_i = 4.5 nM). Acylation was superior to alkylation at the N^{ω}-position (**2.8a** vs. **2.17**, Chart 1), but an exchange of the α-CH_2 group by an oxygen atom, resulting in an ester group, was again less favorable (**2.16**, **2.8a**, Chart 1). Surprisingly, the corresponding N^{ω}-carbamoyl substituted analogs (**2.19**, **2.20**), containing a α-NH group, showed significantly higher affinities. Presumably, the presence of this H-donor group enables additional affinity-enhancing interactions resulting in a different favorable orientation of the flexible amide substituent in **2.19**, **2.20** compared to the acyl residues in **2.8a,b**.

Considering Y_1R affinity and selectivity as well as synthetic feasibility, the N^{ω}-[2,3-^{3}H]propionyl-substituted argininamide **2.8b** ([^{3}H]-UR-MK114), the "hot" form of **2.8a**, was considered most attractive, particularly, since tritiated propionic acid succinimidyl ester is commercially available as a radiolabeling reagent. In principle, this labeling strategy can be transferred to other classes of compounds containing guanidine as a pharmacophoric group.

2.2.1 Chemistry

Alkylguanidines are conventionally prepared from amines and bis-urethane (Boc, Cbz) protected guanidinylation reagents, for example *S*-methyl isothioureas[5], pyrazol-1-carboxamidines[6, 7], or triflylguanidines[8]. Alternatively, guanidine derivatives can be *N*-alkylated with alcohols under Mitsunobu conditions.[9] Acyl-substituted alkylguanidines are accessible, if one of the protecting groups of the guanidinylation reagent is replaced with the pertinent acyl residue.[4] However, such a synthetic route is not well suited for the preparation of a tritiated propionylguanidine, since the radiolabel is preferably introduced in a final one-pot reaction. Therefore, direct attachment of the acyl substituent to the guanidine group in **2.14** (Chart 1) was performed using the N^{ω}-Boc, *O*-*tert*-butyl protected analog **2.7** as a precursor (Scheme 1).



no.	R^1
2.3-**2.7**	4-*tert*-butoxyphenyl
2.8a,**b**	4-hydroxyphenyl

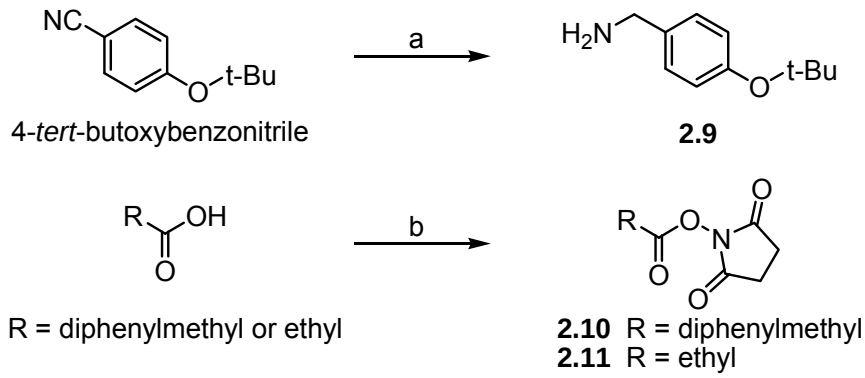
	R^2
2.3-**2.8**	diphenylacetyl

2.8, **2.11**	**a**	**b**
R^3		

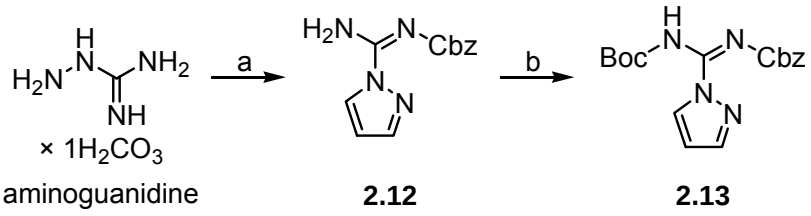
Scheme 1. Synthetic route for the preparation of the N^{ω}-propanoyl argininamides **2.8a,b**.
Reagent and conditions: Reagents and conditions: (a) (1) Cbz-Cl (45 % in toluene), NaOH 0.5 N, K_2CO_3, $CuSO_4$, 20 h, 4 °C to rt; (2) titriplex III, H_2O, 60 min, 100 °C, 83 %; (b) FmocOSu, H_2O/dioxane, 20 h, rt, 96 %; (c) CDI, THF, 20 h, rt, 62 %; (d) diethylamine, DCM, 4 h, rt, 76 %; (e) NEt_3, 1,2-dimethoxyethane, 20 h, 35 °C, 84 %; (f) Pd/C, ammonium formate, MeOH, 20 h, rt, 85 %; (g) (1) NEt_3, CH_2Cl_2, 20 h, rt; (2) Pd/C, H_2, MeOH, 3.5 h, rt, 84 %; (h) (1) NEt_3, MeCN, 20 h, rt; (2) TFA, MeCN, 3 h, 50 °C, 70 % (**2.8a**), 34 % (**2.8b**).

This method afforded the target compounds in good (up to 80 %) yields and has been frequently used in our laboratory to synthesize N^{ω}-acylated arginine derivatives. Carboxylic acids can be attached to the N^{G}-Boc-alkylguanidine scaffold with the aid of coupling reagents from peptide chemistry (EDAC, CDI) or using anhydrides, chlorides and active esters. However, the reaction rate is noticeably slower compared to the coupling with amines.

The precursor **2.7** can be obtained from amine **2.6** by reaction with a guanidinylation reagent containing a Boc and a Cbz protecting group (**2.13**), followed by hydrogenolytic Cbz deprotection. For the preparation of amine **2.6,** starting from *D*-ornithine, the α-amino group was protected with Fmoc, orthogonally to Cbz and the *tert*-butyl phenyl ether.[4] A conversion of the alcohol corresponding to amine **2.6** (obtained via reduction of *D*-glutamate) into acylated guanidines under Mitsunobu condition was reported not to give the desired argininamides[10], and was therefore not explored any longer as an alternative synthetic route. The guanidinylation reagent **2.13** was prepared from aminoguanidine carbonate following reported protocols (Scheme 3).[6] The other required compounds (**2.9**, **2.10**, **2.11**) were prepared according to standard procedures (Scheme 2).



Scheme 2. (a) (1) $LiAlH_4$, diethyl ether, 4 h, reflux; (2) H_2O, 15 % aq. NaOH, 3 h, 0 °C, 98 %; (b) *N*-hydroxysuccinimide, DCC, THF, 20 h, rt; 94 % (**2.10**), 60 % (**2.11a**).



Scheme 3. (a) (1) conc. HCl, 1,1,3,3-tetramethoxypropane, H_2O, 1.5 h, 45 °C; (2) Cbz-Cl, CH_2Cl_2, MeOH, NEt_3, 20 h, 0 °C to rt, 25 %; (b) (1) Boc_2O, DMAP, $CHCl_3$, 20 h, rt; (2) $Mg(ClO_4)_2$, THF, 3 h, 55 °C, 53 %.

Depending on the source of succinimidyl [2,3-^{3}H]-propionate, GE Healthcare (Amersham) and ARC (American Radiolabeled Chemicals, Inc.), the tritiated compound **2.8b** was obtained with specific activities of 97 Ci/mmol and 51 Ci/mmol, respectively. The radioligand **2.8b** was isolated

by HPLC with a radiochemical purity of 99 % (Figure 1, dashed line) and showed a high long-term stability when stored in ethanol at -20 °C over a period of 1.5 years (Figure 1, solid line).

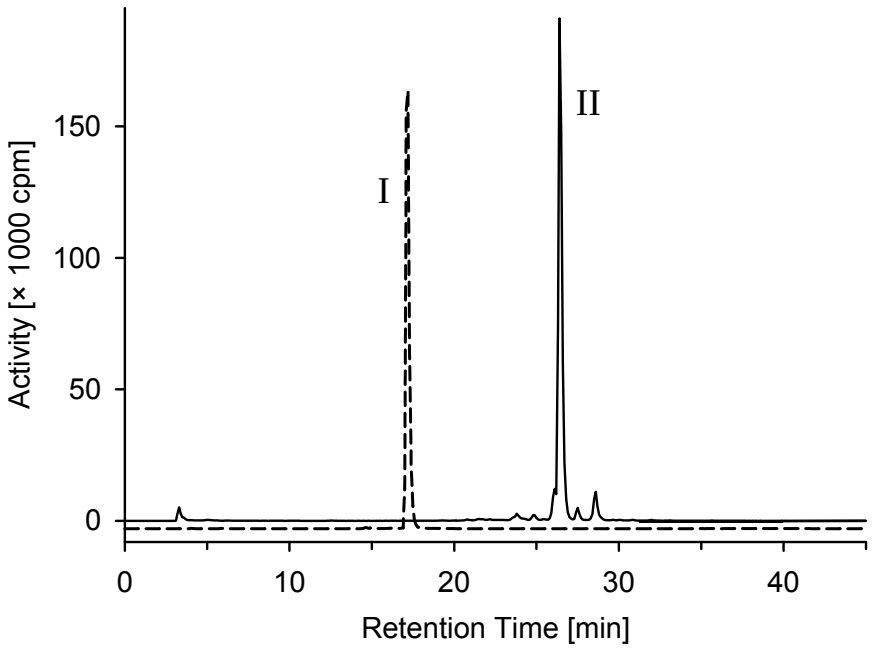


Figure 1. HPLC purity control of the freshly synthesized (I, dashed line) and long-term (18 months) stability control (II, solid line) of the tritiated Y_1R antagonist **2.8b** with radiometric detection. Conditions: I, purity control: eluent: mixtures of acetonitrile + 0.05 % TFA (A) and 0.05 % aq. TFA (B), gradient: 0 to 30 min: A/B 20/80 to 90/10, 30 to 38 min: 90/10, t_R = 16.7 min; II, long term stability control: eluent: mixtures of acetonitrile (A) and 0.05 % aq. TFA (B), gradient: 0 to 30 min: A/B 20/80 to 50/50, 30 to 35 min: 50/50 to 90/10, 35 to 45 min: 90/10, t_R = 26.5 min. The identity of **2.8b** was confirmed by co-injection of larger amounts of the "cold" analog **2.8a**, which allowed UV detection.

2.2.2 Pharmacology: Selectivity, Schild Analysis, Kinetics and Saturation Experiments

The selectivity of the new Y_1R antagonist **2.8a** for human NPY Y_1 over Y_2, Y_4 and Y_5 receptors was proven by flow cytometric binding assays based on fluorescence-labeled pNPY (Y_2R, Y_5R) or [K^4]-hPP (Y_4R) according to previously described methods.[11-13] As also reported for the parent compound BIBP 3226, the *N*$^{\omega}$-propionylated derivative (**2.8a**) shows a clear binding preference for the Y_1R (Table 1).

Table 1. NPY receptor subtype selectivity of **2.8a**.

Y_1 K_i [nM][a]	Y_2 K_i [nM][b]	Y_4 K_i [nM][c]	Y_5 K_i [nM][b]
1.0	> 10,000	> 10,000	> 2,000

[a]Dissociation constant from radioligand competition assay with **2.8b** on SK-N-MC neuroblastoma cells. [b]Flow cytometric binding assay on CHO-Y_2 and HEC-1B-Y_5 cells using Dy-635-pNPY or Cy5-pNPY as labeled ligands (10 nM). [c]Flow cytometric binding assay on CHO-Y_4 cells with Cy5-[K^4]-hPP (5 nM) as fluorescent ligand.

The Y_1R antagonism of **2.8a** was investigated in a fura-2-based Ca^{2+}-assay on HEL cells[14]. Concentration-response curves of pNPY were constructed in the absence and presence of **2.8a** at different concentrations (Figure 2A) and the data were subjected to Schild analysis[15] (Figure 2B). Since the slope in this linear plot nearly equals unity a competitive antagonism of **2.8a** is very likely. The pK_B (in this case identical to pA_2) reflects the affinity of the antagonist and the resulting K_B value (0.8 nM) is in very good agreement with the K_D value (1.2 nM) from saturation binding experiments (Figure 3). A plausible reason for the depressed maxima of the concentration effect curves (Figure 2A) is hemi-equilibrium.[15] Due to the rapid onset of the calcium response in the functional assay, the time window is too narrow to enable an equilibrium among the receptors, the agonist pNPY (slow binding kinetics[16]) and the antagonist **2.8a** (high on- and off-rate, Figure 3).

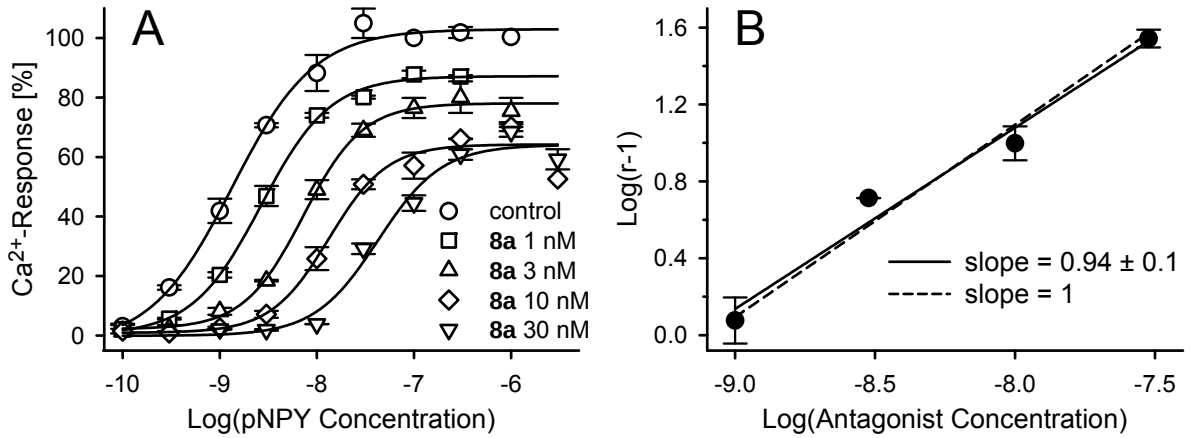


Figure 2. A: Concentration-response curves (CRCs) of pNPY from a fura-2 assay on HEL cells. The presence of **2.8a** led to a parallel rightward shift of the curves. B: Schild regression: log(r-1) plotted against log[antagonist]; the concentration ratios r ($r = 10^{\Delta pEC50}$) were calculated from the rightward shifts (ΔpEC_{50}) of the CRCs in the presence of **2.8a** as shown in Fig. 2A. The slope of the line equals unity, therefore the pA_2 value corresponds to pK_B: $pA_2 \approx pK_B = 9.1$ (slope forced to unity), $K_B = 0.8$ nM. (mean values ± SEM or propagated error, n = 2)

The results of kinetic studies of **2.8b** at 24 °C are presented in Figure 3. Association and dissociation at the Y_1R were in the range of minutes ($k_{on} = 0.19\ min^{-1} \cdot nM^{-1}$, $k_{off} = 0.22\ min^{-1}$) which is typical for small ligands acting as antagonists. The kinetically derived K_D ($k_{off}/k_{on} = 1.1$ nM) is in excellent agreement with the K_D from saturation binding experiments (1.2 nM).

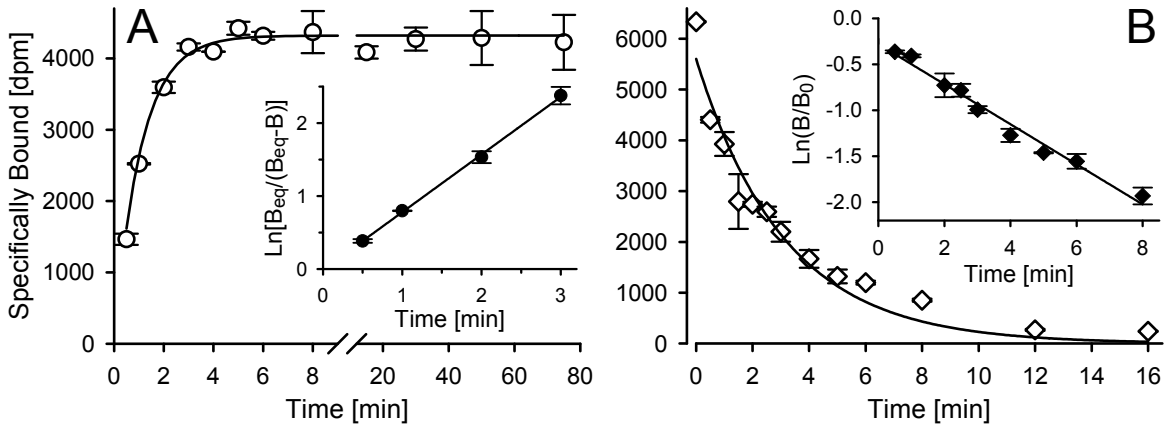


Figure 3. Association and dissociation kinetics for the specific Y_1R binding of **2.8b** on SK-N-MC cells (65th passage) at 24 °C. A: Radioligand (c = 3 nM) association as a function of time, Inset: $\ln[B_{eq}/(B_{eq}-B)]$ versus time, slope = k_{ob} = 0.79 min^{-1}, k_{on} = $(k_{ob} - k_{off})/[L]$ = 0.19 min^{-1} · nM^{-1}. B: Radioligand (pre-incubation: 6 nM) dissociation as a function of time, monophasic exponential decay, $t_{1/2}$ = 2.2 min, Inset: $\ln(B/B_0)$ versus time, slope·(-1) = k_{off} = 0.22 min^{-1}. (mean values ± SEM, n = 3)

Saturation analysis of radioligands provides dual information, the equilibrium dissociation constant K_D and the maximum number of binding sites (B_{max}). Multiple saturation experiments with **2.8b** on SK-N-MC neuroblastoma cells and MCF-7-Y_1 breast cancer cells afforded K_D values of 1.2 nM and 2.9 nM, respectively (Figure 4). The B_{max} value for SK-N-MC cells of about 50,000 sites per cell fit well with data from literature.[17] The breast cancer cells express about 100,000 to 150,000 sites/cell (fluctuations are probably caused by a slightly varying estrogen stimulus provided by the fetal calf serum supplement of the culture medium). Estrogen stimulation of MCF-7-Y_1 cells leads to Y_1R up-regulation up to 300,000 sites/cell. Y_1R up-regulation has been already reported on the mRNA level[18] and could be confirmed on intact cells at the protein level using the radioligand **2.8b** as an NPY Y_1R probe. Based on these results an assay has been developed for the functional characterization of estrogens and antiestrogens (to be reported elsewhere). Binding and functional Y_1R data of **2.8a**/**2.8b** are summarized in Table 2.

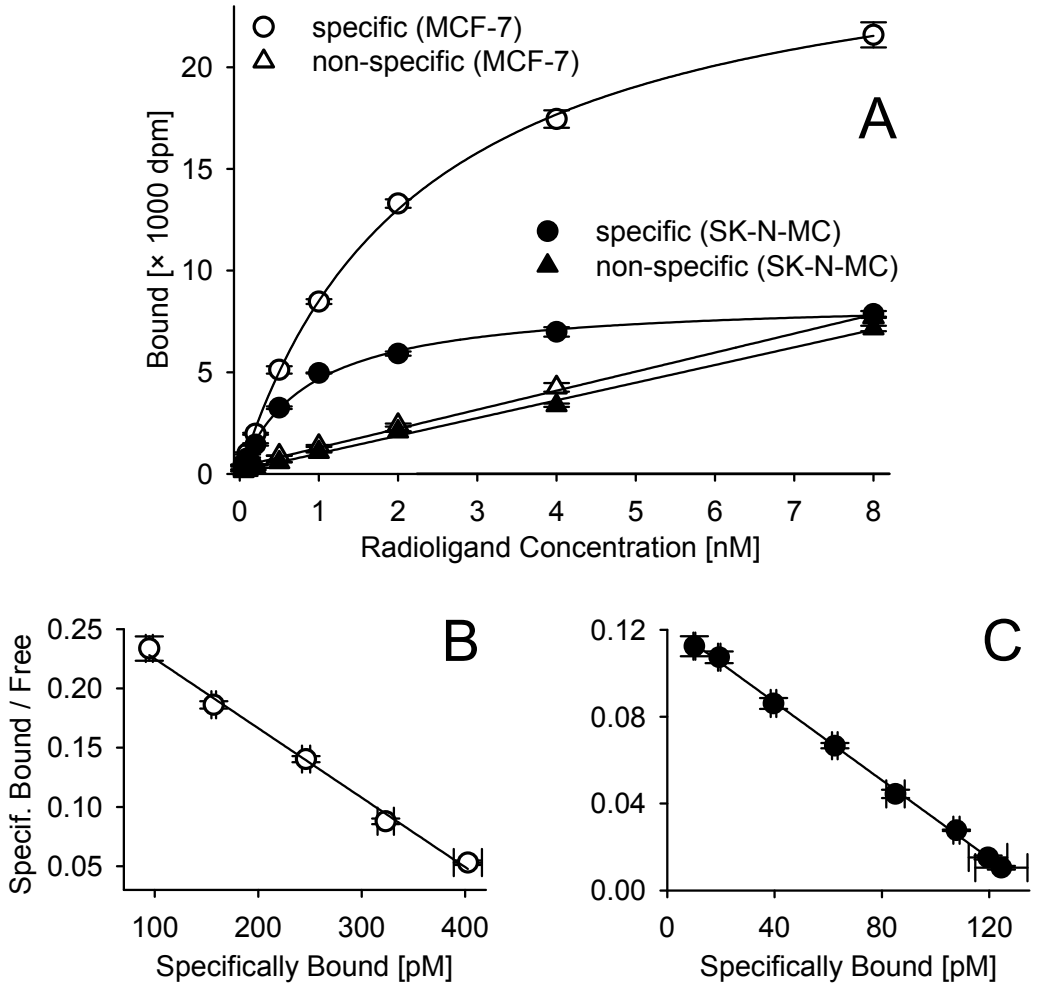


Figure 4. A: Saturation curves for binding of **2.8b** to MCF-7-Y_1 (176th passage) and SK-N-MC (67th passage) cells revealing K_D values of 2.2 nM and 0.85 nM, respectively. Estimated B_{max} (sites/cell[a]): 120,000 (MCF-7-Y_1) and 52,000 (SK-N-MC). [a]The cell number was determined in at least 4-6 wells. B: Scatchard plot for the binding of **2.8b** to MCF-7-Y_1 cells, K_D = -1/slope = 1.7 nM. C: Scatchard plot for the binding to SK-N-MC cells, K_D = -1/slope = 1.1 nM. (mean values ± SEM or propagated error, n = 3)

Table 2. Y_1R binding and functional characteristics of **2.8a**/**2.8b**.

K_D [nM][a]	K_D [nM][b]	K_B [nM][c]	K_D [nM][d]	k_{on} [$min^{-1} \cdot nM^{-1}$][e]	k_{off} [min^{-1}][f]
2.9 ± 0.4	1.2 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	0.19 ± 0.01	0.22 ± 0.01

[a]Equilibrium dissociation constant determined on MCF-7-Y_1 cells, mean value ± SEM from 5 independent experiments, each in triplicate. [b]Equilibrium dissociation constant determined on SK-N-MC cells, mean value ± SEM from 6 independent experiments, each in triplicate. [c]Schild analysis derived dissociation constant of **2.8a**, mean value ± SEM from 2 independent experiments (calcium assay using HEL cells). [d]Kinetically derived dissociation constant ± propagated error. [e]Association rate constant ± standard error from linear regression. [f]Dissociation rate constant ± standard error from linear regression.

2.2.3 Competition Binding Experiments and Autoradiography

Competition binding experiments of **2.8b** with the antagonist BIBP 3226 and the agonist pNPY yielded K_i values (1.5 ± 0.2 nM and 0.5 ± 0.02 nM, resp.) consistent with reported data (Figure 5).[17, 19] In theory the dissociation constant of **2.8a** derived from a competition binding experiment with its hot analog **2.8b** has to be identical with the K_D value of **2.8b**. The experimental data (K_i of **2.8a** = 1.0 nM (Figure 5); K_D of **2.8b** = 1.2 nM and 1.1 nM, resp.) are in good agreement with this requirement.

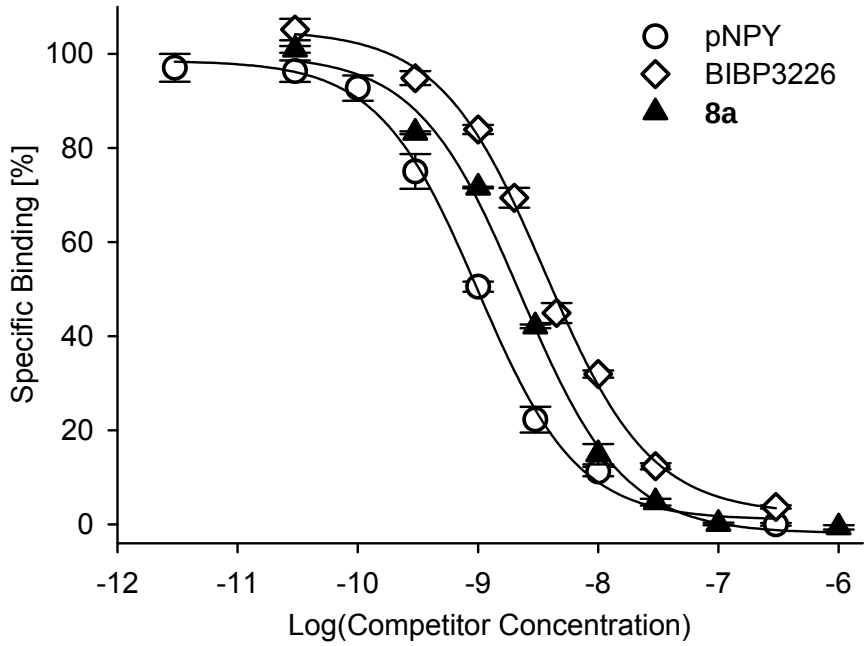


Figure 5. Displacement of the tritiated Y_1R antagonist **2.8b** (c = 1.5 nM) by the agonist pNPY, the antagonist BIBP 3226 and the non-labeled analog **2.8a**. The competition assay was performed on SK-N-MC cells at 24 °C with incubation periods of 20 min (BIBP 3226, **2.8a**) and 120 min (pNPY). BIBP 3226: pIC_{50} = 8.44, K_i = 1.5 nM, pNPY: pIC_{50} = 9.01, K_i = 0.5 nM, **2.8a**: pIC_{50} = 8.65, K_i = 1.0 nM. (mean values ± SEM, n = 3)

To explore the applicability of **2.8b** to autoradiographic binding studies cryosections of a human MCF-7-Y_1 tumor, subcutaneously grown in a nude mouse, and of a rat brain were incubated with the new radioligand. In rat brain Y_1R binding sites were clearly detected in the cerebral cortex in layers I-III (I: molecular layer, II: external granular layer, III: layer of medium-sized pyramidal cells), in the thalamus and in the hippocampus (Figure 6, A and B) as reported from binding studies with [^{125}I][Leu^{31},Pro^{34}]PYY and [^{3}H]BIBP 3226.[20, 21] The results indicate a very high Y_1R expression in the tumor as there is a strong difference between total and non-specific binding (Figure 6C).

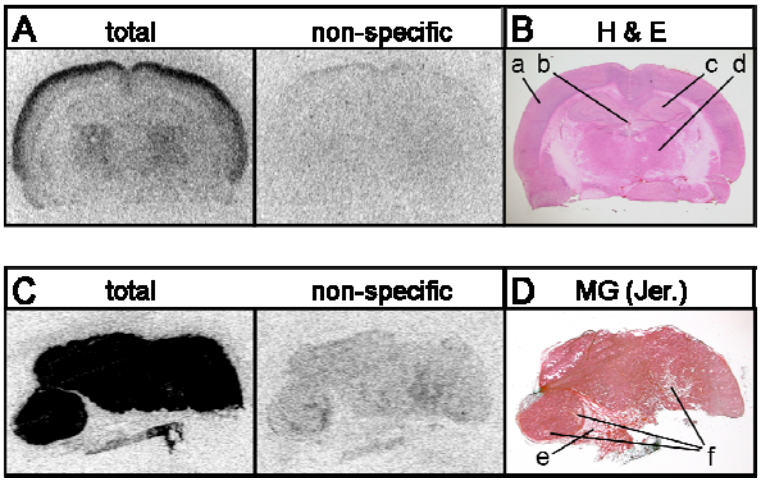


Figure 6. A: Total and non-specific binding of the tritiated Y_1R antagonist **2.8b** to adjacent coronal sections of rat brain (male Wistar rat, 10 month old). B: H & E stained adjacent section, a: cerebral cortex, b: third ventricle, c: hippocampus, d: thalamus. C: Total and non-specific binding of **2.8b** to adjacent tumor sections (s.c. human MCF-7 mammary carcinoma from a NMRI (nu/nu) mouse). D: Masson-Goldner (Jerusalem's modification) stained adjacent tumor section, e: connective and adipose tissue, f: necrotic regions.

2.2.4 Conclusion

N^{ω}-propionylation of BIBP 3226 leads to a highly potent (K_i = 1 nM) and selective Y_1R antagonist (**2.8a**). Direct N^{ω}-acylation of N^{ω}-Boc, *O-tert*-butyl protected BIBP 3226 (**2.7**) allows a convenient preparation of a tritium-labeled radioligand from succinimidyl [2,3-^{3}H]-propionate with specific activities of up to 100 Ci/mmol. Due to practicability and low costs of this labeling strategy, **2.8b** ([^{3}H]-UR-MK114) is a highly attractive alternative to (the formerly commercially available) [^{3}H]-BIBP 3226. **2.8b** has also advantages over radiolabeled peptides addressing the Y_1R due to stability, selectivity, mode of action (antagonist) and costs. [^{3}H]-UR-MK114 can be used for the quantification of Y_1R binding sites as well as for autoradiography experiments on tissues. Because of the attenuated basicity (pK_a = 7 - 8) of the acyl-guanidine moiety **2.8a** is supposed to exhibit an improved pharmacokinetic profile over the strongly basic BIBP 3226 (pK_a ≈ 13). Generally, radioligands capable of penetrating into the brain would provide interesting tools for the study of Y_1R binding by ex vivo (autoradiography) and in vivo (PET) experiments. Recent findings in the NPY Y_2R[10] as well as in the histamine H_2R and H_4R field[22] support the hypothesis that the concept of guanidine-acylguanidine bioisosterism may be of general interest to design receptor ligands including radiotracers with high affinity, reduced basicity and improved pharmacokinetic properties.

2.3 Experimental Section

2.3.1 General Experimental Conditions

Chemicals and solvents were purchased from commercial suppliers (Merck, Darmstadt, Germany; Sigma, München, Germany) and used without further purification unless otherwise stated. *D*-ornithine hydrochloride was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Petroleum ether (40-60 °C) was distilled before use. DMF was stored over 3 Å molecular sieves. Anhydrous reactions were run under an atmosphere of dry nitrogen or argon. Thin layer chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates. For column chromatography silica gel Geduran 60 (Merck, 0.063-0.200 mm) was used. NMR spectra were recorded on an Avance 300 (^{1}H: 300 MHz) and an Avance 600 (^{1}H: 600 MHz, ^{13}C: 150.9 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. Elemental analysis (C, H, N) was performed in-house on a Heraeus Elementar Vario EL III. Mass spectrometry analysis (MS) was performed on a Finnigan ThermoQuest TSQ 7000 (ES-MS), a Finnigan SSQ 710A (EI-MS 70 eV, CI-MS) and Finnigan MAT 95 (LSIMS, HRMS). Melting points were determined with a Büchi 510 melting point apparatus and are uncorrected. Lyophilization was done with a Christ alpha 2-4 LD equipped with a vacuubrand RZ 6 rotary vane vacuum pump. 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 µm; Knauer, Berlin, Germany) at a flow rate of 38 mL/min. Analytical HPLC analysis of non-labeled compounds was performed on a system from Thermo Separation Products composed of a SN400 controller, a P4000 pump, an AS3000 autosampler, a Spectra Focus UV-VIS detector and a RP-column (Eurospher-100 C18, 250 × 4.0 mm, 5 µm; Knauer, Berlin, Germany) at a flow rate of 0.8 mL/min. Tritiated compounds were chromatographically analyzed and purified on a RP-column (Agilent Scalar C18, 250 × 4.6 mm, 5 µm) either with a Shimadzu system C-R4A (pump LC-6A, UV-VIS-spectrophotometric detector SPD-6AV, system controller SCL-6A, Chromatopac C-R4AX) or on a Waters system (two pumps 510, pump control module, 486 UV detector, Packard Radiomatic Flow-1 beta Series A-500 detector, flow rate of liquid szintillator (Quickszint 212; Zinsser Analytic, Frankfurt, Germany): 4 mL/min).

2.3.2 Chemistry: Experimental Protocols and Analytical Data

(*R*)-*N*$^{\delta}$-Benzyloxycarbonylornithine (2.1).[23, 24] Benzyloxycarbonyl chloride (45 % in toluene, 48.5 mL, 130.46 mmol, 1.1 eq) was added to an ice-cold solution of *D*-ornithine hydrochloride (20 g, 118.6 mmol, 1 eq), potassium carbonate (16.4 g, 118.6 mmol, 1 eq) and copper(II)sulfate pentahydrate (14.8 g, 59.3 mmol, 0.5 eq) in aqueous NaOH (0.5 N, 237 mL, 118.6 mmol, 1 eq)

over a period of 15 min. The ice-bath was removed and the reaction was left under stirring 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). Lyophilization yielded a blue-violet powder which was suspended together with Titriplex III (22.1 g, 59.3 mmol, 0.5 eq) in water (200 mL) under vigorous stirring and heated to 100 °C for 60 min. After cooling to 4 °C the solid was separated, washed with ice-cold water (3 × 40 mL) and dried (white solid, 26.8 g, 83 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.35-1.61 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.69 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 2.97 (m, 2H, C$\underline{H}_2$-NH), 3.09 (m, 1H, C$\underline{H}^{\alpha}$), 5.0 (s, 2H, C$\underline{H}_2$-Ph), 7.3-7.4 (m, 5H, Ph); MS (ES, H_2O/MeOH + 10 mM NH_4OAc): *m/z* 267 $[M + H]^+$; $C_{13}H_{18}N_2O_4$ (266.3)

(*R*)-*N*$^{\delta}$-Benzyloxycarbonyl-*N*$^{\alpha}$-(9-fluorenylmethoxycarbonyl)ornithine (2.2).[25] A solution of 9-fluorenylmethyl succinimidyl carbonate (FmocOSu) (26.35 g, 78.1 mmol, 1 eq) in 1,4-dioxane (200 mL) was added to a solution of **2.1** (20.8 g, 78.1 mmol, 1 eq) and sodium carbonate (8.3 g, 78.1 mmol, 1 eq) in a mixture of water and 1,4-dioxane (2.5/1 v/v, 350 mL) over a period of 60 min and the mixture was stirred at rt overnight. About 50 % (volume) of the solvent was evaporated under reduced pressure, the product was precipitated by the addition of 1 M aq. hydrochloric acid (160 mL) and filtered off. Washing with hydrochloric acid (0.1 %, 50 mL) and ice-cold water (50 mL) afforded the product as a white solid (36.6 g, 75 mmol, 96 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.4-1.68 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.73 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 3.0 (m, 2H, C$\underline{H}_2$-NH), 3.91 (m, 1H, C$\underline{H}^{\alpha}$), 4.17-4.31 (m, 3H, C$\underline{H}$-C$\underline{H}_2$ from Fmoc), 5.01 (s, 2H, C$\underline{H}_2$-Ph), 7.26-7.45 (m, 10H, Ph, Fmoc, CH$_2$-N$\underline{H}$), 7.60 (d, 1H, ^{3}J = 7.95 Hz, CO-N$\underline{H}$-CH), 7.73 (d, 2H, ^{3}J = 7.37 Hz, Fmoc), 7.89 (d, 2H, ^{3}J = 7.45 Hz, Fmoc), 12.76 (bs, 1H, COO$\underline{H}$); MS (ES, H_2O/MeOH + 10 mM NH_4OAc): *m/z* 506 $[M + NH_4]^+$, 489 $[M + H]^+$; $C_{28}H_{28}N_2O_6$ (488.5)

(*R*)-*N*$^{\delta}$-Benzyloxycarbonyl-*N*-(4-*tert*-butoxybenzyl)-*N*$^{\alpha}$-(9-fluorenylmethoxycarbonyl)-ornithinamide (2.3). The carboxylic acid **2.2** (37 g, 75.7 mmol, 1 eq) was activated with carbonyldiimidazole (15.4 g, 94.7 mmol, 1.3 eq) in THF (650 mL) for 60 min followed by the addition of **2.9** (13.6 g, 75.7 mmol, 1 eq) in THF (100 mL). Stirring was continued under argon at rt for 20 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in ethyl acetate (1000 mL) under warming. The solution was washed with 0.1 M aq. $KHSO_4$/brine 8/1 (180 mL), water/brine 8/1 (180 mL), 5 % aq. Na_2CO_3/brine 15/1 (160 mL), water/brine 3/1 (200 mL) and brine (150 mL). The solvent was removed under reduced pressure after drying over Na_2SO_4 and filtration. Subsequent purification with column chromatography (eluent: EtOAc/PE 1/1 to EtOAc) afforded the product as a white solid (30.6 g, 47.2 mmol, 62 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.24 (s, 9H, *t*-Bu), 1.34-1.74 (m, 4H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 3.0 (m, 2H, C$\underline{H}_2$-NH), 4.05 (m, 1H, C$\underline{H}^{\alpha}$), 4.17-4.32 (m, 5H, C$\underline{H}$-C$\underline{H}_2$ from Fmoc, C$\underline{H}_2$-Ar-O-*t*-Bu), 5.01 (s, 2H, C$\underline{H}_2$-Ph), 6.88 (d, 2H, ^{3}J = 8.30 Hz, AA'BB'), 7.14 (d, 2H, ^{3}J = 8.42 Hz, AA'BB'),

7.26-7.45 (m, 10H, Ph, Fmoc, CH_2-CH_2-N<u>H</u>), 7.56 (d, 1H, ^{3}J = 8.18 Hz, CO-N<u>H</u>-CH), 7.74 (d, 2H, ^{3}J = 7.24 Hz, Fmoc), 7.89 (d, 2H, ^{3}J = 7.45 Hz, Fmoc), 8.36 (t, 1H, ^{3}J = 5.66, CH-CO-N<u>H</u>-CH_2); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 650 $[M + H]^+$, 667 $[M + NH_4]^+$; $C_{39}H_{43}N_3O_6$ (649.8)

(*R*)-*N*$^\delta$-Benzyloxycarbonyl-*N*-(4-*tert*-butoxybenzyl)ornithinamide (2.4). A solution of **2.3** (30.6 g, 47.1 mmol,) in CH_2Cl_2 (1200 mL), DMF (100 mL) and diethylamine (150 mL) was stirred at rt for 4 h. The volatiles were removed under reduced pressure and the residue was taken up in CH_2Cl_2 (400 mL). The solution was filtered from a quite small amount of insoluble material and the filtrate was subjected to column chromatography (eluent: EtOAc to EtOAc/MeOH 5/1). The product was obtained as a yellow oil (15.3 g, 35.8 mmol, 76 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.26 (s, 9H, *t*-Bu), 1.3-1.63 (m, 4H, CH-C<u>H</u>$_2$-C<u>H</u>$_2$), 2.98 (m, 2H, CH_2-C<u>H</u>$_2$-NH), 3.15 (m, 1H, C<u>H</u>$^\alpha$), 4.23 (d, 2H, ^{3}J = 5.87 Hz, C<u>H</u>$_2$-C_6H_4-O-t-Bu), 5.01 (s, 2H, C<u>H</u>$_2$-Ph), 6.9 (d, 2H, ^{3}J = 8.45 Hz, AA'BB'), 7.02 (bs, 2H, N<u>H</u>$_2$), 7.15 (d, 2H, ^{3}J = 8.47 Hz, AA'BB'), 7.25-7.4 (m, 5H, Ph), 7.64 (t, 1H, CH_2-CH_2-N<u>H</u>), 8.26 (t, 1H, ^{3}J = 5.93, CH-CO-N<u>H</u>-CH_2); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 428 $[M + H]^+$, 855 $[2M + H]^+$; $C_{24}H_{33}N_3O_4$ (427.5)

(*R*)-*N*$^\delta$-Benzyloxycarbonyl-*N*-(4-*tert*-butoxybenzyl)-*N*$^\alpha$-(2,2-diphenylacetyl)ornithinamide (2.5). A solution of **2.10** (14.4 g, 46.5 mmol, 1.3 eq) in 1,2-dimethoxyethane (100 mL) and THF (40 mL) was added to a solution of **2.4** (15.3 g, 35.8 mmol, 1 eq) and NEt_3 (3.6 g, 35.8 mmol, 1 eq) in 1,2-dimethoxyethane (130 mL). The reaction mixture was concentrated under reduced pressure to give a volume of about 80 mL after stirring at 35 °C for 20 h. Water was added (220 mL) and the pH was adjusted to 2.5-3 with $KHSO_4$. The precipitate was separated by filtration, washed with water (60 mL), dried *in vacuo*, dissolved in CH_2Cl_2/EtOAc 3/1 (150 mL) and purified by column chromatography (eluent: CH_2Cl_2/EtOAc 3/1 to EtOAc) affording **2.5** as a white solid (18.8 g, 30.3 mmol, 84 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.27 (s, 9H, *t*-Bu), 1.38 (m, 2H, CH_2-C<u>H</u>$_2$-CH_2), 1.5-1.76 (m, 2H, CH-C<u>H</u>$_2$), 2.98 (m, 2H, CH_2-C<u>H</u>$_2$-NH), 4.24 (m, 2H, C<u>H</u>$_2$-C_6H_4-O-t-Bu), 4.34 (m, 1H, C<u>H</u>$^\alpha$), 5.02 (s, 2H, C<u>H</u>$_2$-Ph), 5.08 (s, 1H, C<u>H</u>-$(Ph)_2$), 6.87, (d, 2H, ^{3}J = 8.45 Hz, AA'BB'), 7.12 (d, 2H, ^{3}J = 8.48 Hz, AA'BB'), 7.1-7.4 (m, 15H, Ph), 8.43 (t, 1H, ^{3}J = 5.78, CH-CO-N<u>H</u>-CH_2), 8.49 (d, 1H, ^{3}J = 8.03, CO-N<u>H</u>-CH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 622 $[M + H]^+$, 639 $[M + NH_4]^+$, 660 $[M + K]^+$; $C_{38}H_{43}N_3O_5$ (621.8)

(*R*)-*N*-(4-*tert*-Butoxybenzyl)-*N*$^\alpha$-(2,2-diphenylacetyl)ornithinamide (2.6).[26] To a suspension of **2.5** (18.5 g, 29.75 mmol, 1 eq) in MeOH (400 mL) were added ammonium formate (22.5 g, 357 mmol, 12 eq) and 10 % Pd/C catalyst (2.7 g). The evolution of gas was monitored with a bubble counter. Compound 5 had been completely dissolved after 4 h. The reaction mixture was left under stirring at rt overnight. The catalyst was removed by filtration, the filtrate was

concentrated under reduced pressure and the residue was taken up in $CHCl_3$ (600 mL). The organic solution was treated with saturated aq. Na_2CO_3 (150 mL, 100 mL) and brine (100 mL), then dried over Na_2SO_4 and filtered. Removal of the solvent yielded an oil which turned to a hardned foam during drying *in vacuo* (12.3 g, 25.2 mmol, 85 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.27 (s, 9H, *t*-Bu), 1.21-1.36 (m, 2H, CH_2-C$\underline{H}_2$-CH_2), 1.48-1.77 (m, 2H, CH-C$\underline{H}_2$), 3.29 (m, 2H, CH_2-C$\underline{H}_2$-NH_2), 4.21 (m, 2H, C$\underline{H}_2$-C_6H_4-O-t-Bu), 4.3 (m, 1H, C$\underline{H}^{\alpha}$), 5.12 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.86 (d, 2H, ^{3}J = 8.49 Hz, AA'BB'), 7.09 (d, 2H, ^{3}J = 8.48 Hz, AA'BB'), 7.16-7.34 (m, 10H, Ph), 8.47 (m, 1H, CO-N$\underline{H}$-CH_2), 8.55 (d, 1H, ^{3}J = 8.03, CO-N$\underline{H}$-CH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 488 $[M + H]^+$; $C_{30}H_{37}N_3O_3$ (487.6)

(*R*)-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-*tert*-butoxycarbonyl-N^{α}-(2,2-diphenylacetyl)argininamide (2.7). Compound **2.6** (3.5 g, 7.18 mmol, 1 eq) and *N*-tert-butoxycarbonyl-*N*'-benzyloxycarbonyl-1*H*-pyrazol-1-carboxamidine (**2.13**) (2.97 g, 8.6 mmol, 1.2 eq) were dissolved in CH_2Cl_2 (50 mL) and NEt_3 (0.36 g, 0.5 ml, 3.59 mmol, 0.5 eq) was added. The reaction mixture was stirred at rt overnight and then subjected to column chromatography (eluent: CH_2Cl_2/EtOAc 20/1 to 3/1, column was packed with 0.5 % NEt_3). The eluate was evaporated to dryness *in vacuo* yielding a hardened foam which was dissolved in MeOH (HPLC grade, 65 mL) in a two-necked round bottom flask equipped with a three-way valve (N_2, H_2). A 10 % palladium-on-charcoal catalyst (0.4 g) was added and a slow stream of hydrogen was led through a glass tube into the vigorously stirred suspension. After depletion of the starting material (3.5 h; control by TLC) the catalyst was removed using a PTFE-filter (25 mm, 0.2 µm). The filtrate was concentrated to give an oil and water (50 mL) was added. Lyophilization afforded the product as a white solid (3.83 g, 6.08 mmol, 84 %). Mp > 85 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d_6, COSY, HSQC): δ (ppm) 1.27 (s, 9H, *t*-Bu), 1.36 (s, 9H, *t*-Bu), 1.4 (m, 2H, CH_2-C$\underline{H}_2$-CH_2), 1.55 (m, 1H, CH-C$\underline{H}_2$), 1.68 (m, 1H, CH-C$\underline{H}_2$), 3.06 (m, 2H, CH_2-C$\underline{H}_2$-NH), 4.21 (dd, 1H, ^{2}J = 15.28 Hz, ^{3}J = 5.96 Hz, C$\underline{H}_2$-C_6H_4-O-t-Bu), 4.25 (dd, 1H, ^{2}J = 15.28 Hz, ^{3}J = 5.96 Hz, C$\underline{H}_2$-C_6H_4-O-t-Bu), 4.34 (m, 1H, C$\underline{H}^{\alpha}$), 5.13 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.87 (d, 2H, ^{3}J = 8.48 Hz, AA'BB'), 7.1 (d, 2H, ^{3}J = 8.49 Hz, AA'BB'), 7.2 (m, 2H, Ph), 7.25-7.32 (m, 8H, Ph), 8.43 (t, 1H, ^{3}J = 5.65 Hz, CO-N$\underline{H}$-CH_2), 8.49 (d, 1H, ^{3}J = 8.03, CO-N$\underline{H}$-CH); ^{13}C-NMR (150 MHz, DMSO-d_6, HSQC, HMBC): δ (ppm) 25.4 (CH_2-$\underline{C}H_2$-CH_2), 28.3 (C-($\underline{C}H_3$)$_3$), 28.5 (C-($\underline{C}H_3$)$_3$), 29.6 (CH-$\underline{C}H_2$), 39.6 (CH_2-$\underline{C}H_2$-NH), 41.5 ($\underline{C}H_2$-C_6H_4-O-t-Bu), 52.5 ($\underline{C}H^{\alpha}$), 55.9 ($\underline{C}$H-$(Ph)_2$), 75.9 ($\underline{C}$-$(CH_3)_3$), 77.7 ($\underline{C}$-$(CH_3)_3$), 123.5 (aryl, AA'BB'), 126.48 (Ph), 126.52 (Ph), 127.6 (Ar AA'BB'), 128.1 (Ph), 128.2 (Ph), 128.4 (Ph), 128.6 (Ph), 133.7 (CH_2-$\underline{C}C_4H_4$C-O-t-Bu), 140.3 (CH-($\underline{C}C_5H_5$)$_2$), 140.5 (CH-($\underline{C}C_5H_5$)$_2$), 153.8 (CH_2-$CC_4H_4\underline{C}$-O-*t*-Bu), 161.1 ($\underline{C}$O-O-t-Bu), 163.1 (guanidine), 170.9 ($\underline{C}$O-NH-CH), 171.3 ($\underline{C}$O-NH-CH_2); Anal. ($C_{36}H_{47}N_5O_5$) calcd. C 68.66, H 7.52, N 11.12, found C 68.41, H 7.74, N 11.05; HRMS: (FAB$^+$, MeOH/Glycerin): *m/z* calcd. for $[C_{36}H_{47}N_5O_5 + H]^+$ 630.3655, found: 630.3646; $C_{36}H_{47}N_5O_5$ (629.8); $C_{36}H_{47}N_5O_5$ (629.8)

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-hydroxybenzyl)-*N*$^{\omega}$-propanoylargininamide (2.8a).

Compounds **2.7** (100 mg, 159 µmol, 1 eq) and **2.11a** (32.5 mg, 191 µmol, 1.2 eq) were dissolved in anhydrous acetonitrile (4 mL), NEt_3 (8 mg, 11 µL, 79 mmol, 0.5 eq) was added and the reaction mixture was stirred under argon at rt overnight. TFA (2 mL) and two drops of water were added and stirring was continued for 4 h. CH_2Cl_2 (20 mL) was added and the solution was concentrated under reduced pressure. Addition of CH_2Cl_2 (30 mL) and evaporation under reduced pressure was repeated twice. The residue (yellow oil) was taken up in a mixture of acetonitrile and 0.1 % aq. TFA (1/1 v/v, 5 mL), filtered (0.2 µm) and injected into the preparative HPLC system (6 injections). Eluent: mixtures of acetonitrile (A) and 0.1 % aq. TFA (B). Gradient: A/B 20/80 to 50/50 in 30 min. Acetonitrile was removed under reduced pressure from the eluate containing the product ($t_R \approx 24$ min). After lyophilization of the remaining aqueous solution the product was obtained as TFA salt as a white fluffy solid (71.6 mg, 111 µmol, 70 %). Mp 166-168 °C; ^{1}H-NMR (600 MHz, DMSO-d_6, ^{1}H-COSY, HSQC): δ (ppm) 1.04 (t, 3H, C$\underline{H}_3$, ^3J = 7.42 Hz), 1.39-1.48 (m, 2H, CH_2-C$\underline{H}_2$-CH_2), 1.54 (m, 1H, CH-C$\underline{H}_2$), 1.67 (m, 1H, CH-C$\underline{H}_2$), 2.43 (q, 2H, C$\underline{H}_2$-CH_3, ^3J = 7.40 Hz), 3.22 (m, 2H, CH_2-C$\underline{H}_2$-NH), 4.12 (dd, 1H, ^2J = 14.82 Hz, ^3J = 5.78 Hz, C$\underline{H}_2$-C_6H_4-OH), 4.16 (dd, 1H, ^2J = 14.81 Hz, ^3J = 5.85 Hz, C$\underline{H}_2$-C_6H_4-OH), 4.33 (m, 1H, C$\underline{H}^{\alpha}$), 5.12 (s, 1H, C$\underline{H}$($Ph)_2$), 6.6 (d, 2H, ^3J = 8.49 Hz, AA'BB'), 6.99 (d, 2H, ^3J = 8.49 Hz, AA'BB'), 7.21 (m, 2H, Ph), 7.26-7.30 (m, 8H, Ph), 8.34 (t, 1H, ^3J = 5.81 Hz, CO-N$\underline{H}$-CH_2), 8.46 (d, 1H, ^3J = 8.10, CO-N$\underline{H}$-CH), 8.67 (bs, 2H, N$\underline{H}_2$), 9.16 (t, 1H, CH_2-CH_2-N$\underline{H}$), 9.28 (s, 1H, N$\underline{H}$-CO-CH_2-CH_3), 11.7 (s, 1H, ArO$\underline{H}$); ^{13}C-NMR (150 MHz, DMSO-d_6, HSQC, HMBC): δ (ppm) 8.2 ($\underline{C}H_3$), 24.3 (CH_2-$\underline{C}H_2$-CH_2), 29.3 (CH-$\underline{C}H_2$), 29.5 ($\underline{C}H_2$-CH_3), 40.4 (CH_2-$\underline{C}H_2$-NH), 41.6 ($\underline{C}H_2$-C_6H_4-OH), 52.3 ($\underline{C}H^{\alpha}$), 55.9 ($\underline{C}$H-$(Ph)_2$), 115.0 (Ar AA'BB'), 126.5 (Ph), 126.6 (Ph), 128.1 (Ph), 128.2 (Ph), 128.4 (Ar AA'BB'), 128.46 (Ph), 128.48 (Ph), 129.1 (CH_2-$\underline{C}C_4H_4$C-OH), 140.3 (CH-($\underline{C}C_5H_5)_2$), 140.5 (CH-($\underline{C}C_5H_5)_2$), 152.9 (guanidine), 156.2 (CH_2-$CC_4H_4\underline{C}$-OH), 170.9 ($\underline{C}$O-NH), 171.0 ($\underline{C}$O-NH), 175.9 ($\underline{C}OCH_2CH_3$); HPLC purity (210 nm): > 99 % (cf. Figure 7); HRMS: (FAB^+, MeOH/glycerol): *m/z* calcd. for $[C_{30}H_{35}N_5O_4 + H]^+$ 530.2767, found: 530.2774; $C_{30}H_{35}N_5O_4 \times C_2F_3HO_2$ (643.6)

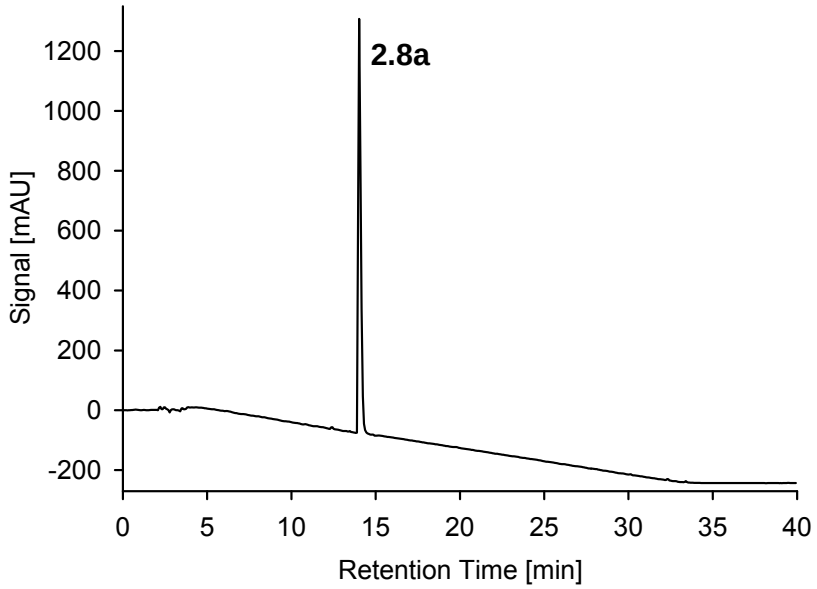


Figure 7. HPLC purity control of **2.8a**. Eluent: mixtures of acetonitrile (A) and 0.05 % aq. TFA (B), gradient: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5, detection: 210 nm, injection: 100 µL of a 50 µM solution of **2.8a** in a mixture of acetonitrile and 0.05 % aq. TFA (20/80) prepared from a 5 mM solution in acetonitrile, t_R = 14.0 min, k = 4.2.

4-*tert*-Butoxybenzylamine (2.9).[27] A solution of 4-*tert*-butoxybenzonitrile (50 g, 285.4 mmol, 1 eq) in anhydrous diethyl ether (200 mL) was dropped to a mechanically stirred suspension of $LiAlH_4$ (25 g, 658.8 mmol, 2.3 eq) in anhydrous diethyl ether (400 mL) over a period of 3 h. The reaction mixture was refluxed for additional 60 min, cooled to 0 °C and carefully quenched by the dropwise addition of water (20 mL), 15 % aq. NaOH (17 mL) and again water (50 mL). Inorganic material was filtered off and washed twice with diethyl ether. The combined filtrate and washings were concentrated to about 150 mL followed by the addition of water (850 mL) and 1 M aq. $KHSO_4$ (about 300 mL) to adjust the pH to 2-3. The aqueous phase was separated, washed with diethyl ether (180 mL) and alkalified with 2 N aq. NaOH (200 mL). The product was extracted with CH_2Cl_2 (three times 450 mL). The extracts were pooled, washed with brine (300 mL) and dried over Na_2SO_4. After filtration and removal of the solvent in high vacuo the product was obtained as a yellowish oil (50.1 g, 299.2 mmol, 98 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.31 (s, 9H, *t*-Bu), 2.03 (s, 2H, N$\underline{H}_2$), 3.8 (s, 2H, C$\underline{H}_2$), 6.93 (d, 2H, ^{3}J = 8.51, AA'BB'), 7.18 (d, 2H, ^{3}J = 8.64, AA'BB'); MS (EI): *m/z* 122.0 [M - $C(CH_3)_3$]$^+$, 123.0 [M - ·$C(CH_3)_3$ + H]$^{\cdot+}$, 164 [M - ·CH_3]$^+$, 179 (M·$^+$); $C_{11}H_{17}NO$ (179.3)

Succinimidyl 2,2-diphenylacetate (2.10).[28, 29] DCC (40.8 g, 197.9 mmol, 1.05 eq) was added to a stirred solution of diphenylacetic acid (40 g, 188.5 mmol, 1 eq) and *N*-hydroxysuccinimide (22.8 g, 197.9 mmol, 1.05 eq) in anhydrous THF (800 mL). After stirring at rt overnight the white solid (DCU) was removed by filtration and the solvent was removed under reduced pressure. Purification with column chromatography (eluent: PE/EtOAc 2/1 to 1/2) yielded the product as a

white semi-crystalline solid (54.8 g, 177.2 mmol, 94 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 2.79 (s, 4H, C<u>H</u>$_2$), 5.36 (s, 1H, C<u>H</u>), 7.26-7.4 (m, 10H, Ph); MS (CI, NH_3): *m/z* 327 $[M + NH_4]^+$; $C_{18}H_{15}NO_4$ (309.3)

Succinimidyl propanoate (2.11a).[30] DCC (3 g, 14.85 mmol, 1.1 eq) was added to a stirred ice-cold solution of propanoic acid (1 g, 1 mL, 13.5 mmol, 1 eq) and *N*-hydroxysuccinimide (1.71 g, 14.85 mmol, 1.1 eq) in anhydrous THF (30 mL). The ice-bath was removed after 2 h and stirring was continued at rt overnight. The white solid (DCU) was removed by filtration and the solvent was removed under reduced pressure. Purification with column chromatography (eluent: PE/EtOAc 3/1 to 1/1) yielded the product as a white solid (1.4 g, 8.2 mmol, 60 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.27 (t, 3H, ^{3}J = 7.51, C<u>H</u>$_3$), 2.63 (q, 2H, ^{3}J = 7.52, C<u>H</u>$_2$-CH_3), 2.82 (s, 4H, C<u>H</u>$_2$-C<u>H</u>$_2$); MS (CI, NH_3): *m/z* 189 $[M + NH_4]^+$; $C_7H_9NO_4$ (171.2)

***N*-Benzyloxycarbonyl-1*H*-pyrazole-1-carboxamidine (2.12).**[26, 31] Concentrated hydrochloric acid (76 mL) was added dropwise to a stirred suspension of aminoguanidine carbonate (80 g, 587.8 mmol, 1.05 eq) in water (120 mL) resulting in a clear solution of aminoguanidine hydrochloride which was added to a gently warmed (45 °C) suspension of 1,1,3,3-tetramethoxypropane (91.9 g, 559.8 mmol, 1 eq) in water (120 mL) over a period of 60 min. The obtained darkly colored solution was stirred for additional 30 min. Since crystallization of 1*H*-pyrazole-1-carboxamidine hydrochloride could not be initiated by the treatment with acetone (as described[26, 31]) or acetonitrile, all solvents were removed by evaporation under reduced pressure and drying *in vacuo*. The darkly colored residue (only soluble in water under warming) was suspended in MeOH/CH_2Cl_2 1/1 (1000 mL) and NEt_3 (178 mg, 244 µL, 1763 mmol, 3 eq) was added. The mixture was cooled to 0 °C, benzyloxycarbonyl chloride (45 % in toluene, 559.8 mmol, 218.5 mL, 1.0 eq) was added over a period of 2.5 h and the reaction was left under stirrung at rt overnight. All volatiles were removed under reduced pressure. To the residue ethyl acetate (1300 mL) and water (400 mL) were added. The two phases were mixed vigorously and then separated. The darkly colored aqueous phase was treated with ethyl acetate (200 mL), the organic layers were combined and washed with saturated aqueous $NaHCO_3$ solution (300 mL), water (300 mL) and brine (300 mL). After drying over Na_2SO_4 and filtration the solvent was removed under reduced pressure and the product was roughly purified by column chromatography (eluent: CH_2Cl_2/EA 3/1). The product was crystallized from CH_2Cl_2 (35.8 g, 146.7 mmol, 25 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 5.13 (s, 2H, C<u>H</u>$_2$-Ph), 6.58 (dd, 1H, ^{3}J = 1.61 Hz, 2.76 Hz, pyrazole), 7.32-7.42 (m, 5H, Ph), 7.92 (dd, 1H, ^{3}J = 1.52 Hz, ^{4}J = 0.60 Hz, pyrazole), 8.45 (dd, 1H, ^{3}J = 2.75 Hz, ^{4}J = 0.48 Hz, pyrazole), 8.97 (bs, 2H, NH_2); MS (CI, NH_3): *m/z* 245 $[M + H]^+$; $C_{12}H_{12}N_4O_2$ (244.2)

***N*-Benzyloxycarbonyl-*N'*-*tert*-butoxycarbonyl-1*H*-pyrazole-1-carboxamidine (2.13).**[32, 33] A solution of **2.12** (35.5 g, 145.3 mmol, 1 eq) and di-*tert*-butyl dicarbonate (76 g, 348 mmol, 2.4 eq) in chloroform (150 mL) was treated with DMAP (3.6 g, 29.1 mmol, 0.2 eq) and stirred overnight. Chloroform (250 mL) was added and the reaction mixture was washed with 0.5 M aq. $KHSO_4$ (100 mL), water (100 mL) and brine (100 mL). The solution was dried over Na_2SO_4, filtered, and the volume was reduced to about 100 mL under reduced pressure. The residue was subjected to column chromatography (eluent: CH_2Cl_2/EtOAc 20/1 to 5/1) to isolate the twofold Boc-protected intermediate as a yellow oil which was treated with $Mg(ClO_4)_2$ (6.2 g, 27.8 mmol, 0.2 eq) in THF (500 mL) at 55 °C for 3 h. The solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2 (1000 mL). The organic solution was washed with water (200 mL) and brine (200 mL), then dried over Na_2SO_4 and filtered. The filtrate was concentrated. Purification with column chromatography (eluent: PE/EtOAc 5/1 to 1/1, column packed with 0.5 % NEt_3) afforded the product as a highly viscous yellow oil (26.7 g, 77.5 mmol, 53 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.44 (s, 9H, *t*-Bu) 5.14 (s, 2H, C$\underline{H_2}$-Ph), 6.60 (dd, 1H, ^3J = 1.61 Hz, 2.76 Hz, pyrazole), 7.32-7.44 (m, 5H, Ph), 7.88 (dd, 1H, ^3J = 1.58 Hz, ^4J = 0.53 Hz, pyrazole), 8.37 (dd, 1H, ^3J = 2.79 Hz, ^4J = 0.53 Hz, pyrazole); MS (CI, NH_3): *m/z* 345 $[M + H]^+$, 245 $[M - Boc + H]^+$; $C_{17}H_{20}N_4O_4$ (344.4)

2.3.3 Synthesis of N^{ω}-([2,3-^{3}H]-Propionyl)-BIBP 3226 ([^{3}H]-UR-MK114, 2.8b)

2.8b was prepared from two different batches of succinimidyl [2,3-^{3}H]-propionate (**2.11b**), one from GE Healthcare (München, Germany, formerly Amersham Biosciences) provided in toluene (185 MBq/5 mL, a_s = 3.48 TBq/mmol), and a second one from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA) provided in ethyl acetate (185 MBq/1 mL, a_s = 2.22 TBq/mmol). The labeling reaction was performed in a 1.5-mL Eppendorf reaction vessel (screw top) in acetonitrile using 40 eq of the labeling precursor **2.7**.

(*R*)-N^{α}-(2,2-Diphenylacetyl)-*N*-(4-hydroxybenzyl)-N^{ω}-([2,3-^{3}H]propanoyl)argininamide (2.8b).

(1) Synthesis of **2.8b** from 185 MBq of succinimidyl [2,3-^{3}H]-propanoate (**2.11b**) (a_s = 3.48 TBq/mmol, GE Healthcare). Compound **2.7** (1.34 mg, 2.13 µmol, 40 eq) was dissolved in anhydrous acetonitrile (30 µL) yielding a concentration of 70.9 mM, and NEt_3 was diluted in acetonitrile to a concentration of 0.55 µL NEt_3/22.5 µL. Compound **2.7** (70.9 mM in acetonitrile, 15 µL, 1.064 µmol, 20 eq) and NEt_3 (7.5 µL of the obove described solution in acetonitrile, 1.33 µmol, 25 eq) were added to **2.11b** (9.3 µg, 0.053 µmol, 1 eq) in toluene (5 mL), and the solvent was removed with a slight stream of nitrogen (blown through a PTFE filter, 13 mm, 0.2 µm, nylon) over a period of 2 h. The 5-mL glas vial was rinsed three times with acetonitrile (100 µL

each), the washings were combined in a 1.5-mL Eppendorf reaction vessel (screw top) and **2.7** (70.9 mM in acetonitrile, 15 µL, 1.064 µmol, 20 eq) as well as NEt_3 (15 µL of the above described solution in acetonitrile, 2.66 µmol, 50 eq) were added. The reaction mixture was concentrated to about 80 µL in a vacuum concentrator and stirred with a magnetic micro stirrer at 35 °C overnight. TFA (100 µL) was added and the reaction mixture was allowed to stand under stirring at 60 °C for 2 h. MeOH (400 µL) was added and the volume was reduced to about 20 µL in a vacuum concentrator (at 40 °C). The residue was diluted with a mixture of acetonitrile and 0.05 % aq. TFA (25/75 v/v, 300 µL) and the product was isolated with a Shimadzu HPLC system (4 injections). Eluent: mixtures of acetonitrile plus 0.04 % TFA (A) and 0.05 % aq. TFA (B), gradient: 0 to 22 min: A/B 25/75 to 53/47, 22 to 24 min: 53/47 to 95/5, 24 to 29 min: 95/5. The product was eluted at 18.9 min and collected in a 1.5-mL Eppendorf reaction vessel (screw top), which was put into the vacuum concentrator between the injections. The combined product fractions were evaporated to dryness in a vacuum concentrator (about 5 h), the residue was dissolved in ethanol (500 µL), transferred into the clean 5-mL Amersham glass vial and combined with the washings (160 µL and 140 µL).

Quantification: A five point calibration was performed with **2.8a** (0.5, 0.75, 1.0, 1.25 and 1.5 µM, inj. vol.: 100 µL). Eluent: mixtures of acetonitrile plus 0.04 % TFA (A) and 0.05 % aq. TFA (B), gradient: 0 to 17 min: A/B 20/80 to 50/50, 17 to 19 min: 50/50 to 90/10, 19 to 24 min: 90/10 (t_R = 18.7 min). The solutions for injection were prepared in a mixture of acetonitrile and 0.05 % aq. TFA (20/80) less than 3 min prior to the injection. All standard solutions were prepared separately from a 100 µM solution of **2.8a** in a mixture of acetonitrile and 0.05 % aq. TFA (20/80), which was freshly prepared for each standard from a 10 mM solution of **2.8a** in DMSO. 6.6 µL of the ethanolic solution (total volume: 800 µL) were diluted with 103.4 µL of a mixture of acetonitrile and 0.05 % aq. TFA (20/80) in duplicate and 100 µL of each solution were analyzed by HPLC. The molarity of the ethanolic solution of **2.8b** was calculated from the mean of the peak areas and the linear calibration curve obtained from the peak areas of the standards. Yield: 9.6 µg (0.018 µmol, 34 %[a]).

Determination of the specific activity: four aliquots (1 µL) of the ethanolic solution were diluted with 499 µL of acetonitrile and 10 µL of the 1:500-dilutions were counted in 3 mL of Rothiszint eco plus (Roth, Karlsruhe, Germany) in a LS 6500 liquid scintillation counter (Beckmann-Coulter, München; Germany). Calculated specific activity: 3.6 TBq/mmol (97.3 Ci/mmol). The activity concentration was adjusted to 18.5 MBq/mL (0.5 mCi/mL) by the addition of ethanol (3311 µL) with a TFA additive (100 µM) to the remaining solution yielding a concentration of 4.4 µM. The radioligand **2.8b** was stored at –20 °C.

([a]The actual efficiency of the labeling reaction was about 60 % related to the amount of activity which was available after the toluene removal.)

(2) Synthesis of **2.8b** from 74 MBq of succinimidyl [2,3-^{3}H]-propanoate (**2.11b**) (a_s = 2.22 TBq/mmol, American Radiolabeled Chemicals). Compound **2.7** (0.84 mg, 1.33 µmol, 40 eq) was dissolved in anhydrous acetonitrile (60 µL) yielding a concentration of 22.2 mM, and NEt_3 was diluted in acetonitrile to a concentration of 0.18 µL NEt_3/10 µL. Compound **2.7** (22.2 mM in acetonitrile, 20 µL, 0.44 µmol, 13.3 eq) was added to **2.11b** (5.84 µg, 0.033 µmol, 1 eq, 74 MBq) in ethyl acetate (0.4 mL) in a 1.5-mL Eppendorf reaction vessel (screw top). The solvent was removed in a vacuum concentrator (40 °C) over a period of 50 min. **2.7** (22.2 mM in acetonitrile, 40 µL, 0.89 µmol, 26.7 eq) and NEt_3 (10 µL of the above described solution in acetonitrile, 1.33 µmol, 40 eq) were added. The reaction mixture was vigorously blended (vortexer) for 1 min, briefly centrifuged and stirred with a magnetic micro stirrer at rt overnight. For deprotection TFA (60 µL) was added and stirring was continued for 3 h at 40 °C. For HPLC analysis 2 µL of the "deprotection mixture" were diluted (1:20) with 38 µL of acetonitrile/0.05 % aq. TFA (50/50), and 2 µL of this solution were diluted (1:50) with 98 µL of acetonitrile/0.05 % aq. TFA (20/80). This solution was completely injected into the Waters HPLC-system and analyzed with UV- and radiometric detection (radiometric: see Figure 8B). The residual "deprotection mixture" (≈ 98 µL) was diluted with acetonitrile/0.05 % aq. TFA (10/90, 260 µL) and combined with the remaining 1:20-diluted "deprotection mixture" (≈ 38 µL). The product was isolated with a Waters HPLC system (3 injections) using the conditions specified for the analysis of the "deprotection mixture". For this purpose radiometric detection was not performed (Figure 8A). The product was eluted at 25.4 to 26.1 min and collected in a 1.5-mL Eppendorf reaction vessel (screw top), which was put into the vacuum concentrator between the injections. The combined product fractions were evaporated to dryness in a vacuum concentrator (about 5 h), the residue was dissolved in 200 µL of ethanol containing TFA (100 µM) and transferred to a clean 5-mL Amersham glass vial together with the washings (3 × 100 µL).

Quantification: A five point calibration was performed with **2.8a** (0.3, 0.5, 0.75, 1.0 and 1.5 µM, inj. vol.: 100 µL). Eluent: mixtures of acetonitrile + 0.05 % TFA (A) and 0.05 % aq. TFA (B), gradient for the 1.5 µM standard: 0 to 30 min: A/B 20/80 to 90/10, 30 to 38 min: 90/10 (retention time of **2.8a**: 16.7 min), gradient for the other standards: 0 to 12 min: A/B 30/70 to 50/50, 12 to 12.5 min: 50/50 to 90/10, 12.5 to 18 min: 90/10 (retention time of **2.8a**: 12.85 min). The solutions for injection were prepared in acetonitrile/0.05 % aq. TFA (20/80) less than 3 min prior to the injection. All standard solutions were prepared from a 40 µM solution of **2.8a** (in acetonitrile/0.05 % aq. TFA 20/80), which was freshly made for each standard solution from a 2 mM stock solution of **2.8a** in acetonitrile/water 80/20. Two aliquots (4.0 µL) of the ethanolic solution (total volume: 500 µL) of the product were diluted with 100 µL of acetonitrile/0.05 % aq. TFA (20/80), and 100 µL were analyzed by HPLC. Whereas one sample was only used for quantification of the product by UV detection the second sample was additionally monitored radiometrically to determine radiochemical purity (Figure 1). The molarity of the ethanolic

solution of **2.8b** was calculated from the mean of the peak areas and the linear calibration curve obtained from the peak areas of the standards. Yield: 5.86 µg (0.011 µmol, 33 %).

Determination of the specific activity: 1.5 µL of the ethanolic solution were diluted with 448.5 µL of a mixture of acetonitrile and water (65/35) in triplicate, and 9 µL of the 1:300-dilutions were counted in Rothiszint™ eco plus (3 mL). Calculated specific activity: 1.9 TBq/mmol (51 Ci/mmol). The activity concentration was adjusted to 9.25 MBq/mL (0.25 mCi/mL) by addition of ethanol (1696.5 µL) containing TFA (100 µM) to the residual solution yielding a molarity of 4.9 µM. The radioligand **2.8b** was stored at –20 °C.

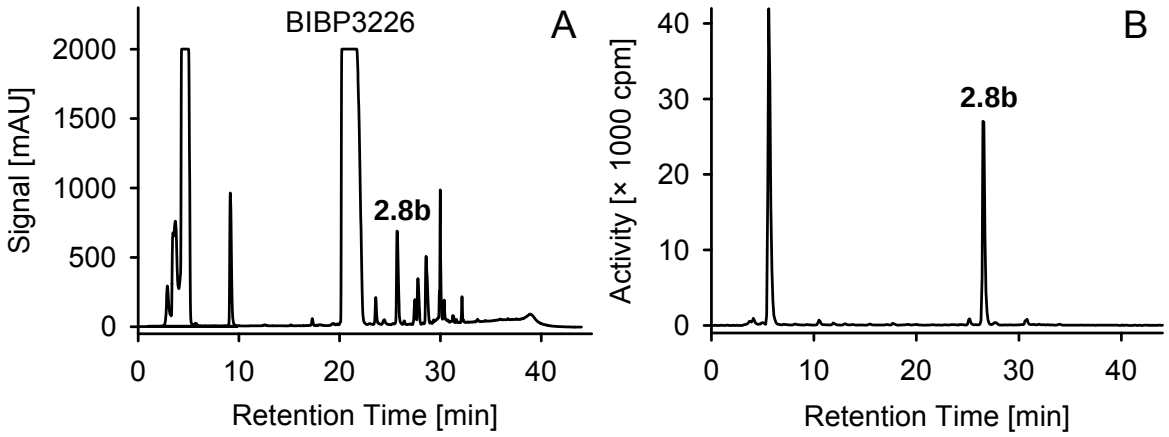


Figure 8. A: Analytical HPLC run for isolation of radioligand **2.8b**; detection: UV (220 nm); t_R = 25.8 min. B: HPLC analysis of the "deprotection mixture" containing the non-purified radioligand **2.8b**; radiometric detection, t_R = 26.4 min. Eluent (for A and B): mixtures of acetonitrile + 0.05 % TFA (A) and 0.05 % aq. TFA (B); gradient (for A and B): 0 to 26 min: A/B 20/80 to 47/53, 26 to 27 min: 47/53 to 90/10, 27 to 35 min: 90/10.

2.3.4 Pharmacology: Experimental Protocols

General. Unless otherwise noted, all chemicals (analytical grade) were obtained from commercial suppliers. Millipore water was used throughout. Dy-635-pNPY was synthesized as described previously.[34] [K^4]-hPP was synthesized in the laboratory of Prof. Beck-Sickinger (University of Leipzig, Germany) and labeled with the cyanine dye Cy5 in our laboratory.[35] Porcine NPY (pNPY) was prepared in-house in the laboratory of Dr. C. Cabrele. The fura-2 AM (Calbiochem/Merck Biosciences, Beeston, UK) stock solution (1 mM) was prepared in anhydrous DMSO. Pluronic F-127 (Calbiochem/Merck Biosciences, Beeston, UK) was dissolved in DMSO to a concentration of 20 %. The buffer for the Y_1R binding studies of **2.8b** on SK-N-MC cells, MCF-7 cells and tissue sections was prepared by the addition of BSA (1 % for binding on cells, 0.2 % for binding on tissue sections) and bacitracin (50 µg/mL) 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), $Mg_2SO_4 \cdot 7H_2O$ (1.2 mM), and $NaHCO_3$ (25 mM). This HEPES buffer without the additives was used to wash the cells and tissue sections previous to and after radioligand binding. The loading buffer (pH 7.4) for the determination of intracellular Ca^{2+} mobilization in HEL cells was

prepared by dissolving NaCl (120 mM), KCl (5 mM), $MgCl_2 \cdot 6H_2O$ (2 mM), $CaCl_2 \cdot 2H_2O$ (1.5 mM), HEPES (25 mM), and glucose (10 mM). The lysis solution for the radioligand binding assays consisted of urea (8 M), acetic acid (3 M) and Triton-X-100 (1 %) in water. Stock solutions of **2.8a** (10 mM in DMSO, 2 mM in acetonitrile/water 80/20), BIBP 3226 (10 mM in DMSO) and pNPY (1 mM in DMSO) were stored at –20 °C. Tritium counting was done in 3 mL of Rothiszint eco plus with a Beckman LS 6500 beta-counter. Animals were taken from the livestock breeding of the University of Regensburg.

Cell culture. HEL and SK-N-MC cells were cultured as described elsewhere.[36, 37] HEL cells were subcultured by 1:6-dilution with fresh culture medium 24 h prior to the fura Ca^{2+}-assay. MCF-7-Y_1[a] cells were maintained in MEM (Sigma), supplemented with 5 % FCS (Biochrom AG, Berlin, Germany). The CHO cells transfected with the Y_2R, $G_{\alpha qi5}$ and aequorin were cultured as previously described.[34] CHO cells transfected with the Y_4R, $G_{\alpha qi5}$ and aequorin were cultured under the same conditions. HEC-1B-Y_5 cells were cultured as previously described.[38]
([a]This cell line originated from MCF-7 cells in the 157th passage and shows a higher Y_1R expression (factor 2 – 3) over the original MCF-7 cells.)

Radioligand binding assay. The radioligand binding assay was essentially performed as described[36]. Cells were seeded in 24-well plates 2 or 3 days prior to the experiment. On the day of the experiment confluency of the cells was at least 70 %. The culture medium was removed, the cells were washed once with buffer (500 µL), and covered with binding buffer (200 µL). Binding buffer (25 µL) and binding buffer (25 µL) with **2.8b** (10-fold concentrated) was added for total binding. For non-specific binding and displacement of **2.8b** binding buffer (25 µL) with the competing agent (pNPY, **2.14** (BIBP 3226) or **2.8a**, 10-fold concentrated) and binding buffer (25 µL) with **2.8b** (10-fold concentrated) was added. During incubation at room temperature (22 – 25 °C) the plates were gently shaken. After incubation (saturation experiments and competition with **2.14** or **2.8a**: 20 min; competition with pNPY: 2 h) the binding buffer was removed, the cells were washed twice with buffer (500 µL, 4 °C, ≤ 30 s), covered with lysis solution (200 µL) and the plates were gently shaken for at least 30 min. The lysis solution was transferred into 6-mL scintillation vials filled with scintillator (3 mL) and the dishes were washed once with lysis solution (100 µL). In case of the investigation of the dissociation kinetics the cells were incubated with binding buffer (250 µL) containing BIBP 3226 (100-fold higher concentrated than the previously used radioligand) after the incubation with radioligand (6 nM) and subsequent washing (twice 500 µL, 4 °C). After different periods of time the cells were washed again twice with buffer (500 µL, 4 °C) followed by the addition of lysis solution. Assays were performed in triplicate.

Fura-2 assay on HEL cells (Schild analysis). The fura assay was performed with HEL cells as previously described[39] using an LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany). Concentration-response curves of pNPY were constructed in the presence of **2.8a** at different concentrations. The signal intensities were related to the maximal intensity obtained from a pNPY effect curve in the absence of **2.8a**.

Autoradiography. Subcutaneous MCF-7-Y_1 tumors were established in female NMRI (nu/nu) mice bearing an estrogen depot[40] by subcutaneous injection of a cell suspension in culture medium without FCS (4 Mio cells/50 µL, 171st in vitro passage). The tumor and the rat brain (male Wistar rat, 10 month) were taken from the animals (brain: 24 h prior to experiment, tumor: 48 h prior to experiment), immediately frozen in Tissue-Tek with the help of dry ice, and stored at -18 °C (brain) and at -78 °C (tumor). Cryosections (12 µm) were obtained at -16 °C with a 2800 Frigocut E freezing microtome (Reichert-Jung/Leica, Germany). Two adjacent sections were mounted on a microscopic slide (Superfrost Plus, 75 × 25 × 1 mm), put 1 min into a chamber of 100 % humidity and then carefully covered with binding buffer or fixed for 20 s in an alcoholic formaldehyde fixative (40 mL of 37 % formaldehyde, 360 mL of 95 % ethanol and 0.2 g calcium acetate[41]). The binding buffer was removed (after a period of less than 60 min under cooling) by putting the slides uprightly on a paper towel (≈ 1 min). For total binding the sections were covered with binding buffer (about 800 to 1000 µL for one slide) containing **2.8b** (3 nM) and for non-specific binding with binding buffer containing **2.8b** (3 nM), **2.8a** (30 nM) and pNPY (300 nM)[a]. The sections were incubated at room temperature (22 - 25 °C) for a period of 8 min (tumor) or 30 min (brain). After incubation the binding buffer was removed, the slides were immerged three times into buffer split to 3 vessels (4 °C, 10 s) and finally rinsed with distilled water (4 °C, 3 s). The slides were put uprightly on a paper towel for 1 min and then dried in horizontal position in a desiccator over P_4O_{10}. The slides were set in close contact with a tritium sensitive screen (PerkinElmer, 192 × 125 mm) using an X-ray film cassette and stored in a dark room for 10 d. The autoradiographic image was generated from the tritium screen using a phosphorimager (Cyclone Storage Phosphor System, Packard).

The fixed sections were stained with haematoxylin and eosin (brain) or using the Masson-Goldner (Jerusalem's modification) staining procedure (tumor): H & E: rinsing (demineralized H_2O), Mayer's haemalum solution (Merck) 1:3 diluted in water (11 min), rinsing (tap water), 1 % aq. acetic acid (3 × immersion), running tap water (10 min), rinsing (H_2O_{demin}), eosin standard solution (2 min), running H_2O_{demin} (5 min); Masson-Goldner (Jerusalem's modification): Weigert's iron-haematein (45 s), rinsing (H_2O_{demin}), running tap water (10 min), differentiation with 200 mL of H_2O_{demin} + 20 mL of 2 M aq. hydrochloric acid (15 s), running tap water (10 min), rinsing (H_2O_{demin}), 0.5 % aq. phosphotungstic acid (15 s), running H_2O_{demin} (10 min), acid fuchsine-Ponceau (30 s), 1 % aq. acetic acid (3 × immersion), phosphoric acid-Orange G (10 s), 1 % aq. acetic acid (3 × immersion), 0.2 % light green (3.5 min), 1 % aq. acetic acid (3 × immersion), 96

% aq. ethanol (2 × 3 min), 100 % ethanol (2 × 3 min), 100 % xylene (3 min). Entellan (Merck) was used for covering.

([a]**2.8b** was displaced with pNPY in combination with **2.8a** to determine non-specific binding in tissue structures inaccessible to pNPY.)

Flow cytometric binding assays. Flow cytometric binding studies at the Y_2R (CHO cells transfected with the Y_2R, $G\alpha_{qi5}$ and aequorin)[42] and at the Y_5R (expressed by HEC-1B cells transfected with the Y_5R)[34, 43] were performed as previously described. The cell density in loading buffer was 1 Mio/mL (Y_2R) or 0.5 Mio/mL (Y_5R). For Y_2R binding studies Dy-635-pNPY (10 nM) was used instead of Cy5-pNPY (10 nM).

Flow cytometric binding studies at the Y_4R (expressed by CHO cells transfected with the Y_4R, $G\alpha_{qi5}$ and aequorin)[35] were performed as reported[44] with the following variations: Cy5-[K^4]-hPP[35] (5 nM) was used instead of S0586-[K^4]-hPP (10 nM). The cell density in loading buffer was 10^6/mL. Non-specific binding was determined in the presence of hPP (100 nM). The stock solution of Cy5-[K^4]-hPP was prepared in 10 mM HCl + 0.1 % BSA (500 nM).

All flow cytometric assays were performed in triplicate on a FACSCalibur™ flow cytometer (Becton Dickinson, Heidelberg, Germany), equipped with an argon laser (488 nm) and a red diode laser (635 nm). **2.8a** was tested at a concentration of 1 µM and 10 µM (for Y_2R binding also 30 µM). Whereas no displacement of the fluorescent ligands was observed in case of the Y_2R and Y_4R by **2.8a** (10 µM), displacement at the Y_5R was around 50 %.

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Chapter 3

Bivalent Argininamide-Type NPY Y_1 Receptor Antagonists

3.1 Introduction

Today, a large number of G-protein coupled receptors (GPCRs), for instance adrenergic receptors, $GABA_B$ receptors, dopamine receptors, opioid receptors, adenosine receptors, neuropeptide Y receptors, etc.[1, 2] are supposed to form homo- or hetero-oligomers, and there is growing evidence that particularly GPCR hetero-oligomerization is implicated in the modulation of physiological responses. Various methods were developed to study the existence of GPCR dimers and higher oligomers. Co-immunoprecipitation allows the detection of GPCR dimers in cell lysates after co-expression of differentially epitope-tagged forms of a GPCR.[3, 4] Distinct pairs of individually non-functional receptor mutants showed reconstituted function when co-expressed.[5-7] Resonance energy transfer techniques (FRET, BRET) are frequently used to study GPCR oligomerization, but they are compromised by a low signal-to-noise ratio due to an overlap of the emission spectra with cellular auto-fluorescence. Another limitation is the occurrence of intracellular FRET additional to FRET on the cell surface, which is of interest. These problems could be overcome with time-resolved FRET (TR-FRET)[8], a technology, where epitope tagged receptors are labeled with an antibody, carrying a long-lived fluorescence donor such as europium cryptate and an appropriate acceptor, respectively.[9] Recently, this technique has been improved by the fusion of the *N*-terminal receptor domain with a snap protein. The snap protein, exerting O^6-guanine nucleotide alkyltransferase activity, serves for a covalent attachment of TR-FRET-compatible fluorophores to the receptor.[10] These techniques require the expression of GPCRs which are either epitope-tagged or fused to a protein.

The use of bivalent ligands as an alternative to the afore-mentioned methods offers the possibility to investigate native GPCRs expressed endogenously by normal wild-type cells. Such compounds, defined as two pharmacophoric entities linked through a spacer, are anticipated to bring more insight into the phenomenon of GPCR dimerization. Addressing GPCR dimers by homo or hetero bivalent ligands is challanging because the bivalent ligands have to fulfill numerous special requirements. One prerequisite is the appropriate length of the linker, which should be at least as long as the distance between the binding sites. For instance, Portoghese proposed a distance of 22 to 32 Å (corresponding to 15-22 atoms) for the opioid receptor family.[11, 12] This value is a rule of thumb as on one hand, the distance between the

binding sites of a GPCR dimer depends on the orientation of the two receptor monomers contacting each other, on the other hand the linker may span the transmembrane domains, the shortest distance between the binding sites, or it may form an extracellular loop requiring remarkably longer spacers.

Another critical issue is the binding behavior of bivalent ligands, which can occupy both binding sites of two receptor monomers simultaneously. Do we stringently have to expect a dramatic increase in affinity (factor of 50 and more) over mono-site binding? Such observations are limited to a few cases.[13, 14] This leads to a key question, the thermodynamic profile of a bivalent ligand, which addresses two binding sites simultaneously. The binding affinity, reflected by the dissociation constant K_i, is related to the Gibbs free energy ($\Delta G = RT\ln K_i$). The released Gibbs free energy upon complex formation (ligand binding) results from entropic and enthalpic contributions ($\Delta G = \Delta H - T\Delta S$). In favorable cases the overall binding enthalpy (ΔH_{total}) of a polyvalent ligand can be more negative than the sum of the enthalpies, which result from a single docking of pharmacophoric moieties.[15] Since a simultaneous binding of two pharmacophoric entities of a bivalent ligand is entropically disfavored, the loss of entropy will more or less compensate the gain in enthalpy. Particularly for very flexible linkers (high number of freely rotatable single bonds), the binding will be remarkably diminished due to considerable vibrational and rotational restrictions.[15, 16] Positive entropic contributions arising from the release of water molecules are assumed to be less important for these considerations since such entropic terms were reported to have usually similar values in monovalent and polyvalent systems.[15] This argumentation can be subjected to the potential case that one pharmacophoric entity of a bivalent ligand binds orthosterically and the second simultaneously to an allosteric site. The observed affinity in the latter constellation is supposed to be comparatively lower since it is likely that the binding to the allosteric site is less strong compared to the orthosteric docking. Nevertheless, ligand binding can be favorably altered through allosteric interactions of the second pharmacophoric entity as becomes obvious, e.g. from twin drug approaches. In close relation to bivalent ligands twin drugs are composed of two drug entities (different or identical) combined by a linker, no linker or in overlap mode. Twin drugs were developed, e.g. for adrenergic, muscarinic, opioid and serotonergic receptors.[17-21] In most cases they do not intend to address two binding sites simultaneously, but in numerous cases twin drugs proved to be more potent and differentially profiled in their pharmacological properties. Therefore, these twin drug approaches give evidence that interaction of the second, not specifically bound pharmacophoric moiety with allosteric sites can considerably trigger the pharmacological profile.

In close connection to allosteric modulation cooperativity effects are usually discussed. For instance, the phenomenon that binding of one pharmacophoric moiety facilitates the binding of the second pharmacophore is termed positive cooperativity.

Also critical is the chemical nature of the spacers. Most bivalent ligand approaches are based on the use of linkers with well-balanced hydrophilic and lipophilic properties which are attained,

e.g. by the implication of ethylene glycol and amide moieties. Purely aliphatic spacers would lead to solubility problems and strong membrane binding. The connection of the linker and the recognition unit is also important. The spacer has to be attached in a manner that binding affinity is affected as little as possible. On one hand the linker should be sufficiently flexible for sterical adaption, but on the other hand a too flexible linker is entropically disfavored.

To explore if bivalent ligans can contribute to bring more insight into the presumed association of GPCRs, a series of bivalent neuropeptide Y (NPY) Y_1 receptor antagonists with spacer lengths between 8 and 36 atoms were synthesized. The Y_1 receptor, belonging to the superfamily of G-protein coupled receptors, was recently proposed to dimerize[2] and a twin drug, the irreversible alpha-adrenoceptor antagonist benextramine, was reported previously to interact with NPY binding sites.[22, 23]

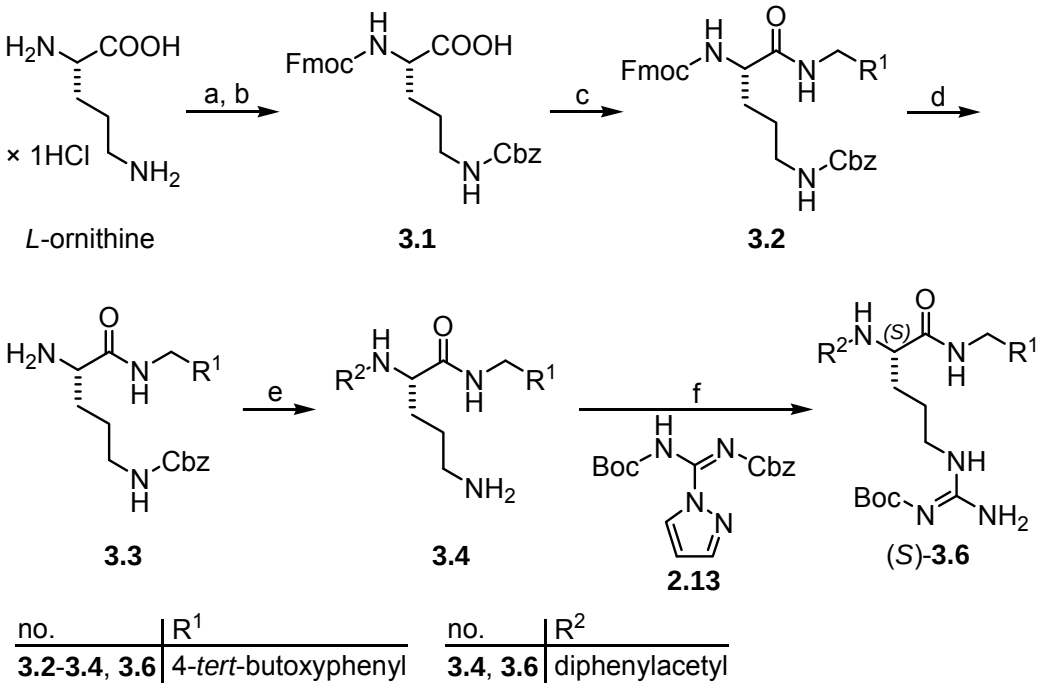
3.2 Results and Discussion

The presented bivalent Y_1R antagonists are derived from the (*R*)-argininamide BIBP 3226, the first highly potent and selective Y_1R antagonist.[24] Advantage was taken of the experience in the synthesis and pharmacological characterization of N^{ω}-substituted BIBP 3226 analogs. Among them, acylated and carbamoylated derivatives turned out to be more favorable in terms of retaining binding affinity. In some cases the affinity could be increased over that of the parent argininamide.[25] The distomer of BIBP 3226, the (*S*)-enantiomer (referred to as BIBP 3435), was reported to be more than 1000 times less potent than the eutomer.[24] Therefore, sets of (*R,R*)-, (*R,S*)- and (*S,S*)-configured bivalent ligands with identical physicochemical properties were prepared (Scheme 2) in order to facilitate conclusions from binding data. This approach was considered advantageous compared to strategies, where analogs of monovalent ligands with bisected or capped linkers are used as reference compounds, as any additional structural element (the linker and the second pharmacophore, if it does not bind specifically to a second site), which is attached to the orthosterically bound pharmacophoric entity, will undergo non-specific interactions with the receptor protein or parts of the cell membrane.

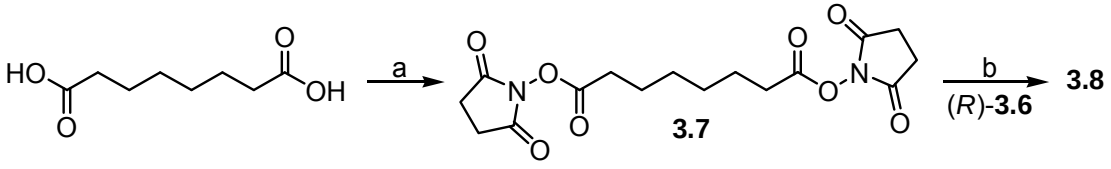
3.2.1 Chemistry

Synthetic strategies aimed at compounds of maximal purity on the low mg scale rather than at the optimization of yields and synthetic routes. The bivalent ligands (**3.8**, **3.22** – **3.25**, **3.29**, **3.37** – **3.41**) were prepared in a straightforward manner using standard protecting group chemistry as depicted in Schemes 2 - 5. A synthesis focused, click chemistry and BIBP 3226 based approach for the preparation of bivalent Y_1R antagonists has already been reported.[26] The linkers between the two BIBP 3226 moieties are composed of aliphatic dicarboxylic acids and

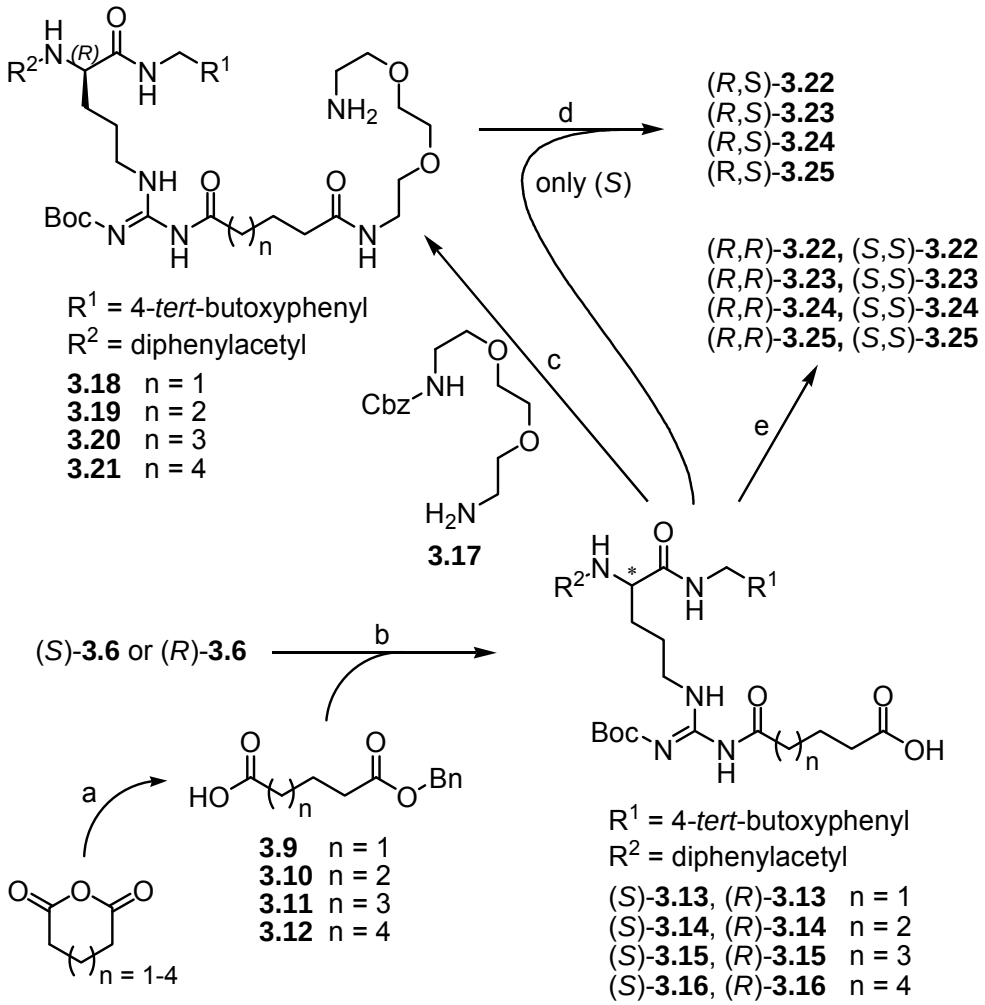
3,6-dioxa-octane-1,8-diamine as well as *N*,*N*'-bis(2-aminoethyl)-ethane-1,2-diamine (branched linkers) providing a hydrophilic-lipophilic balance. A linkage to the ω-nitrogen of the Y_1R antagonist BIBP 3226 and its less active *S*-enantiomer BIBP 3435 via carboxylic groups yielding N^{ω}-acylated BIBP 3226 and BIBP 3435 derivatives, respectively, was chosen. The synthesis of the guanidinylating reagent **2.13** as well as the N^{ω}-Boc, *O*-*tert*-butyl protected BIBP 3226 building block *(R)*-**3.6** (cp. to **2.7**) was described in chapter 2, and the respective *S*-enantiomer (*S*)-**3.6** was prepared following this protocol (Scheme 1). The non-protected terminal guanidine nitrogen ($N^{\omega'}$) of this building block reacts similar to amines, but at lower reaction rates. N^{ω}-acylation can be achieved by the aid of coupling reagents from peptide chemistry (EDAC, CDI) or using anhydrides, chlorides and active esters. Final deprotection with TFA and purification with preparative HPLC yielded the bivalent ligands with purities > 90 %. Mono-Boc protected *N*,*N*'-bis(2-aminoethyl)ethane-1,2-diamine (**3.30**) was successfully prepared according to a published protocol (Scheme 5).[27]



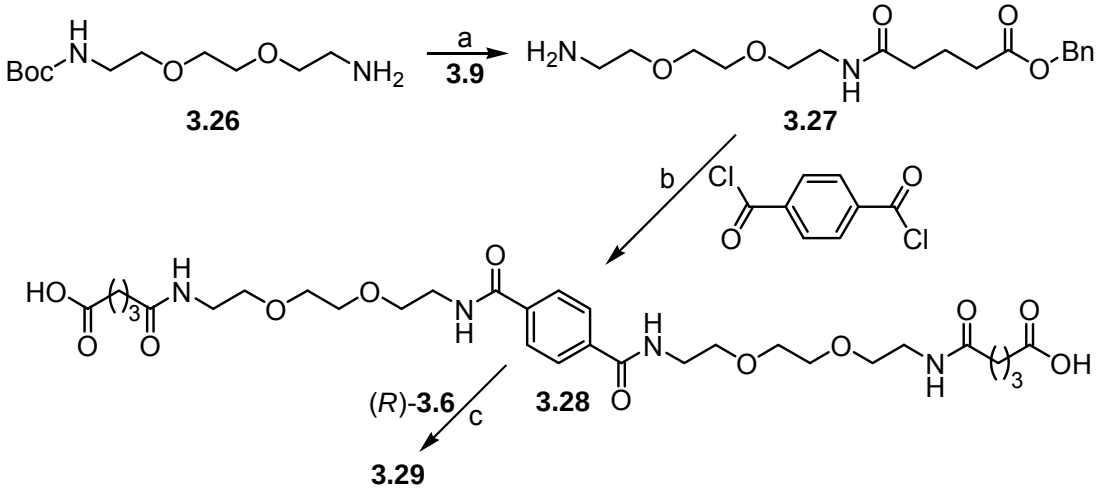
Scheme 1. Synthesis of the BIBP 3435 building block (*S*)-**3.6**.
Reagents and conditions: (a) (1) Cbz-Cl (45 % in toluol), NaOH 0.5 N, K_2CO_3, $CuSO_4$, 4 °C to rt, 20 h; (2) Titriplex III, H_2O, 100 °C, 60 min; (b) FmocOSu, H_2O/Dioxan, rt, 20 h, 85 %; (c) 4-*tert*-butoxybenzylamine, CDI, THF, rt, 20 h, 60 %; (d) diethylamine, DCM, rt, 4 h, 76 %; (e) (1) diphenylacetic acid succinimidyl ester, NEt_3, 1,2-dimethoxyethane, 35 °C, 20 h; (2) Pd/C, ammonium formate, MeOH, rt, 20 h, 82 %; (f) (1) NEt_3, CH_2Cl_2, rt, 20 h; (2) Pd/C, H_2, MeOH, rt, 3.5 h, 87 %.



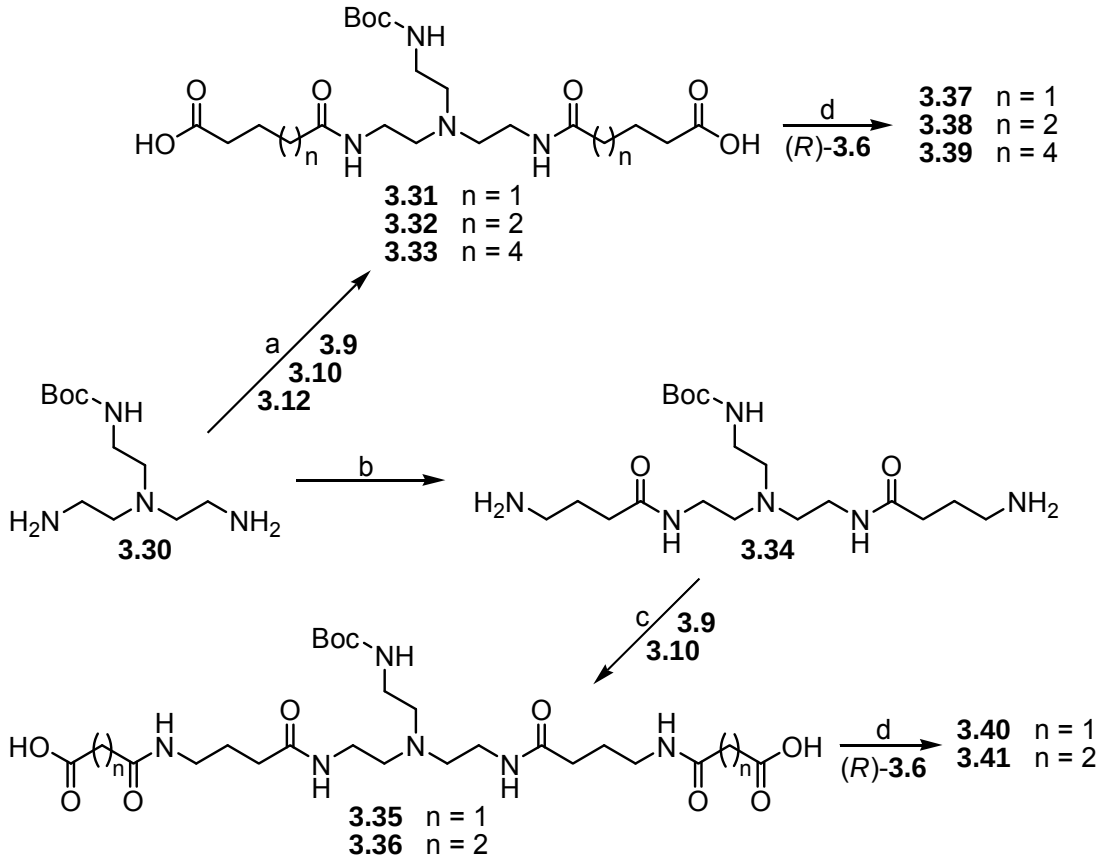
Scheme 2. Synthesis of the bivalent ligand **3.8**.
Reagents and conditions: (a) NHS, DCC, THF, 0 °C to rt, 20 h, 42 %; (b) (1) NEt_3, MeCN, rt, 20 h; (2) MeCN/TFA 1/1 (v/v), H_2O, rt, 4 h, 57 %.



Scheme 3. Synthesis of the bivalent ligands **3.22** - **3.25**.
Reagents and conditions: (a) (1) alkanedioic acid, acetic anhydride, *m*-xylene, reflux, 2 h; (2) BnOH, 60 °C, 20 h, 30-77 %; (b) (1) **3.9** - **3.12**, oxalyl chloride, DMF, CH_2Cl_2, reflux, 2 h; (2) **3.6**, NEt_3, CH_2Cl_2, rt, 20 h; (3) Pd/C, H_2, MeOH, rt, 20 h, 55-65 %; (c) (1) **3.13** - **3.16** (only *R*-configuration), EDAC, CH_2Cl_2, rt, 20 h; (2) Pd/C, H_2, MeOH, rt, 20 h, 45-55 %; (d) (1) EDAC, CH_2Cl_2, rt, 20 h; (2) CH_2Cl_2/TFA 1/1 (v/v), H_2O, rt, 2.5 h, 19-26 %; (e) (1) 3,6-dioxaoctane-1,8-diamine, EDAC, CH_2Cl_2, rt, 20 h; (2) CH_2Cl_2/TFA 1/1 (v/v), H_2O, rt, 2.5 h, 14-32 %.

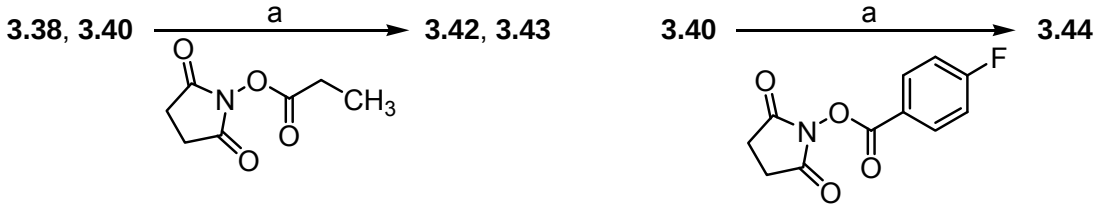


Scheme 4. Synthesis of the bivalent ligand **3.29**.
Reagents and conditions: (a) (1) EDAC, CH_2Cl_2, rt, 20 h; (2) CH_2Cl_2/TFA 1/1 (v/v), rt, 3 h, 55 %; (b) (1) NEt_3, CH_2Cl_2, rt, 20 h; (2) Pd/C, H_2, MeOH, rt, 84 %; (c) (1) CDI, DMF, rt, 20 h; (2) CH_2Cl_2/TFA 1/1 (v/v), rt, 2.5 h, 21 %.



Scheme 5. Synthesis of the bivalent ligands **3.37** - **3.41**.
Reagents and conditions: (a) (1) EDAC, CH_2Cl_2, rt, 20 h; (2) Pd/C, H_2, MeOH, rt, 58-75 %; (b) (1) *N*-benzyloxycarbonyl-γ-aminobutyric acid, EDAC, CH_2Cl_2, rt, 20 h; (2) Pd/C, H_2, MeOH, rt, 89 %; (c) (1) EDAC, CH_2Cl_2, rt, 20 h; (2) Pd/C, H_2, MeOH, rt, 53-60 %; (d) (1) CDI, DMF, rt, 20 h; (2) CH_2Cl_2/TFA 1/1 (v/v), rt, 2.5 h, 12-37 %.

The potential bivalent radioligands **3.42** - **3.44** were synthesized from the fully deprotected amine precursors **3.38** and **3.40** using NHS esters of the pertinent carboxylic acids, which are standard reagents for a tritium or fluorine-18 labeling (Scheme 6). Usually, the most profitable strategy for the preparation of radioligands is a labeling at the final step.



Scheme 6. Synthesis of the potential bivalent tritium ligands **3.42** and **3.43** as well as the potential bivalent PET ligand **3.44**.
Reagents and conditions: (a) DMF, NEt_3, 3 h, rt, 55-68 %.

The fluorescence labeling of the bivalent amine precursors **3.38** - **3.40** yielding the fluorescent ligands **4.45** – **4.47** is described in chapter 4.

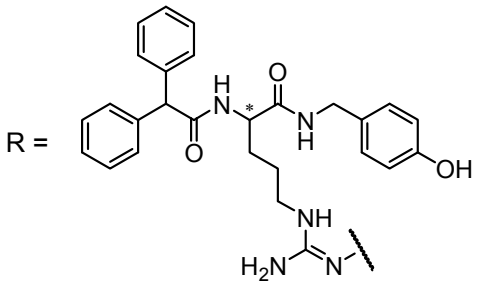
3.2.2 Pharmacology: Y_1R Antagonism and Binding

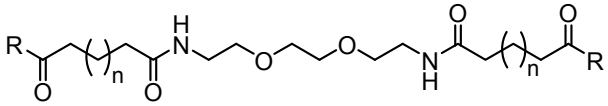
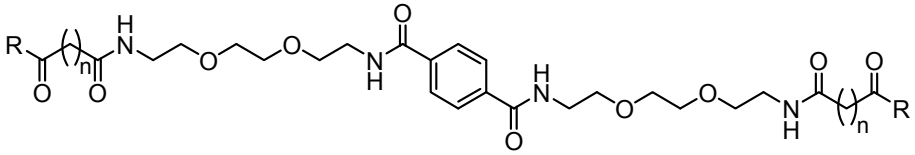
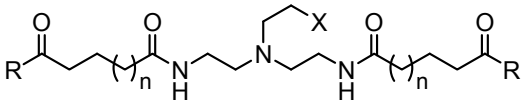
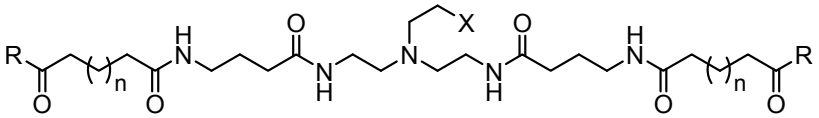
The Y_1R antagonistic activity of the bivalent Y_1R antagonists was determined by inhibition of the neuropeptide Y induced mobilization of intracellular Ca^{2+} in human erythroleukemia (HEL) cells.[28] [^{3}H]-UR-MK114 (cf. chapter 2) served as radioligand for binding studies on SK-N-MC cells (ca. 50,000 sites/cell) as well as MCF-7-Y_1 cells (ca. 250,000 sites/cell, when stimulated with 1 nM β-estradiol for 48 h, cf. chapter 2). Data for Y_1R antagonism and binding are summarized in Table 1. Except from the (*S*,*S*)-enantiomers all bivalent ligands proved to be potent Y_1R antagonists with affinities in the nM range, whereas Y_1R affinities at SK-N-MC cells were slightly higher (factor 2 - 3) than at MCF-7-Y_1 cells as was also observed for other BIBP 3226 analogs (cf. chapters 2, 4 and 5). Among the four sets of (*R*,*R*)-, (*R*,*S*)- and (*S*,*S*)-configured bivalent Y_1R antagonists the triplet with the shortest spacer length (20 atoms) revealed a moderate difference (factor 4 - 6) in binding affinity between the (*R*,*R*)- and (*R*,*S*)-diastereomer ((*R,R*)-**3.22** vs. (*R*,*S*)-**3.22**: 8/34 nM on SK-N-MC cells and 22/150 nM on MCF-7-Y_1 cells). The other (*R*,*R*)- and (*R*,*S*)-isomer pairs showed almost no difference between affinities (factor 1 - 2, cf. Table 1). The preferential binding of (*R*,*R*)-**3.22** may be a hint to a bridging of two Y_1R monomers. However, this finding must not be over-interpreted as the competition curves were not bi-phasic and no statistically significant difference in the Hill-slope from one was observed (Figure 1). Nevertheless, this gives reason to prepare further sets of (*R*,*R*)- and (*R*,*S*)-stereoisomers with structurally modified linkers (i.e. more rigid linkers) around 20 atoms of length and to conduct supporting investigations, e.g. with the help of bivalent radioligands as discussed below. A successive truncation of compound (*R*,*R*)-**3.22** to monovalent ligands containing the whole linker and fragments of the linker, would be an approach to study the impact of the nature of the linker on affinity.

Surprisingly there was only a small fluctuation in binding affinity (9 to 63 nM on SK-N-MC cells) among the (*R,R*)-configured ligands over the wide span in spacer length, ranging from 8 to 36 atoms. The bivalent ligand with the longest linker of 36 atoms even proved to be one of the most potent compound (K_i = 16 nM). Thus, linker length and structural diversity seem to be less decisive for binding affinity, and it is difficult to evaluate the effect of the second pharmacophoric moiety. Does it enhance binding while interacting with allosteric sites? As quite long and flexible linkers were used, a potential increase in affinity due to the simultaneous occupation of an allosteric pocket could be entropically balanced or even over-compensated (discussed in the introduction). On the contrary, unfavorable allosteric interactions, which hinder the specific binding of the orthosterically bound pharmacophoric entitiy are less probable due to the long distance between both pharmacophoric moieties, which are linked through quite long flexible spacers. To some extend this could be the case for compound **3.8** with a short linker of eight atoms having the lowest affinity in this series (Table 1).

Table 1. Structures, Y_1R antagonistic activities (IC_{50}) and affinities (K_i) of the bivalent Y_1R antagonists.

R =



No.	Config.	X	n	Spacer[a]	IC_{50} [nM][b]	K_i [nM][c]	K_i [nM][d]
			R-CO-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CO-R				
3.8	*R,R*		-	8	23±6	63±13	--
							
3.22	*R,R*		1	20	15±2	9±3*	22±4
	R,S		1	20	40±15	34±2	150±22
	S,S		1	20	1500±470	1800±300	--
3.23	*R,R*		2	22	31±9	23±5	62±15
	R,S		2	22	65±10	26±3	96±7
	S,S		2	22	890±70	1300±70	--
3.24	*R,R*		3	24	18±4	15±2	42±3
	R,S		3	24	20±3	19±3	52±14
	S,S		4	24	1600±100	2600±800	--
3.25	*R,R*		4	26	10±3	41±5	95±24
	R,S		4	26	13±2	65±7	280±96
	S,S		4	26	1440±170	2500±90	--
							
3.29	*R,R*		3	36	97±3	16±4	31±6
							
3.37	*R,R*	NH_2	1	17	28±3	30±4	34±8
3.38	*R,R*	NH_2	2	19	15±3	16±1	43±3
3.42	*R,R*	NHCO-CH_2CH_3	2	19	23±3	49±4	--
3.39	*R,R*	NH_2	4	23	20±5	34±5	47±1
							
3.40	*R,R*	NH_2	1	27	20±4	17±2	29±5
3.43	*R,R*	NHCO-CH_2CH_3	1	27	41±3	18±3	--
3.44	*R,R*	NH-4-F-benzoyl	1	27	21±1	34±1	--
3.41	*R,R*	NH_2	2	29	19±5	11±3	27±3
			H−R (BIBP3226/3435)				
BIBP 3226	*R*		-	--	10±1	1.3±0.2	--
BIBP 3435 (**3.45**)	*S*		-	--	3400±120	200±47	--

[a]Number of atoms which contribute to spacer length. [b]Inhibition of 10 nM NPY induced $[Ca^{2+}]_i$ mobilization in HEL cells; mean values ± SEM from two independent experiments. [C]Dissociation constant from the displacement of [^{3}H]-UR-MK114 (K_D = 1.2 nM, c = 1.5 nM) on SK-N-MC cells; mean values ± SEM from two independent experiments performed in triplicate. [d]Dissociation constant from the displacement of [^{3}H]-UR-MK114 (K_D = 2.9 nM, c = 2 nM) on MCF-7 cells; mean values ± SEM from two independent experiments performed in duplicate. *mean value ± SEM from four independent experiments performed in triplicate using three independently prepared stock solutions.

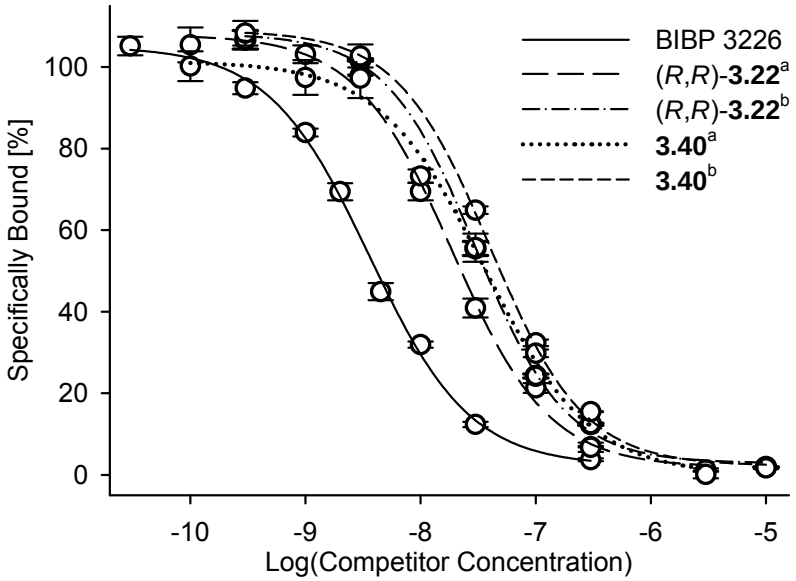


Figure 1. Displacement of [^{3}H]-UR-MK114 (SK-N-MC cells[a]: K_D = 1.2 nM, c = 1.5 nM; MCF-7-Y_1 cells[b]: K_D = 2.9 nM, c = 2 nM) by BIBP 3226 as well as the bivalent ligands (*R*,*R*)-**3.22** and **3.40** on SK-N-MC[a] cells and MCF-7-Y_1[b] cells.

It is very likely that simply the attachment of the linkers to BIBP 3226 leads to a decrease in affinity as it was e.g. observed, for N^{ω}-acetylated BIBP 3226 (K_i = 12 nM).[25] Moreover, the attachment of fluorescent dyes through a linker to BIBP 3226 can lead to a considerable decrease in affinity (K_i > 100 nM), indicating unfavorable allosteric interactions of the bulky fluorophore.[29] Therefore, with respect to the observed affinity range of 9 – 63 nM (K_i) a nearly neutral behavior of the second pharmacophoric entity with respect to enhancement or mitigation of binding affinity is assumed.

All (*S*,*S)*-enantiomers exhibited significantly lower Y_1R antagonistic acitivities, but not as low as reported[24] due to the presence of an impurity of 2.7 % of the eutomer BIBP 3226 as determined by capillary electrophoresis (using γ-cyclodextrin) for BIBP 3435 (**3.45**). **3.45** was obtained by N^{ω}- and *O-tert*-butyl deprotection of the building block (*S*)-**3.6** and revealed a remarkable activity (K_i = 200 nM, Table 1). *D*-ornithine, the starting material for the synthesis of (*S*)-**3.6** (Scheme 1), was determined to be enantiomerically pure (investigated by HPLC after derivatization with ortho-phthalaldehyde and *N*-acetyl-*L*-cysteine[30]). Consequently, racemization occurred during the synthesis of BIBP 3435 (**3.45**).

Selectivity data were not determined for two reasons. Firstly, there was no bivalent Y_1R antagonist with outstanding characteristics, and secondly the selectivity was already confirmed for monovalent fluorescence labeled BIBP 3226 derivatives, where the fluorophore is attached to the guanidine in the same way through alkanoyl linkers.[29] Nevertheless, the selectivity profile of fluorescence labeled or radiolabeled bivalent Y_1R antagonists such as the potential radioligand **3.43** has to be determined if such ligands are used as pharmacological tools.

With the intension to extend the experimental applicability of the bivalent ligands as pharmacological tools, branched linkers, bearing a primary amino group for radio- and fluorescence labeling, were introduced. The bivalent Y_1R antagonist **3.43**, one of the most potent bivalent compounds (K_i = 18 nM), can be easily prepared as a tritiated ligand from **3.40** and commercially available [2,3-^{3}H]-propanoic acid succinimidyl ester. Complementary to binding experiments with the monovalent Y_1R antagonist [^{3}H]-UR-MK114, such a radiolabeled bivalent ligand should be a valuable tool for investigations on the stoichiometry of Y_1R binding sites.

The fluorescence labeling of amine precursors **3.38** – **3.40** resulted only in a moderate decrease in affinity, and two bivalent fluorescent ligands proved to be well suited for an imaging of Y_1 receptors on MCF-7-Y_1 cells using confocal microscopy (cf. chapter 4).

3.2.3 Summary and Conclusion

A wide range of linker lengths (shortest: 8-membered chain, longest: 36-membered chain) was chosen for the preparation of bivalent Y_1R antagonists derived from the highly potent and selective Y_1R antagonist BIBP 3226. The most potent bivalent ligand, consisting of a 20-atom spacer, revealed a moderately lower Y_1R affinity for the (*R*,*S*)-stereoisomer than for the (*R*,*R*)-enantiomer (factor of 4-6), which was not observed for other pairs of (*R*,*R*)- and (*R*,*S*)-configured bivalent ligands (Table 1). This can be interpreted as hint to the bridging of two Y_1 receptor monomers, but further investigations based on complementary tools are needed. Since quite flexible linkers were used in this work, it is possible that the enhanced binding of the (*R*,*R*)-enantiomer ((*R,R*)-**3.22**) is mitigated by negative entropic effects. However, the preparation of further sets of (*R*,*R*)- and (*R*,*S*)-stereoisomers with more rigid linkers comprising around 20 atoms could be promising.

Interestingly, among the (*R,R*)-configured antagonists only minor differences in affinities (9 to 63 nM) with respect to spacer length and chemical diversity were observed (Table 1). The successful preparation of bivalent Y_1R antagonists with branched linkers, where radio- and fluorescence labels can be attached, is especially worth mentioning. Such a potential bivalent radioligand had an affinity of 18 nM (K_i), which is probably high enough to perform investigations on the stoichiometry of binding sites in combination with monovalent radioligands. Notwithstanding the presented work paved the way for the future development of high affiniy bivalent ligands with improved structure of the linker entitiy (length, rigidity).

3.3 Experimental Section

3.3.1 General Experimental Conditions

Unless otherwise stated, chemicals and solvents were purchased from commercial suppliers and used without further purification. *L*-Ornithine hydrochloride was obtained from Merck (Darmstadt, Germany). Millipore water was used throughout for the preparation of buffers and HPLC eluents. Petroleum ether (40-60 °C) was distilled before use. DMF was stored over a molecular sieve (3 Å). Anhydrous reactions were run under an atmosphere of dry nitrogen or argon. Thin layer chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates. For column chromatography silica gel Geduran 60 (Merck, 0.063-0.200 mm) was used. NMR spectra were recorded on a Bruker Avance 300 (1H: 300 MHz) and a Bruker Avance 600 (1H: 600 MHz, 13C: 150.9 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. Low resolution mass spectrometry analysis (MS) was performed in-house on a Finnigan ThermoQuest TSQ 7000 (ES-MS) and a Finnigan SSQ 710A (EI-MS 70 eV, CI-MS). High resolution mass spectrometry for compound **3.8** was performed on a LTQ Orbitrap Discovery (Thermo Fisher Scientific, Waltham, MA, USA). Other high resolution mass spectra were acquired with an Ultraflex III MALDI-TOF/TOF 200 systen mass spectrometer (2007, BrukerDaltonics) equipped with a nitrogen laser emitting at 337 nm. The spectra were calibrated using an external calibration with Peptide Calibration Standard II (BrukerDaltonics).
Melting points were determined with a Büchi 510 melting point apparatus and are uncorrected. Lyophilisation was done with a Christ alpha 2-4 LD equipped with a vacuubrand RZ 6 rotary vane vacuum pump. Fluorescence spectra were recorded with a Cary Eclipse spectrofluorimeter (Varian Inc., Mulgrave, Victoria, Australia).
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 µm) at a flow rate of 38 mL/min. Mixtures of acetonitrile and 0.1 % aq. TFA were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure (final pressure: 60 mbar) at 40 °C prior to lyophilization. Analytical HPLC analysis was performed on three different systems (Waters, Merck, Thermo Separation Products) which are listed in detail below. Mixtures of acetonitrile (A) and 0.05 % aq. TFA (B) were used as mobile phase. Helium degassing was used throughout.
The buffer for the Y_1R binding studies on SK-N-MC cells and MCF-7-Y_1 cells was prepared by the 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), $Mg_2SO_4 \cdot 7H_2O$ (1.2 mM), and $NaHCO_3$ (25 mM). HEPES buffer without additives was used to wash the cells prior to and after radioligand binding. The loading buffer (pH 7.4) for the determination of the mobilization of intracellular Ca^{2+} in HEL cells was prepared by dissolving NaCl (120 mM), KCl (5

mM), $MgCl_2 \cdot 6H_2O$ (2 mM), $CaCl_2 \cdot 2H_2O$ (1.5 mM), HEPES (25 mM), and glucose (10 mM). The lysis solution for the radioligand binding assays consisted of urea (8 M), acetic acid (3 M) and Triton-X-100 (1 %) in water. Stock solutions of all compounds were prepared in DMSO at concentrations of 5 or 10 mM and stored at –20 °C. Tritium counting was done in 3 mL of a liquid scintillator (Rothiszint^{TM} eco plus) with a Beckman LS 6500 beta-counter.

Analytical HPLC systems:
System 1: Waters; composed of a 600S controller and pump, a Waters degasser, a temperature control module, a 717 plus autosampler and a 2487 UV-detector; flow rate: 0.7 mL/min
System 2: Merck; composed of a L-5000 controller, a 655A-12 pump, a 655A-40 autosampler and a L-4250 UV-VIS detector; flow rate: 0.7 mL/min
System 3: Thermo Separation Products; composed of a SN400 controller, a P4000 pump, a degasser (Degassex DG-4400, phenomenex), an AS3000 autosampler and a Spectra Focus UV-VIS detector; flow rate: 0.8 mL/min

Applied gradients:
Gradient 1: 0 to 25 min: A/B 25/75 to 65/35, 25 to 27 min: 65/35 to 95/5, 27 to 33 min: 95/5
Gradient 2: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5
With the exception of compound **3.8** all bivalent Y_1R antagonists were analyzed with gradient 2 on a Eurospher-100 C18 (250 × 4 mm, 5 µm, Knauer, Berlin, Dueren, Germany). In case of compound **3.8** a Nucleodur 100-5 C18 ec (250 × 4 mm, 5 µm, Macherey-Nagel, Germany) served as reversed-phase column.

3.3.2 Chemistry: Experimental Protocols and Analytical Data

For synthesis of compounds **3.1** – **3.6** the experimental protocols as for the preparation of **2.1** – **2.7** (chapter 2) were used.

(*S*)-N^{δ}-Benzyloxycarbonyl-N^{α}-(9-fluorenylmethoxycarbonyl)ornithine (3.1). White solid, 85 % (74 g); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.4-1.69 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.75 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 3.02 (m, 2H, C$\underline{H}_2$-NH), 3.92 (m, 1H, C$\underline{H}^{\alpha}$), 4.16-4.31 (m, 3H, C$\underline{H}$-C$\underline{H}_2$ Fmoc), 5.01 (s, 2H, C$\underline{H}_2$-Ph), 7.25-7.45 (m, 10H, Ph, Fmoc, CH$_2$-N$\underline{H}$), 7.60 (d, 1H, ^{3}J = 7.94 Hz, CO-N$\underline{H}$-CH), 7.73 (d, 2H, ^{3}J = 7.38 Hz, Fmoc), 7.89 (d, 2H, ^{3}J = 7.45 Hz, Fmoc), 12.76 (bs, 1H, COO$\underline{H}$); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 489 $[M + H]^+$; $C_{28}H_{28}N_2O_6$ (488.5)

(*S*)-N^{δ}-Benzyloxycarbonyl-*N*-(4-*tert*-butoxybenzyl)-N^{α}-(9-fluorenylmethoxycarbonyl)-ornithinamide (3.2). White solid, 60 % (50 g); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.24 (s, 9H, *t*-Bu), 1.34-1.74 (m, 4H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 3.0 (m, 2H, C$\underline{H}_2$-NH), 4.05 (m, 1H, C$\underline{H}^{\alpha}$), 4.17-4.32 (m, 5H, C$\underline{H}$-C$\underline{H}_2$ Fmoc, C$\underline{H}_2$-C_6H_4-O-t-Bu), 5.01 (s, 2H, C$\underline{H}_2$-Ph), 6.88 (d, 2H, ^{3}J = 8.30 Hz, AA'BB'), 7.14 (d, 2H, ^{3}J = 8.42 Hz, AA'BB'), 7.26-7.45 (m, 10H, Ph, Fmoc, CH_2-CH_2-N$\underline{H}$), 7.56 (d, 1H, ^{3}J = 8.18 Hz, CO-N$\underline{H}$-CH), 7.74 (d, 2H, ^{3}J = 7.24 Hz, Fmoc), 7.89 (d, 2H, ^{3}J = 7.45 Hz, Fmoc), 8.36 (t, 1H, ^{3}J = 5.66, CH-CO-N$\underline{H}$-CH_2); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 650 $[M + H]^+$; $C_{39}H_{43}N_3O_6$ (649.8)

(*S*)-N^{δ}-Benzyloxycarbonyl-*N*-(4-*tert*-butoxybenzyl)ornithinamide (3.3). Yellow oil, 76 % (25 g); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.32 (s, 9H, *t*-Bu), 1.6 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.85 (m, 1H, CH-C$\underline{H}_2$-CH_2), 3.21 (m, 2H, CH_2-C$\underline{H}_2$-NH), 3.43 (m, 1H, C$\underline{H}^{\alpha}$), 4.37 (d, 2H, ^{3}J = 5.86 Hz, C$\underline{H}_2$- C_6H_4-O-*t*-Bu), 5.06 (s, 2H, C$\underline{H}_2$-Ph), 6.93 (d, 2H, ^{3}J = 8.51 Hz, AA'BB'), 7.15 (d, 2H, ^{3}J = 8.53 Hz, AA'BB'), 7.28-7.37 (m, 5H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 428 $[M + H]^+$; $C_{24}H_{33}N_3O_4$ (427.5)

(*S*)-*N*-(4-*tert*-Butoxybenzyl)-N^{α}-(2,2-diphenylacetyl)ornithinamide (3.4). White solid, 82 % (5.6 g); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.31 (s, 9H, *t*-Bu), 1.48-1.78 (m, 4H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 2.9 (m, 2H, CH_2-C$\underline{H}_2$-NH_2), 4.32 (m, 2H, C$\underline{H}_2$-C_6H_4-O-*t*-Bu), 4.54 (m, 1H, C$\underline{H}^{\alpha}$), 4.97 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.89 (d, 2H, ^{3}J = 8.52 Hz, AA'BB'), 7.1 (d, 2H, ^{3}J = 8.49 Hz, AA'BB'), 7.18-7.37 (m, 10H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 488 $[M + H]^+$; $C_{30}H_{37}N_3O_3$ (487.6)

(*S*)-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-(4-*tert*-butoxycarbonyl)-N^{α}-(2,2-diphenylacetyl)arginin-amide (3.6). White solid, 87 % (6.2 g); mp > 85 °C (decomp.); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.32 (s, 9H, *t*-Bu), 1.37-1.54 (m, 11H, *t*-Bu, CH_2-C$\underline{H}_2$-CH_2), 1.64 (m, 1H, CH-C$\underline{H}_2$), 1.74 (m, 1H, CH-C$\underline{H}_2$), 3.09 (m, 2H, CH_2-C$\underline{H}_2$-NH), 4.19 (dd, 1H, ^{2}J = 14.92 Hz, ^{3}J = 4.3 Hz, C$\underline{H}_2$-C_6H_4-O-*t*-Bu), 4.31 (dd, 1H, ^{2}J = 14.71 Hz, ^{3}J = 4.52 Hz, C$\underline{H}_2$-C_6H_4-O-*t*-Bu), 4.52 (m, 1H, C$\underline{H}^{\alpha}$), 4.97 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.88 (d, 2H, ^{3}J = 8.52 Hz, AA'BB'), 7.02 (d, 2H, ^{3}J = 8.53 Hz, AA'BB'), 7.18-7.34 (m, 10H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 630 $[M + H]^+$; $C_{36}H_{47}N_5O_5$ (629.8)

Octanedioic acid disuccinimidylester (3.7). DCC (2.81 g, 13.64 mmol, 2.2 eq) was added to an ice-cold, stirred solution of octanedioic acid (1.08 g, 6.2 mmol, 1 eq) and *N*-hydroxysuccinimide (1.57 g, 13.64 mmol, 2.2 eq) in anhydrous THF (45 mL). The mixture was stirred under cooling for 60 min. The ice-bath was removed and stirring was continued at rt overnight. The white solid (DCU) was separated by filtration and the solvent was removed under reduced pressure. Purification with column chromatography (eluent: PE/EtOAc) yielded the

product as a white solid (0.95 g, 2.59 mmol, 42 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.46 (m, 4H, CH_2-CH_2-C<u>H_2</u>-C<u>H_2</u>-CH_2-CH_2), 1.76 (m, 4H, CH_2-C<u>H_2</u>-CH_2-CH_2-C<u>H_2</u>-CH_2), 2.61 (t, 4H, ^{3}J = 7.37, C<u>H_2</u>-CH_2-CH_2-CH_2-CH_2-C<u>H_2</u>), 2.83 (s, 8H, CO-C<u>H_2</u>-C<u>H_2</u>-CO); MS (CI, NH_3): *m/z* 386 [M + NH_4]$^+$; $C_{16}H_{20}N_2O_8$ (368.3)

(*R,R*)-*N,N'*-Bis{[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]-amino(amino)methylene}octandiamide (3.8). (*R*)-**3.6** (100 mg, 159 µmol, 2.5 eq) and **3.7** (23.4 mg, 63.5 µmol, 1 eq) were dissolved in anhydrous acetonitrile (5 mL). NEt_3 (19.3 mg, 26.4 µL, 190.6 µmol, 3 eq) was added and the reaction mixture was stirred under argon at rt overnight. TFA (5 mL) and 4 drops of water were added and stirring was continued for 4 h. The mixture was concentrated under reduced pressure and MeOH (20 mL) was added twice, each time followed by evaporation. The residue (colorless oil) was taken up in a mixture of acetonitrile and 0.1 % aq. TFA (1/1, 4 mL), filtered (0.2 µm) and injected into the preparative HPLC system (4 injections). Acetonitrile was removed under reduced pressure prior to lyophilization. White fluffy solid (47.8 mg, 36.4 µmol, 57 %); mp 148-151 °C; ^{1}H-NMR (600 MHz, DMSO-d_6, COSY, HSQC): δ (ppm) 1.3 (bs, 4H, CH_2-CH_2-C<u>H_2</u>-C<u>H_2</u>-CH_2-CH_2), 1.44 (m, 4H, CH_2-C<u>H_2</u>-CH_2-CH_2-C<u>H_2</u>-CH_2), 1.56 (m, 6H, CH-C<u>H_2</u>-C<u>H_2</u>), 1.68 (m, 2H, CH-C<u>H_2</u>-CH_2), 2.41 (t, 4H, ^{3}J = 7.22 Hz, C<u>H_2</u>-CO), 3.22 (m, 4H, CH_2-CH_2-C<u>H_2</u>-NH), 4.12 (dd, 2H, ^{2}J = 14.78 Hz, ^{3}J = 5.68 Hz, C<u>H_2</u>-ArOH), 4.17 (dd, 2H, ^{2}J = 14.79 Hz, ^{3}J = 5.83 Hz, C<u>H_2</u>-ArOH), 4.33 (m, 2H, CH^{α}), 5.12 (s, 2H, C<u>H</u>-$(Ph)_2$), 6.67 (d, 4H, ^{3}J = 8.43 Hz, AA'BB'), 7.0 (d, 4H, ^{3}J = 8.41 Hz, AA'BB'), 7.22 (m, 4H, Ph), 7.28 (m, 16H, Ph), 8.35 (t, 2H, ^{3}J = 5.76 Hz, CO-N<u>H</u>-CH_2), 8.47 (d, 2H, ^{3}J = 8.06 Hz, CO-N<u>H</u>-CH), 8.57 (bd, 4H, N<u>H_2</u>), 9.22 (s, 2H, N<u>H</u>), 9.31 (s, 2H, N<u>H</u>), 11.83 (s, 2H, ArO<u>H</u>); RP-HPLC (210 nm, system 3): 100 % (t_R = 15.3 min, k = 5.6); HRMS: (ES): *m/z* calcd. for [$C_{62}H_{73}N_{10}O_8$ + H]$^+$ 1085.5613, found: 1085.5604; $C_{62}H_{72}N_{10}O_8$ × $C_4H_2F_6O_4$ (1313.3)

Glutaric acid monobenzylester (3.9). Glutaric anhydride (10 g, 87.6 mmol, 1 eq) was treated with benzyl alcohole (11.4 g, 105 mmol, 1.2 eq) at 60 °C overnight. Column chromatography afforded the product as a white solid (15 g, 67.6 mmol, 77 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.78 (m, 2H, CH_2-C<u>H_2</u>-CH_2), 2.41 (m, 4H, C<u>H_2</u>-CH_2-C<u>H_2</u>), 5.12 (s, 2H, C<u>H_2</u>-Ph), 7.32-7.37 (m, 5H, Ph); $C_{12}H_{14}O_4$ (222.2)

General procedure for the synthesis of mono-Cbz-protected alkanedioic acids 3.10 - 3.12

The pertinent alkanedioic acid (1 eq) and acetic anhydride (4 eq) were heated under reflux in xylene (40 mL for 0.5 mmol of dioic acid) for 2 h. All volatiles were removed under reduced pressure and the pale yellow solid was treated with benzyl alcohole (1.2 eq) at 60 °C overnight. Column chromatography afforded the product as a white solid.

Hexanedioic monobenzylester (3.10). 62 % (10 g); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.69 (m, 4H, CH_2-C<u>H_2</u>-C<u>H_2</u>-CH_2), 2.39 (m, 4H, C<u>H_2</u>-CH_2-CH_2-C<u>H_2</u>), 5.12 (s, 2H, C<u>H_2</u>-Ph), 7.33-7.38 (m, 5H, Ph); MS (EI): *m/z* 236 ($M^{\cdot+}$); $C_{13}H_{16}O_4$ (236.3)

Heptanedioic acid monobenzylester (3.11). 51 % (4 g); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.37 (m, 2H, CH_2-CH_2-C<u>H_2</u>-CH_2-CH_2), 1.65 (m, 4H, CH_2-C<u>H_2</u>-CH_2-C<u>H_2</u>-CH_2), 2.35 (m, 4H, C<u>H_2</u>-CH_2-CH_2-CH_2-C<u>H_2</u>), 5.12 (s, 2H, C<u>H_2</u>-Ph), 7.33-7.38 (m, 5H, Ph); MS (EI): *m/z* 250 $(M^{\cdot+})^{\cdot}$; $C_{14}H_{18}O_4$ (250.3)

Octanedioic acid monobenzylester (3.12). 30 % (2.7 g); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.34 (m, 4H, CH_2-CH_2-C<u>H_2</u>-C<u>H_2</u>-CH_2-CH_2), 1.64 (m, 4H, CH_2-C<u>H_2</u>-CH_2-CH_2-C<u>H_2</u>-CH_2), 2.34 (m, 4H, C<u>H_2</u>-CH_2-CH_2-CH_2-CH_2-C<u>H_2</u>), 5.11 (s, 2H, C<u>H_2</u>-Ph), 7.31-7.37 (m, 5H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 265 $[M + H]^+$; $C_{15}H_{20}O_4$ (264.3)

General procedure for the synthesis of carboxylic acids 3.13 - 3.16

The benzyl ester **3.9**-**3.12** (1 eq) was dissolved in CH_2Cl_2. DMF (1 drop) and oxalyl chloride (1.3 eq) were added, the mixture was refluxed for 2 h and the volatiles were removed under reduced pressure. The residue was taken up in CH_2Cl_2 and **3.6** as well as NEt_3 (3 eq) were added. The mixture was allowed to stand at rt overnight. The intermediate was purified by column chromatography (CH_2Cl_2/EtOAc) prior to hydrogenation at rt and atmospheric pressure in MeOH using hydrogen and a 10 % Pd/C catalyst.

(*R*)- and (*S*)-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-(4-*tert*-butoxycarbonyl)-N^{ω}-(4-carboxybutanoyl)-N^{α}-(2,2-diphenylacetyl)argininamide ((*R*)-3.13, (*S*)-3.13). 60-65 % (≈ 200 mg); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.31 (s, 9H, *t*-Bu), 1.4-1.95 (bm, 15H, *t*-Bu, CH-C<u>H_2</u>-C<u>H_2</u>, CH_2-C<u>H_2</u>-CH_2-COOH), 2.15-2.45 (m, 4H, C<u>H_2</u>-CO), 2.4 (m, 2H, C<u>H_2</u>-CO), 3.24 (m, 2H, CH_2-CH_2-C<u>H_2</u>-NH), 4.29 (m, 2H, C<u>H_2</u>-ArOH), 4.52 (m, 1H, CH^{α}), 4.98 (s, 1H, C<u>H</u>-$(Ph)_2$), 6.9 (d, 2H, ^{3}J = 8.48 Hz, AA'BB'), 7.05 (d, 2H, ^{3}J = 8.48 Hz, AA'BB'), 7.16-7.32 (m, 10H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 744 $[M + H]^+$; $C_{41}H_{53}N_5O_8$ (743.9)

(*R*)- and (*S*)-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-(4-*tert*-butoxycarbonyl)-N^{ω}-(5-carboxyheptanoyl)-N^{α}-(2,2-diphenylacetyl)argininamide ((*R*)-3.14, (*S*)-3.14). 55-60 % (≈ 120 mg); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.33 (s, 9H, *t*-Bu), 1.4-1.75 (bm, 16H, *t*-Bu, CH-C<u>H_2</u>-C<u>H_2</u>, CH_2-C<u>H_2</u>-C<u>H_2</u>-CH_2), 1.82 (m, 1H, CH-C<u>H_2</u>-CH_2), 2.2 (m, 2H, C<u>H_2</u>-CO), 2.4 (m, 2H, C<u>H_2</u>-CO), 3.3 (m, 2H, CH_2-CH_2-C<u>H_2</u>-NH), 4.32 (m, 2H, C<u>H_2</u>-ArOH), 4.42 (m, 1H, CH^{α}), 5.05 (s, 1H, C<u>H</u>-$(Ph)_2$), 6.9 (d, 2H, ^{3}J = 8.12 Hz, AA'BB'), 7.12 (d, 2H, ^{3}J = 8.12 Hz, AA'BB'), 7.2-7.35 (m, 10H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 758 $[M + H]^+$; $C_{42}H_{55}N_5O_8$ (757.9)

(*R*)- and (*S*)-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-(4-*tert*-butoxycarbonyl)-N^{ω}-(6-carboxyhexanoyl)-N^{α}-(2,2-diphenylacetyl)argininamide ((*R*)-3.15**, (*S*)-**3.15).** 55-60 % (≈ 120 mg); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.2-1.7 (m, 27H, 2 × *t*-Bu, CH-C<u>H</u>$_2$-C<u>H</u>$_2$, CH_2-C<u>H</u>$_2$-C<u>H</u>$_2$-C<u>H</u>$_2$-CH_2), 1.77 (m, 1H, CH-C<u>H</u>$_2$-CH_2), 2.1-2.4 (m, 4H, C<u>H</u>$_2$-CO), 3.24 (m, 2H, CH_2-CH_2-C<u>H</u>$_2$-NH), 4.24 (m, 2H, C<u>H</u>$_2$-ArOH), 4.6 (m, 1H, CH^{α}), 4.97 (s, 1H, C<u>H</u>-$(Ph)_2$), 6.86 (d, 2H, ^{3}J = 8.39 Hz, AA'BB'), 7.03 (d, 2H, ^{3}J = 8.28 Hz, AA'BB'), 7.1-7.3 (m, 10H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 772 $[M + H]^+$; $C_{43}H_{57}N_5O_8$ (771.9)

(*R*)- and (*S*)-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-(4-*tert*-butoxycarbonyl)-N^{ω}-(7-carboxyheptanoyl)-N^{α}-(2,2-diphenylacetyl)argininamide ((*R*)-3.16**, (*S*)-**3.16).** 55-65 % (≈ 150 mg); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.24-1.43 (m, 22H, 2 × *t*-Bu, CH_2-CH_2-C<u>H</u>$_2$-C<u>H</u>$_2$-CH_2-CH_2), 1.52-1.73 (m, 7H, CH-C<u>H</u>$_2$-C<u>H</u>$_2$, CH_2-C<u>H</u>$_2$-CH_2-CH_2-C<u>H</u>$_2$-CH_2), 1.8 (m, 1H, CH-C<u>H</u>$_2$-CH_2), 2.24-2.35 (m, 4H, C<u>H</u>$_2$-CO), 3.25 (m, 2H, CH_2-CH_2-C<u>H</u>$_2$-NH), 4.3 (m, 2H, C<u>H</u>$_2$-ArOH), 4.6 (m, 1H, CH^{α}), 4.98 (s, 1H, C<u>H</u>-$(Ph)_2$), 6.89 (d, 2H, ^{3}J = 8.45 Hz, AA'BB'), 7.08 (d, 2H, ^{3}J = 8.47 Hz, AA'BB'), 7.18-7.33 (m, 10H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 786 $[M + H]^+$; $C_{44}H_{59}N_5O_8$ (786.0)

***N*-Cbz-3,6-dioxaoctane-1,8-diamine (3.17).** 3,6-dioxa-octane-1,8-diamine (3 g, 20.2 mmol, 1 eq) and Na_2CO_3 (10.7 g, 101 mmol, 5 eq) were dissolved in water (450 mL) and acetonitrile (150 mL). A solution of benzyl chloroformate (3.45 g, 20.2 mmol, 1 eq) in acetonitrile (150 mL) was added dropwise over a period of 3 h. Acetonitrile was removed under reduced pressure, the product was extracted with CH_2Cl_2 and purified by column chromatography (CH_2Cl_2/MeOH/NH_4OH). 47 % (2.7 g, 9.6 mmol); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 2.03 (s, 2H, N<u>H</u>$_2$), 2.83 (s, 2H, O-CH_2-C<u>H</u>$_2$-NH_2), 3.36 (m, 2H, CH_2), 3.47 (t, 2H, ^{3}J = 5.16 Hz, CH_2), 3.5-3.63 (m, 6H, 6 × CH_2), 5.07 (s, 2H, C<u>H</u>$_2$-Ph), 7.27-7.35 (m, 5H, Ph); MS (CI, NH_3): *m/z* 283 $[M + H]^+$; $C_{14}H_{22}N_2O_4$ (282.3)

General procedure for the synthesis of amines 3.18 - 3.21

The carboxylic acids (*R*)-**3.13** - (*R*)-**3.16** (1 eq) and **3.17** were dissolved in CH_2Cl_2, EDAC (1.3 eq) was added and the mixture was kept under stirring at rt for 20 h. The intermediate was purified by column chromatography (EtOAc/MeOH) prior to hydrogenation at rt and atmospheric pressure in MeOH using hydrogen and a 10 % Pd/C catalyst.

(*R*)-N^{ω}-[5-(8-Amino-3,6-dioxaoctyl)amino-5-oxo-pentanoyl]-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-(4-*tert*-butoxycarbonyl)-N^{α}-(2,2-diphenylacetyl)argininamide (3.18). Light brown resin; 50 % (130 mg); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.31 (s, 9H, *t*-Bu), 1.44 (s, 9H, *t*-Bu), 1.52 (m, 3H, CH-C<u>H</u>$_2$-C<u>H</u>$_2$), 1.6-1.95 (bm, 3H, CH-C<u>H</u>$_2$-CH_2, CO-CH_2-C<u>H</u>$_2$-CH_2-CO), 2.23 (m, 4H, C<u>H</u>$_2$-

CO), 3.13 (t, 2H, ^3J = 6.89 Hz, C$\underline{H}_2$-NH_2), 3.36 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH, NH-C$\underline{H}_2$-CH_2-O), 3.5-3.67 (m, 8H, C$\underline{H}_2$-O-C$\underline{H}_2$-C$\underline{H}_2$-O-C$\underline{H}_2$-), 4.32 (s, 2H, C$\underline{H}_2$-ArOH), 4.41 (m, 1H, CH^{α}), 5.08 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.9 (d, 2H, ^3J = 8.54 Hz, AA'BB'), 7.15 (d, 2H, ^3J = 8.59 Hz, AA'BB'), 7.2-7.33 (m, 10H, Ph); $C_{47}H_{67}N_7O_9$ (874.1)

(*R*)-N^{ω}-[6-(8-Amino-3,6-dioxaoctyl)amino-6-oxo-hexanoyl]-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-(4-*tert*-butoxycarbonyl)-N^{α}-(2,2-diphenylacetyl)argininamide (3.19). Light brown resin; 55 % (64 mg); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.3 (s, 9H, *t*-Bu), 1.42 (s, 9H, *t*-Bu), 1.51 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.65 (m, 4H, CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2), 1.83 (m, 1H, CH-C$\underline{H}_2$-CH_2), 2.26 (m, 2H, C$\underline{H}_2$-CO), 2.37 (m, 2H, C$\underline{H}_2$-CO), 2.98 (t, 2H, C$\underline{H}_2$-NH_2), 3.2-3.44 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH, NH-C$\underline{H}_2$-CH_2-O), 3.47-3.65 (m, 8H, C$\underline{H}_2$-O-C$\underline{H}_2$-C$\underline{H}_2$-O-C$\underline{H}_2$), 4.3 (m, 2H, C$\underline{H}_2$-ArOH), 4.58 (m, 1H, CH^{α}), 5.07 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.87 (d, 2H, ^3J = 8.52 Hz, AA'BB'), 7.08 (d, 2H, ^3J = 8.51 Hz, AA'BB'), 7.18-7.31 (m, 10H, Ph); $C_{48}H_{69}N_7O_9$ (888.1)

(*R*)-N^{ω}-[7-(8-Amino-3,6-dioxaoctyl)amino-7-oxo-heptanoyl]-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-(4-*tert*-butoxycarbonyl)-N^{α}-(2,2-diphenylacetyl)argininamide (3.20). Light brown resin; 45 % (83 mg); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.23-1.55 (m, 23H, 2 × *t*-Bu, CH-C$\underline{H}_2$-C$\underline{H}_2$, CH_2-CH_2-C$\underline{H}_2$-CH_2-CH_2), 1.56-1.73 (m, 4H, CH_2-C$\underline{H}_2$-CH_2-C$\underline{H}_2$-CH_2), 1.81 (m, 1H, CH-C$\underline{H}_2$-CH_2), 2.1 (t, 2H, C$\underline{H}_2$-CO), 2.37 (t, 2H, C$\underline{H}_2$-CO), 2.97 (t, 2H, C$\underline{H}_2$-NH_2), 3.2-3.43 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH, NH-C$\underline{H}_2$-CH_2-O), 3.46-6.0 (m, 8H, C$\underline{H}_2$-O-C$\underline{H}_2$-C$\underline{H}_2$-O-C$\underline{H}_2$), 4.29 (m, 2H, C$\underline{H}_2$-ArOH), 4.57 (m, 1H, CH^{α}), 5.06 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.85 (d, 2H, ^3J = 8.54 Hz, AA'BB'), 7.07 (d, 2H, ^3J = 8.53 Hz, AA'BB'), 7.16-7.32 (m, 10H, Ph); $C_{49}H_{71}N_7O_9$ (902.1)

(*R*)-N^{ω}-[8-(8-Amino-3,6-dioxaoctyl)amino-8-oxo-octanoyl]-*N*-(4-*tert*-Butoxybenzyl)-N^{ω}-(4-*tert*-butoxycarbonyl)-N^{α}-(2,2-diphenylacetyl)argininamide (3.21). Light brown resin; 45 % (60 mg); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.24-1.42 (m, 22H, 2 × *t*-Bu, CH_2-CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-CH_2), 1.48-1.73 (m, 7H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CH_2-C$\underline{H}_2$-CH_2-CH_2-C$\underline{H}_2$-CH_2), 1.83 (m, 1H, CH-C$\underline{H}_2$-CH_2), 2.2 (t, 2H, C$\underline{H}_2$-CO), 2.37 (t, 2H, C$\underline{H}_2$-CO), 2.72 (t, 2H, C$\underline{H}_2$-NH_2), 3.23 (m, 2H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.41 (m, 2H, CH_2), 3.53 (m, 2H, CH_2), 3.58 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 3.57 (t, 2H, CH_2), 4.32 (m, 2H, C$\underline{H}_2$-ArOH), 4.57 (m, 1H, CH^{α}), 4.99 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.8 (d, 2H, ^3J = 8.5 Hz, AA'BB'), 7.07 (d, 2H, ^3J = 8.5 Hz, AA'BB'), 7.17-7.32 (m, 10H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 458.5 $[M + 2H]^{2+}$, 917 $[M + H]^+$; $C_{50}H_{73}N_7O_9$ (916.2)

General procedure for the synthesis of bivalent ligands 3.22 - 3.25 (meso forms)

The carboxylic acids (*S*)-**3.13** - (*S*)-**3.16** (1 eq) and amine **3.18** - **3.21** (1.1 eq) were dissolved in CH_2Cl_2. EDAC (1.5 eq) was added and the mixture was kept under stirring at rt for 20 h. TFA (1

volume equivalent) and water (3 drops) were added, the mixture was allowed to stand for 2.5 h and then it was concentrated under reduced pressure. CH_2Cl_2 was added three times, each time followed by evaporation under reduced pressure. The product was purified using preparative HPLC.

(*R,S*)-5,5'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-5,5'-dioxo-*N*,*N*'-(ethylenedioxydiethylenediyl)dipentanamide ((*R*,*S*)-**3.22).** White solid; 23 % (8 mg); mp > 94 °C (decomp.); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.48-2.0 (bm, 12H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-CO), 2.27 (t, 4H, ^{3}J = 7.24 Hz, C$\underline{H}_2$-CO), 2.49 (t, 4H, ^{3}J = 7.34 Hz, C$\underline{H}_2$-CO), 3.25 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.35 (t, 4H, ^{3}J = 5.32 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.53 (t, 4H, ^{3}J = 5.46 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.60 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.21 (d, 2H, ^{2}J = 14.72 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.78 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^{\alpha}$), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.54 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.45 Hz, AA'BB'), 7.20-7.33 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 97 % (t_R = 20.1 min, k = 6.1); HRMS: (MALDI): *m/z* calcd. for $[C_{70}H_{87}N_{12}O_{12} + H]^+$ 1287.6566, found: 1287.6585; $C_{70}H_{86}N_{12}O_{12} \times C_4H_2F_6O_4$ (1515.6)

(*R,S*)-6,6'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-6,6'-dioxo-*N*,*N*'-(ethylenedioxydiethylenediyl)dihexanamide ((*R*,*S*)-**3.23).** White solid; 26 % (13.5 mg); mp 77-79 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.48-1.91 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-CO), 2.23 (t, 4H, ^{3}J = 6.74 Hz, C$\underline{H}_2$-CO), 2.47 (t, 4H, ^{3}J = 6.61 Hz, C$\underline{H}_2$-CO), 3.25 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.35 (t, 4H, ^{3}J = 5.56 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.53 (t, 4H, ^{3}J = 5.42 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.59 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.20 (d, 2H, ^{2}J = 14.64 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.76 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^{\alpha}$), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.56 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.57 Hz, AA'BB'), 7.21-7.33 (m, 20H, Ph); RP-HPLC (220 nm, system 2): 99 % (t_R = 17.1 min, k = 4.9); HRMS: (MALDI): *m/z* calcd. for $[C_{72}H_{91}N_{12}O_{12} + H]^+$ 1315.6879, found: 1315.6879; $C_{72}H_{90}N_{12}O_{12} \times C_4H_2F_6O_4$ (1543.6)

(*R,S*)-7,7'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-7,7'-dioxo-*N*,*N*'-(ethylenedioxydiethylenediyl)diheptanamide ((*R*,*S*)-**3.24).** White solid; 24 % (14 mg); mp > 77 °C (decomp.); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.28-1.44 (m, 4H, CO-CH_2-CH_2-C$\underline{H}_2$-CH_2-CH_2-CO), 1.47-1.9 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-C$\underline{H}_2$-CH_2-CO), 2.20 (t, 4H, ^{3}J = 7.31 Hz, C$\underline{H}_2$-CO), 2.45 (t, 4H, ^{3}J = 7.22 Hz, C$\underline{H}_2$-CO), 3.25 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.34 (t, 4H, ^{3}J = 5.35 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.52 (t, 4H, ^{3}J = 5.36 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.59 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.21 (d, 2H, ^{2}J = 14.92 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.87 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^{\alpha}$), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.53 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.46 Hz, AA'BB'), 7.19-7.34 (m,

20H, Ph); RP-HPLC (220 nm, system 1): 95 % (t_R = 20.8 min, k = 6.4); HRMS: (MALDI): *m/z* calcd. for $[C_{74}H_{95}N_{12}O_{12} + H]^+$ 1343.7192, found: 1343.7204; $C_{74}H_{94}N_{12}O_{12} \times C_4H_2F_6O_4$ (1571.7)

(*R,S*)-8,8'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-8,8'-dioxo-*N,N*'-(ethylenedioxydiethylenediyl)dioctanamide ((*R*,*S*)-3.25). White solid; 19 % (12 mg); mp 78-80 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.27-1.44 (m, 8H, CO-CH_2-CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-CH_2-CO), 1.49-1.9 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-CH_2-C$\underline{H}_2$-CH_2-CO), 2.19 (t, 4H, ^{3}J = 7.48 Hz, C$\underline{H}_2$-CO), 2.44 (t, 4H, ^{3}J = 7.25 Hz, C$\underline{H}_2$-CO), 3.26 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.34 (t, 4H, ^{3}J = 5.79 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.53 (t, 4H, ^{3}J = 5.65 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.6 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.21 (d, 2H, ^{2}J = 14.87 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.77 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^{\alpha}$), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.57 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.56 Hz, AA'BB'), 7.21-7.32 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 98 % (t_R = 21.6 min, k = 6.7); HRMS: (MALDI): *m/z* calcd. for $[C_{76}H_{99}N_{12}O_{12} + H]^+$ 1371.7505, found: 1371.7490; $C_{76}H_{98}N_{12}O_{12} \times C_4H_2F_6O_4$ (1599.7)

General procedure for the synthesis of bivalent ligands 3.22 - 3.25 (*R,R*- and *S,S*-configuration)

The carboxylic acid (*S*)-**3.13** - (*S*)-**3.16** or (*R*)-**3.13** - (*R*)-**3.16** (2 eq) and 3,6-dioxa-octane-1,8-diamine (1 eq) were dissolved in CH_2Cl_2. EDAC (2.5 eq) was added and the mixture was kept under stirring at rt for 20 h. TFA (1 volume equivalent) and water (3 drops) were added, the mixture was allowed to stand for 2.5 h and then it was concentrated under reduced pressure. CH_2Cl_2 was added three times, each time followed by evaporation under reduced pressure. The product was purified using preparative HPLC.

(*R,R*)-5,5'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-5,5'-dioxo-*N,N*'-(ethylenedioxydiethylenediyl)dipentanamide ((*R*,*R*)-3.22). White solid; 26 % (19.9 mg); mp > 94 °C (decomp.); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.48-1.98 (bm, 12H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-CO), 2.26 (t, 4H, ^{3}J = 7.36 Hz, C$\underline{H}_2$-CO), 2.49 (t, 4H, ^{3}J = 7.31 Hz, C$\underline{H}_2$-CO), 3.24 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.34 (t, 4H, ^{3}J = 5.41 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.52 (t, 4H, ^{3}J = 5.51 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.59 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.19 (d, 2H, ^{2}J = 14.62 Hz, C$\underline{H}_2$-ArOH), 4.26 (d, 2H, ^{2}J = 14.64 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^{\alpha}$), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.54 Hz, AA'BB'), 7.04 (d, 4H, ^{3}J = 8.53 Hz, AA'BB'), 7.21-7.30 (m, 20H, Ph); RP-HPLC (220 nm, system 2): 99 % (t_R = 17.4 min, k = 5.0); HRMS: (MALDI): *m/z* calcd. for $[C_{70}H_{87}N_{12}O_{12} + H]^+$ 1287.6566, found: 1287.6561; $C_{70}H_{86}N_{12}O_{12} \times C_4H_2F_6O_4$ (1515.6)

(*S,S*)-5,5'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-5,5'-dioxo-*N,N*'-(ethylenedioxydiethylenediyl)dipentanamide

((*S,S***)-3.22).** White solid; 22 % (16 mg); mp > 94 °C (decomp.); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.48-1.98 (bm, 12H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH$_2$-C$\underline{H}_2$-CH$_2$-CO), 2.27 (t, 4H, ^{3}J = 7.31 Hz, C$\underline{H}_2$-CO), 2.49 (t, 4H, ^{3}J = 7.26 Hz, C$\underline{H}_2$-CO), 3.24 (m, 4H, CH$_2$-CH$_2$-C$\underline{H}_2$-NH), 3.34 (t, 4H, ^{3}J = 5.58 Hz, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.52 (t, 4H, ^{3}J = 5.52 Hz, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.59 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.19 (d, 2H, ^{2}J = 14.58 Hz, C$\underline{H}_2$-ArOH), 4.26 (d, 2H, ^{2}J = 14.60 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^{\alpha}$), 5.07 (s, 2H, C$\underline{H}$-(Ph)$_2$), 6.7 (d, 4H, ^{3}J = 8.55 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.53 Hz, AA'BB'), 7.20-7.31 (m, 20H, Ph); RP-HPLC (220 nm, system 2): 96 % (t_R = 17.2 min, k = 4.9); HRMS: (MALDI): *m/z* calcd. for $[C_{70}H_{87}N_{12}O_{12} + H]^+$ 1287.6566, found: 1287.6552; $C_{70}H_{86}N_{12}O_{12} \times C_4H_2F_6O_4$ (1515.6)

(*R,R*)-6,6'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-6,6'-dioxo-*N,N*'-(ethylenedioxydiethylenediyl)dihexanamide

((*R,R***)-3.23).** White solid; 25 % (20 mg); mp 77-79 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.46-1.91 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH$_2$-C$\underline{H}_2$-C$\underline{H}_2$-CH$_2$-CO), 2.23 (t, 4H, ^{3}J = 6.63 Hz, C$\underline{H}_2$-CO), 2.47 (t, 4H, ^{3}J = 6.42 Hz, C$\underline{H}_2$-CO), 3.25 (m, 4H, CH$_2$-CH$_2$-C$\underline{H}_2$-NH), 3.35 (t, 4H, ^{3}J = 5.54 Hz, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.52 (t, 4H, ^{3}J = 5.52 Hz, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.59 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.21 (d, 2H, ^{2}J = 14.58 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.52 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^{\alpha}$), 5.07 (s, 2H, C$\underline{H}$-(Ph)$_2$), 6.69 (d, 4H, ^{3}J = 8.56 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.55 Hz, AA'BB'), 7.21-7.32 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 97 % (t_R = 20.3 min, k = 6.2); HRMS: (MALDI): *m/z* calcd. for $[C_{72}H_{91}N_{12}O_{12} + H]^+$ 1315.6879, found: 1315.6867; $C_{72}H_{90}N_{12}O_{12} \times C_4H_2F_6O_4$ (1543.6)

(*S,S*)-6,6'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-6,6'-dioxo-*N,N*'-(ethylenedioxydiethylenediyl)dihexanamide

((*S,S***)-3.23).** White solid; 27 % (23.7 mg); mp 77-79 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.47-1.9 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH$_2$-C$\underline{H}_2$-C$\underline{H}_2$-CH$_2$-CO), 2.23 (t, 4H, ^{3}J = 6.41 Hz, C$\underline{H}_2$-CO), 2.47 (t, 4H, ^{3}J = 6.52 Hz, C$\underline{H}_2$-CO), 3.25 (m, 4H, CH$_2$-CH$_2$-C$\underline{H}_2$-NH), 3.35 (t, 4H, ^{3}J = 5.48 Hz, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.52 (t, 4H, ^{3}J = 5.49 Hz, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.59 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.20 (d, 2H, ^{2}J = 14.61 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.59 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^{\alpha}$), 5.07 (s, 2H, C$\underline{H}$-(Ph)$_2$), 6.7 (d, 4H, ^{3}J = 8.55 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.53 Hz, AA'BB'), 7.18-7.32 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 94 % (t_R = 20.4 min, k = 6.2); HRMS: (MALDI): *m/z* calcd. for $[C_{72}H_{91}N_{12}O_{12} + H]^+$ 1315.6879, found: 1315.6878; $C_{72}H_{90}N_{12}O_{12} \times C_4H_2F_6O_4$ (1543.6)

(*R,R*)-7,7'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-7,7'-dioxo-*N*,*N*'-(ethylenedioxydiethylenediyl)diheptanamide ((*R*,*R*)-**3.24).** White solid; 32 % (34.6 mg); mp > 77 °C (decomp.); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.31-1.42 (m, 4H, CO-CH_2-CH_2-C$\underline{H}_2$-CH_2-CH_2-CO), 1.47-1.9 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-C$\underline{H}_2$-CH_2-CO), 2.20 (t, 4H, ^{3}J = 7.39 Hz, C$\underline{H}_2$-CO), 2.45 (t, 4H, ^{3}J = 7.35 Hz, C$\underline{H}_2$-CO), 3.25 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.34 (t, 4H, ^{3}J = 5.72 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.52 (t, 4H, ^{3}J = 5.55 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.59 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.20 (d, 2H, ^{2}J = 14.61 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.56 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^\alpha$), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.55 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.55 Hz, AA'BB'), 7.19-7.32 (m, 20H, Ph); RP-HPLC (220 nm, system 2): 99 % (t_R = 18.1 min, k = 5.2); HRMS: (MALDI): *m/z* calcd. for $[C_{74}H_{95}N_{12}O_{12} + H]^+$ 1343.7192, found: 1343.7176; $C_{74}H_{94}N_{12}O_{12} \times C_4H_2F_6O_4$ (1571.7)

(*S,S*)-7,7'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-7,7'-dioxo-*N*,*N*'-(ethylenedioxydiethylenediyl)diheptanamide ((*S*,*S*)-**3.24).** White solid; 24 % (22 mg); mp > 77 °C (decomp.); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.28-1.43 (m, 4H, CO-CH_2-CH_2-C$\underline{H}_2$-CH_2-CH_2-CO), 1.46-1.9 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-C$\underline{H}_2$-CH_2-CO), 2.20 (t, 4H, ^{3}J = 7.38 Hz, C$\underline{H}_2$-CO), 2.45 (t, 4H, ^{3}J = 7.32 Hz, C$\underline{H}_2$-CO), 3.24 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.34 (t, 4H, ^{3}J = 5.66 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.52 (t, 4H, ^{3}J = 5.55 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.59 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.20 (d, 2H, ^{2}J = 14.60 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.65 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^\alpha$), 5.08 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.56 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.56 Hz, AA'BB'), 7.19-7.33 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 91 % (t_R = 20.8 min, k = 6.4); HRMS: (MALDI): *m/z* calcd. for $[C_{74}H_{95}N_{12}O_{12} + H]^+$ 1343.7192, found: 1343.7211; $C_{74}H_{94}N_{12}O_{12} \times C_4H_2F_6O_4$ (1571.7)

(*R,R*)-8,8'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-8,8'-dioxo-*N*,*N*'-(ethylenedioxydiethylenediyl)dioctanamide ((*R*,*R*)-**3.25).** White solid; 14 % (8 mg); mp 78-80 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.28-1.44 (m, 8H, CO-CH_2-CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-CH_2-CO), 1.49-1.9 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-CH_2-C$\underline{H}_2$-CH_2-CO), 2.19 (t, 4H, ^{3}J = 7.12 Hz, C$\underline{H}_2$-CO), 2.44 (t, 4H, ^{3}J = 7.23 Hz, C$\underline{H}_2$-CO), 3.26 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.34 (t, 4H, ^{3}J = 5.72 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.53 (t, 4H, ^{3}J = 5.49 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.6 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.21 (d, 2H, ^{2}J = 14.34 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.38 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^\alpha$), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.57 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.56 Hz, AA'BB'), 7.2-7.35 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 93 % (t_R = 23.2 min, k = 7.2); HRMS: (MALDI): *m/z* calcd. for $[C_{76}H_{99}N_{12}O_{12} + H]^+$ 1371.7505, found: 1371.7502; $C_{76}H_{98}N_{12}O_{12} \times C_4H_2F_6O_4$ (1599.7)

(*S,S*)-8,8'-Bis({[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methylene}amino)-8,8'-dioxo-*N*,*N*'-(ethylenedioxydiethylenediyl)dioctanamide ((*S*,*S*)-**3.25).** White solid; 19 % (12.5 mg); mp 78-80 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.27-1.44 (m, 8H, CO-CH_2-CH_2-C$\underline{H_2}$-C$\underline{H_2}$-CH_2-CH_2-CO), 1.47-1.9 (bm, 16H, CH-C$\underline{H_2}$-C$\underline{H_2}$, CO-CH_2-C$\underline{H_2}$-CH_2-CH_2-C$\underline{H_2}$-CH_2-CO), 2.19 (t, 4H, ^{3}J = 7.44 Hz, C$\underline{H_2}$-CO), 2.44 (t, 4H, ^{3}J = 7.36 Hz, C$\underline{H_2}$-CO), 3.25 (m, 4H, CH_2-CH_2-C$\underline{H_2}$-NH), 3.34 (t, 4H, ^{3}J = 5.60 Hz, C$\underline{H_2}$ from O-CH_2-CH_2-NH), 3.52 (t, 4H, ^{3}J = 5.53 Hz, C$\underline{H_2}$ from O-CH_2-CH_2-NH), 3.59 (s, 4H, O-C$\underline{H_2}$-C$\underline{H_2}$-O), 4.20 (d, 2H, ^{2}J = 14.56 Hz, C$\underline{H_2}$-ArOH), 4.27 (d, 2H, ^{2}J = 14.58 Hz, C$\underline{H_2}$-ArOH), 4.42 (m, 2H, CH^{α}), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.56 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.55 Hz, AA'BB'), 7.19-7.33 (m, 20H, Ph); RP-HPLC (220 nm, system 2): 90 % (t_R = 20.6 min, k = 6.0); HRMS: (MALDI): *m/z* calcd. for $[C_{76}H_{99}N_{12}O_{12} + H]^+$ 1371.7505, found: 1371.7519; $C_{76}H_{98}N_{12}O_{12} \times C_4H_2F_6O_4$ (1599.7)

***N*-*tert*-butoxycarbonyl-3,6-dioxa-octane-1,8-diamine (3.26).** A solution of di-*tert*-butyl dicarbonate (12 g, 55 mmol, 1 eq) in CH_2Cl_2 (100 mL) was added dropwise to a solution of 3,6-dioxa-octane-1,8-diamine (15.2 g, 102.7 mmol, 1.9 eq) and diisopropylethylamine (7.1 g, 55 mmol, 1 eq) in CH_2Cl_2 (400 mL) over a period of 60 min. The mixture was stirred for another 2 h, the solvent was removed under reduced pressure and the product was isolated by column chromatography (CH_2Cl_2/MeOH/NH_4OH). 48 % (6.5 g, 26.2 mmol); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.39 (s, 9H, *t*-Bu), 2.44 (s, 2H, N$\underline{H_2}$), 2.85 (t, 2H, ^{3}J = 5.08 Hz, O-CH_2-C$\underline{H_2}$-NH_2), 3.26 (m, 2H, CH_2), 3.49 (m, 4H, 2 × CH_2), 3.57 (m, 4H, 2 × CH_2); MS (CI, NH_3): *m/z* 249 $[M + H]^+$; $C_{11}H_{24}N_2O_4$ (248.3)

***N*-(4-(Benzyloxycarbonyl)butanoyl)-3,6-dioxa-octane-1,8-diamine (3.27). 3.9** (1 g, 4.5 mmol, 1 eq) and **3.26** (1.1 g, 4.5 mmol, 1 eq) were dissolved in CH_2Cl_2. EDAC (1 g, 5.4 mmol, 1.2 eq) was added and the mixture was kept under stirring at rt for 20 h. The intermediate was purified by column chromatography (EtOAc/MeOH) prior to deprotection with CH_2Cl_2/TFA 1/1 (v/v). 55 % (1.15 g, 2.48 mmol); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.95 (m, 2H, CH_2-C$\underline{H_2}$-CH_2), 2.23 (t, 2H, ^{3}J = 7.35 Hz, C$\underline{H_2}$-CO), 2.41 (t, 2H, ^{3}J = 7.32 Hz, C$\underline{H_2}$-CO), 2.95 (t, 2H, ^{3}J = 4.64 Hz, O-CH_2-C$\underline{H_2}$-NH_2), 3.4 (m, 2H, C$\underline{H_2}$-NH), 3.47-3.64 (m, 8H, C$\underline{H_2}$-O-C$\underline{H_2}$-C$\underline{H_2}$-O-C$\underline{H_2}$), 5.09 (s, 2H, C$\underline{H_2}$-Ph), 7.28-7.36 (m, 5H, Ph); MS (CI, NH_3): *m/z* 353 $[M + H]^+$; $C_{18}H_{28}N_2O_5 \times C_2HF_3O_2$ (466.4)

***N*,*N*'-Bis(*N*-(4-carboxybutanoyl)-8-amino-3,6-dioxa-octyl)-terephthalamide (3.28). 3.27** (0.9 g, 1.93 mmol, 2 eq) was dissolved in CH_2Cl_2. NEt_3 (98 mg, 134 µL, 0.97 mmol, 1 eq) and therephthaloyl dichloride (0.2 g, 0.965 mmol, 1 eq) were added and the mixture was allowed to stand at rt overnight. The intermediate was purified by column chromatography (EtOAc/MeOH) prior to hydrogenation at rt and atmospheric pressure in MeOH using hydrogen and a 10 % Pd/C catalyst. 84 % (0.53 g, 0.81 mmol); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.86 (m, 4H, CH_2-C$\underline{H_2}$-CH_2), 2.22 (t, 4H, ^{3}J = 7.44 Hz, C$\underline{H_2}$-CO), 2.30 (t, 4H, ^{3}J = 7.41 Hz, C$\underline{H_2}$-CO), 3.34 (m,

4H, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.53 (t, 4H, ^3J = 5.53 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.56-3.71 (m, 16H, C$\underline{H}_2$ from O-CH_2-CH_2-NH, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 7.91 (s, 4H, CO-$C_6\underline{H}_4$-CO); $C_{30}H_{46}N_4O_{12}$ (654.7)

(*R,R*)-*N,N*'-Bis(N-{5-[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methyleneamino-5-oxopentanoyl}-8-amino-3,6-dioxa-octyl)-terephthalamide (3.29). A solution of **3.28** (21.5 mg, 33 µmol, 1 eq) and CDI (13.3 mg, 82.4 µmol, 2.5 eq) in DMF was stirred for 30 min. (*R*)-**3.6** (52 mg, 82.4 µmol, 2.5 eq) was added and the mixture was kept under stirring at rt for 20 h. DMF was evaporated (45 °C, 5-10 mbar), CH_2Cl_2/TFA 1/1 (v/v, 2 mL) as well as water (2 drops) were added, and the mixture was allowed to stand for 2.5 h. The mixture was concentrated under reduced pressure and CH_2Cl_2 was added three times, each time followed by evaporation under reduced pressure. The product was purified using preparative HPLC. White solid; 21 % (12.4 mg, 6.92 µmol); mp 85-87 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.48-1.96 (bm, 12H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-CO), 2.24 (t, 4H, ^3J = 7.32 Hz, C$\underline{H}_2$-CO), 2.46 (t, 4H, ^3J = 7.27 Hz, C$\underline{H}_2$-CO), 3.26 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.32 (t, 4H, ^3J = 5.55 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.52 (t, 4H, ^3J = 5.56 Hz, C$\underline{H}_2$ from O-CH_2-CH_2-NH), 3.55-3.68 (m, 16H, C$\underline{H}_2$ from O-CH_2-CH_2-NH, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.21 (d, 2H, ^2J = 14.56 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^2J = 14.68 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^{\alpha}$), 5.06 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^3J = 8.57 Hz, AA'BB'), 7.05 (d, 4H, ^3J = 8.58 Hz, AA'BB'), 7.2-7.33 (m, 20H, Ph), 7.89 (s, 4H, CO-$C_6\underline{H}_4$-CO); RP-HPLC (220 nm, system 2): 91 % (t_R = 16.3 min, k = 4.6); HRMS: (MALDI): *m/z* calcd. for $[C_{84}H_{105}N_{14}O_{16} + H]^+$ 1565.7833, found: 1565.7824; $C_{84}H_{104}N_{14}O_{16} \times C_4H_2F_6O_4$ (1793.9)

***N,N*-Bis(2-aminoethyl)-*N*'-Boc-ethane-1,2-diamine (3.30).** A solution of di-*tert*-butyl dicarbonate (1.49 g, 6.8 mmol, 1 eq) in CH_2Cl_2 (300 mL) was added dropwise to a cooled (-78 °C) solution of *N*,*N*-bis(2-aminoethyl)-ethane-1,2-diamine (10 g, 68.3 mmol, 10 eq) in CH_2Cl_2 (1000 mL) over a period of 2 h. The mixture was allowed to warm up to rt and stirring was continued at rt overnight. The solvent was removed under reduced pressure and the product was isolated by column chromatography (CH_2Cl_2/MeOH/NH_4OH). Jellowish oil; 82 % (1.38 g, 5.6 mmol); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.44 (s, 9H, *t*-Bu), 2.53 (m, 6H, N-C$\underline{H}_2$-CH_2-NHCO), 2.76 (t, 4H, ^3J = 5.98, C$\underline{H}_2$-NH_2), 3.18 (m, 2H, C$\underline{H}_2$-NHBoc); MS (CI, NH_3): *m/z* 247 $[M + H]^+$; $C_{11}H_{26}N_4O_2$ (246.35)

General procedure for the synthesis of linkers 3.31 - 3.33

3.9, **3.10** or **3.12** (2 eq) and **3.30** (1 eq) were dissolved in CH_2Cl_2. EDAC (2.2 eq) was added and the mixture was kept under stirring at rt for 20 h. The intermediate was purified by column chromatography (EtOAc/MeOH) prior to hydrogenation at rt and atmospheric pressure in MeOH using hydrogen and a 10 % Pd/C catalyst.

***N*,*N*-Bis(*N*-(4-carboxybutanoyl)-2-aminoethyl)-*N'*-Boc-ethane-1,2-diamine (3.31).** 72 % (150 mg); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.44 (s, 9H, *t*-Bu), 1.90 (m, 4H, CH_2-C$\underline{H_2}$-CH_2), 2.3 (m, 8H, C$\underline{H_2}$-CO), 2.7 (m, 6H, N-C$\underline{H_2}$-CH_2-NHCO), 3.14 (t, 2H, ^{3}J = 6.22 Hz, C$\underline{H_2}$-NHBoc), 3.29 (t, 4H, ^{3}J = 6.34, C$\underline{H_2}$-NHCO); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 475 $[M + H]^+$; $C_{21}H_{38}N_4O_8$ (474.55)

***N*,*N*-Bis(*N*-(5-carboxypentanoyl)-2-aminoethyl)-*N'*-Boc-ethane-1,2-diamine (3.32).** 75 % (165 mg); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.46 (s, 9H, *t*-Bu), 1.63 (m, 8H, CH_2-C$\underline{H_2}$-C$\underline{H_2}$-CH_2), 2.3 (m, 8H, C$\underline{H_2}$-CO), 3.4 (m, 8H, CH_2 from N-CH_2-CH_2-NH), 3.53 (m, 4H, CH_2 from N-CH_2-CH_2-NH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 503 $[M + H]^+$; $C_{23}H_{42}N_4O_8$ (502.6)

***N*,*N*-Bis(*N*-(7-carboxyheptanoyl)-2-aminoethyl)-*N'*-Boc-ethane-1,2-diamine (3.33).** 58 % 90 mg); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.36 (m, 8H, CH_2-CH_2-C$\underline{H_2}$-C$\underline{H_2}$-CH_2-CH_2), 1.44 (s, 9H, *t*-Bu), 1.61 (m, 8H, CH_2-C$\underline{H_2}$-CH_2-CH_2-C$\underline{H_2}$-CH_2), 2.25 (m, 8H, C$\underline{H_2}$-CO), 2.67 (m, 6H, N-C$\underline{H_2}$-CH_2-NHCO), 3.12 (t, 2H, ^{3}J = 6.18 Hz, C$\underline{H_2}$-NHBoc), 3.26 (t, 4H, ^{3}J = 6.12 Hz, C$\underline{H_2}$-NHCO); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 559 $[M + H]^+$; $C_{27}H_{50}N_4O_8$ (558.7)

***N*,*N*-Bis(*N*-(4-aminobutanoyl)-2-aminoethyl)-*N'*-Boc-ethane-1,2-diamine (3.34). 3.30** (0.28 g, 1.16 mmol, 1 eq) and *N*-benzyloxycarbonyl-γ-butyric acid (0.55 g, 2.3 mmol, 2 eq) were dissolved in CH_2Cl_2. EDAC (2.2 eq) was added and the mixture was kept under stirring at rt for 20 h. The intermediate was purified by column chromatography (EtOAc/MeOH) prior to hydrogenation at rt and atmospheric pressure in MeOH using hydrogen and a 10 % Pd/C catalyst. 89 % (0.37 g, 0.43 mmol); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.43 (s, 9H, *t*-Bu), 1.8 (m, 4H, CH_2-C$\underline{H_2}$-CH_2), 2.34 (t, 4H, ^{3}J = 7.0, C$\underline{H_2}$-CO), 2.53 (m, 6H, N-C$\underline{H_2}$-CH_2-NHCO), 2.76 (t, 4H, ^{3}J = 6.55, C$\underline{H_2}$-NH_2), 3.12 (m, 2H, C$\underline{H_2}$-NHBoc), 3.28 (m, 4H, C$\underline{H_2}$-NHCO); MS (ES, MeOH + 10 mM NH_4OAc): *m/z* 417 $[M + H]^+$; $C_{19}H_{40}N_6O_4$ (416.6)

General procedure for the synthesis of linkers 3.35 - 3.36

3.9 or **3.10** (2 eq) and **3.34** (1 eq) were dissolved in CH_2Cl_2. EDAC (2.2 eq) was added and the mixture was kept under stirring at rt for 20 h. The intermediate was purified by column chromatography (EtOAc/MeOH) prior to hydrogenation at rt and atmospheric pressure in MeOH using hydrogen and a 10 % Pd/C catalyst.

***N*,*N*-Bis(*N*-(*N*-(4-carboxybutanoyl)-4-aminobutanoyl)-2-aminoethyl)-*N'*-Boc-ethane-1,2-diamine (3.35).** 60 % (220 mg); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.43 (s, 9H, *t*-Bu), 1.73-1.94 (m, 8H, CH_2-C$\underline{H}_2$-CH_2), 2.2-2.36 (m, 12H, C$\underline{H}_2$-CO), 2.69 (m, 6H, N-C$\underline{H}_2$-CH_2-NHCO), 3.13 (t, 2H, ^{3}J = 6.23 Hz, C$\underline{H}_2$-NHBoc), 3.20 (t, 4H, ^{3}J = 6.93 Hz, C$\underline{H}_2$-NHCO), 3.29 (t, 4H, ^{3}J = 6.24 Hz, C$\underline{H}_2$-NHCO); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 645 $[M + H]^+$; $C_{29}H_{52}N_6O_{10}$ (644.8)

***N*,*N*-Bis(*N*-(*N*-(5-carboxypentanoyl)-4-aminobutanoyl)-2-aminoethyl)-*N'*-Boc-ethane-1,2-diamine (3.36).** 53 % (95 mg); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.37 (s, 9H, *t*-Bu), 1.58 (m, 8H, CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2), 1.75 (m, 4H, CO-CH_2-C$\underline{H}_2$-CH_2-NH), 2.14-2.34 (m, 12H, C$\underline{H}_2$-CO), 3.15 (m, 6H, N-C$\underline{H}_2$-CH_2-NHCO), 3.33 (m, 4H, C$\underline{H}_2$-NHCO), 3.43 (m, 2H, C$\underline{H}_2$-NHBoc), 3.54 (m, 4H, C$\underline{H}_2$-NHCO); MS (ES, MeOH + 10 mM NH_4OAc): *m/z* 673 $[M + H]^+$; $C_{31}H_{56}N_6O_{10}$ (672.8)

General procedure for the synthesis of bivalent ligands 3.37 - 3.41

A solution of **3.31**, **3.32**, **3.33**, **3.35** or **3.36** (1 eq) and CDI (2.2 eq) in DMF was stirred for 30-40 min. (*R*)-**3.6** (2.2 eq) was added and the mixture was kept under stirring at rt for 20 h. DMF was evaporated (45 °C, 5-10 mbar), CH_2Cl_2/TFA 1/1 (2 mL) as well as water (2-3 drops) were added, and the mixture was allowed to stand for 2.5 h. The mixture was concentrated under reduced pressure and CH_2Cl_2 was added three times, each time followed by evaporation under reduced pressure. The product was purified using preparative HPLC.

(*R*,*R*)-*N*,*N*-Bis(*N*-{5-[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methyleneamino-5-oxopentanoyl}-2-aminoethyl)-ethane-1,2-diamine (3.37). White solid; 37 % (30 mg); mp 102-104 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.47-2.0 (bm, 12H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-CO), 2.29 (t, 4H, ^{3}J = 7.38 Hz, C$\underline{H}_2$-CO), 2.51 (t, 4H, ^{3}J = 7.28 Hz, C$\underline{H}_2$-CO), 2.69 (t, 4H, ^{3}J = 6.08, N-C$\underline{H}_2$-CH_2-NHCO), 2.85 (t, 2H, ^{3}J = 5.61, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 3.03 (t, 2H, ^{3}J = 5.46, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 3.26 (m, 8H, CH_2-CH_2-C$\underline{H}_2$-NH, N-CH_2-C$\underline{H}_2$-NHCO), 4.20 (d, 2H, ^{2}J = 14.64 Hz, C$\underline{H}_2$-ArOH), 4.26 (d, 2H, ^{2}J = 14.57 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH^{α}), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.54 Hz, AA'BB'), 7.04 (d, 4H, ^{3}J = 8.53 Hz, AA'BB'), 7.19-7.32 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 98 % (t_R = 18.4

min, k = 5.5); HRMS: (MALDI): *m/z* calcd. for $[C_{70}H_{89}N_{14}O_{10} + H]^+$ 1285.6886, found: 1285.6867; $C_{70}H_{88}N_{14}O_{10} \times C_8H_4F_{12}O_8$ (1741.6)

(*R,R*)-*N*,*N*-Bis(*N*-{6-[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methyleneamino-6-oxohexanoyl}-2-aminoethyl)-ethane-1,2-diamine (3.38). White solid; 34 % (36 mg); mp 116-118 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.48-1.9 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2), 2.23 (t, 4H, ^{3}J = 6.44 Hz, C$\underline{H}_2$-CO), 2.48 (t, 4H, ^{3}J = 6.53 Hz, C$\underline{H}_2$-CO), 2.64 (t, 4H, ^{3}J = 6.35, N-C$\underline{H}_2$-CH_2-NHCO), 2.79 (t, 2H, ^{3}J = 5.65, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 2.99 (t, 2H, ^{3}J = 5.50, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 3.25 (m, 8H, CH_2-CH_2-C$\underline{H}_2$-NH, N-CH_2-C$\underline{H}_2$-NHCO), 4.20 (d, 2H, ^{2}J = 15.16 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 15.08 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH^{α}), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.57 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.54 Hz, AA'BB'), 7.2-7.32 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 97 % (t_R = 18.7 min, k = 5.6); HRMS: (MALDI): *m/z* calcd. for $[C_{72}H_{93}N_{14}O_{10} + H]^+$ 1313.7199, found: 1313.7201; $C_{72}H_{92}N_{14}O_{10} \times C_8H_4F_{12}O_8$ (1769.7)

(*R,R*)-*N*,*N*-Bis(*N*-{8-[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methyleneamino-8-oxo-octanoyl}-2-aminoethyl)-ethane-1,2-diamine (3.39). White solid; 24 % (16 mg); mp 104-105 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.28-1.43 (m, 8H, CH_2-CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-CH_2),1.48-1.9 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CH_2-C$\underline{H}_2$-CH_2-CH_2-C$\underline{H}_2$-CH_2), 2.20 (t, 4H, ^{3}J = 7.44 Hz, C$\underline{H}_2$-CO), 2.45 (t, 4H, ^{3}J = 7.29 Hz, C$\underline{H}_2$-CO), 2.63 (t, 4H, ^{3}J = 6.67, N-C$\underline{H}_2$-CH_2-NHCO), 2.78 (t, 2H, ^{3}J = 5.97, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 2.99 (t, 2H, ^{3}J = 5.82, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 3.25 (m, 8H, CH_2-CH_2-C$\underline{H}_2$-NH, N-CH_2-C$\underline{H}_2$-NHCO), 4.21 (d, 2H, ^{2}J = 14.33 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.55 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH^{α}), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.55 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.53 Hz, AA'BB'), 7.18-7.33 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 92 % (t_R = 18.8 min, k = 5.7); HRMS: (MALDI): *m/z* calcd. for $[C_{76}H_{101}N_{14}O_{10} + H]^+$ 1369.7825, found: 1369.7808; $C_{76}H_{100}N_{14}O_{10} \times C_8H_4F_{12}O_8$ (1825.8)

(*R,R*)-*N*,*N*-Bis[*N*-(*N*-{5-[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]-amino(amino)methyleneamino-5-oxopentanoyl}-4-aminobutanoyl)-2-aminoethyl]-ethane-1,2-diamine (3.40). White solid; 35 % (114 mg); mp 100-102 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.48-2.0 (bm, 16H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$-CH_2-CO, CO-CH_2-C$\underline{H}_2$-CH_2-NH), 2.23 (m, 8H, C$\underline{H}_2$-CO), 2.50 (t, 4H, ^{3}J = 7.25 Hz, C$\underline{H}_2$-CO), 2.65 (t, 4H, ^{3}J = 5.76, N-C$\underline{H}_2$-CH_2-NHCO), 2.81 (t, 2H, ^{3}J = 5.86, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 2.99 (t, 2H, ^{3}J = 5.71, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 3.17 (t, 4H, ^{3}J = 7.15, C$\underline{H}_2$-NHCO), 3.26 (m, 8H, CH_2-CH_2-C$\underline{H}_2$-NH, C$\underline{H}_2$-NHCO), 4.21 (d, 2H, ^{2}J = 14.55 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.57 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH^{α}), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.49 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.47 Hz, AA'BB'), 7.2-7.32 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 95 % (t_R = 17.8 min, k = 5.3); HRMS: (MALDI): *m/z*

calcd. for $[C_{78}H_{103}N_{16}O_{12} + H]^+$ 1455.7941, found: 1455.7950; $C_{78}H_{102}N_{16}O_{12} \times C_8H_4F_{12}O_8$ (1911.8)

(*R,R*)-*N*,*N*-Bis[*N*-(*N*-{6-[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]-amino(amino)methyleneamino-6-oxohexanoyl}-4-aminobutanoyl)-2-aminoethyl]-ethane-1,2-diamine (3.41). White solid; 12 % (5.7 mg); mp 71-73 °C; ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.48-1.9 (bm, 20H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2, CO-CH_2-C$\underline{H}_2$-CH_2-NH), 2.22 (m, 8H, C$\underline{H}_2$-CO), 2.48 (t, 4H, ^{3}J = 6.37 Hz, C$\underline{H}_2$-CO), 2.72 (t, 4H, ^{3}J = 6.63, N-C$\underline{H}_2$-CH_2-NHCO), 2.89 (m, 2H, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 3.05 (t, 2H, C$\underline{H}_2$ from N-CH_2-CH_2-NH_2), 3.18 (t, 4H, ^{3}J = 7.07, C$\underline{H}_2$-NHCO), 3.27 (m, 8H, CH_2-CH_2-C$\underline{H}_2$-NH, C$\underline{H}_2$-NHCO), 4.21 (d, 2H, ^{2}J = 14.75 Hz, C$\underline{H}_2$-ArOH), 4.27 (d, 2H, ^{2}J = 14.58 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 2H, CH$^\alpha$), 5.07 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 4H, ^{3}J = 8.58 Hz, AA'BB'), 7.05 (d, 4H, ^{3}J = 8.59 Hz, AA'BB'), 7.18-7.33 (m, 20H, Ph); RP-HPLC (220 nm, system 1): 90 % (t_R = 18.7 min, k = 5.6); HRMS: (MALDI): *m/z* calcd. for $[C_{80}H_{107}N_{16}O_{12} + H]^+$ 1483.8254, found: 1483.8251; $C_{80}H_{106}N_{16}O_{12} \times C_8H_4F_{12}O_8$ (1939.9)

General procedure for the synthesis of bivalent ligands 3.42 - 3.44

The precursor **3.38** or **3.40** (1.05 eq) was dissolved in DMF (200 µL) and NEt_3 (5 eq) as well as the NHS ester of the pertinent acid (1 eq) in DMF (20 µL) were added. After an incubation period of 3 h at rt, 10 % aq. TFA (corresponding to 5 eq. of TFA) was added. The mixture was diluted with acetonitrile and water to give an injectable solution for purification with preparative HPLC.

(*R,R*)-*N*,*N*-Bis(*N*-{6-[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]amino-(amino)methyleneamino-6-oxohexanoyl}-2-aminoethyl)-*N*'-propanoyl-ethane-1,2-diamine (3.42). From **3.38** and propionic acid succinimidyl ester; 68 % (9.9 mg); mp 115-118 °C; ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 0.98 (t, 3H, ^{3}J = 7.58 Hz, C$\underline{H}_3$), 1.36-1.47 (m, 4H, CH-CH_2-C$\underline{H}_2$-CH_2), 1.52 (m, 10H, CH-C$\underline{H}_2$-CH_2, CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2), 1.67 (m, 2H, CH-C$\underline{H}_2$-CH_2), 2.12 (m, 6H, C$\underline{H}_2$-CO), 2.41 (t, 4H, ^{3}J = 6.44 Hz, C$\underline{H}_2$-CO), 3.22 (m, 10H, CH_2-CH_2-C$\underline{H}_2$-NH, N-C$\underline{H}_2$-CH_2-NH), 3.38 (s, 6H, N-CH_2-C$\underline{H}_2$-NH), 4.13 (dd, 2H, ^{2}J = 14.79 Hz, ^{3}J = 5.65 Hz, C$\underline{H}_2$-ArOH), 4.17 (dd, 2H, ^{2}J = 14.67 Hz, ^{3}J = 5.91 Hz, C$\underline{H}_2$-ArOH), 4.33 (m, 2H, CH$^\alpha$), 5.11 (s, 2H, C$\underline{H}$-$(Ph)_2$), 6.66 (d, 4H, ^{3}J = 8.49 Hz, AA'BB'), 6.99 (d, 4H, ^{3}J = 8.50 Hz, AA'BB'), 7.21 (m, 4H, Ph), 7.27 (m, 16H, Ph), 8.12 (m, 3H, N$\underline{H}$, N$\underline{H}$CO-CH_2-CH_3), 8.33 (t, 2H, ^{3}J = 5.84 Hz, CO-N$\underline{H}$-CH_2), 8.45 (d, 2H, ^{3}J = 8.11 Hz, CO-N$\underline{H}$-CH), 8.59 (s, 4H, N$\underline{H}_2$), 8.94 (s, 2H, N$\underline{H}$), 9.26 (s, 2H, N$\underline{H}$), 9.58 (s, 1H, N$\underline{H}^+$), 11.41 (s, 2H, ArO$\underline{H}$); RP-HPLC (210 nm, system 3): 99 % (t_R = 13.8 min, k = 4.1); HRMS: (MALDI): *m/z* calcd. for $[C_{75}H_{97}N_{14}O_{11} + H]^+$ 1369.7461, found: 1369.7481; $C_{75}H_{96}N_{14}O_{11} \times C_6H_3F_9O_6$ (1711.7)

(*R,R*)-*N*,*N*-Bis[*N*-(*N*-{5-[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]-amino(amino)methyleneamino-5-oxopentanoyl}-4-aminobutanoyl)-2-aminoethyl]-*N'*-propanoyl-ethane-1,2-diamine (3.43). From **3.40** and propionic acid succinimidyl ester; 62 % (12.6 mg); mp > 96 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 0.99 (t, 3H, ^{3}J = 7.58 Hz, C$\underline{H}_3$), 1.36-1.48 (m, 4H, CH-CH$_2$-C$\underline{H}_2$-CH$_2$), 1.53 (m, 2H, CH-C$\underline{H}_2$-CH$_2$), 1.6 (m, 4H, CO-CH$_2$-C$\underline{H}_2$-CH$_2$-NH), 1.67 (m, 2H, CH-C$\underline{H}_2$-CH$_2$), 1.76 (m, 4H, CO-CH$_2$-C$\underline{H}_2$-CH$_2$-CO), 2.11 (m, 10H, C$\underline{H}_2$-CO), 2.41 (t, 4H, ^{3}J = 7.36 Hz, C$\underline{H}_2$-CO), 3.01 (m, 4H, CO-CH$_2$-CH$_2$-C$\underline{H}_2$-NH), 3.22 (m, 10H, CH$_2$-CH$_2$-C$\underline{H}_2$-NH, N-C$\underline{H}_2$-CH$_2$-NH), 3.38 (s, 6H, N-CH$_2$-C$\underline{H}_2$-NH), 4.1 (dd, 2H, ^{2}J = 14.76 Hz, ^{3}J = 5.61 Hz, C$\underline{H}_2$-ArOH), 4.17 (dd, 2H, ^{2}J = 14.78 Hz, ^{3}J = 5.86 Hz, C$\underline{H}_2$-ArOH), 4.33 (m, 2H, CH$^\alpha$), 5.11 (s, 2H, C$\underline{H}$-(Ph)$_2$), 6.66 (d, 4H, ^{3}J = 8.49 Hz, AA'BB'), 6.99 (d, 4H, ^{3}J = 8.52 Hz, AA'BB'), 7.22 (m, 4H, Ph), 7.27 (m, 16H, Ph), 7.83 (t, 2H, ^{3}J = 5.51 Hz, CO-CH$_2$-CH$_2$-CH$_2$-N$\underline{H}$), 8.12 (s, 3H, N$\underline{H}$, N$\underline{H}$CO-CH$_2$-CH$_3$), 8.33 (t, 2H, ^{3}J = 5.83 Hz, CO-N$\underline{H}$-CH$_2$), 8.45 (d, 2H, ^{3}J = 8.11 Hz, CO-N$\underline{H}$-CH), 8.54 (bs, 4H, N$\underline{H}_2$), 8.86 (s, 2H, N$\underline{H}$), 9.26 (s, 2H, N$\underline{H}$), 9.51 (s, 1H, N$\underline{H}^+$), 11.29 (s, 2H, ArO$\underline{H}$); RP-HPLC (210 nm, system 3): 99 % (t_R = 13.2 min, k = 3.9); HRMS: (MALDI): *m/z* calcd. for $[C_{81}H_{107}N_{16}O_{13} + H]^+$ 1511.8204, found: 1511.8210; $C_{81}H_{106}N_{16}O_{13} \times C_6H_3F_9O_6$ (1853.9)

(*R,R*)-*N*,*N*-Bis[*N*-(*N*-{5-[4-diphenylacetamido-4-(4-hydroxybenzylaminocarbonyl)butyl]-amino(amino)methyleneamino-5-oxopentanoyl}-4-aminobutanoyl)-2-aminoethyl]-*N'*-(4-fluorobenzoyl)-ethane-1,2-diamine (3.44). From **3.40** and 4-fluorobenzoic acid succinimidyl ester; 55 % (8.75 mg); mp 117-118 °C; ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.36-1.48 (m, 4H, CH-CH$_2$-C$\underline{H}_2$-CH$_2$), 1.49-1.62 (m, 6H, CH-C$\underline{H}_2$-CH$_2$, CO-CH$_2$-C$\underline{H}_2$-CH$_2$-NH), 1.66 (m, 2H, CH-C$\underline{H}_2$-CH$_2$), 1.76 (m, 4H, CO-CH$_2$-C$\underline{H}_2$-CH$_2$-CO), 2.1 (m, 8H, C$\underline{H}_2$-CO), 2.41 (t, 4H, ^{3}J = 7.38 Hz, C$\underline{H}_2$-CO), 2.99 (m, 4H, CO-CH$_2$-CH$_2$-C$\underline{H}_2$-NH), 3.21 (m, 4H, CH-CH$_2$-CH$_2$-C$\underline{H}_2$-NH), 3.3 (s, 4H, N-C$\underline{H}_2$-CH$_2$-NH), 3.41 (s, 6H, N-CH$_2$-C$\underline{H}_2$-NH, N-C$\underline{H}_2$-CH$_2$-NH), 3.61 (s, 2H, N-CH$_2$-C$\underline{H}_2$-NH), 4.1 (dd, 2H, ^{2}J = 14.79 Hz, ^{3}J = 5.64 Hz, C$\underline{H}_2$-ArOH), 4.17 (dd, 2H, ^{2}J = 14.78 Hz, ^{3}J = 5.89 Hz, C$\underline{H}_2$-ArOH), 4.33 (m, 2H, CH$^\alpha$), 5.11 (s, 2H, C$\underline{H}$-(Ph)$_2$), 6.66 (d, 4H, ^{3}J = 8.49 Hz, AA'BB'), 6.99 (d, 4H, ^{3}J = 8.49 Hz, AA'BB'), 7.22 (m, 4H, Ph), 7.25-7.34 (m, 18H, Ph, 4-F-Ph), 7.82 (t, 2H, ^{3}J = 5.52 Hz, CO-CH$_2$-CH$_2$-CH$_2$-N$\underline{H}$), 7.9 (dd, 2H, ^{3}J = 8.65 Hz, ^{3}J = 5.55 Hz, 4-F-Ph), 8.15 (s, 2H, N$\underline{H}$), 8.33 (t, 2H, ^{3}J = 5.84 Hz, CO-N$\underline{H}$-CH$_2$), 8.45 (d, 2H, ^{3}J = 8.12 Hz, CO-N$\underline{H}$-CH), 8.54 (bs, 4H, N$\underline{H}_2$), 8.74 (s, 1H, N$\underline{H}$CO-Ar-F), 8.86 (s, 2H, N$\underline{H}$), 9.26 (s, 2H, N$\underline{H}$), 9.54 (s, 1H, N$\underline{H}^+$), 11.29 (s, 2H, ArO$\underline{H}$); RP-HPLC (210 nm, system 3): 100 % (t_R = 13.9 min, k = 4.1); HRMS: (MALDI): *m/z* calcd. for $[C_{85}H_{106}FN_{16}O_{13} + H]^+$ 1577.8109, found: 1577.8107; $C_{85}H_{105}FN_{16}O_{13} \times C_6H_3F_9O_6$ (1919.9)

(S)-N^α-(2,2-Diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide (3.45). TFA (50 % v/v) and water (0.5-1 % v/v) were added to a solution of (*S*)-**3.6** in CH_2Cl_2. The mixture was allowed to stand for 3 hours at rt, CH_2Cl_2 (500 % v/v) was added and the mixture was concentrated under

reduced pressure. CH_2Cl_2 was added two times, each time followed by evaporation under reduced pressure. The product was purified using preparative HPLC. 91 % (35 mg); mp > 65 °C (decomp.); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.38 (m, 2H, CH_2-C<u>H</u>$_2$-CH_2), 1.51 (m, 1H, CH-C<u>H</u>$_2$), 1.66 (m, 1H, CH-C<u>H</u>$_2$), 3.05 (m, 2H, CH_2-C<u>H</u>$_2$-NH), 4.11 (dd, 1H, ^{2}J = 14.86 Hz, ^{3}J = 5.81 Hz, C<u>H</u>$_2$-C_6H_4-OH), 4.18 (dd, 1H, ^{2}J = 15.16 Hz, ^{3}J = 5.85 Hz, C<u>H</u>$_2$-C_6H_4-OH), 4.32 (m, 1H, CH^{α}), 5.13 (s, 1H, C<u>H</u>-$(Ph)_2$), 6.67 (d, 2H, ^{3}J = 8.51 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.43 Hz, AA'BB'), 7.19-7.34 (m, 10H, Ph), 7.44 (t, 1H, ^{3}J = 5.69, guanidine), 8.39 (t, 1H, ^{3}J = 5.91 Hz, CO-N<u>H</u>-CH_2), 8.50 (d, 1H, ^{3}J = 8.08, CO-N<u>H</u>-CH), 9.31 (s, 1H, guanidine); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 474 $[M + H]^+$; $C_{27}H_{31}N_5O_3 \times C_2H_1F_3O_2$ (587.6)

3.3.3 Analysis of the Enantiomeric Purity of BIBP 3226 and BIBP 3435 (3.45) with Capillary Electrophoresis (CE)

CE was performed on a BioFocus™ 3000 capillary electrophoresis system (BioRad, Hercules, CA, USA) using an uncoated fused silica capillary (75 / 70.4 cm × 50 µm i.d.) and a sodium phosphate buffer (125 mM, pH 2.5) enriched with γ-cyclodextrin (0.1 M) and urea (5 M). The capillary temperature was set to 30 °C and the voltage to 20.0 kV. UV-detection was performed at 210 nm. An equimolar mixture of BIBP 3435 and BIBP 3226 (racemic mixture) was analyzed followed by the analysis of BIBP 3435 and BIBP 3435 spiked with the racemic mixture. The difference in migration times of BIBP 3226 and BIBP 3435 was about 2 min Absolute migration times varied between 80 and 90 min. The enantiomeric purity of BIBP 3435 was determined to amount to an ee of 97.2 % (2.7 % were identified as the active stereoisomer BIBP 3226). The enantiomeric purity of BIBP 3226 (obtained through deprotection of building block **2.7** with TFA, cf. chapter 2) was similarly determined to be 99.3 % ee.

3.3.4 Pharmacology: Cell Culture, Fura-2 Assay and Competition Binding Assay

Cell culture. HEL and SK-N-MC cells were cultured as described elsewhere.[31, 32] HEL cells were subcultured by 1:6-dilution with fresh culture medium 24 h prior to the Fura Ca^{2+}-assay. MCF-7-Y_1 cells[a] were maintained in MEM (Sigma, Deisenhofen, Germany), supplemented with 5 % FCS (Biochrom AG, Berlin, Germany).

[a]This cell line was subcloned from MCF-7 cells in passage 157th and shows a higher Y_1R expression (by a factor 2 -3 higher than that of the MCF-7 cells (ATCC number HTB 22)).

Fura-2 assay on HEL cells. The Fura assay was performed with HEL cells as previously described[28] using a Perkin-Elmer LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany).

Radioligand competition binding assay. The procedure performed in the radioligand binding assay was deduced from a previously described protocol[31] and [^{3}H]-UR-MK114 (**2.8b**, cf. chapter 2) was used instead [^{3}H]-propionyl-pNPY. Radioligand concentration was set to 1.5 nM for SK-N-MC cells (K_D = 1.2 nM) and 2 nM for MCF-7 cells (K_D = 2.9 nM). Cells were seeded in 24-well plates 2 or 3 days prior to the experiment. MCF-7-Y_1^a cells were seeded in culture medium with 1 % β-Estradiol in order to up-regulate the Y_1 receptor. On the day of the experiment confluency of the cells was at least 70 %. The culture medium was sucked off, cells were washed once with buffer (500 µL) and covered with binding buffer (200 µL for SK-N-MC, 400 µL for MCF-7 cells). Binding buffer (25/50 µL) and binding buffer (25/50 µL) with radioligand (ten fold concentrated) was added for total binding. For non-specific binding and competition of test compounds with radioligand binding buffer (25/50 µL) with the competing agent (pNPY or test substance, ten fold concentrated) and binding buffer (25/50 µL) with radioligand (ten fold concentrated) was added. During incubation at room temperature (22 – 25 °C) the plates were gently shaken. After incubation (20 min for SK-N-MC, 30 min for MCF-7-Y_1 cells) the binding buffer was removed, the cells were washed twice with buffer (500 µL, 4 °C, ≤ 30 s) and covered with lysis solution (200 µL). The plates were gently shaken for at least 30 min. The lysis solution was transferred into 6-mL scintillation vials filled with scintillator (3 mL) and the dishes were washed once with lysis solution (100 µL). Assays were performed in triplicate or duplicate.

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Chapter 4

Fluorescently Labeled Y_1 Receptor Antagonists

4.1 Introduction

Fluorescence-based techniques offer highly attractive alternatives to radiometric assays for the investigation of ligand-receptor interactions. Consequently, there is a growing demand for appropriate fluorescent probes, and many fluorescent ligands have become available in the last decade.[1] Fluorescence-labeled compounds are preferred over radioligands in terms of safety precautions and waste disposal, and in contrast to radiotracers, fluorescent ligands are applicable to fluorescence microscopy and flow cytometry, powerful techniques, which have become routine in many laboratories. For instance, by combination of fluorescent dyes emitting at distinct wavelengths, multiparametric assays become possible.[2, 3] Compared to radioligands, however, a major challenge in the preparation of fluorescent ligands is to retain the binding affinity, particularly when small ligands are labeled with bulky fluorophores. A straightforward and frequently successful approach for investigations on peptidergic G-protein coupled receptors (GPCRs) consists in fluorescence labeling of the peptide. In the case of peptides, the decrease in affinity is often only moderate, although a factor of ten or higher compared to the parent peptide is not exceptional.[4] Many fluorescent peptide ligands have been reported up to now, e.g. derivatives of formyl peptide,[5] insulin,[6] epidermal growth factor[7, 8] or neuropeptide Y (NPY) analogs.[9] The synthesis of non-peptidic receptor subtype selective fluorescent antagonists is more challenging, particularly in the field of aminergic GPCRs, although a growing number of low molecular weight fluorescent GPCR ligands with adequate binding affinity is reported in the literature.[1, 10-14]

For the detection and investigation of only one receptor subtype on cells and in tissues selective potent fluorescent ligands are required. High affinity low molecular weight antagonists are superior to peptides concerning selectivity, kinetics, stability and mode of action. In most cases peptidic ligands are receptor agonists and can cause receptor desensitization and internalization, which may hamper the determination of binding kinetics and lead to a high non-specific fluorescence background in binding assays. In addition, peptides often need a long period of time to reach binding equilibrium, and they can be (rapidly) degraded by enzymes.

The lack of a selective low molecular weight fluorescent probe for the NPY Y_1 receptor stimulated us to search for fluorescent ligands starting from the (*R*)-argininamide BIBP 3226, a potent and selective NPY Y_1R antagonist.[15] Previous investigations on N^{ω}-substituted analogs of this argininamide in our laboratory revealed a preference for electron-withdrawing

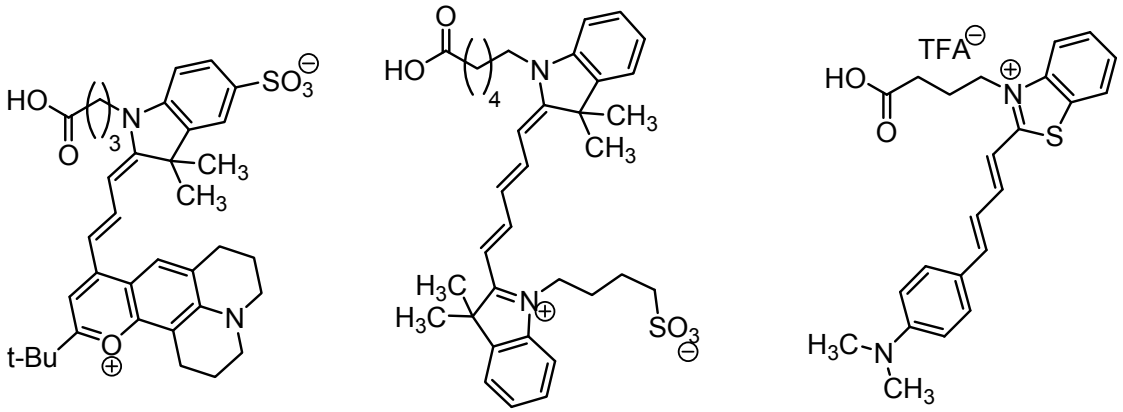
substituents (alkanoyl, carbamoyl) in terms of retaining or even increasing the Y_1R affinity over the parent compound.[16, 17] This guanidine-acylguanidine bioisosterism, also found in the NPY Y_2R[18] as well as in the histamine H_2R field[19], enables the preparation of highly potent radioligands (cf. chapter 2) as well as fluorescent ligands as described below.

4.2 Results and Discussion

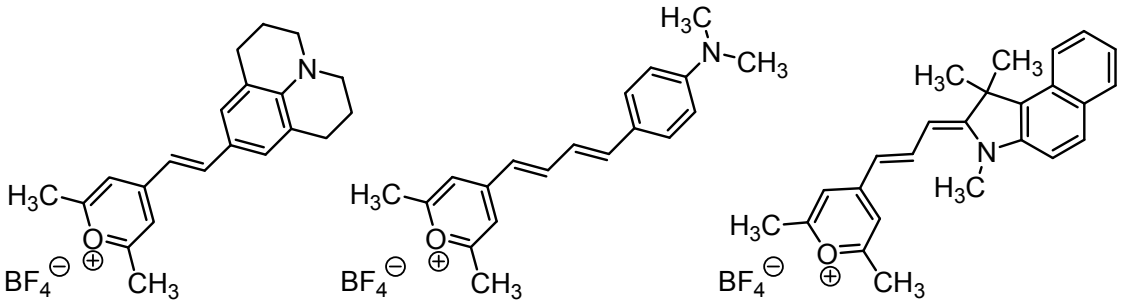
4.2.1 Chemistry

With respect to potential use in cellular assays red fluorescent dyes (emission wavelength > 590 nm) were selected to reduce the background fluorescence and thus to improve the signal-to-noise ratio. Preferably, we used the new class of pyrylium dyes[20, 21] (Py-1, Py-5 and Py-6), as well as the Cy5-related cyanine dye S0436 and the dye Dy-635 with an unsymmetrical hemicyanine structure consisting of an indol and a benzopyrane moiety (Figure 1). In addition, two fluorescent ligands were prepared by labeling with the benzothiazole derivative DE99 (Figure 1), a dye derived from reported push-pull conjugated benzothiazolium compounds[22]. DE99 was synthesized by Daniela Erdmann in our research group as part of her dissertation project (to be reported elsewhere).

The fluorescent dyes S0436, Dy-635 and DE99 are excitable at 635 nm with a red diode laser, used as light source in commercial flow cytometers. Fluorescence emission can be detected around 660 nm. The pyrylium dyes Py-1, Py-5 and Py-6 belong to the new class of “chameleon labels”, which has been developed for the staining and quantification of picomolar concentrations of proteins.[21] These dyes as well as the benzothiazole derivative DE99 are relatively low molecular weight compounds (about 300 - 400 g/mol), and are therefore anticipated to be well suited for coupling to small molecules. An additional advantage of pyrylium labeled fluorescent probes consists in their excitability with the widely used 488 nm laser, a standard component of flow cytometers and confocal microscopes.



Dy-635 **S0436** **DE99**



Py-1 **Py-5** **Py-6**

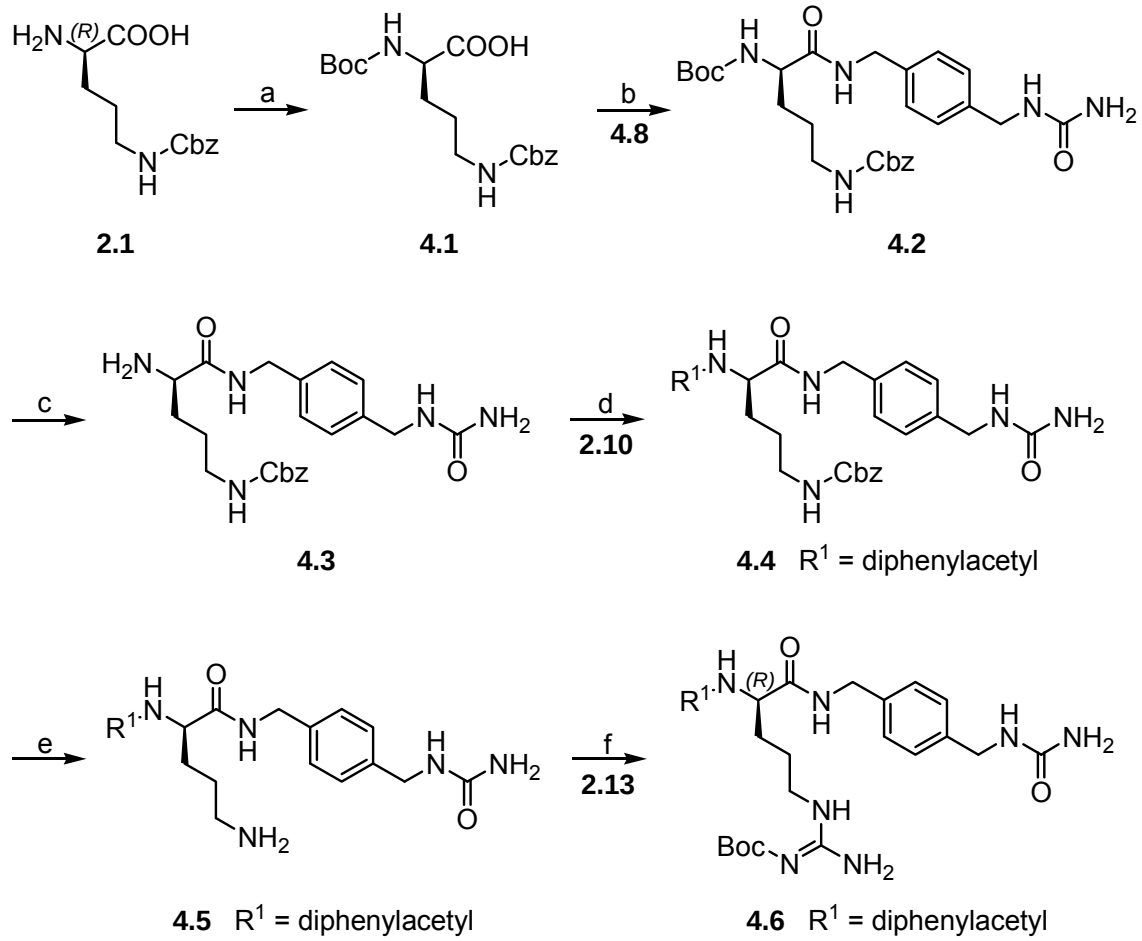
Figure 1. Structures of the dyes which were used for the preparation of the fluorescent Y_1R antagonists (**4.28** - **4.47**).

The dyes S0436, Dy-635 and DE99 contain a carboxylated side chain, which enables coupling to amines. S0436 and Dy-635 were purchased as succinimidyl esters, and DE99 was coupled as NHS ester or with the aid of coupling reagents (CDI, EDAC) (Scheme 7). Pyrylium dyes react very rapidly with primary amines at pH 8-9 at room temperature to form positively charged *N*-substituted pyridinium adducts.[23] The ring transformation is accompanied by a hypsochromic shift of the absorption maximum by more than 100 nm. Thus, the coupling reaction can be followed visually due to a change in color from blue to red.

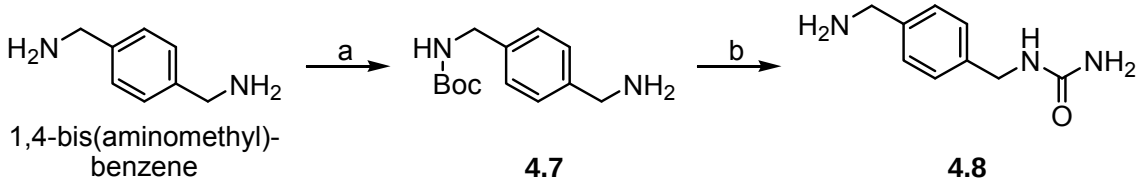
All synthesized fluorescent ligands, except **4.32**, are derivatives of the (*R*)-argininamide BIBP 3226 (Schemes 6 – 8). **4.32** represents a derivative of the (*R*)-argininamide BIBO 3304 (**6.6**, cf. chapter 6), another highly potent and selective Y_1R antagonist.[24] The synthesis of the BIBP 3226 building block **2.7** is described in chapter 2. The BIBO 3304 moiety (building block **4.6**) was built up straight forward using standard protecting group chemistry (Scheme 1). The urea entity was obtained from mono Boc-protected 1,4-bis(aminomethyl)benzene (**4.7**) and potassium cyanate, a common route for the synthesis of *N*-substituted ureas[25-28] (Scheme 2). When the urea moiety was integrated (compound **4.2**) subsequent synthesis of building block **4.6** was hampered by solubility problems. Intermediate **4.4** (Scheme 1) exhibited lowest solubility, i. e. the compound was only slightly soluble in MeOH (under heating) and insoluble in methylene chloride, acetonitrile, 1,2-dimethoxyethane, THF, 1,4-dioxane, propan-2-ol, ethanol

and in mixtures of water with water-miscible solvents. Nevertheless, building block **4.6** was obtained in good yield (Scheme 1).

The previously described synthesis of BIBO 3304 started with N^{ω}-nitro protected *D*-arginine, which was first treated with diphenylacetyl choride and then amidated with *N*-(4-aminomethyl-benzyl)urea (**4.8**) using TBTU as coupling reagent.[29] Final cleavage of the N^{ω}-nitro group (Pd, H_2 at 5 bar, 80 % aq. acetic acid, 40 °C) yielded the argininamide BIBO 3304.



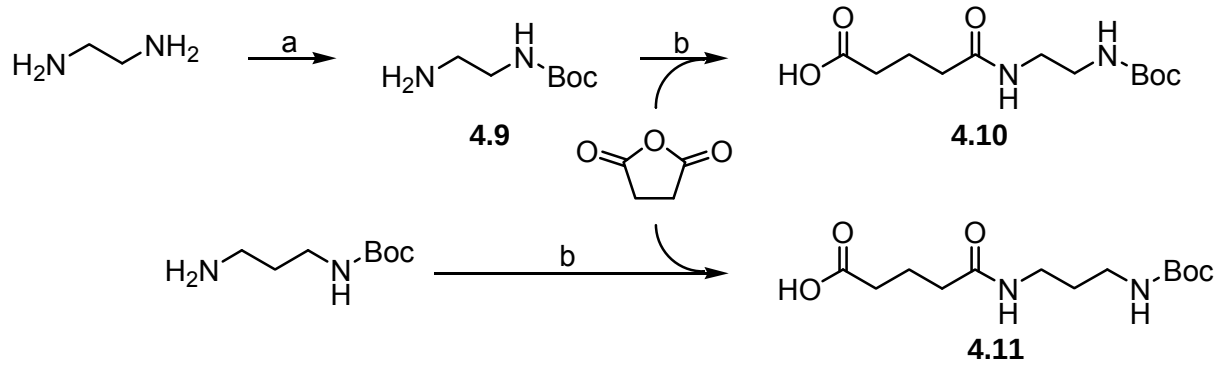
Scheme 1. Synthesis of N^{G}-Boc-protected BIBO 3304 (**4.6**).
Reagents and conditions: (a) K_2CO_3, di-*tert*-butyl dicarbonate, 1,4-dioxane, H_2O, rt, 20 h, 92 %; (b) CDI, NEt_3, DMAP, DMF, 0 °C/45 °C/rt, 20 h, 85 %; (c) AcCl, MeOH, rt, 2 h, 100 %; (d) NEt_3, DMAP, DMF, rt, 20 h, 83 %; (e) Pd/C, H_2, CH_3COOH, MeOH, 45 °C, 2.5 h, 88 %; (f) (1) NEt_3, MeOH, CH_2Cl_2, rt, 20 h; (2) Pd/C, H_2, CH_3COOH, MeOH, rt, 6.5 h, 70 %.



Scheme 2. Synthesis of *N*-(4-aminomethyl-benzyl)urea (**4.8**).
Reagents and conditions: (a) di-*tert*-butyl dicarbonate, 1 M aq. NaOH, 1,4-dioxane, 0 °C/rt, 20 h, 42 %; (b) (1) potassium cyanate, H_2O, EtOH, 1 M aq. HCl, reflux, 2 h; (2) AcCl, MeOH, rt, 2.5 h, 88 %.

The fluorophores were linked to the guanidine group of the argininamides via ω-aminoalkanoyl or ω-aminoalkylcarbamoyl spacers (Schemes 6 – 8) with a length of 6 – 17 atoms. ω-

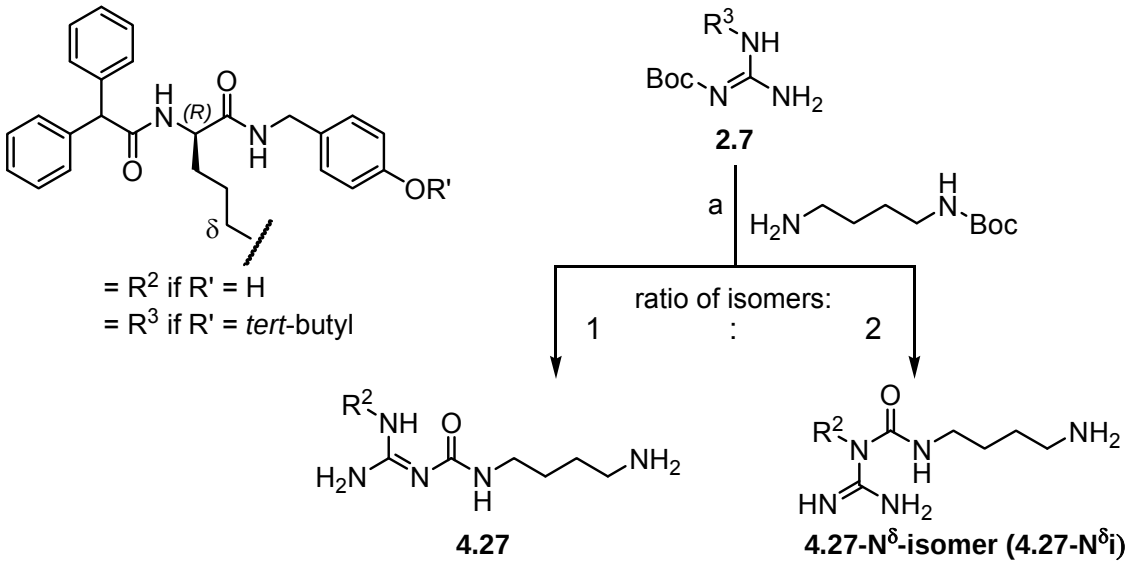
Aminoalkanoyl linkers containing glycol ether moieties or amide groups were used in order to retain a certain water solubility of the compounds. The linkers **4.10**, **4.11**, **4.16** and **4.17** were not commercially available; the synthesis is depicted in Schemes 3 and 6.



Scheme 3. Synthesis of the carboxylic acids **4.10** and **4.11**.
Reagents and conditions: (a) di-*tert*-butyl dicarbonate, $CHCl_3$, -15 °C/rt, 20 h, 97 %; (b) CH_2Cl_2, rt, 60 min, 97 %.

Except for compounds **4.26**, **4.27** and the bivalent amino precursors **3.38** – **3.40** (cf. chapter 3), all amino precursors (**4.18** – **4.25**) were prepared via direct attachment of the *N*-protected (Boc, Cbz) ω-aminocarboxylic acids to N^{ω}-Boc, *O*-*tert*-butyl protected BIBP 3226 (**2.7**, cf. chapter 2) and N^{G}-Boc protected BIBO 3304 (**4.6**) respectively (Scheme 6). The labeling precursor N^{ω}-6-aminohexanoyl-BIBP 3226 (**4.21**) proved to be sufficiently stable under basic conditions in contrast to its lower homolog, the N^{ω}-5-aminopentanoyl derivative, which is cleaved by intramolecular aminolysis into BIBP 3226 and valerolactam. This decomposition takes place within seconds as demonstrated by kinetic investigations on aminopentanoyl-guanidine as a model compound.[30] By contrast, the formation of a seven-membered ring, namely ε-lactam, by cleavage of **4.21** is considerably less favored.

Amino precursor **4.26** was prepared from the N^{ω}-(7-carboxyheptanoyl), $N^{\omega'}$-Boc, *O*-*tert*-butyl protected argininamide **3.16** (cf. chapter 3) as shown in Scheme 6. The N^{ω}-carbamoylated amine precursor **4.27** was initially prepared from *N*-Boc-protected butane-1,4-diamine and the building block **2.7** (Scheme 4) following a reported protocol for the transformation of amines into isocyanates with the aid of triphosgene.[31] Unfortunately, this procedure preferentially gave the N^{δ}-substituted compound (**4.27-N$^{\delta}$i**) instead of the desired N^{ω}-substituted argininamide derivative (Scheme 4, Figure 2). This is presumably facilitated by the small size of the isocyanate entity (acting as electrophile), but other factors such as different nucleophilicity of the δ-nitrogen and the ω-nitrogen, seem to play a role as well. Therefore, the precursor **4.27** was prepared from the guanidinylating reagent **4.13** and amine **2.6** (cf. chapter 2) as depicted in Schemes 5 and 6. *S*-methyl-isothiourea (**4.12**) was prepared according to the methods described by A. Brennauer.[18]



Scheme 4. Synthesis of the amine precursor **4.27** via direct carbamoylation of N^{ω}-Boc, *O*-*tert*-butyl protected BIBP 3226 (**2.7**).
Reagents and conditions: (a) (1) triphosgene, DIEA, CH_2Cl_2, rt, 2.5 h; (2) CH_2Cl_2/TFA 1/1, rt, 1.5 h, total yield: 50 %. **4.27** and **4.27-N$^{\delta}$i** differ in the ^{1}H-NMR signal of the δ-CH_2 group (**4.27**: δ = 3.19 ppm, **4.27-N$^{\delta}$i**: δ = 3.57 ppm).

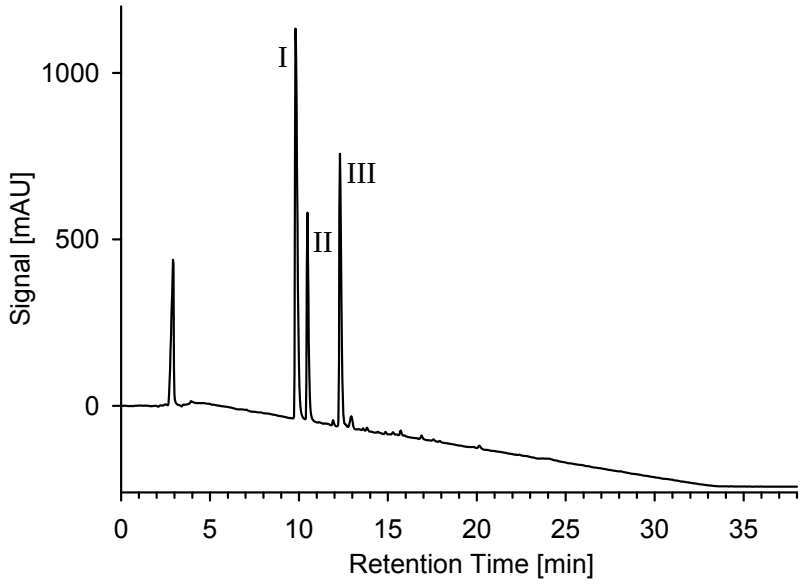
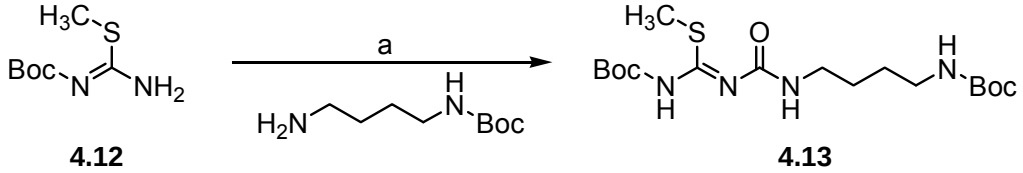
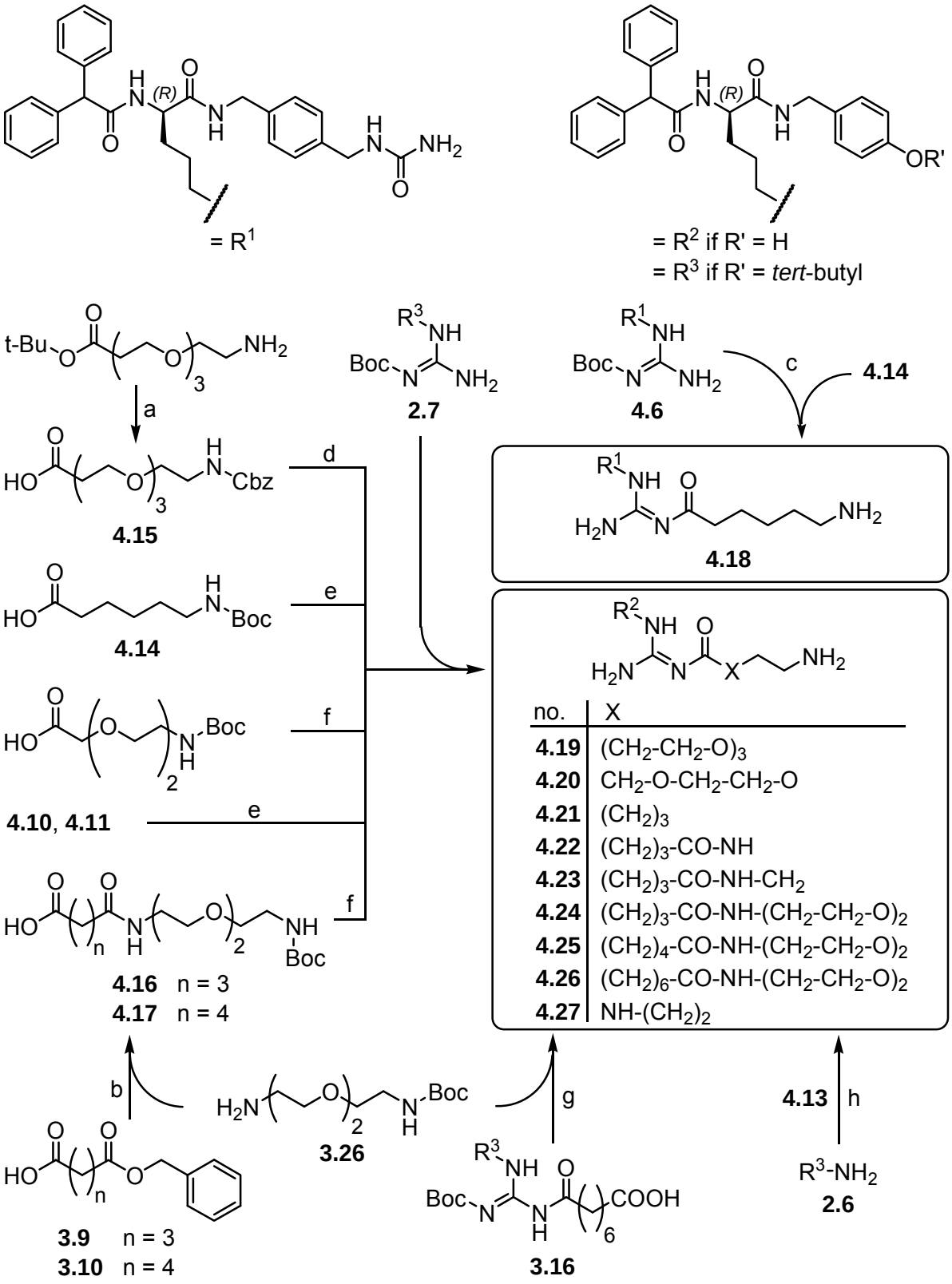


Figure 2. HPLC control of the reaction mixture (after deprotection) of the synthesis of amino precursor **4.27** from *N*-Boc-protected butane-1,4-diamine and the BIBP 3226 building block **2.7** (Scheme 4). Conditions: column: Eurospher-100 C18 (250 × 4 mm, 5 µm), eluent: mixtures of acetonitrile (A) and 0.05 % aq. TFA (B), gradient: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5, flow rate: 0.8 mL/min, UV-detection: 210 nm. I: **4.27-N$^{\delta}$i** (t_R = 9.8 min), II: **4.27** (t_R = 10.5 min), III: BIBP 3226 (t_R = 12.3 min).

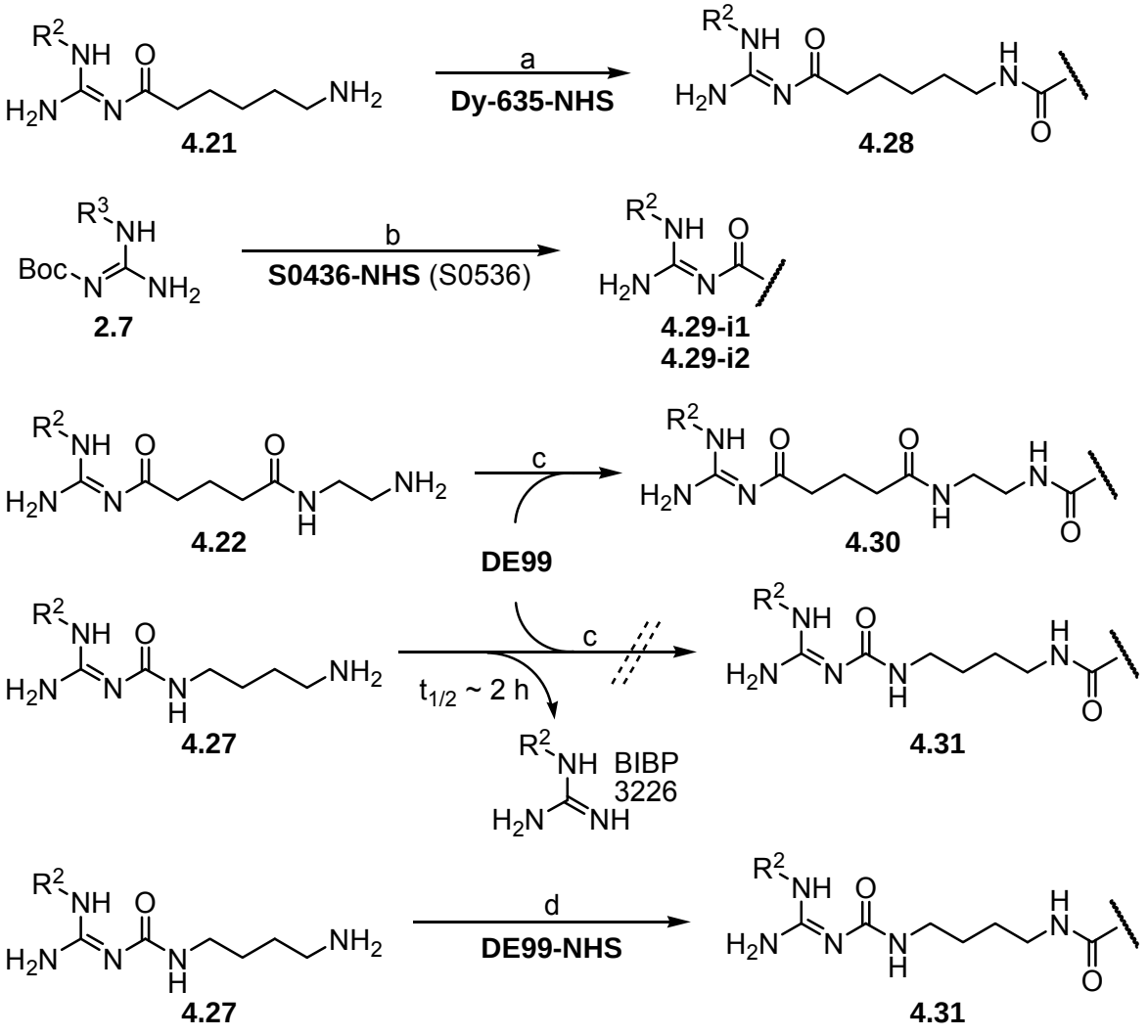


Scheme 5. Synthesis of the guanidinylation reagent **4.13**. Reagents and conditions: (a) triphosgene, DIEA, CH_2Cl_2, rt, 3 h, 75 %.



Scheme 6. Synthesis of the amine precursors **4.18** – **4.27**.
Reagents and conditions: (a) (1) Cbz-Cl, NEt_3, CH_2Cl_2, 0 °C/rt, 2.5 h; (2) CH_2Cl_2/TFA 4/1, rt, 3 h, 88 %; (b) (1) EDAC, CH_2Cl_2, rt, 20 h; (2) Pd/C, H_2, MeOH, rt, 63-66 %; (c) (1) CDI, NEt_3, acetonitrile, 35 °C, 20 h; (2) acetonitrile/TFA, 50 °C, 1.5 h, 36 %; (d) (1) CDI, NEt_3, CH_2Cl_2, rt, 20 h; (2) Pd/C, H_2, MeOH, rt; (3) CH_2Cl_2/TFA 10/1, rt, 20 h, 36 %; (e) (1) CDI, NEt_3, CH_2Cl_2, rt, 20 h; (2) CH_2Cl_2/TFA/H_2O 18/6/1, rt, 3 h, 64-94 %; (f) (1) CDI, NEt_3, CH_2Cl_2, rt, 20 h; (2) CH_2Cl_2/TFA, rt, 2.5-4 h, 44-64 %; (g) (1) EDAC, CH_2Cl_2, rt, 20 h; (2) CH_2Cl_2/TFA, rt, 2 h, 43 %; (h) (1) $HgCl_2$, DIEA, DMF, rt, 20 h; (2) CH_2Cl_2/TFA 1/1, rt, 2 h, 74 %.

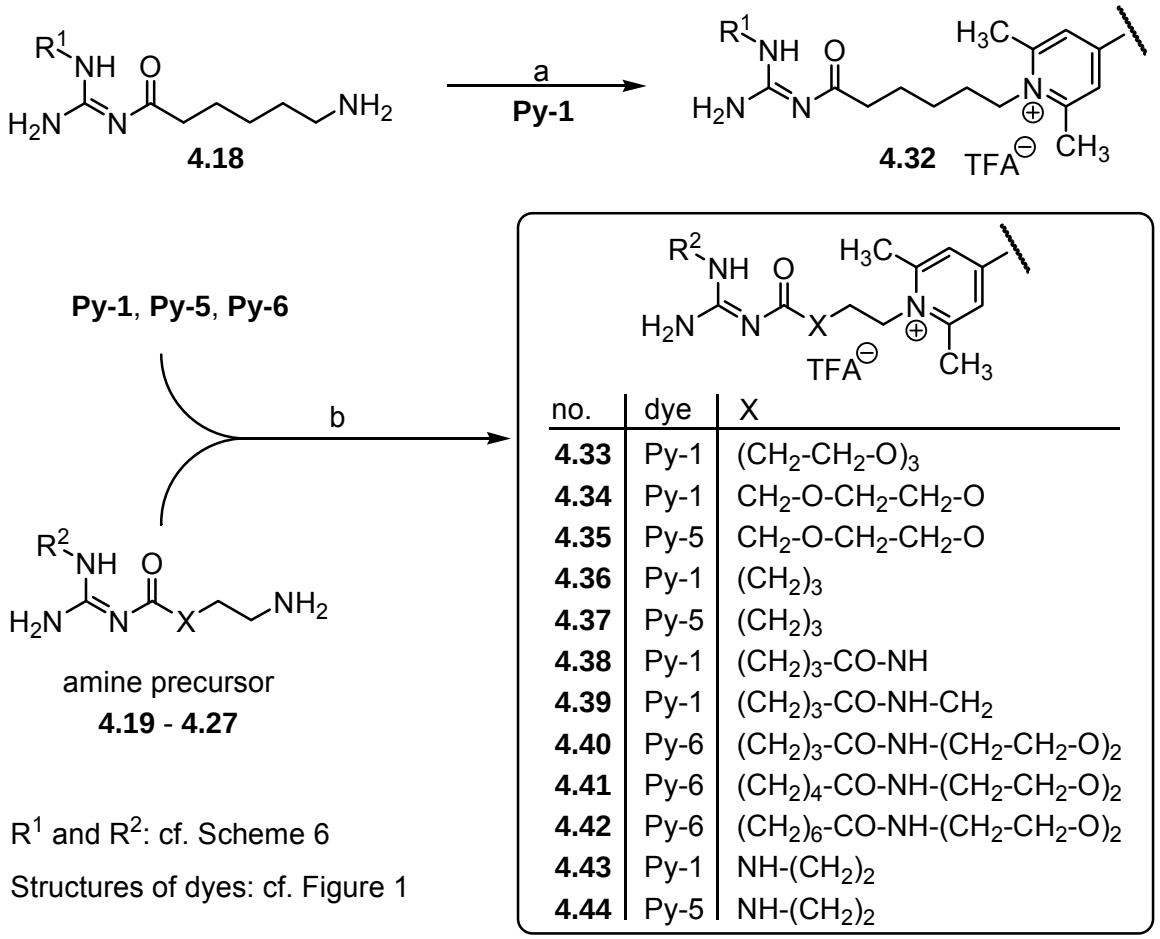
Except for **4.29,** all fluorescent Y_1R antagonists were prepared by coupling the fluorophores with completely deprotected amine precursors **4.18** – **4.27** (Scheme 6 – 8) and **3.38** – **4.40** (Scheme 9). The synthesis of the bivalent Y_1R antagonists **3.38** – **3.40** is described in chapter 3. Labeling of these bivalent amine precursors with Py-1 afforded the fluorescence labeled bivalent ligands in satisfactory yields, but labeling of the precursor **3.40** with DE99 or DE99-NHS failed totally (Scheme 9). This was also observed for the higher homolog **3.41** (cf. chapter 3, Scheme 5).



Scheme 7. Synthesis of the fluorescent Y_1R ligands **4.28** – **4.31**.
Reagents and conditions: (a) NEt_3, acetonitrile, rt, 3.5 h, 23 %; (b) (1) NEt_3, acetonitrile, rt, 20 h; (2) acetonitrile/TFA 1/1, rt, 2 h, 20 % (isomer 1 (**4.29-i1**): 11 %, isomer 2 (**4.29-i2**): 9 %, cf. Figure 3); (c) CDI or EDAC, NEt_3, DMF, rt, 2.5 - 7 h, 23 % for **4.30**, 0.3 % for **4.31**; (d) NEt_3, DMF, rt, 3 h, 24 %.

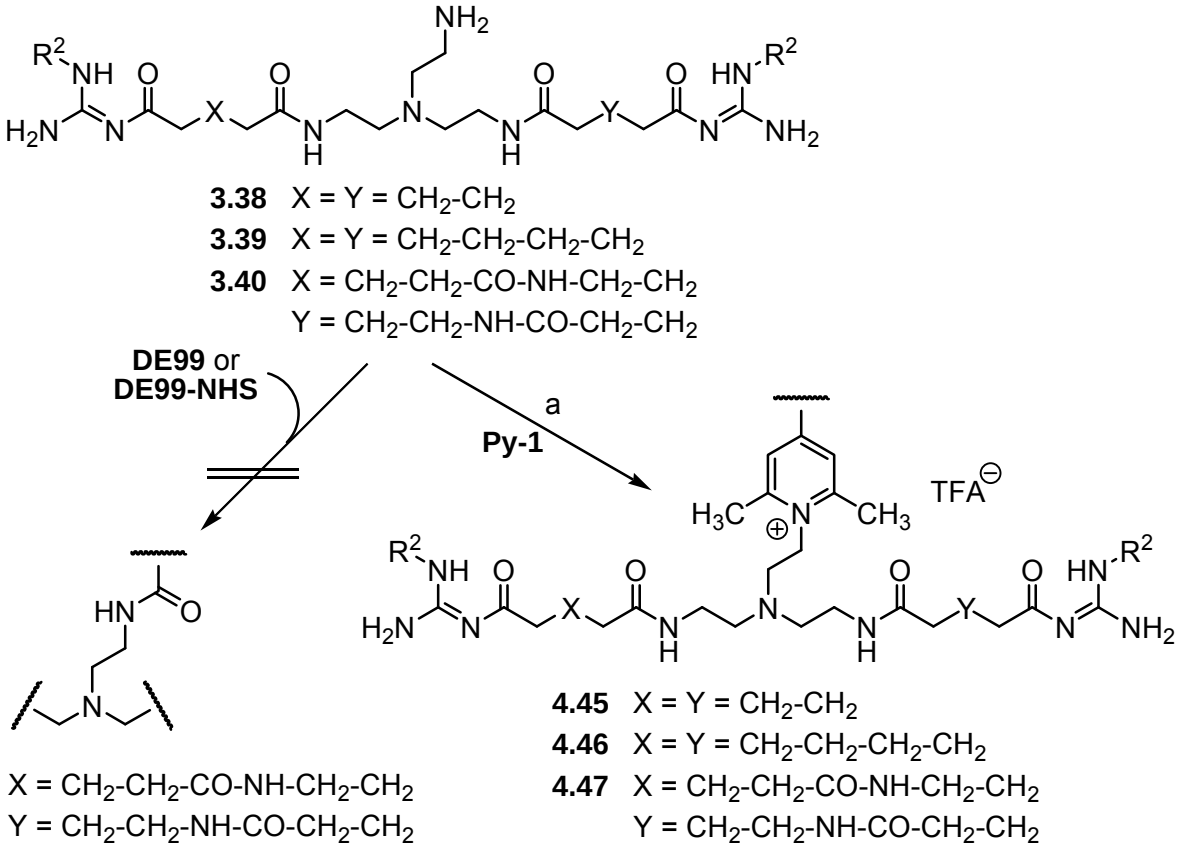
Activation of DE99 with carbonyldiimidazole in dimethylformamide and subsequent treatment with amine precursor **4.22** in the presence of triethylamine (Scheme 7) gave the fluorescent ligand **4.30** in satisfactory yield (23 %), whereas the synthesis of **4.31** from the carbamoyl precursor **4.27** nearly failed under the same conditions (yield: 0.3 %). Using EDAC instead of carbonyldiimidazole led to the same unsactisfactory result (Scheme 7). This can be explained

by the decomposition of precursor **4.27** into BIBP 3226 in the presence of carbonyldiimidazole or EDAC (Scheme 7) as analyzed by HPLC. By contrast labeling of amine **4.27** with DE99-NHS afforded **4.31** in 24 % yield (Scheme 7).



no.	dye	X
4.33	Py-1	$(CH_2\text{-}CH_2\text{-}O)_3$
4.34	Py-1	$CH_2\text{-}O\text{-}CH_2\text{-}CH_2\text{-}O$
4.35	Py-5	$CH_2\text{-}O\text{-}CH_2\text{-}CH_2\text{-}O$
4.36	Py-1	$(CH_2)_3$
4.37	Py-5	$(CH_2)_3$
4.38	Py-1	$(CH_2)_3\text{-}CO\text{-}NH$
4.39	Py-1	$(CH_2)_3\text{-}CO\text{-}NH\text{-}CH_2$
4.40	Py-6	$(CH_2)_3\text{-}CO\text{-}NH\text{-}(CH_2\text{-}CH_2\text{-}O)_2$
4.41	Py-6	$(CH_2)_4\text{-}CO\text{-}NH\text{-}(CH_2\text{-}CH_2\text{-}O)_2$
4.42	Py-6	$(CH_2)_6\text{-}CO\text{-}NH\text{-}(CH_2\text{-}CH_2\text{-}O)_2$
4.43	Py-1	$NH\text{-}(CH_2)_2$
4.44	Py-5	$NH\text{-}(CH_2)_2$

Scheme 8. Synthesis of the fluorescent Y_1R ligands **4.32** – **4.44**.
Reagents and conditions: (a) NEt_3, acetonitrile, H_2O, rt, 2 h, 33 %; (b) NEt_3, acetonitrile, DMF, rt, 1-2 h; 13-56 %.



Scheme 9. Synthesis of the fluorescent Y_1R ligands **4.45** – **4.47** and failed labeling of **3.40** with DE99. Reagents and conditions: (a) NEt_3, acetonitrile, DMF, rt, 1-2 h; 26-28 %; (b) DE99 activated in DMF with CDI (30 min) followed by the addition of amino precursor and NEt_3, rt; or: DE99-NHS, DMF, NEt_3, rt.

For the preparation of compound **4.29** the succinimidyl ester of the fluorescent dye S0436 was coupled with N^{ω}-Boc, *O-tert*-butyl protected BIBP 3226 (**2.7**, cf. chapter 2) followed by a deprotection step (acetonitrile, TFA; Scheme 7). Interestingly, two different products were isolated. Both samples exhibited the expected molecular mass in MS analysis. Presumably, an isomerization of the fluorescent entity in S0436 (Figure 1) was induced under the acidic deprotection conditions. The isomers were referred to as **4.29-i1** (exhibits the lower retention time when analyzed with reversed-phase chromatography, Figure 3) and **4.29-i2** (exhibits a higher retention time, Figure 3). An acylation of the δ-nitrogen of the building block **2.7** is not very likely, since acylation of **2.7** with succinimidyl propionate (chapter 2) as well as acylation with carboxylic acids using CDI (preparation of amino precursors **4.19** – **4.23**, Scheme 6) gave no remarkable amounts of N^{δ}-acylated side products.

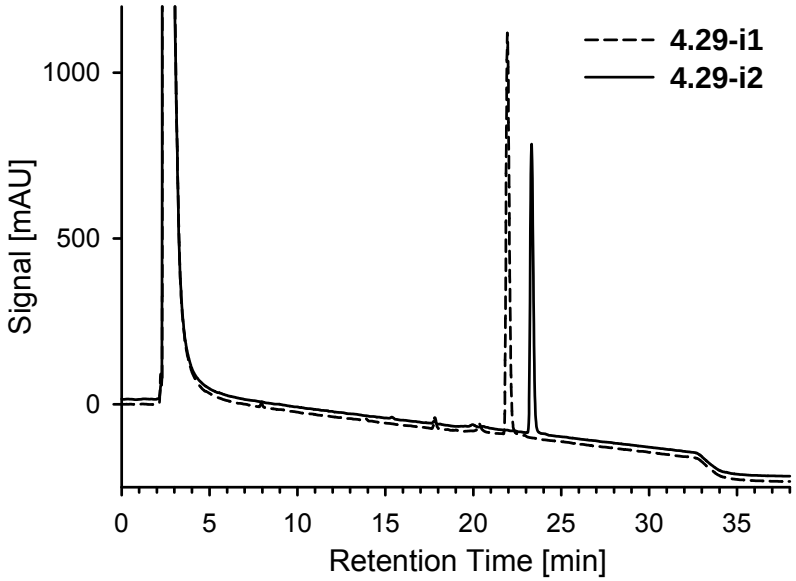
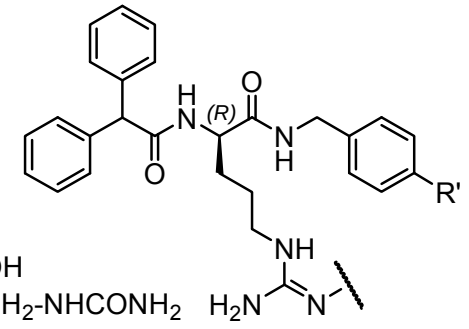


Figure 3. HPLC purity control of the fluorescent Y_1R ligands **4.29-i1** and **4.29-i2**. Conditions: column: Nucleodur 100-5 C18 (250 × 4 mm, 5 µm), eluent: mixtures of acetonitrile (A) and 0.05 % aq. TFA (B), gradient: 0 to 30 min: A/B 20/80 to 75/25, 30 to 31 min: 75/25 to 95/5, 31 to 38 min: 95/5, flow rate: 0.8 mL/min, UV-detection: 210 nm. t_R (**4.29-i1**) = 21.9 min; t_R (**4.29-i2**) = 23.3 min.

4.2.2 Y_1 Receptor Antagonism, Affinity and Selectivity

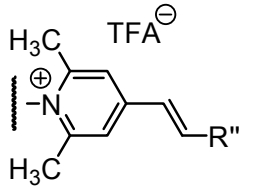
The fluorescent Y_1R antagonists as well as the amino precursors **4.21** and **4.27** were investigated for Y_1R antagonism in a spectrofluorimetric Ca^{2+}-assay (Fura-2 assay) on human erythroleukemia (HEL) cells[32] and in a radioligand binding assay on SK-N-MC neuroblastoma cells using [^{3}H]-UR-MK114 (cf. chapter 2) as radioligand (Table 1). Y_1R binding of the DE99 labeled ligand **4.30** and the Py-1 labeled antagonist **4.34** was additionally determined on MCF-7-Y_1 breast cancer cells. All fluorescent ligands were able to antagonize the effect of the agonist pNPY on HEL cells in the lower and middle nanomolar range except the S0436 labeled ligand (**4.29**) with an IC_{50}-value of about 1 µM and a corresponding K_b value of about 150 nM for both isomers (Table 1). Radioligand competition experiments on SK-N-MC cells revealed that most of the fluorescent Y_1R antagonists are potent Y_1R ligands with dissociation binding constants in the two-digit nanomolar range (Table 1). The lowest affinity showed the Dy-635 labeled ligand **4.28** with a K_i value of 570 nM. Compound **4.34** proved to be the most potent ligand on SK-M-MC cells (K_i = 13 nM), but binds on MCF-7-Y_1 cells with the same affinity as fluorescent ligand **4.30** (K_i = 52 nM and 57 nM, resp.). The lower affinity (factor 2 – 3) at Y_1 receptors expressed on MCF-7 cells over Y_1 receptors on SK-N-MC cells is a general phenomenon which was also observed for the radioligand [^{3}H]-UR-MK114 (K_D = 1.2 nM and 2.9 nM, resp.), for potential PET ligands described in chapter 5 as well as for a large number of bivalent Y_1R antagonists (chapter 3).

Table 1. Structures, Y_1 receptor antagonism and binding data of BIBP 3226, BIBO 3304 (**6.6**), amine precursors **4.21**, **4.27** and fluorescent ligands **4.28** - **4.47**.

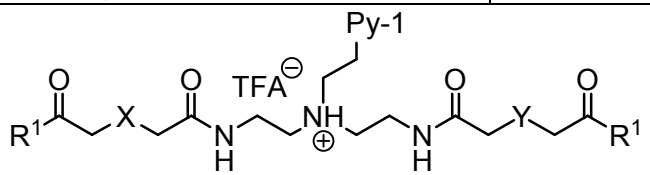


= R^1 if R' = OH
= R^2 if R' = CH_2-$NHCONH_2$

Py-1/5/6* =



R": depends on type of the Py-dye (cf. Figure 1)

Compd.	Structure	IC_{50} (K_b) [nM][a]		K_i[b] (K_i[c]) [nM]
BIBP 3226	R^1-H	10±1	(1.5)	1.3±0.2
6.6	R^2-H (BIBO 3304)	4.6±1	(0.7)	0.25±0.01
4.21	R^1-CO-$(CH_2)_5$-NH_2	620±130	(95)	19±5
4.27	R^1-CONH-$(CH_2)_4$-NH_2	110±3	(16)	4.3±0.5
4.27-N$^{\delta}$i	confer Scheme 4	3300±790	(510)	38±3
4.28	R^1-CO-$(CH_2)_5$-NH-Dy-635	110±11	(18)	570±270
4.29-i1	R^1-S0436	950±300	(150)	144±56
4.29-i2	R^1-S0436	990±270	(153)	40±7
4.30	R^1-CO-$(CH_2)_3$-CONH-$(CH_2)_2$-NH-DE99	16±1	(2.4)	42±5 (57±15)
4.31	R^1-CONH-$(CH_2)_4$-NH-DE99	4.6±1	(0.7)	26±2
4.32	R^2-CO-$(CH_2)_5$-Py-1	79±2	(12)	23±3
4.33	R^1-CO-$(CH_2$-CH_2-$O)_3$-CH_2-CH_2-Py-1	27±2	(4.1)	97±6
4.34	R^1-CO-CH_2-$(O$-CH_2-$CH_2)_2$-Py-1	170±35	(26)	13±4 (52±7)
4.35	R^1-CO-CH_2-$(O$-CH_2-$CH_2)_2$-Py-5	74±5	(11)	24±3
4.36	R^1-CO-$(CH_2)_5$-Py-1	7.0±2	(1.1)	30±2
4.37	R^1-CO-$(CH_2)_5$-Py-5	24±4	(3,7)	50±4
4.38	R^1-CO-$(CH_2)_3$-CONH-$(CH_2)_2$-Py-1	39±2	(6.0)	22±5
4.39	R^1-CO-$(CH_2)_3$-CONH-$(CH_2)_3$-Py-1	73±3	(11)	38±2
4.40	R^1-CO-$(CH_2)_3$-CONH-$(CH_2$-CH_2-$O)_2$-$(CH_2)_2$-Py-6	44±8	(6.8)	96±16
4.41	R^1-CO-$(CH_2)_4$-CONH-$(CH_2$-CH_2-$O)_2$-$(CH_2)_2$-Py-6	52±6	(8.0)	130±40
4.42	R^1-CO-$(CH_2)_6$-CONH-$(CH_2$-CH_2-$O)_2$-$(CH_2)_2$-Py-6	50±17	(7.8)	130±27
4.43	R^1-CONH-$(CH_2)_4$-Py-1	47±8	(7.2)	140±10
4.44	R^1-CONH-$(CH_2)_4$-Py-5	49±4	(7.6)	150±15
				
4.45	X = Y = CH_2-CH_2	7.7±1	(1.2)	61±8
4.46	X = Y = CH_2-CH_2-CH_2-CH_2	3.6±1	(0.6)	100±10
4.47	X = CH_2-CH_2-CONH-CH_2-CH_2 Y = CH_2-CH_2-NHCO-CH_2-CH_2	6.5±2	(1.0)	56±4

The fluorescent dyes Dy-635, S0436 and DE99 are bound via the carboxylic groups. For structures of dyes see Figure 1. [a]Inhibition of 10 nM pNPY induced $[Ca^{2+}]_i$ mobilization in HEL cells; mean values ± SEM from two independent experiments. [b,c]Dissociation constant determined from the displacement of [^{3}H]-UR-MK114 (SK-N-MC cells: K_D = 1.2 nM, MCF-7-Y_1 cells: K_D = 2.9 nM) on [b]SK-N-MC and [c]MCF-7-Y_1 cells; mean values ± SEM from two independent experiments performed in triplicate. *In this Table "Py" designates the pyridinium adducts of the pyrylium dyes.

Interestingly, the BIBO 3304 derivative **4.32** and the BIBP 3326 derivative **4.36**, differing only in their pharmacophoric moiety, exhibited nearly identical affinities (K_i = 23 nM and 30 nM, resp., Table 1) even though, comparing the parent compounds, BIBO 3304 is superior to BIBP 3226 by a factor of about five. As described in chapter 6, the potential radioligand N^G-propionyl-BIBO 3304 (**6.7**) (K_i = 7.8 nM, cf. chapter 6) has even lower Y_1R affinity than [^{3}H]-N^ω-propionyl-BIBP (**2.8b**) (K_D = 1.2 nM, cf. chapter 2). Therefore, BIBO 3304 seems to interact with the Y_1 receptor in a manner that N^ω-substitution is less tolerated compared to BIBP 3226.

The amino precursor **4.27**, the carbamoyl analog of precursor **4.21**, is more potent than **4.21** by a factor of about four (K_i = 4.3 nM and 19 nM, resp.). The same was found for N^ω-ethylaminocarbonyl-BIBP 3226[16] which has about four times higher Y_1R affinity than the corresponding alkanoyl analog, N^ω-butanoyl-BIBP 3226 (**6.2**, cf. chapter 6) (K_i values: 0.17 nM and 0.74 nM, resp.). However, this does not allow the conclusion that N^ω-carbamoylation of BIBP 3226 is generally superior to N^ω-acylation. For instance, the fluorescent ligands **4.43** and **4.44** (K_i = 140 nM and 150 nM, Table 1) have markedly lower affinity than compound **4.36** (K_i = 30 nM), the “carba analogue” of **4.43**. Therefore it is possible that the presence of the carbamoyl NH-group implicates a different orientation of the N^ω-substituent which is less compatible with the attachment of bulky entities such as fluorophores.

The K_i and K_b values from binding and functional assays, respectively, are not identical. There are diverse possible reasons for these discrepancies. First, the Fura-2 Ca^{2+}-assay is performed in a time window (a few minutes) in which the antagonists, exhibiting a high on- and off-rate (compare association and dissociation kinetics of [^{3}H]-UR-MK114 (**2.8b**) in chapter 2), can nearly reach equilibrium conditions, whereas the agonist pNPY is characterized by slow binding kinetics.[33] Moreover, also the structure of the compounds seems to play a role: The K_b value of most BIBP 3226 derived fluorescent ligands is significantly lower than the K_i value, but a good correlation between K_b and K_i value was found e.g. for BIBP 3226, BIBO 3304 and the BIBO 3304 derived ligand **4.32**. In case of amine precursors **4.21**, **4.27** and **4.27-N$^\delta$i** the K_b value was even higher than the K_i value.

The Y_1 receptor selectivity was exemplary confirmed for compound **4.36** and **4.38** in flow cytometric binding studies using Cy5, Dy-635 and S0486 labeled peptides (Cy5-pNPY, Dy-635-pNPY, S0586-[K^4]hPP, Cy5-[K^4]hPP) and cells expressing human Y_1, Y_2, Y_4 and Y_5 receptors (Table 2). It is not possible to determine selectivity data for the Dy-635, S0436 as well as DE99 labeled ligands using these assays due to the excitability of Dy-635, S0436 and DE99 with the red diode laser (635 nm). For these compounds radiolabeled pNPY or PP analogs have to be used.

Table 2. NPY receptor subtype selectivity of the fluorescent ligands **4.36** and **4.38**.

Comp.	Y_1 K_i [nM][a]	Y_1 K_i [nM][b]	Y_2 K_i [nM][c]	Y_4 K_i [nM][d]	Y_5 K_i [nM][c]
4.36	30	7	> 1,500	> 500	> 1,000
4.38	22	--	> 3,000	> 2,000	> 5,000

[a]K_i values from radioligand competition assay with [^{3}H]-UR-MK114 (c = 1.5 nM) on SK-N-MC neuroblastoma cells. [b]Flow cytometric binding assay on HEL using Cy5-pNPY as labelled ligand. [c]Flow cytometric binding assay on CHO-Y_2 and HEC-1B-Y_5 cells using Dy-635-pNPY or Cy5-pNPY as labeled ligands (10 nM). [d]Flow cytometric binding assay on CHO-Y_4 cells with S0586-[K^4]-hPP (10 nM for **4.36**) or Cy5-[K^4]-hPP (5 nM for **4.38**) as fluorescent ligand.

4.2.3 Fluorescence Properties of the Fluorescently Labeled Y_1R Antagonists

The fluorescence properties of the labeled Y_1R antagonists are summarized in Table 3. The fluorescence quantum yields were determined (reference: cresyl violet perchlorate) 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. Additionally, the quantum yield of most compounds was also determined in ethanol to examine the influence of the polarity of the solvent.

Table 3. Spectroscopic properties of the fluorescent Y_1R antagonists **4.28** - **4.47**: Influence of the polarity of the solvent (PBS pH 7 vs. ethanol) and protein (BSA) on the quantum yield Φ (reference: cresyl violet perchlorate) as well as excitation/emission maxima.

		PBS		PBS + 1 % BSA		EtOH	
Compd.	Dye	λ_{ex} / λ_{em}	Φ [%]	λ_{ex} / λ_{em}	Φ [%]	λ_{ex} / λ_{em}	Φ [%]
4.28	Dy-635	505,643 / 668	0.4	505,659 / 673	36	--	--
4.29-i1	S0436	644 / 662	2.1	667 / 676	59	--	--
4.29-i2	S0436	646 / 663	7.5	608 / 676	62	--	--
4.30	DE99	583 / 704	1.8	600 / 680	25	595 / 706	6.6
4.31	DE99	580 / 705	1.1	608 / 676	29	595 / 705	6.9
4.32	Py-1	509 / 646	0.6	527 / 614	21	--	--
4.33	Py-1	508 / 640	1.1	525 / 613	45	521 / 633	2.5
4.34	Py-1	506 / 640	1.4	518 / 610	50	519 / 633	2.3
4.35	Py-5	470 / 708	4.6	488 / 643	38	509 / 708	20
4.36	Py-1	508 / 647	0.7	540 / 614	56	--	--
4.37	Py-5	458 / 709	2.0	488 / 644	34	504 / 706	21
4.38	Py-1	514 / 643	1.6	524 / 610	48	520 / 633	2.4
4.39	Py-1	505 / 642	0.8	526 / 612	49	522 / 632	2.1
4.40	Py-6	533 / 607	7.6	543 / 596	48	541 / 609	23
4.41	Py-6	534 / 607	7.7	541 / 596	47	542 / 609	21
4.42	Py-6	534 / 607	4.9	542 / 596	48	540 / 609	23
4.43	Py-1	504 / 640	0.5	517 / 604	52	517 /630	2.4
4.44	Py-5	456 / 707	2.4	489 / 643	34	497 / 705	24
4.45	Py-1	545 / 644	0.7	527 / 612	37	525 / 634	2.8
4.46	Py-1	540 / 644	0.6	526 / 610	23	525 / 635	2.7
4.47	Py-1	543 / 642	0.5	526 / 611	50	524 / 633	3.0

Generally, the pharmacophoric entity (BIBP 3226 or BIBO 3304) and the chemical structure of the linkers, connecting pharmacophore and fluorophore, have almost no effect on the fluorescence properties. Only the bivalent ligands show a significant bathochromic shift of the excitation maximum in PBS (≈ 543 nm) compared to the monovalent fluorescent ligands labeled with Py-1 (λ_{ex} ≈ 507 nm).

All fluorescent ligands hold the highest quantum yield in PBS with 1 % BSA (up to ≈ 60 % for S0436 and ≈ 50 % quantum yield for Py-1 and Py-6 labeled compounds) and a very low quantum yield in pure PBS (< 8 %). In some cases the quantum yield increased by a factor higher than 50 after addition of BSA. Reasons for this phenomenon have to be intermolecular interactions, particularly hydrophobic and electrostatic interactions, of the fluorophores with the protein. Moreover, binding of the fluorescent ligands to proteins can be regarded as a kind of rigidization, which generally leads to an increase in quantum yield. Therefore, when BSA free buffers are used for binding assays with these fluorescent ligands, the fluorescence could increase in the receptor bound state. However, an increase in fluorescence intensity could also result form non-specific interactions of the ligand with other proteins or with the cell membrane. It is noticeable that the high quantum yields in ethanol are quite high (≈ 22 %) for the Py-5 and Py-6 labeled ligands in relation to the quantum yields of these compounds in PBS with 1 % BSA (Table 3) and compared to the compounds labeled with other fluorescent dyes. Obviously, ligands labeled with Py-5 or Py-6 are more sensitive to hydrophobic interactions than those labeled with Py-1 or DE99 (quantum yield in ethanol < 7 %). The two isomers of the fluorescent ligand **4.29** (cf. 4.2.1) do not differ significantly in their fluorescence properties.

The excitation and corrected emission spectra of various fluorescence labeled antagonists in PBS containing 1 % of BSA are depicted in Figure 4. The largest Stoke's shift show the Py-5 labeled compounds followed by Py-1 and DE99 labeled ligands. S0436, Dy-635 and DE99 labeled fluorescent ligands can be excited with the red diode laser at 635 nm. DE99 is additionally excitable at 488 nm (argon laser), but with lower efficiency. Py-1, Py-5 and Py-6 labeled compounds can be excited at 488 nm (highest efficiency for Py-5).
Thus, the fluorescence properties of all synthesized fluorescent ligands enable an application to flow cytometric equilibrium binding studies and confocal microscopy.

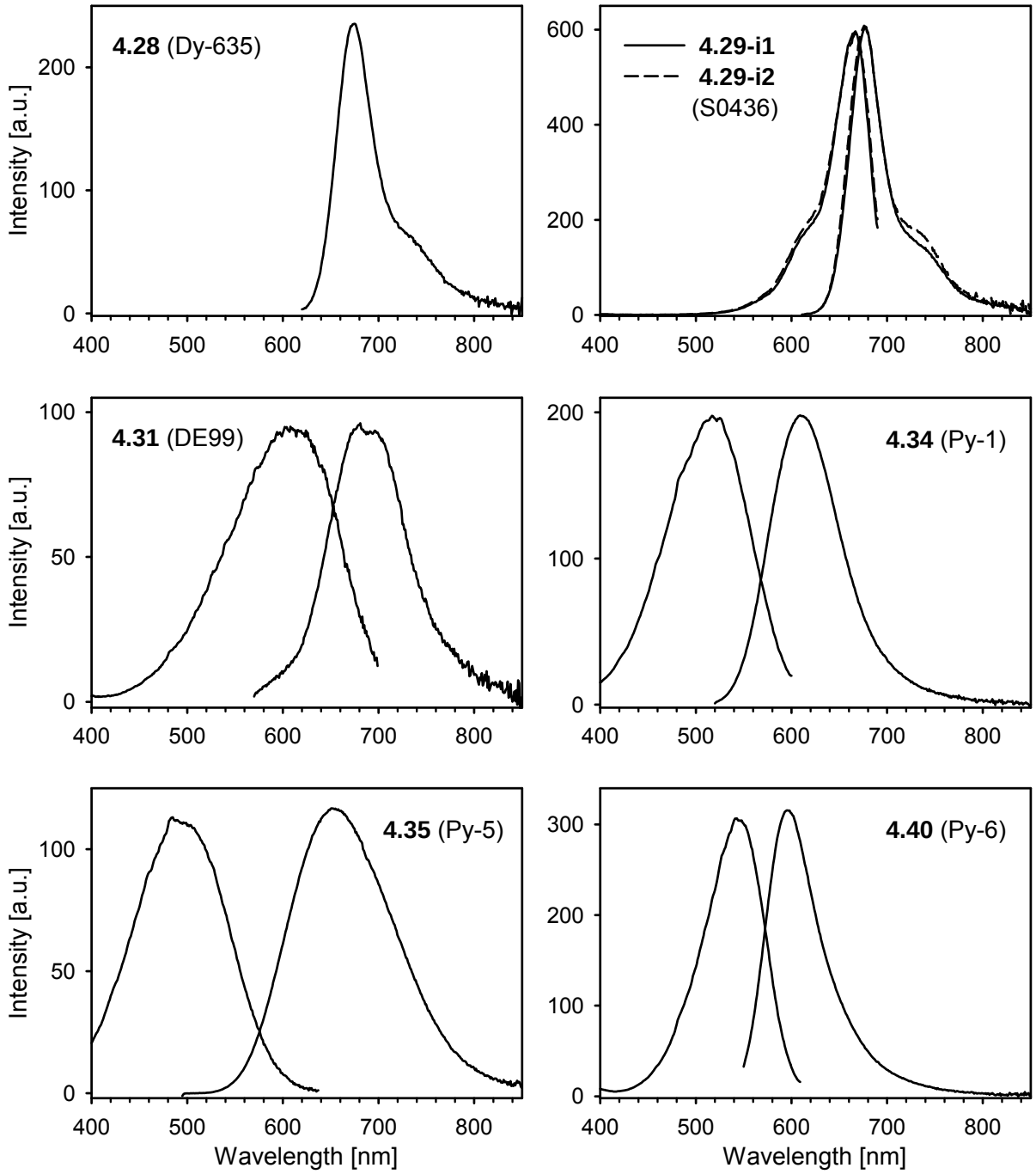


Figure 4. Excitation and corrected emission spectra in PBS + 1 % BSA of a Dy-635, S0436, DE99, Py-1, Py-5 and Py-6 labeled Y_1R antagonist (recorded at 22 °C).

4.2.4 Application of the Fluorescent Y_1R Antagonists to Confocal Microscopy and Flow Cytometry

For binding studies with confocal microscopy and flow cytometry only fluorescent Y_1R antagonists with K_i values of 50 nM and lower (SK-N-MC cells) were used. In view of the potential diagnostic value of fluorescent GPCR ligands in microscopy, steroid hormone-sensitive MCF-7 breast cancer cells were selected as a model, since these cells were reported to express the Y_1R.[34, 35] As a MCF-7 subclone showing higher Y_1 receptor expression than the wild-type MCF-7 cells (ATCC number HTB 22) was recently established in our laboratory, this cell line (designated MCF-7-Y_1) was used for confocal microscopy experiments. As shown in Figures 5 and 6**,** a clear difference between total and non-specific binding was obtained for the Py-1 labeled Y_1R antagonists **4.36**, **4.38**, **4.34**, **4.32**, **4.45** and **4.47** as well as for the Py-5 labeled ligand **4.35** and the DE99 labeled compounds **4.30** and **4.31**. Non-specific binding was determined in the presence of the non-fluorescent Y_1R antagonist BIBP 3226. The amount of specifically bound fluorescent ligand varies between low (**4.32**, Fig. 5, panel D) to very high (**4.34**, Fig. 5, panel C; **4.47**, Fig. 6, panel I). In principle, this could be due to differing binding constants on Y_1R expressed on MCF-7-Y_1 cells (only determined for **4.30** and **4.34**, Table 1) or fluctuations in the receptor density. Generally, the monovalent ligands were significantly enriched intracellularly after incubation periods longer than 15 min, as exemplary shown in Figure 6 (panel F) for the DE99 labeled Y_1R antagonist **4.30**. Penetration into the cell nuclei (apparent as dark areas) was not observed. By contrast, the bivalent fluorescent ligands **4.45** and **4.47** were not detected inside the cells under the same conditions, as becomes obvious from Figure 6 (panels H and I) (images acquired after an incubation period of 23 min).

Binding experiments with the S0436 labeled ligand **4.29-i2** (isomer with the higher affinity, cf. Table 1) revealed no clear difference between total and non-specific binding (Figure 7), presumably due low affinity for Y_1 receptors expressed on MCF-7-Y_1 cells (not determined) or to unusually high non-specific binding.

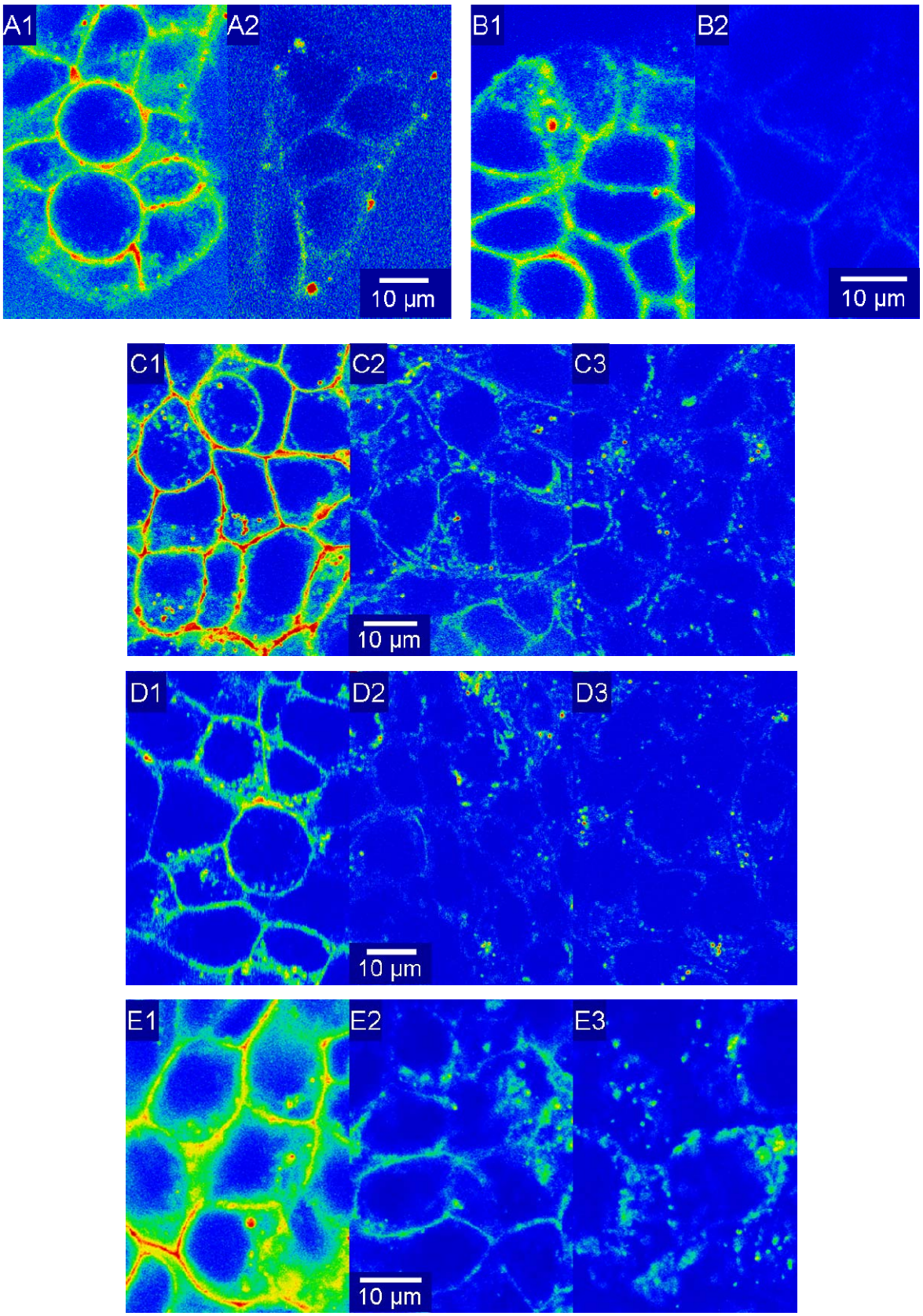


Figure 5. Binding of the fluorescent ligands **4.36** (panel A), **4.38** (panel B), **4.34** (panel C), **4.32** (panel D) and **4.35** (panel E) to Y_1R constitutively expressed in the cell membrane of MCF-7-Y_1 tumor cells, visualized by confocal microscopy. 1 designates total binding, 2 non-specific binding (in the presence of BIBP 3226 at 70-160 fold higher concentrations than the fluorescent ligands), 3 autofluorescence. A: **4.36** (60 nM, 6 min), B: **4.38** (40 nM, 9 min), C: **4.34** (60 nM, 6 min), D: **4.32** (60 nM, 9 min), E: **4.35** (30 nM, 15 min). Cells were incubated with the fluorescent ligands at room temperature in Leibowitz L15 culture medium. All images were acquired with a Zeiss Axiovert 200 M microscope.

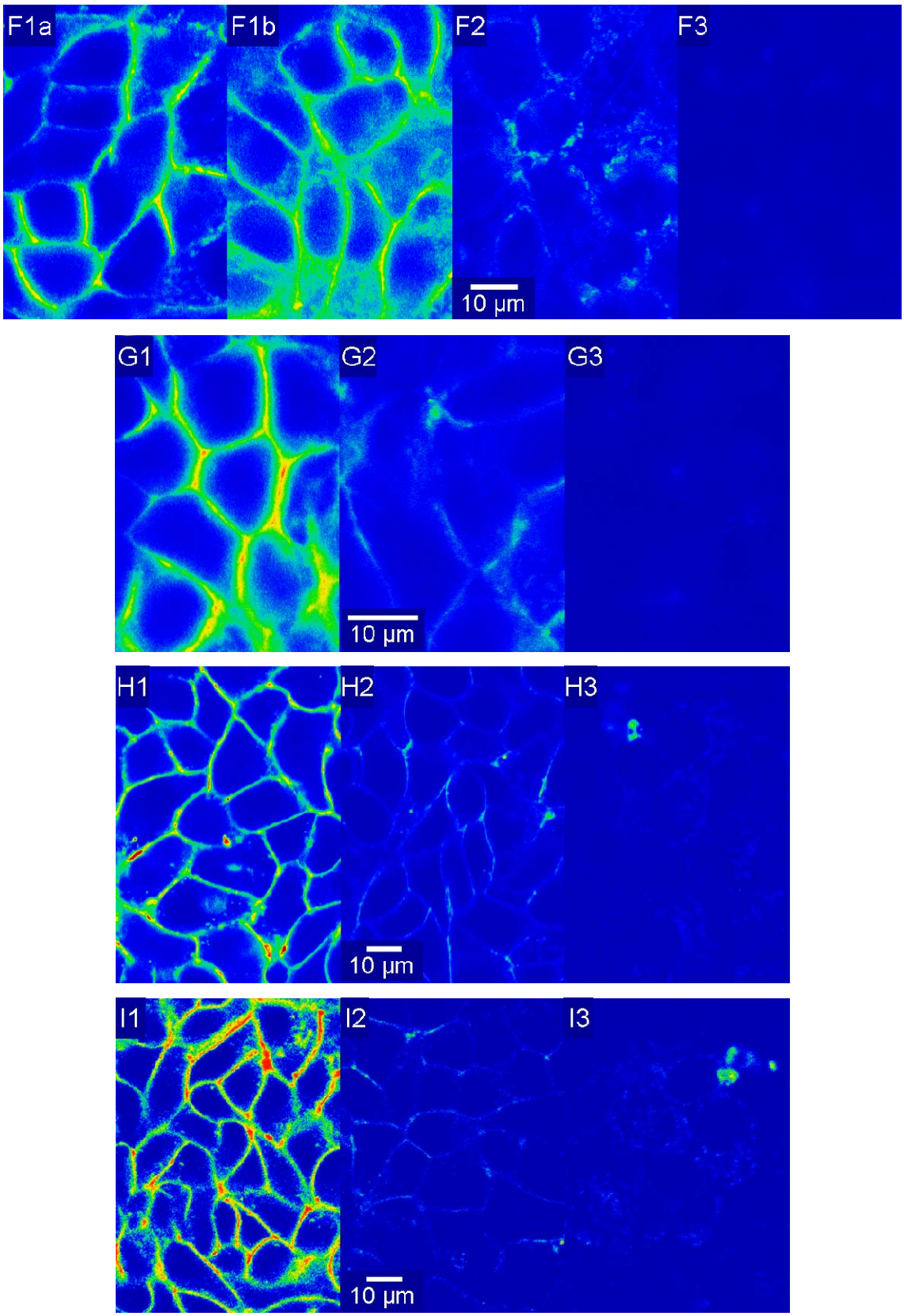


Figure 6. Binding of the fluorescent ligands **4.30** (panel F), **4.31** (panel G), **4.45** (panel H) and **4.47** (panel I) to Y_1R constitutively expressed in the cell membrane of MCF-7-Y_1 tumor cancer cells, visualized by confocal microscopy. 1 designates total binding, 2 non-specific binding (in the presence of BIBP 3226, 70-125 fold concentrated), 3 autofluorescence. F: **4.30** (70 nM, 1a: 10 min, 1b: 20 min), G: **4.31** (70 nM, 7 min), H: **4.45** (80 nM, 23 min), I: **4.47** (80 nM, 23 min). Cells were incubated with the fluorescent ligands at room temperature in Leibowitz L15 culture medium. All images were acquired with a Zeiss Axiovert 200 M microscope.

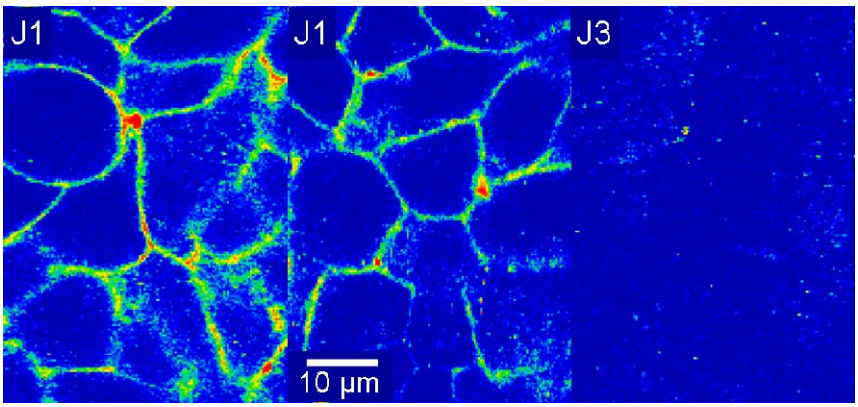


Figure 7. Binding of the fluorescent ligand **4.29-i2** to Y_1R expressing MCF-7-Y_1 tumor cells, visualized by confocal microscopy. J1: total binding (60 nM, 7 min), J2: non-specific binding (60 nM, 9 min) in the presence of BIBP 3226 (5 µM), J3: autofluorescence. All images were acquired with a Zeiss Axiovert 200 M microscope after 7-9 min of incubation time at room temperature in Leibowitz L15 culture medium.

With respect to the application of the fluorescent ligands in flow cytometric binding studies HEL cells, which are constitutively expressing Y_1 receptors and can be easily grown in suspension culture, as well as MCF-7-Y_1 cells were used. Displacement curves of the Py-1 labeled Y_1R antagonist **4.36** with BIBP 3226 are shown in Figure 8. From the data recorded in the fluorescence channels Fl-2 (585 ± 21 nm, Figure 8A) and Fl-3 (> 670 nm, Figure 8B) K_i values of 3.5 nM and 4.6 nM were calculated. Thus, in principle both fluorescence channels are suited for binding studies with Y_1R antagonist **4.36**. The apparent K_i value of compound **4.36** (K_i = 7 nM, Table 2) from the flow cytometric selectivity assay (Table 2) was used for the calculation of the K_i values for the model compound BIBP 3226.

As shown in Figure 9A, flow cytometric saturation binding experiments with fluorescent ligand **4.31** using MCF-7-Y_1 breast cancer cells yielded saturation curves with low non-specific binding and a K_D value of 5.3 ± 0.7 nM (mean value ± SEM, two independent experiments performed in duplicate). Displacement of the fluorescent ligand **4.31** with BIBP 3226 yielded a K_i value of 2.2 nM calculated with the determined K_D value of 5.3 nM.

These data determined for the model compound BIBP 3226 (K_i = 3.5 nM and 4.6 nM, resp., as well as 2.2 nM) are in good accordance with the value (K_i = 1.3 nM) determined with [^{3}H]-UR-MK114 (chapter 2) as well as with previously reported K_i values of 7 nM and 5.1 nM, determined in radioligand competition studies.[15, 36]

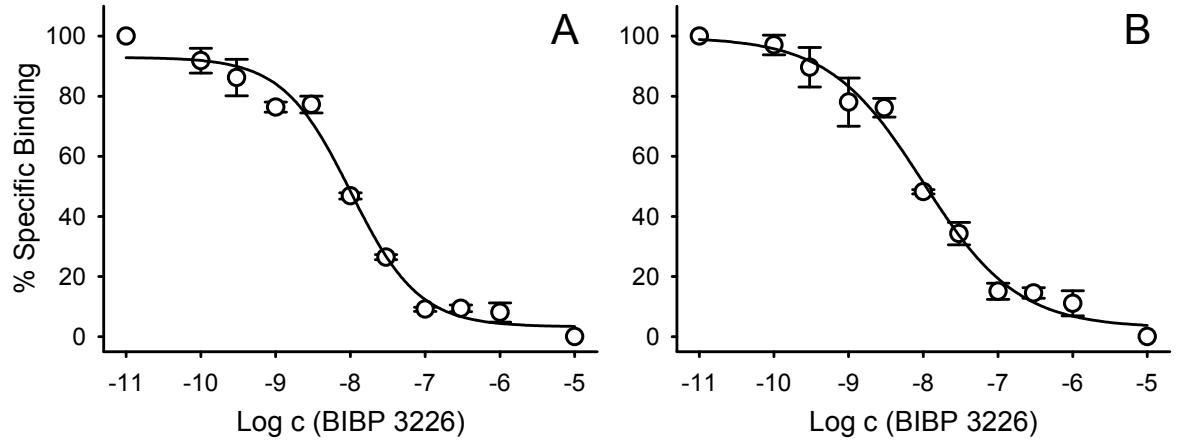


Figure 8. Flow cytometric Y_1R binding assay with antagonist **4.36** at human erythroleukemia cells (HEL cells). The geometrical mean values of fluorescence intensities obtained from the competition assay were converted to percentage inhibition according to the procedure described in detail previously.[2, 3] Displacement of **4.36** (10 nM) by increasing concentrations of BIBP 3226 was registered in fluorescence channels Fl-2 (585 ± 21 nm, Figure 4A) and Fl-3 (> 670 nm, Figure 4B), respectively. Samples were incubated at room temperature for 15 min. Calculated K_i values: 3.5 nM (A) and 4.6 nM (B). (mean values ± SEM, n = 3)

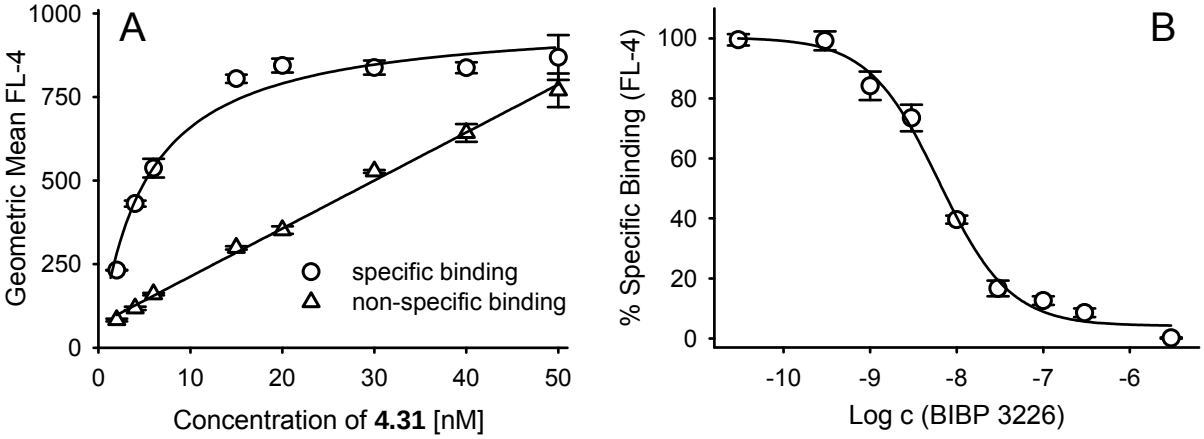


Figure 9. A: Flow cytometric saturation binding experiment with fluorescent ligand **4.31** at human MCF-7-Y_1 breast cancer cellls. Unspecific binding was determined in the presence of BIBP 3226 (500-fold excess). Determined K_D value: 5.0 ± 0.6 nM. B: Flow cytometric Y_1R competition assay with **4.31** (10 nM) and BIBP 3226 using MCF-7-Y_1 cells. The geometrical mean values of fluorescence intensities (fluorescence channel Fl-4) obtained from the competition assay were converted to percentage inhibition values according to the procedure described in detail previously.[2, 3] Samples were incubated for 15 min at room temperature. Calculated K_i value: 2.2 nM. (mean values ± SEM, A: n = 2, B: n = 3)

4.2.5 Summary and Conclusion

The design of fluorescent Y_1R ligands was based on the application of the guanidine-acylguanidine bioisosteric approach to the argininamide-type Y_1R antagonists BIBP 3226 and BIBO 3304. The guanidine group was linked to fluorophores via ω-aminoacyl spacers of different lengths and chemical nature. Such derivatives proved to be potent and selective fluorescence labeled Y_1R antagonists, although - depending on the fluorescent dye - a more or less pronounced decrease in activity compared to the parent argininamide was noticed.

The low molecular weight pyrylium dyes, the smallest red-fluorescent fluorophores known, turned out to be well suited for the fluorescence labeling of Y_1R antagonists. In some cases the decrease in affinity compared to the parent compound BIBP 3226 was less than a factor of 10 - 20 (**4.31**, **4.34**, **4.35** and **4.38**, Table 1) which is a very good result for fluorescence labeling of a small ligand. Functionalization of amine precursors **4.22** and **4.27** with the benzothiazolium derivative DE99 (Figure 1) yielded fluorescent probes (**4.30** and **4.31**) for the Y_1 receptor with favorable characteristics comparable to those of the pyrylium dye labeled compounds. The coupling of the dyes Py-1 and Py-5 (Figure 1) to amine precursor **4.20** (Scheme 6) provided the most potent fluorescent ligands (**4.34** and **4.35**, Table 1). With respect to future work, this result suggests labeling of the precursor **4.20** with DE99, as this dye proved to be superior to Py-1 and Py-5 in terms of retaining the binding affinity (cf. compounds **4.31**, **4.43** and **4.44**, Table 1). The attachment of DE99 to precursor **4.20** will probably result in a high affinity fluorescent ligand with decreased lipophilicity and consequently lower membrane binding and cell penetration.

The majority of the most potent fluorescent Y_1R antagonists (K_i = 10 – 50 nM) was successfully applied in confocal microscopy and flow cytometric equilibrium binding studies. Such fluorescent antagonists are anticipated to enable more detailed investigations of association kinetics at the NPY Y_1R with - compared to agonists - less interference with receptor desensitization and internalization processes. Therefore, these fluorescent probes pave the way to optical detection of NPY Y_1 receptors on cells, in tissues and organs, as demonstrated on MCF-7-Y_1 breast cancer cells in Figures 5 and 6.

4.3 Experimental Section

4.3.1 General Experimental Conditions

Unless otherwise noted, chemicals and solvents were purchased from commercial suppliers and used without further purification. The glycol-derived spacers 12-amino-4,7,10-trioxa-dodecanoic acid *tert*-butylester and *N*-Boc-8-amino-3,6-dioxa-octanoic acid dicyclohexylamine salt were purchased from Fluka (Sigma-Aldrich Chemie GmbH, Munich, Germany). *D*-Ornithine hydrochloride was obtained from Iris Biotech GmbH (Marktredwitz, Germany). Bovine serum albumin (BSA) was from Serva (Heidelberg, Germany). The tetrafluoroborate salts of the pyrylium dyes Py-1, Py-5 and Py-6 were synthesized in the Institute of Analytical Chemistry, Chemo- and Biosensors at the University of Regensburg[20, 21] (note: these dyes are commercially available from Active Motif Chromeon, www.activemotif.com). The activated (NHS ester) fluorescent dyes S0536 (S0436-NHS) and Dy-635-NHS were obtained from FEW Chemicals (Bitterfeld-Wolfen, Germany) and Dyomics (Jena, Germany), respectively. Dy-635-pNPY and Cy5-pNPY were synthesized as described previously.[3] [K^4]-hPP was synthesized in the laboratory of Prof. Beck-Sickinger (University of Leipzig, Germany) and labeled with the cyanine dyes Cy5 and S0586 (FEW chemicals (Bitterfeld-Wolfen, Germany) in our laboratory.[37] Porcine NPY (pNPY) was prepared in-house in the laboratory of Dr. C. Cabrele. The fura-2 AM (Calbiochem/Merck Biosciences, Beeston, UK) stock solution (1 mM) was prepared in DMSO. Pluronic F-127 (Calbiochem/Merck Biosciences, Beeston, UK) was dissolved in DMSO to obtain a concentration of 20 %.

Millipore water was used throughout for the preparation of buffers and HPLC eluents. Petroleum ether (40-60 °C) was distilled before use. DMF was stored over a molecular sieve (3 Å). Anhydrous reactions were run under an atmosphere of dry nitrogen or argon.

Thin layer chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates. For column chromatography silica gel Geduran 60 (Merck, 0.063-0.200 mm) was used. NMR spectra were recorded on a Bruker Avance 300 (1H: 300 MHz) and a Bruker Avance 600 (1H: 600 MHz, 13C: 150.9 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. Mass spectrometry analysis (MS) was performed in-house on a Finnigan ThermoQuest TSQ 7000 (ES-MS) and a Finnigan SSQ 710A (EI-MS 70 eV, CI-MS). Lyophilization was done with a Christ alpha 2-4 LD equipped with a vacuubrand RZ 6 rotary vane vacuum pump.

Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18 (20 × 21 mm, 5 µm; Macherey-Nagel, Germany) and a Eurospher-100 C18 (250 × 32 mm, 5 µm; Knauer, Germany) served as RP-columns at flow rates of 24 and 38 mL/min, respectively. Mixtures of acetonitrile and 0.1 % aq. TFA were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure (final pressure: 60 mbar) at 40 °C prior to lyophilization. Analytical

HPLC analysis was performed on a system from Thermo Separation Products (composed of a SN400 controller, a P4000 pump, a degasser (Degassex DG-4400, phenomenex), an AS3000 autosampler and a Spectra Focus UV-VIS detector). An Eurospher-100 C18 (250 × 4 mm, 5 µm, Knauer, Germany) or a Nucleodur 100-5 C18 ec (250 × 4 mm, 5 µm, Macherey-Nagel, Germany) served as RP-columns. Mixtures of acetonitrile (A) and 0.05 % aq. TFA (B) were used as mobile phase. Helium degassing, an oven temperature of 30 °C and a flow rate of 0.8 mL/min were used throughout. Solutions for injection (concentrations in the two-digit µM range) were prepared in a mixture of A and B corresponding to the mixture at the beginning of the gradient. The following gradients were applied for analytical HPLC analysis:

Gradient 1: 0 to 30 min: A/B 20/80 to 75/25, 30 to 31 min: 75/25 to 95/5, 31 to 35 min: 95/5

Gradient 2: 0 to 30 min: A/B 20/80 to 60/40, 30 to 32 min: 60/40 to 95/5, 32 to 36 min: 95/5

Gradient 3: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5

The preparation of the buffer and the lysis solution for Y_1R binding studies on SK-N-MC cells and MCF-7 cells as well as the loading buffer for the determination of the mobilization of intracellular Ca^{2+} in HEL cells are described in chapter 3.

4.3.2 Chemistry: Experimental Protocols and Analytical Data

(*R*)-N^{δ}-Benzyloxycarbonyl-N^{α}-(*tert*-butoxycarbonyl)ornithine (4.1).[38] Compound **2.1** (22 g, 82.6 mmol, 1 eq; cf. chapter 2) was dissolved/suspended in an aqueous solution (250 mL) of potassium carbonate (12.56 g, 90.9 mmol, 1.1 eq) and 1,4-dioxane (100 mL) was added. Di-*tert*-butyl dicarbonate (18.93 g, 86.75 mmol, 1.05 eq) was added dropwise in 1,4-dioxane (200 mL) over a period of 60 min. The mixture was stirred at rt overnight, then concentrated under reduced pressure to a volume of about 150 mL. Water (50 mL) was added and the pH was adjusted to 2-3 by the addition of 1 M aq. hydrochloric acid (about 150 mL). The product was extracted with ethyl acetate (400 mL and 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 sodium sulfate. Filtration and evaporation of the solvent yielded a yellowish oil which turned into a foam that hardened to a solid during drying *in vacuo* (27.8 g, 75.9 mmol, 92 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.38 (s, 9H, *tert*-butyl), 1.4-1.59 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.65 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 2.98 (m, 2H, C$\underline{H}_2$-NH), 3.83 (m, 1H, C$\underline{H}^{\alpha}$), 5.0 (s, 2H, C$\underline{H}_2$-Ph), 7.07 (d, 1H, ^{3}J = 7.98 Hz, CO-N$\underline{H}$-CH), 7.25 (t, 1H, ^{3}J = 5.49 Hz, CO-N$\underline{H}$-CH$_2$), 7.34 (m, 5H, Ph), 12.42 (s, 1H, COO$\underline{H}$); $C_{18}H_{28}N_2O_6$ (366.4)

(*R*)-N^{δ}-Benzyloxycarbonyl-N^{α}-*tert*-butoxycarbonyl-*N*-(4-ureidomethylbenzyl)ornithinamide (4.2). Compound **4.1** (7.93 g, 21.65 mmol, 1 eq) was activated with carbonyldiimidazole (3.86 g, 23.8 mmol, 1.1 eq) in DMF (80 mL) at 0 °C for 60 min. NEt_3 (3.3 g, 32.5 mmol, 1.5 eq), DMAP

(1.3 g, 10.8 mmol, 0.5 eq) and **4.8** (4.67 g, 21.65 mmol, 1 eq) were added in DMF (250 mL) as suspension. The mixture was slowly warmed up to 45 °C yielding an almost clear solution. Warming was finished after 30 min and the mixture was stirred at rt overnight. Glacial acetic acid (2 mL, about 1.5 eq) was added and DMF was removed under reduced pressure at 45 °C yielding an oily residue which was dried *in vacuo*. Purification by column chromatography yielded the product as a yellowish hard solid (9.75 g, 18.48 mmol, 85 %). ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.43 (s, 9H, *tert*-butyl), 1.46-1.66 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.74 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 3.11 (m, 2H, CH$_2$-C$\underline{H}_2$-NH), 4.04 (m, 1H, C$\underline{H}^{\alpha}$), 4.25 (s, 2H, C$\underline{H}_2$-NH-CO-NH$_2$), 4.33 (m, 2H, NH-C$\underline{H}_2$-Ar), 5.04 (s, 2H, C$\underline{H}_2$-Ph), 7.22 (m, 4H, CH$_2$-C$_6\underline{H}_4$-CH$_2$), 7.28-7.35 (m, 5H, Ph); MS (ES, MeOH + 10 mM NH_4OAc): *m/z* 528 $[M + H]^+$; $C_{27}H_{37}N_5O_6$ (527.6)

(*R*)-*N*$^{\delta}$-Benzyloxycarbonyl-*N*-(4-ureidomethyl-benzyl)ornithinamide (4.3). Compound **4.2** (9 g, 17.1 mmol) was dissolved in MeOH (250 mL) and acetyl chloride (30 mL) 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). Lyophilization afforded the product as a white solid (7.9 g, 17.1 mmol, 100 %); Solubility checked for DMF (+), MeOH (+), acetonitrile (-), THF (-), ethyl acetate (-), dimethoxyethane (-) and CH_2Cl_2 (-); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.57 (m, 2H, CH-CH$_2$-C$\underline{H}_2$), 1.72-2.04 (bm, 2H, CH-C$\underline{H}_2$-CH$_2$), 2.98 (m, 1H, C$\underline{H}^{\alpha}$), 3.14 (m, 2H, CH$_2$-C$\underline{H}_2$-NH), 4.34-4.42 (m, 4H, C$\underline{H}_2$-Ar-C$\underline{H}_2$), 5.05 (s, 2H, C$\underline{H}_2$-Ph), 7.26-7.36 (m, 9H, Ph, CH$_2$-C$_6\underline{H}_4$-CH$_2$); MS (ES, MeOH + 10 mM NH_4OAc): *m/z* 428 $[M + H]^+$; $C_{22}H_{29}N_5O_4$ × HCl (464.0)

(*R*)-*N*$^{\delta}$-Benzyloxycarbonyl-*N*$^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-ureidomethylbenzyl)ornithinamide (4.4).[29] Compound **4.3** (7.8 g, 16.8 mmol, 1 eq) was dissolved in anhydrous DMF (100 mL). NEt_3 (2.6 g, 3.5 mL, 25.2 mmol, 1.5 eq), DMAP (1 g, 8.4 mmol, 0.5 eq) and **2.10** (6.2 g, 20.2 mmol, 1.2 eq, cf. chapter 2) were added and the mixture was kept under stirring for 20 h at rt. Glacial acetic acid (3 mL) was added and DMF was removed under reduced pressure at 45 °C yielding a light yellow-brown solid which was dried *in vacuo*. The solid was insoluble or poorly soluble in MeOH, CH_2Cl_2, acetonitrile, dimethoxyethane, THF, 1,4-dioxane, propan-2-ol, ethanol and in mixtures with water of the water-miscible solvents. Therefore the solid material was mechanically crushed to small pellets and suspended in acetonitrile (2500 mL). The solid turned into a white fluffy solid during heating (70 °C) and treatment with ultrasound (30 min). Separation by filtration and intensive washing with acetonitrile (2 × 250 mL) and water (3 × 150 mL) yielded the product as a white solid (8.7 g, 14 mmol, 83 %). Solubility: insoluble or poorly soluble in above listed solvents; ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.37 (m, 2H, CH-CH$_2$-C$\underline{H}_2$), 1.44-1.72 (bm, 2H, CH-C$\underline{H}_2$-CH$_2$), 2.95 (m, 2H, CH$_2$-C$\underline{H}_2$-NH), 4.1-4.26 (m, 4H, C$\underline{H}_2$-Ar-C$\underline{H}_2$), 4.31 (m, 1H, C$\underline{H}^{\alpha}$), 5.0 (s, 2H, C$\underline{H}_2$-Ph), 5.12 (s, 1H, C$\underline{H}$-(Ph)$_2$), 5.52 (bs, 2H, N$\underline{H}_2$), 6.38 (t,

1H, NH-CO), 7.15 (m, 4H, CH_2-C_6H_4-CH_2), 7.18-7.38 (m, 16H, Ph, NH-CO), 8.44 (m, 2H, 2 × NH-CO); MS (ES, acetonitrile/TFA): *m/z* 622 $[M + H]^+$; $C_{36}H_{39}N_5O_5$ (621.7)

(*R*)-N^{α}-(2,2-Diphenylacetyl)-*N*-(4-ureidomethyl-benzyl)ornithinamide (4.5).[29] Compound **4.4** (2.7 g, 4.3 mmol) was suspended in MeOH (300 mL). 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 (430 mg) were added and hydrogen was lead through the vigorously stirred mixture at 45 °C for 2.5 h. At this time the white solid had disappeared. After 3 h the catalyst was removed by filtration, volatiles were removed under reduced pressure and the residue was suspended in water (50 mL) at 40 °C. Lyophilization afforded the product as a white solid (2.1 g, 3.8 mmol, 88 %); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.4-1.63 (m, 3H, CH-CH_2-CH_2), 1.63-1.78 (m, 1H, CH-CH_2-CH_2), 2.76 (m, 2H, CH_2-NH_2), 4.1-4.29 (m, 4H, CH_2-Ar-CH_2), 4.35 (m, 1H, CH^{α}), 5.13 (s, 1H, CH-$(Ph)_2$), 5.54 (bs, 2H, NH_2), 6.45 (t, 1H, NH-CO), 7.15 (m, 4H, CH_2-C_6H_4-CH_2), 7.2-7.35 (m, 10H, Ph), 7.74 (bs, 3H, NH_3^+), 8.51 (t, 1H, NH-CO), 8.58 (d, 1H, CO-NH-CH); MS (ES, acetonitrile/TFA): *m/z* 488 $[M + H]^+$; $C_{28}H_{33}N_5O_3$ × $C_2H_4O_2$ (547.6)

(*R*)-N^{ω}-*tert*-Butoxycarbonyl-N^{α}-(2,2-diphenylacetyl)-*N*-(4-ureidomethylbenzyl)argininamide (4.6). Compound **4.5** (2.05 g, 3.74 mmol, 1 eq) was suspended in MeOH (30 mL). NEt_3 (0.57 g, 0.78 mL, 5.6 mmol, 1.5 eq) and **2.13** (1.55 g, 4.5 mmol, 1.5 eq, cf. chapter 2) were added in CH_2Cl_2 (10 mL) and the mixture was kept under stirring at rt for 20 h. The suspension turned into a cloudy solution during the first 30 min of the reaction. Volatiles were removed under reduced pressure and purification of the intermediate with column chromatography (CH_2Cl_2/MeOH 100/1 to 10/1) yielded a white solid which was dried *in vacuo* and dissolved in MeOH (180 mL). Glacial acetic acid (0.25 mL) and a 10 % Pd/C catalyst (400 mg) were added and hydrogen was lead through the vigorously stirred mixture for 6.5 h. The catalyst was removed by filtration, volatiles were removed under reduced pressure and the residue was suspended in water (60 mL). Lyophilization afforded the product as a white solid (1.8 g, 2.6 mmol, 70 %); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.27-1.45 (m, 11H, CH-CH_2-CH_2, *tert*-butyl), 1.45-1.72 (bm, 2H, CH-CH_2-CH_2), 3.05 (m, 2H, CH_2-CH_2-NH), 4.13 (d, 2H, ^{3}J = 5.99 Hz, CH_2-Ar), 4.23 (d, 2H, ^{3}J = 5.79 Hz, CH_2-Ar), 4.35 (m, 1H, CH^{α}), 5.12 (s, 1H, CH-$(Ph)_2$), 5.52 (s, 2H, NH_2), 6.38 (t, 1H, NH-CH_2), 7.15 (m, 4H, CH_2-C_6H_4-CH_2), 7.2-7.34 (m, 10H, Ph), 8.48 (m, 2H, 2 × NH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 630 $[M + H]^+$; $C_{34}H_{43}N_7O_5$ × $C_2H_4O_2$ (689.7)

***N*-*tert*-Butoxycarbonyl-1,4-bis(aminomethyl)benzene (4.7).**[39] 1,4-Bis(aminomethyl)benzene (30 g, 220.3 mmol, 1 eq) was dissolved/suspended in 1 M aq. NaOH (220 mL, 220.3 mmol, 1 eq). 1,4-dioxane was added (250 mL) and the slolution was cooled in an ice-water bath. Di-*tert*-butyl dicarbonate (48.1 g, 220.3 mmol, 1 eq) was added dropwise in 1,4-dioxane (150 mL) over

a period of 2 h. The ice-water bath was removed and the mixture was allowed to stand at rt overnight. The volume was reduced to about 250 mL under reduced pressure. The white solid was separated by filtration, washed twice with ice-cold water (2 × 100 mL) and dried *in vacuo*. White solid (47.6 g of a mixture of **4.7** and di-Boc-protected 1,4-bis(aminomethyl)benzene (≈ 55:45), 22 g (93.1 mmol, 42 %) related to **4.7**); ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.44 (s, 24H, *tert*-butyl, **4.7** and "di-Boc"), 3.84 (s, 2H, C$\underline{H}_2$-NH_2), 4.19 (s, 3.3H, C$\underline{H}_2$-NH, "di-Boc"), 4.20 (s, 2H, C$\underline{H}_2$-NH, **4.7**), 7.2 (s, 3.3H, C$\underline{H}^{Ar}$, "di-Boc"), 7.29 (m, 4H, C$\underline{H}^{Ar}$, **4.7**); MS (CI, NH_3): *m/z* 254 $[M + NH_4]^+$, 354 ["di-Boc" + $NH_4]^+$; $C_{13}H_{20}N_2O_2$ (236.2)

***N*-(4-Aminomethylbenzyl)urea (4.8).**[29] Compound **4.6** (19.6 g, 83.1 mmol, 1 eq; 42.5 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 (7 g, 86.3 mmol, 1.04 eq) was added and the mixture was refluxed for 75 min. 1 M aq. hydrochloric acid (8 mL) was added to adjust the pH to 7, followed by the addition of 4.67 g potassium cyanate (4.67 g, 57.6 mmol, 0.7 eq). Reflux was continued for 2 h, then the mixture was stirred at rt overnight (final pH: ≈ 8) and 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*. A suspension was prepared in a mixture of MeOH and CH_2Cl_2 1/10 (2000 mL) and subjected to column chromatography (CH_2Cl_2/MeOH 20/1 to 7.5/1). The isolated Boc-protected intermediate (well soluble in MeOH, poorly soluble in water, acetonitrile, ethyl acetate and CH_2Cl_2) 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). Lyophilization afforded the product as a white solid (15.8 g, 73.3 mmol, 88 %). ^{1}H-NMR (300 MHz, MeOH-d_4/D_2O 80/20): δ (ppm) 4.06 (s, 2H, C$\underline{H}_2$-NH_2), 4.26 (s, 2H, C$\underline{H}_2$-NH), 7.34 (m, 4H, CH^{Ar}); MS (CI, NH_3): *m/z* 197 $[M + NH_4]^+$, 180 $[M + H]^+$; $C_{14}H_{21}N_3O_3$ × HCl (215.8)

***N*-*tert*-Butoxycarbonyl-ethane-1,2-diamine (4.9).**[40] Ethane-1,2-diamine (36.1 g, 0.6 mol, 12 eq) was dissolved in chloroform (450 mL) and the solution was cooled to -15 °C. A solution of di-*tert*-butyl dicarbonate (10.9 g, 50 mmol, 1eq) was added dropwise over a period of 4 h. Stirring was continued overnight and the mixture was allowed to slowly warm up to rt. The solution was washed three times with alkalified brine (3 × 130 mL of brine + 5 mL of 1 M aq. NaOH), then with brine (130 mL) and water (100 mL). Drying over sodium sulfate, filtration and removal of the solvent under reduced pressure yielded the product as yellow oil (7.82 g, 48.8 mmol, 97 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.42 (s, 9H, *tert*-Bu), 2.77 (t, 2H, ^3J = 5.91 Hz, CH_2), 3.14 (m, 2H, CH_2); $C_7H_{16}N_2O_2$ (160.2)

4-(2-*tert*-Butoxycarbonylaminoethyl)aminocarbonylbutanoic acid (4.10).[41] Glutaric anhydride (2.28 g, 20 mmol, 1 eq) was added to a solution of amine **4.9** (3.5 g, 22 mmol, 1.1 eq) in CH_2Cl_2 (20 mL) and the mixture was stirred at rt for 60 min. CH_2Cl_2 (150 mL) was added prior to washing with aq. NH_4Cl solution (two times 30 mL of a saturated solution + 10 mL of water) and brine (40 mL). Drying over sodium sulfate, filtration and removal of the solvent under reduced pressure yielded the product as highly viscous yellowish oil (5.4 g, 19.6 mmol, 98 %). ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.43 (s, 9H, *tert*-Bu), 1.88 (p, 2H, ^3J = 7.24 Hz, CH_2-C$\underline{H}_2$-CH_2), 2.24 (t, 2H, ^3J = 7.46 Hz, C$\underline{H}_2$-CO), 2.32 (t, 2H, ^3J = 7.38 Hz, C$\underline{H}_2$-CO), 3.14 (m, 2H, C$\underline{H}_2$-NH), 3.24 (m, 2H, C$\underline{H}_2$-NH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 273 $[M - H]^-$; $C_{12}H_{22}N_2O_5$ (274.3)

4-(3-*tert*-Butoxycarbonylaminopropyl)aminocarbonylbutanoic acid (4.11).[41] Glutaric anhydride (0.31 g, 2.73 mmol, 1 eq) in CH_2Cl_2 (5 mL) was added dropwise to a solution of *N*-Boc-propane-1,3-diamine (0.5 g, 2.87 mmol, 1.05 eq) in CH_2Cl_2 (1.5 mL) over a period of 5 min. The mixture was stirred at rt for 60 min. Chloroform (40 mL) was added prior to washing with aq. NH_4Cl solution (two times 8 mL of a saturated solution + 2 mL of water) and brine (10 mL). Drying over sodium sulfate, filtration and removal of the solvent under reduced pressure yielded the product as highly viscous yellowish oil (0.8 g, 2.78 mmol, 97 %). ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.43 (s, 9H, *tert*-Bu), 1.63 (p, 2H, ^3J = 6.79 Hz, NH-CH_2-C$\underline{H}_2$-CH_2-NH), 1.88 (p, 2H, ^3J = 7.22 Hz, CO-CH_2-C$\underline{H}_2$-CH_2-CO), 2.24 (t, 2H, ^3J = 7.47 Hz, CH_2-CO), 2.32 (t, 2H, ^3J = 7.37 Hz, CH_2-CO), 3.06 (t, 2H, ^3J = 6.78 Hz, C$\underline{H}_2$-NH), 3.19 (t, 2H, ^3J = 6.87 Hz, C$\underline{H}_2$-NH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 287 $[M - H]^-$; $C_{13}H_{24}N_2O_5$ (288.3)

***N*-*tert*-Butoxycarbonyl-*N'*-[*N*-(4-*tert*-butoxycarbonylaminobutyl)aminocarbonyl]-*S*-methylisothiourea (4.13).** The reaction was carried out in an argon purged 50 mL two-necked round bottom flask equipped with a pressure equalizing addition funnel. The flask and the funnel were baked out in a nitrogen atmosphere prior to the reaction. A solution of *N*-Boc-butane-1,4-diamine (250 mg, 1.33 mmol, 1 eq) and diisopropylethylamine (480 mg, 3.7 mmol, 2.8 eq) in anhydrous CH_2Cl_2 (10 mL) was added dropwise to a solution of triphosgene (197 mg, 0.66 mmol, 0.5 eq) in anhydrous CH_2Cl_2 (5 mL) over a period of 30 min. *N*-Boc-*S*-methylisothiourea (505 mg, 2.66 mmol, 2 eq) was added and stirring was continued for 2.5 h. The volume was reduced under reduced pressure to about 3 mL and the mixture was directly subjected to column chromatography (CH_2Cl_2/EtOAc 50/1 to 5/1). The separation of the product from the excess of the urea was quite difficult due to nearly identical TLC R_f-values (CH_2Cl_2/EtOAc 5/1). Therefore the use of 1 eq of *N*-Boc-*S*-methylisothiourea is recommended. Highly viscous yellowish oil (403 mg, 1 mmol, 75 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.27-1.53 (m, 22H, 2 × *tert*-Bu, CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2), 2.28 (s, 3H, C$\underline{H}_3$), 2.89 (m, 2H, C$\underline{H}_2$-NH), 3.01 (m, 2H,

C$\underline{H}_2$-NH), 6.80 (t, 1H, ^{3}J = 5.17 Hz, CH$_2$-N$\underline{H}$), 7.79 (t, 1H, ^{3}J = 5.79 Hz, CH$_2$-N$\underline{H}$), 12.44 (s, 1H, N$\underline{H}$); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 405 $[M + H]^+$; $C_{17}H_{34}N_4O_5S$ (406.5)

12-(Benzyloxycarbonylamino)-4,7,10-trioxadodecanoic acid (4.15). 12-Amino-4,7,10-trioxadodecanoic acid *tert*-butylester (1 g, 3.6 mmol, 1 eq) was dissolved in CH_2Cl_2 (10 mL). NEt_3 (0.73 g, 1 mL, 7.2 mmol, 2 eq) was added and the solution was cooled to 0 °C. Benzyl chloroformate (0.68 g, 3.97 mmol, 1.1 eq) was added dropwise in CH_2Cl_2 (10 mL) over a period of 30 min. The ice-water bath was removed and the mixture was allowed to stand at rt for 2 h. The intermediate was purified by column chromatography prior to ester cleavage in CH_2Cl_2/TFA 4/1 (v/v, 25 mL) for 3 h. CH_2Cl_2 (20 mL) was added three times, each time followed by evaporation under reduced pressure. Drying *in vacuo* yielded the product as reddish oil (1.13 g, 3.17 mmol, 88 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 2.43 (t, 2H, ^{3}J = 6.35 Hz, CH$_2$-C$\underline{H}_2$-COOH), 3.14 (m, 2H, C$\underline{H}_2$-NH), 3.41 (t, 2H, ^{3}J = 5.94 Hz, O-C$\underline{H}_2$-CH$_2$-NH), 3.48 (m, 8H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 3.59 (t, 2H, ^{3}J = 6.35 Hz, C$\underline{H}_2$-CH$_2$-COOH), 5.01 (s, 2H, C$\underline{H}_2$-Ar), 7.27 (m, 1H, N$\underline{H}$), 7.3-7.4 (m, 5H, Ph); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 356 $[M + H]^+$, 354 $[M - H]^-$; $C_{17}H_{25}NO_7$ (355.4)

General procedure for the synthesis of acids 4.16 and 4.17.

N-Boc-3,6-dioxaoctane-1,8-diamine (1 eq, cf. chapter 3) and the pertinent alkanedioic acid monobenzylester (1 eq) were dissolved in dry CH_2Cl_2. EDAC (1.2 eq) was added and the mixture was kept under stirring at rt for 20 h. The intermediate was purified by column chromatography (EtOAc/MeOH) prior to hydrogenation at rt and atmospheric pressure in MeOH using hydrogen and a 10 % Pd/C catalyst.

4-(8-*tert*-Butoxycarbonylamino-3,6-dioxaoctyl)aminocarbonylbutanoic acid (4.16). From glutaric acid monobenzylester **3.9** (cf. chapter 3); 63 % (460 mg); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.43 (s, 9H, *t*-Bu), 1.94 (m, 2H, CH$_2$-C$\underline{H}_2$-CH$_2$), 2.31 (t, 2H, C$\underline{H}_2$-CO), 2.39 (t, 2H, C$\underline{H}_2$-CO), 3.31 (m, 2H, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.47 (m, 2H, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.51-3.63 (m, 8H, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH, O-C$\underline{H}_2$-C$\underline{H}_2$-O); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 363 $[M + H]^+$; $C_{16}H_{30}N_2O_7$ (362.4)

5-(8-*tert*-Butoxycarbonylamino-3,6-dioxaoctyl)aminocarbonylpentanoic acid (4.17). From adipic acid monobenzylester **3.10** (cf. chapter 3); 66 % (260 mg); ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.36 (s, 9H, *t*-Bu), 1.6 (m, 4H, CH$_2$-C$\underline{H}_2$-C$\underline{H}_2$-CH$_2$), 2.17 (t, 2H, C$\underline{H}_2$-CO), 2.28 (t, 2H, C$\underline{H}_2$-CO), 3.25 (m, 2H, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.39 (m, 2H, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH), 3.45-3.63 (m, 8H, C$\underline{H}_2$ from O-CH$_2$-CH$_2$-NH, O-C$\underline{H}_2$-C$\underline{H}_2$-O); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 377 $[M + H]^+$; $C_{17}H_{32}N_2O_7$ (376.5)

(*R*)-N^{ω}-(6-Aminohexanoyl)-N^{α}-(2,2-diphenylacetyl)-*N*-(4-ureidomethylbenzyl)argininamide (4.18). Carbonyldiimidazole (56 mg, 0.35 mmol, 2.8 eq) was added to a solution of 6-aminohexanoic acid (71.3 mg, 0.31 mmol, 2.5 eq) in anhydrous acetonitrile (2 mL). The mixture was stirred for 40 min at rt and then added to a suspension of **4.6** (85 mg, 0.12 mmol, 1 eq) and NEt_3 (62.3 mg, 0.62 mmol, 5 eq) in acetonitrile (10 mL). The precipitate almost disappeared completely during heating to 40 °C for 2 h. The mixture was stirred at 35 °C overnight, then TFA (10 mL) was added and the mixture was allowed to stand at 50 °C for 1.5 h. MeOH (20 mL) was added three times each time followed by evaporation under reduced pressure. Purification with preparative HPLC afforded the product as a white fluffy solid (39 mg, 45 µmol, 36 %). ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) 1.44 (m, 2H, CH_2-CH_2-C$\underline{H}_2$-CH_2-CH_2), 1.5-1.77 (bm, 7H, CH_2-C$\underline{H}_2$-CH_2-C$\underline{H}_2$-CH_2, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.84 (m, 1H, CH-C$\underline{H}_2$-CH_2), 2.5 (t, 2H, ^{3}J = 7.34 Hz, C$\underline{H}_2$-CO), 2.91 (t, 2H, ^{3}J = 7.59 Hz, C$\underline{H}_2$-NH_2), 3.25 (m, 2H, CH_2-C$\underline{H}_2$-NH), 4.26 (s, 2H, C$\underline{H}_2$-NH-CO-NH_2), 4.32 (m, 2H, CH-CO-NH-C$\underline{H}_2$-Ar), 4.42 (m, 1H, C$\underline{H}^{\alpha}$), 5.08 (s, 1H, C$\underline{H}$-$(Ph)_2$), 7.15-7.34 (m, 14H, Ar); MS (ES, TFA/acetonitrile): m/z 322 $[M + 2H]^{2+}$, 643 $[M + H]^+$; $C_{35}H_{46}N_8O_4$ × $C_4H_2F_6O_4$ (870.8)

(*R*)-N^{ω}-(12-Amino-4,7,10-trioxadodecanoyl)-N^{α}-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)-argininamide (4.19). A solution of **4.15** (300 mg, 0.84 mmol, 1.05 eq) and CDI (156 mg, 0.96 mmol, 1.2 eq) in CH_2Cl_2 (10 mL) was stirred for 30 min. **2.7** (506 mg, 0.8 mmol, 1 eq) and NEt_3 (40.7 mg, 56 µL, 0.4 mmol, 0.5 eq) were added and the mixture was kept under stirring at rt for 20 h. Volatiles were removed under reduced pressure and the intermediate was purified by column chromatography (CH_2Cl_2/MeOH 50/1 to 10/1) prior to hydrogenation at rt and atmospheric pressure in MeOH (20 mL) using hydrogen and a 10 % Pd/C catalyst (120 mg). The catalyst was removed by filtration and removal of the solvent *in vacuo* yielded a highly viscous colorless oil which was dissolved in CH_2Cl_2/TFA 10/1 (v/v). The mixture was allowed to stand at rt overnight and was subsequently concentrated under reduced pressure. Purification with preparative HPLC afforded the product as a white fluffy solid (264 mg, 0.29 µmol, 36 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.32-1.6 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.66 (m, 1H, CH-C$\underline{H}_2$-CH_2), 2.68 (t, 2H, ^{3}J = 5.94 Hz, C$\underline{H}_2$-CO), 2.96 (m, 2H, C$\underline{H}_2$-NH_2), 3.24 (m, 2H, CH_2-C$\underline{H}_2$-NH), 3.54 (m, 10H, O-C$\underline{H}_2$-C$\underline{H}_2$-O, O-C$\underline{H}_2$-CH_2-NH_2), 3.68 (t, 2H, ^{3}J = 6.04 Hz, C$\underline{H}_2$-CH_2-CO), 4.1 (dd, 1H, ^{2}J = 15.17 Hz, ^{3}J = 5.92 Hz, C$\underline{H}_2$-ArOH), 4.19 (dd, 1H, ^{2}J = 15.09 Hz, ^{3}J = 5.81 Hz, C$\underline{H}_2$-ArOH), 4.34 (m, 1H, CH^{α}), 5.12 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.67 (d, 2H, ^{3}J = 8.50 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.46 Hz, AA'BB'), 7.18-7.35 (m, 10H, Ph), 7.75 (bs, 3H, NH_3^+), 8.38 (t, 1H, ^{3}J = 5.67 Hz, CO-N$\underline{H}$-CH_2), 8.50 (d, 1H, ^{3}J = 8.08 Hz, CO-N$\underline{H}$-CH), 8.68 (s, 2H, NH_2), 9.07 (m, 1H, NH), 9.3 (s, 1H, NH), 11.63 (s, 1H, ArO$\underline{H}$); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 677 $[M + H]^+$, 339 $[M + 2H]^{2+}$; $C_{36}H_{48}N_6O_7$ × $C_4H_2F_6O_4$ (904.8)

(*R*)-N^{ω}-(8-Amino-3,6-dioxaoctanoyl)-N^{α}-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)arginin-amide (4.20). *N*-Boc-8-amino-3,6-dioxaoctanoic acid × dicyclohexylamine (300 mg) was dissolved in dimethoxyethane (10 mL). The amine was precipitated by the addition of 37 % hydrochloric acid (≈ 60 µL) and removed by filtration. Removal of the solvent *in vacuo* yielded a yellow oil (200 mg, 0.76 mmol, 1 eq) which was dissolved in anhydrous CH_2Cl_2 (3 mL). Carbonyldiimidazole (148 mg, 0.91 mmol, 1.2 eq) was added and the mixture was allowed to stand at rt for 30 min. CH_2Cl_2 (15 mL), **2.7** (478 mg, 0.76 mmol, 1 eq) and NEt_3 (77 mg, 105 µL, 0.76 mmol, 1 eq) were added and the mixture was kept under stirring at rt for 20 h. TFA (10 mL) was added, the mixture was allowed to stand at rt for 4 h and then it was concentrated under reduced pressure. MeOH (20 mL) was added twice each time followed by evaporation under reduced pressure. Purification with preparative HPLC afforded the product as a white fluffy solid (300 mg, 0.35 mmol, 47 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.32-1.61 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.68 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 2.98 (m, 2H, C$\underline{H}_2$-NH$_2$), 3.25 (m, 2H, CH$_2$-C$\underline{H}_2$-NH), 3.61 (m, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 3.71 (m, 2H, O-C$\underline{H}_2$-CH$_2$-NH$_2$), 4.1 (dd, 1H, ^{2}J = 15.35 Hz, ^{3}J = 6.10 Hz, C$\underline{H}_2$-ArOH), 4.18 (dd, 1H, ^{2}J = 15.36 Hz, ^{3}J = 6.19 Hz, C$\underline{H}_2$-ArOH), 4.23 (s, 2H, O-C$\underline{H}_2$-CO), 4.33 (m, 1H, CH$^{\alpha}$), 5.13 (s, 1H, C$\underline{H}$-(Ph)$_2$), 6.67 (d, 2H, ^{3}J = 8.51 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.52 Hz, AA'BB'), 7.18-7.33 (m, 10H, Ph), 7.8 (bs, 3H, NH_3^+), 8.39 (t, 1H, ^{3}J = 5.83 Hz, CO-N$\underline{H}$-CH$_2$), 8.51 (d, 1H, ^{3}J = 8.05 Hz, CO-N$\underline{H}$-CH), 8.81 (s, 2H, NH$_2$), 9.12 (m, 1H, NH), 9.32 (s, 1H, NH), 10.98 (s, 1H, ArO$\underline{H}$); MS (ES, acetonitrile/TFA): *m/z* 310 $[M + 2H]^{2+}$, 619 $[M + H]^+$; $C_{33}H_{42}N_6O_6 \times C_4H_2F_6O_4$ (846.8)

General procedure for the synthesis of amines 4.21 to 4.23

Carbonyldiimidazole (1.6 eq) was added to a solution of the pertinent carboxcylic acid (1.5 eq) in CH_2Cl_2 (2-3 mL) and the mixture was allowed to stand at rt for 30-40 min. **2.7** (1 eq) and NEt_3 (0.3 eq) were added and the mixture was kept under stirring at rt for 20 h. The intermediate was purified by column chromatography (**4.21**: CH_2Cl_2/EtOAc 5/1 to 1/1, **4.22** and **4.23**: CH_2Cl_2/EtOAc 2/1 to EtOAc/MeOH 50/1) prior to deprotection with CH_2Cl_2/TFA/water 18/6/1 (12.5 mL) for 3 h. CH_2Cl_2 (20 mL) was added three times each time followed by evaporation under reduced pressure. The oily residue was dissolved in water (20 mL) and lyophilized to afford the product as a white fluffy solid.

(*R*)-N^{ω}-(6-Aminohexanoyl)-N^{α}-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide (4.21).[18] From 6-aminohexanoic acid (**4.14**); 94 % (490 mg); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.32 (m, 2H, C$\underline{H}_2$-CH$_2$-CH$_2$-NH$_2$), 1.37-1.6 (m, 7H, CH-C$\underline{H}_2$-C$\underline{H}_2$, C$\underline{H}_2$-CH$_2$-C$\underline{H}_2$-CH$_2$-NH$_2$), 1.67 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 2.42 (t, 2H, ^{3}J = 7.33 Hz, C$\underline{H}_2$-CO), 2.76 (m, 2H, C$\underline{H}_2$-NH$_2$), 3.24 (m, 2H, CH$_2$-C$\underline{H}_2$-NH), 4.1 (dd, 1H, ^{2}J = 15.20 Hz, ^{3}J = 5.93 Hz, C$\underline{H}_2$-ArOH), 4.18 (dd, 1H, ^{2}J = 15.08 Hz, ^{3}J = 6.12 Hz, C$\underline{H}_2$-ArOH), 4.33 (m, 1H, CH$^{\alpha}$), 5.13 (s, 1H, C$\underline{H}$-(Ph)$_2$), 6.67 (d, 2H, ^{3}J =

8.51 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.50 Hz, AA'BB'), 7.18-7.33 (m, 10H, Ph), 7.63 (bs, 3H, N$\underline{H}_3^+$), 8.38 (t, 1H, ^{3}J = 5.84 Hz, CO-N$\underline{H}$-CH$_2$), 8.5 (d, 1H, ^{3}J = 8.05 Hz, CO-N$\underline{H}$-CH), 8.6 (s, 2H, N$\underline{H}_2$), 8.95 (m, 1H, N$\underline{H}$), 9.3 (s, 1H, N$\underline{H}$), 11.4 (s, 1H, ArO$\underline{H}$); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 587 $[M + H]^+$, 294 $[M + 2H]^{2+}$; $C_{33}H_{42}N_6O_4 \times C_4H_2F_6O_4$ (814.7)

(*R*)-*N*$^{\omega}$-[4-(2-Aminoethyl)aminocarbonylbutanoyl]-*N*$^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide (4.22). From **4.10**; 71 % (320 mg); ^{1}H-NMR (300 MHz, DMSO-d$_6$): δ (ppm) 1.32-1.6 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.67 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 1.78 (m, 2H, CO-CH$_2$-C$\underline{H}_2$-CH$_2$-CO), 2.16 (t, 2H, ^{3}J = 7.43 Hz, C$\underline{H}_2$-CO), 2.44 (t, 2H, ^{3}J = 6.85 Hz, C$\underline{H}_2$-CO), 2.83 (m, 2H, C$\underline{H}_2$-NH$_2$), 3.25 (m, 4H, NH-C$\underline{H}_2$-CH$_2$-NH$_2$, CH$_2$-CH$_2$-C$\underline{H}_2$-NH), 4.1 (dd, 1H, ^{2}J = 15.17 Hz, ^{3}J = 6.22 Hz, C$\underline{H}_2$-ArOH), 4.18 (dd, 1H, ^{2}J = 14.89 Hz, ^{3}J = 6.02 Hz, C$\underline{H}_2$-ArOH), 4.34 (m, 1H, CH$^{\alpha}$), 5.13 (s, 1H, C$\underline{H}$-(Ph)$_2$), 6.67 (d, 2H, ^{3}J = 8.55 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.51 Hz, AA'BB'), 7.18-7.33 (m, 10H, Ph), 7.69 (bs, 3H, N$\underline{H}_3^+$), 8.0 (t, 1H, ^{3}J = 5.7 Hz, CO-N$\underline{H}$-CH$_2$), 8.38 (t, 1H, ^{3}J = 6.15 Hz, CO-N$\underline{H}$-CH$_2$), 8.52 (m, 3H, CO-N$\underline{H}$-CH, N$\underline{H}_2$), 8.85 (m, 1H, N$\underline{H}$), 9.3 (s, 1H, N$\underline{H}$), 11.28 (s, 1H, ArO$\underline{H}$); MS (ES, acetonitrile/TFA): *m/z* 315.5 $[M + 2H]^{2+}$, 630 $[M + H]^+$; $C_{34}H_{43}N_7O_5 \times C_4H_2F_6O_4$ (857.7)

(*R*)-*N*$^{\omega}$-[4-(3-Aminopropyl)aminocarbonylbutanoyl]-*N*$^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide (4.23). From **4.11**; 64 % (310 mg); ^{1}H-NMR (300 MHz, DMSO-d$_6$): δ (ppm) 1.32-1.6 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.65 (m, 3H, CH-C$\underline{H}_2$-CH$_2$, C$\underline{H}_2$-CH$_2$-NH$_2$), 1.77 (m, 2H, CO-CH$_2$-C$\underline{H}_2$-CH$_2$-CO), 2.14 (t, 2H, ^{3}J = 7.36 Hz, C$\underline{H}_2$-CO), 2.43 (t, 2H, ^{3}J = 7.0 Hz, C$\underline{H}_2$-CO), 2.76 (m, 2H, C$\underline{H}_2$-NH$_2$), 3.1 (m, 2H, NH-C$\underline{H}_2$-CH$_2$-CH$_2$-NH$_2$, 3.24 (m, 2H, CH-CH$_2$-CH$_2$-C$\underline{H}_2$-NH), 4.1 (dd, 1H, ^{2}J = 15.19 Hz, ^{3}J = 6.0 Hz, C$\underline{H}_2$-ArOH), 4.18 (dd, 1H, ^{2}J = 15.12 Hz, ^{3}J = 5.90 Hz, C$\underline{H}_2$-ArOH), 4.34 (m, 1H, CH$^{\alpha}$), 5.12 (s, 1H, C$\underline{H}$-(Ph)$_2$), 6.67 (d, 2H, ^{3}J = 8.49 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.49 Hz, AA'BB'), 7.18-7.33 (m, 10H, Ph), 7.65 (bs, 3H, N$\underline{H}_3^+$), 7.98 (t, 1H, ^{3}J = 5.5 Hz, CO-N$\underline{H}$-CH$_2$), 8.38 (t, 1H, ^{3}J = 5.69 Hz, CO-N$\underline{H}$-CH$_2$), 8.5 (d, 1H, CO-N$\underline{H}$-CH), 8.56 (s, 2H, N$\underline{H}_2$), 8.85 (m, 1H, N$\underline{H}$), 9.3 (s, 1H, N$\underline{H}$), 11.32 (s, 1H, ArO$\underline{H}$); MS (ES, acetonitrile/TFA): *m/z* 322.5 $[M + 2H]^{2+}$, 644 $[M + H]^+$; $C_{35}H_{45}N_7O_5 \times C_4H_2F_6O_4$ (871.7)

General procedure for the synthesis of amines 4.24 and 4.25

Carbonyldiimidazole (1.2 eq) was added to a solution of acid **4.16** or **4.17** (1 eq) in CH_2Cl_2 and the mixture was stirred for 30 min at rt. Compound **2.7** (1eq, cf chapter 2) was added and stirring was continued at rt overnight. The intermediate was purified by column chromatography (CH_2Cl_2/EtOAc) prior to deprotection with CH_2Cl_2/TFA 1/1 (v/v) for 2.5 h. CH_2Cl_2 (5 vol. parts) was added three times, each time followed by evaporation *in vacuo*. The product was purified using preparative HPLC.

(*R*)-*N*$^{\omega}$-[4-(8-Amino-3,6-dioxaoctyl)aminocarbonylbutanoyl]-*N*$^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide (4.24). From **4.16**; white solid; 64 % (192 mg); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.34-1.6 (bm, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.66 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 1.76 (p, 2H, CO-CH$_2$-C$\underline{H}_2$-CH$_2$-CO, ^{3}J = 7.26 Hz), 2.13 (t, 2H, ^{3}J = 7.28 Hz, C$\underline{H}_2$-CO), 2.42 (t, 2H, ^{3}J = 7.29 Hz, C$\underline{H}_2$-CO), 2.97 (m, 2H, C$\underline{H}_2$-NH$_2$), 3.21 (m, 4H, CH$_2$-CH$_2$-C$\underline{H}_2$-NH, NH-C$\underline{H}_2$-CH$_2$-O), 3.4 (t, 2H, ^{3}J = 6.09 Hz, O-C$\underline{H}_2$-CH$_2$-NH$_2$), 3.5-3.61 (m, 6H, C$\underline{H}_2$-O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.14 (m, 2H, C$\underline{H}_2$-ArOH), 4.33 (m, 1H, C$\underline{H}^{\alpha}$), 5.12 (s, 1H, C$\underline{H}$-(Ph)$_2$), 6.67 (d, 2H, ^{3}J = 8.45 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.44 Hz, AA'BB'), 7.18-7.33 (m, 10H, Ph), 7.77 (bs, 2H, N$\underline{H}_2$), 7.92 (t, 1H, ^{3}J = 5.62 Hz, CO-N$\underline{H}$-CH$_2$), 8.39 (t, 1H, ^{3}J = 5.89 Hz, CO-N$\underline{H}$-CH$_2$), 8.5 (d, 1H, ^{3}J = 8.01 Hz, CO-N$\underline{H}$-CH), 8.64 (s, 2H, N$\underline{H}_2$), 9.03 (s, 1H, N$\underline{H}$), 9.31 (s, 1H, N$\underline{H}$), 11.54 (s, 1H, ArO$\underline{H}$); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 718 $[M + H]^+$, 359.5 $[M + 2H]^{2+}$; $C_{38}H_{51}N_7O_7 \times C_4H_2F_6O_4$ (945.9)

(*R*)-*N*$^{\omega}$-[5-(8-Amino-3,6-dioxaoctyl)aminocarbonylpentanoyl]-*N*$^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide (4.25). From **4.17**; white solid; 44 % (135 mg); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.34-1.6 (bm, 7H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CH$_2$-C$\underline{H}_2$-C$\underline{H}_2$-CH$_2$), 1.66 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 2.08 (t, 2H, ^{3}J = 6.20 Hz, C$\underline{H}_2$-CO), 2.42 (t, 2H, ^{3}J = 6.14 Hz, C$\underline{H}_2$-CO), 2.97 (m, 2H, C$\underline{H}_2$-NH$_2$), 3.2 (m, 4H, CH$_2$-CH$_2$-C$\underline{H}_2$-NH, NH-C$\underline{H}_2$-CH$_2$-O), 3.39 (t, 2H, ^{3}J = 6.06 Hz, O-C$\underline{H}_2$-CH$_2$-NH$_2$), 3.5-3.62 (m, 6H, C$\underline{H}_2$-O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.14 (m, 2H, C$\underline{H}_2$-ArOH), 4.33 (m, 1H, C$\underline{H}^{\alpha}$), 5.12 (s, 1H, C$\underline{H}$-(Ph)$_2$), 6.67 (d, 2H, ^{3}J = 8.47 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.47 Hz, AA'BB'), 7.18-7.33 (m, 10H, Ph), 7.77 (bs, 2H, N$\underline{H}_2$), 7.88 (t, 1H, ^{3}J = 5.51 Hz, CO-N$\underline{H}$-CH$_2$), 8.38 (t, 1H, ^{3}J = 5.85 Hz, CO-N$\underline{H}$-CH$_2$), 8.5 (d, 1H, ^{3}J = 7.96 Hz, CO-N$\underline{H}$-CH), 8.66 (s, 2H, N$\underline{H}_2$), 9.08 (s, 1H, N$\underline{H}$), 9.31 (s, 1H, N$\underline{H}$), 11.6 (s, 1H, ArO$\underline{H}$); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 732 $[M + H]^+$, 366.5 $[M + 2H]^{2+}$; $C_{39}H_{53}N_7O_7 \times C_4H_2F_6O_4$ (959.9)

(*R*)-*N*$^{\omega}$-[7-(8-Amino-3,6-dioxaoctyl)aminocarbonylheptanoyl-*N*$^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide (4.26). The carboxylic acid **3.16** (370 mg, 0.47 mmol, 1 eq; cf. chapter 3) and amine **3.26** (140 mg, 0.56 mmol, 1.2 eq; cf. chapter 3) were dissolved in CH_2Cl_2 (5 mL). EDAC (108 mg, 0.56 mmol, 1.2 eq) was added and the mixture was kept under stirring at rt for 20 h. TFA (4 mL) was added and stirring was continued for 2.5 h. CH_2Cl_2 (30 mL) was added three times, each time followed by evaporation under reduced pressure. The product was purified using preparative HPLC. Light brown resin; 43 % (200 mg, 0.2 mmol); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.25 (m, 4H, CH$_2$-CH$_2$-C$\underline{H}_2$-C$\underline{H}_2$-CH$_2$-CH$_2$), 1.35-1.6 (m, 7H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CH$_2$-C$\underline{H}_2$-CH$_2$-CH$_2$-C$\underline{H}_2$-CH$_2$), 1.66 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 2.05 (t, 2H, ^{3}J = 7.38 Hz, C$\underline{H}_2$-CO), 2.4 (t, 2H, ^{3}J = 7.34 Hz, C$\underline{H}_2$-CO), 2.97 (m, 2H, C$\underline{H}_2$-NH$_2$), 3.2 (m, 4H, CH$_2$-CH$_2$-C$\underline{H}_2$-NH, NH-C$\underline{H}_2$-CH$_2$-O), 3.39 (t, 2H, ^{3}J = 6.06 Hz, O-C$\underline{H}_2$-CH$_2$-NH$_2$), 3.5-3.61 (m, 6H, C$\underline{H}_2$-O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.14 (m, 2H, C$\underline{H}_2$-ArOH), 4.33 (m, 1H, C$\underline{H}^{\alpha}$), 5.12 (s, 1H, C$\underline{H}$-(Ph)$_2$), 6.67 (d, 2H, ^{3}J = 8.44 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.45 Hz, AA'BB'), 7.18-7.34 (m, 10H, Ph), 7.76 (bs, 2H, N$\underline{H}_2$), 7.84 (t, 1H, ^{3}J = 5.49 Hz, CO-N$\underline{H}$-CH$_2$), 8.39 (t, 1H, ^{3}J = 5.80 Hz, CO-N$\underline{H}$-CH$_2$), 8.5 (d, 1H, ^{3}J =

7.92 Hz, CO-NH-CH), 8.62 (s, 2H, NH_2), 8.99 (s, 1H, NH), 9.31 (s, 1H, NH), 11.45 (s, 1H, ArOH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 380.5 $[M + 2H]^{2+}$, 760 $[M + H]^+$; $C_{41}H_{57}N_7O_7 \times C_4H_2F_6O_4$ (988.0)

(*R*)-N^{ω}-[(4-Aminobutyl)aminocarbonyl]-N^{α}-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)-argininamide (4.27). 4.13 (178 mg, 0.44 mmol, 1 eq) and **2.6** (215 mg, 0.44 mmol, 1 eq) were dissolved in anhydrous DMF (10 mL). $HgCl_2$ (197 mg, 0.66 mmol, 1.5 eq) and diisopropylethylamine (114 mg, 0.88 mmol, 2 eq) were added, the mixture was stirred at rt overnight and then concentrated under reduced pressure (final pressure: 1 mbar) at 43 °C. The intermediate was purified by column chromatography (CH_2Cl_2/EtOAc 4/1 to 1/2) prior to deprotection with CH_2Cl_2/TFA 1/1 (4 mL) and three drops of water (2 h). CH_2Cl_2 (20 mL) was added three times, each time followed by evaporation under reduced pressure. Uptake of the oily residue in water (20 mL) and lyophilization afforded the product as a white fluffy solid (266 mg, 0.33 mmol, 74 %). ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.34-1.56 (m, 7H, CH-CH_2-CH_2, CH_2-CH_2-CH_2-NH_2), 1.66 (m, 1H, CH-CH_2-CH_2), 2.78 (m, 2H, CH_2-NH_2), 3.11 (m, 2H, NH-CH_2-CH_2-CH_2-CH_2), 3.19 (m, 2H, CH-CH_2-CH_2-CH_2-NH), 4.11 (dd, 1H, ^{2}J = 14.78 Hz, ^{3}J = 5.57 Hz, CH_2-ArOH), 4.17 (dd, 1H, ^{2}J = 14.76 Hz, ^{3}J = 5.78 Hz, CH_2-ArOH), 4.33 (m, 1H, CH^{α}), 5.11 (s, 1H, CH-$(Ph)_2$), 6.66 (d, 2H, ^{3}J = 8.4 Hz, AA'BB'), 6.99 (d, 2H, ^{3}J = 8.4 Hz, AA'BB'), 7.21 (m, 2H, Ph), 7.28 (m, 8H, Ph), 7.54 (m, 1H, CO-NH-CH_2), 7.66 (bs, 3H, NH_3^+), 8.35 (m, 1H, CO-NH-CH_2), 8.4 (bs, 2H, NH_2), 8.47 (d, 1H, ^{3}J = 8.08 Hz, CO-NH-CH), 8.9 (s, 1H, NH), 9.27 (s, 1H, NH), 9.96 (s, 1H, ArOH); MS (ES, acetonitrile/TFA): *m/z* 294.5 $[M + 2H]^{2+}$, 588 $[M + H]^+$; $C_{32}H_{41}N_7O_4 \times C_4H_2F_6O_4$ (815.7)

Compound 4.21 labeled with Dy-635 (4.28). A solution of Dy-635-NHS (1 mg, 1.32 µmol, 1 eq) in acetonitrile (200 µL) was added to a solution of **4.21** (2.16 mg, 2.65 µmol, 2 eq) and NEt_3 (1.1 mg, 1.5 µL, 10.6 µmol, 8 eq) in acetonitrile (300 µL). The reaction was stopped by addition of 10 % aq. TFA (10 µL) after 3.5 h of incubation at room temperature and the solvent was evaporated. The residue was dissolved in a mixture of acetonitrile (300 µL) and 0.05 % aqueous TFA (300 µL) and the product was purified by analytical HPLC (column: Nucleodur 250 × 4 mm, 7 injections). Yield: 23 % (0.44 mg, 0.304 µmol); RP-HPLC (Nucleodur 250 × 4 mm, 210 nm, gradient 1): 94 % (t_R = 25.1 min, k = 9.9); MS (ES, H_2O/acetonitrile): m/z 1227.9 M^+, 614.5 $[M^+ + H]^{2+}$; $C_{71}H_{87}N_8O_9S \times C_4H_1F_6O_4$ (1455.6)

***N*$^{\omega}$-S0436-labeled BIBP 3226 (4.29).** A solution of S0436-NHS (S0536) (5.4 mg, 7.69 µmol, 1 eq) in acetonitrile (1 mL) was added to a solution of **2.7** (9.6 mg, 15.24 µmol, 2 eq) and NEt_3 (6.2 mg, 8.5 µL, 61.6 µmol, 8 eq) in acetonitrile (2 mL). The mixture was incubated for 20 h at room temperature. TFA (3 mL) and water (0.1 mL) were added and the mixture was allowed to stand for 2 h at room temperature prior to concentration to a volume of about 1 mL. Acetonitrile

(800 µL) and water (800 µL) was added and the product (both isomers) was isolated with preparative HPLC (column: Nucleodur 250 × 21 mm, 3 injections). Yield: 20 % (total: 1.8 mg, 1.54 µmol; 0.8 mg of isomer 1, 1 mg of isomer 2); RP-HPLC (Nucleodur 250 × 4 mm, 210 nm, gradient 1): isomer 1: 95 % (t_R = 21.9 min, k = 8.5), isomer 2: 99 % (t_R = 23.3 min, k = 9.1); MS (ES, H_2O/acetonitrile): isomer 1 (**4.29-i1**): m/z 1060.7 M^+, 531.0 $[M^+ + H]^{2+}$, isomer 2 (**4.29-i2**): m/z 531.0 $[M^+ + H]^{2+}$, 1060.7 M^+; $C_{62}H_{74}N_7O_7S \times C_4H_1F_6O_4$ (1288.4)

Compound 4.22 labeled with DE99 (4.30). Carbonyldiimidazole (1.1 mg, 6.6 µmol, 1.3 eq) was added to a solution of **4.22** (6.5 mg, 7.6 µmol, 1.5 eq) in anhydrous DMF (500 µL) and the mixture was stirred for 40 min at rt. **DE99** (2 mg, 5.1 µmol, 1eq) and NEt_3 (1.8 mg, 2.5 µL, 17.8 µmol, 3.5 eq) were added and stirring was continued at rt for 6.5 h. The reaction was stopped by addition of 10 % aq. TFA (corresponding to 10 eq of TFA) and the product was purified with preparative HPLC (Eurospher, 250 × 32 mm). Yield: 23 % (1.43 mg, 1.16 µmol); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 95 % (t_R = 16.9 min, k = 5.3); MS (ES, acetonitrile/TFA): m/z 502.9 $[M^+ + H]^{2+}$, 1004.6 M^+; $C_{57}H_{66}N_9O_6S \times C_4H_1F_6O_4$ (1232.3)

Compound 4.27 labeled with DE99 (4.31). 4.27 (5.6 mg, 6.9 µmol, 1.6 eq) and DE99-NHS × CF_3COO^- (2.6 mg, 4.3 µmol, 1 eq) were dissolved in anhydrous DMF (150 µL). NEt_3 (1.7 mg, 2.4 µL, 17.2 µmol, 4 eq) was added and the mixture was stirred for 3 h at rt. The reaction was stopped by addition of 10 % aq. TFA (corresponding to 4 eq of TFA) and the product was purified with preparative HPLC (Eurospher, 250 × 32 mm). Yield: 24 % (1.23 mg, 1.03 µmol); RP-HPLC (Eurospher 250 × 4 mm, 220 nm, gradient 3): 94 % (t_R = 18.5 min, k = 5.9); MS (ES, acetonitrile/TFA): m/z 481.5 $[M^+ + H]^{2+}$, 962 M^+; $C_{55}H_{64}N_9O_5S \times C_4H_1F_6O_4$ (1190.2)

Compound 4.18 labeled with Py-1 (4.32). 4.18 (9.3 mg, 10.7 µmol, 2.1 eq) and NEt_3 (5.1 mg, 7.1 µL, 50.9 µmol, 10 eq) were dissolved in a mixture of acetonitrile (1 mL) and water (100 µL) followed by the addition of Py-1 × 1 BF_4^- (2 mg, 5.1 µmol, 1 eq) in acetonitrile (200 µL). The reaction was stopped by addition of 10 % aq. TFA (corresponding to 10 eq TFA) after an incubation period of 2 h at rt. The product was purified with preparative HPLC (column: Eurospher-100 C18 250 × 32 mm), Yield: 33 % (1.75 mg, 1.7 µmol); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 82 % (t_R = 19.7 min, k = 6.3); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): m/z 465.5 $[M^+ + H]^{2+}$, 930 M^+; $C_{56}H_{68}N_9O_4 \times C_4H_1F_6O_4$ (1158.2)

General procedure for the synthesis of fluorescent ligands 4.33 - 4.47

The pertinent amine precursor (2-3 eq) and NEt_3 (6-10 eq) was dissolved in a mixture of acetonitrile and DMF (≈ 10-20 % DMF v/v, total volume: 300-600 µL) followed by the addition of the pyrylium dye × 1 BF_4^- (1 eq) in DMF (≈ 25-50 µL). The reaction was stopped by addition of

10 % aq. TFA (corresponding to 6-10 eq TFA) after an incubation period of 1-2 h at rt. The product was purified with preparative HPLC (column: Eurospher, 250 × 32 mm, except for **4.36**).

Compound 4.19 labeled with Py-1 (4.33). Yield: 47 % (2.84 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 92 % (t_R = 21.7 min, k = 7.0); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): m/z 482.5 $[M^+ + H]^{2+}$, 964 M^+; $C_{57}H_{70}N_7O_7 \times C_4H_1F_6O_4$ (1192.2)

Compound 4.20 labeled with Py-1 (4.34). Yield: 56 % (1.63 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 97 % (t_R = 19.4 min, k = 6.2); MS (ES, TFA/acetonitrile): m/z 453.5 $[M^+ + H]^{2+}$, 906 M^+; $C_{54}H_{64}N_7O_6 \times C_4H_1F_6O_4$ (1134.1)

Compound 4.20 labeled with Py-5 (4.35). Yield: 23 % (1.58 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 96 % (t_R = 16.8 min, k = 5.2); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): m/z 440.5 $[M^+ + H]^{2+}$, 880 M^+; $C_{52}H_{62}N_7O_6 \times C_4H_1F_6O_4$ (1108.1)

Compound 4.21 labeled with Py-1 (4.36). Purification with preparative HPLC: Nucleodur 250 × 21 mm; Yield: 42 % (5.2 mg); RP-HPLC (Nucleodur 250 × 4 mm, 210 nm, gradient 2): 99 % (t_R = 25.2 min, k = 10.0); MS (ES, H_2O/acetonitrile): m/z 437.5 $[M^+ + H]^{2+}$, 874 M^+; $C_{54}H_{64}N_7O_4 \times C_4H_1F_6O_4$ (1102.1)

Compound 4.21 labeled with Py-5 (4.37). Yield: 15 % (1.3 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 96 % (t_R = 18.1 min, k = 5.7); MS (ES, H_2O/acetonitrile/MeOH + 10 mM NH_4OAc): m/z 424.5 $[M^+ + H]^{2+}$, 848 M^+; $C_{52}H_{62}N_7O_4 \times C_4H_1F_6O_4$ (1076.1)

Compound 4.22 labeled with Py-1 (4.38). Yield: 53 % (3.07 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 96 % (t_R = 18.5 min, k = 5.9); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): m/z 459 $[M^+ + H]^{2+}$, 917 M^+; $C_{55}H_{65}N_8O_5 \times C_4H_1F_6O_4$ (1145.2)

Compound 4.23 labeled with Py-1 (4.39). Yield: 36 % (2.15 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 96 % (t_R = 18.9 min, k = 6.0); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): m/z 466 $[M^+ + H]^{2+}$, 931 M^+; $C_{56}H_{67}N_8O_5 \times C_4H_1F_6O_4$ (1159.2)

Compound 4.24 labeled with Py-6 (4.40). Yield: 15 % (1.51 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 97 % (t_R = 21.3 min, k = 6.9); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): m/z 528.5 $[M^+ + H]^{2+}$, 1055.6 M^+; $C_{63}H_{75}N_8O_7 \times C_4H_1F_6O_4$ (1283.3)

Compound 4.25 labeled with Py-6 (4.41). Yield: 16 % (1.68 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 96 % (t_R = 21.5 min, k = 7.0); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): m/z 535.5 $[M^+ + H]^{2+}$, 1069.7 M^+; $C_{64}H_{77}N_8O_7 \times C_4H_1F_6O_4$ (1297.3)

Compound 4.26 labeled with Py-6 (4.42). Yield: 13 % (1.43 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 89 % (t_R = 21.7 min, k = 7.0); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): m/z 549.5 $[M^+ + H]^{2+}$, 1097.7 M^+; $C_{66}H_{81}N_8O_7 \times C_4H_1F_6O_4$ (1325.4)

Compound 4.27 labeled with Py-1 (4.43). Yield: 26 % (1.94 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 97 % (t_R = 20.3 min, k = 6.5); MS (ES, TFA/acetonitrile): m/z 438 $[M^+ + H]^{2+}$, 875 M^+; $C_{53}H_{63}N_8O_4 \times C_4H_1F_6O_4$ (1103.1)

Compound 4.27 labeled with Py-5 (4.44). Yield: 23 % (0.94 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 99 % (t_R = 17.4 min, k = 5.4); MS (ES, TFA/acetonitrile): m/z 425 $[M^+ + H]^{2+}$, 849 M^+; $C_{51}H_{61}N_8O_4 \times C_4H_1F_6O_4$ (1077.1)

Compound 3.38 labeled with Py-1 (4.45). Yield: 26 % (1.32 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 98 % (t_R = 18.0 min, k = 5.7); MS (ES, TFA/acetonitrile): m/z 801.1 $[M^+ + H]^{2+}$, 543.5 $[M^+ + 2H]^{3+}$; $C_{93}H_{114}N_{15}O_{10} \times C_8H_4F_{12}O_8$ (2057.1)

Compound 3.39 labeled with Py-1 (4.46). Yield: 28 % (1.54 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 90 % (t_R = 18.8 min, k = 6.0); MS (ES, TFA/acetonitrile): m/z 553.2 $[M^+ + 2H]^{3+}$, 829.2 $[M^+ + H]^{2+}$; $C_{97}H_{122}N_{15}O_{10} \times C_8H_4F_{12}O_8$ (2113.2)

Compound 3.40 labeled with Py-1 (4.47). Yield: 28 % (1.53 mg); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 3): 93 % (t_R = 16.7 min, k = 5.2); MS (ES, TFA/acetonitrile): m/z 872.2 $[M^+ + H]^{2+}$, 581.9 $[M^+ + 2H]^{3+}$; $C_{99}H_{124}N_{17}O_{12} \times C_8H_4F_{12}O_8$ (2199.3)

4.3.3 Fluorescence Spectroscopy and Determination of Quantum Yields

Determination of quantum yields was performed with a Cary Eclipse spectrofluorimeter and Cary 100 UV/VIS photometer (Varian Inc., Mulgrave, Victoria, Australia). The photomultiplier voltage of the Cary Eclipse spectrofluorimeter was set to 400 V throughout. Recording of excitation spectra was performed with an excitation slit of 5 nm and an emission slit of 10 nm. Emission spectra depicted in Figure 4 were recorded with an excitation slit of 10 nm and an emission slit of 5 nm.

For the determination of quantum yields cresyl violet perchlorate (Acros Organics, Geel, Belgium) was used as a red fluorescent standard, for which a quantum yield of 54 % in ethanol was reported in the literature[42]. It is important to use the perchlorate, because only this salt is provided with sufficiently high purity.

Spectra were recorded in glass cuvettes (Hellma, 100-OS, 10 × 10 mm) or in acryl cuvettes (10 × 10 mm, Ref. 67.755, Sarstedt, Nümbrecht, Germany). The use of the latter was less time consuming due to the one-way use and solutions had not to be transferred into another cuvette between recording of fluorescence and absorption spectra. Quantum yields proved to be nearly unaffected by the material of the cuvette (glass/acryl). First of all, the concentrations of the fluorescent ligands for the determination of the quantum yield was determined. For this purpose an absorption spectrum was recorded with a concentration in the range of 2-6 µM. For the determination of quantum yields solutions with absorbances between 0.1 and 0.2 at the excitation wavelength were used. The excitation wavelength was chosen as close to the absorption maximum as possible or at a plateau of the absorption spectrum (e.g. S0436 labeled compounds). It was strictly avoided to excite the fluorescent compounds in flank of the excitation spectrum.

Solutions of the fluorescent ligands in PBS, PBS + 1 % BSA or ethanol were freshly prepared from 1 or 2 mM stock solutions (DMSO) of the compounds and immediately protected from light. Fluorescence spectra were recorded at three different slit adjustments (excitation/emission): 5/5 nm, 10/5 nm and 10/10 nm. Spectra of the cresyl violet standard were only recorded in ethanol. For the determination of reference spectra, the pure solvents with the same DMSO content, but without fluorescent compound, were used. The solutions were always maintained in the dark.

The emission spectra were recorded within 15 - 20 min at a temperature of 22 °C using the medium scan rate. The filter settings were “auto” for the excitation and “open” for the emission filter. The emission starting point was set 10 nm above the excitation wavelength for the slit adjustments 5/5 nm and 10/5 nm as well as 15 nm above the excitation wavelength for the slit combination 10/10 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 net spectra were integrated up to 850 nm.

The absorbance at the excitation wavelength was determined by recording absorption spectra immediatly 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:

$$\Phi_{F(X)} = (A_s/A_x)\,(F_x/F_s)\,(n_x/n_s)^2\,\Phi_{F(S)}$$

where A_s is the absorbance and F_s the integral of the corrected emission spectrum of the cresyl violet standard solution. A_x and F_x stand for the absorbance and the integral of the corrected emission spectrum of the fluorescent ligand. The refraction indices of the solvents for the

fluorescent ligands and the cresyl violet standard are denoted n_x and n_s, respectively. $\Phi_{F(S)}$ is the reported quantum yield of cresyl violet, in this case 54 %.

4.3.4 Pharmacology: Cell Culture, Fura-2 Assay and Competition Binding Assay

Cell culture. HEL and SK-N-MC cells were cultured as described elsewhere.[43, 44] HEL cells were subcultured by 1:6-dilution with fresh culture medium 24 h prior to the fura Ca^{2+}-assay. MCF-7-Y_1[a] cells were maintained in MEM (Sigma), supplemented with 5 % FCS (Biochrom AG, Berlin, Germany). The CHO cells, transfected with the human Y_2R, $G_{\alpha qi5}$ and aequorin, were cultured as previously described.[3] CHO cells, transfected with the human Y_4R, $G_{\alpha qi5}$ and aequorin, were cultured under the same conditions. HEC-1B cells, transfected with the human Y_5R, were cultured as previously described.[45]

[a]This cell line was established from MCF-7 cells (ATCC number HTB 22) in the 157th passage and shows 2 - 3-fold higher Y_1R expression than the original MCF-7 cells.

Fura-2 assay on HEL cells and radioligand competition binding assay. The Fura assay was performed with HEL cells as previously described[32] using a Perkin-Elmer LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany). Radioligand competition experiments with [^{3}H]-UR-MK114 (cf. chapter 2) were performed as described in chapter 3.

4.3.5 Pharmacology: Flow Cytometric Binding Experiments

All flow cytometric assays were performed with a FACSCalibur^{TM} flow cytometer (Becton Dickinson, Heidelberg, Germany), equipped with an argon laser (488 nm) and a red diode laser (635 nm).

Selectivity assay with fluorescent ligand 4.36. The selectivity assay was essentially performed as previously described[3] with the following modifications: due to the interfering fluorescence emission of compound **4.36** in channel Fl-3 of the flow cytometer, the indicator dye fura red was not used for cell labeling. Therefore, only two cell types were mixed. One mixture contained fluo-4 loaded HEL cells (constitutively expressing the Y_1R) and unloaded CHO cells (transfected with the Y_2R, $G\alpha q_{i5}$ and aequorin). The other mixture consisted of fluo-4 loaded HEL cells and unloaded Y_5R transfected HEC-1B cells. Fluo-4 loading of the HEL cells was essentially performed as previously described for fura-2[43], but with a fluo-4 concentration of 0.75 µM. The cell density in the final mixture was 10^6 cells/mL for the HEL cells, $0.7 \cdot 10^6$ cells/mL for the CHO cells and $0.6 \cdot 10^6$ cells/mL for the HEC-1B cells. Every cell suspension contained

bacitracin (0.1 g/l) in order to inhibit protease activity. 490 µL of cell suspension were added to the reaction vessels containing 5 µL of the respective 100-fold concentrated stock solutions (cy5-pNPY in 10 mM HCl + 0.1 % BSA and compound **4.36** in DMSO). Compound **4.36** was used in the range between 0.1 nM and 10 µM, cy5-pNPY at a concentration of 10 nM. All samples were prepared in triplicates. After incubation at room temperature for 60-90 min, the samples were measured at the highest flow rate until 20,000 gated events were counted.

Flow cytometric binding studies with 4.38 at the Y_2R and Y_5R. Flow cytometric binding studies with **4.38** at the Y_2R (CHO cells, transfected with the Y_2R, $G\alpha_{qi5}$ and aequorin)[46], and at the Y_5R (expressed by HEC-1B cells transfected with the Y_5R)[3, 47] were performed as previously described. The cell density in loading buffer was 10^6 cells/mL (Y_2R) or $0.5 \cdot 10^6$ cells/mL (Y_5R). For Y_2R binding studies Dy-635-pNPY (10 nM) was used instead of Cy5-pNPY (10 nM).

Flow cytometric binding studies at the Y_4R. For the determination of the Y_4R affinity of compound **4.36** and **4.38**, a confluent culture of CHO cells transfected with the Y_4R, $G_{\alpha qi5}$ and aequorin was trypsinized and resuspended in loading buffer containing 1 % BSA. After centrifugation at 1,000 rpm for 5 min, the cells were suspended in loading buffer + 1% BSA to a density of $2.5 \cdot 10^5$ cells/mL (**4.36**) or 10^6 cells/mL (**4.38**). Bacitracin was added (0.1 g/l) in order to prevent protease-mediated degradation of the peptide ligands. Compound **4.36** was tested at the same concentrations as in the aforementioned selectivity assay, compound **4.38** at a concentration of 1 and 10 µM. In both cases 100-fold concentrated stock solutions in DMSO were used. An S0586-labeled derivative of the human pancreatic polypeptide (S0586-[K^4]-hPP) was used as fluorescent ligand (10 nM) to determine the affinity of **4.36**, and Cy5-[K^4]-hPP[37] (5 nM) was used to determine the affinity of **4.38**. Stock solutions (S0586-[K^4]-hPP: 1 µM, Cy5-[K^4]-hPP: 0.5 µM) were prepared in 10 mM HCl + 0.1 % BSA. 490 µL of the cell suspension were added to the reaction vessels containing 5 µL of the respective stock solutions of S0586-[K^4]-hPP or Cy5-[K^4]-hPP and the test compounds. Non-specific binding was determined in the presence of hPP (100 nM). All samples were prepared in triplicates. The samples were incubated for 90 min at room temperature and measured at the highest flow rate (**4.36**: 1 min, **4.38**: until 20,000 gated events were counted), detecting the S0586-[K^4]-hPP or Cy5-[K^4]-hPP fluorescence emission in the Fl-4 channel.

Displacement of fluorescent ligand 4.36 by BIBP 3226. The displacement of antagonist **4.36** by BIBP 3226 (Figure 8) was determined on HEL cells in loading buffer without BSA using a cell density of $2.5 \cdot 10^5$ cells/mL. The non-fluorescent antagonist BIBP 3226 was used at final concentrations between 0.1 nM and 1 µM. For the determination of non-specific binding BIBP 3226 (10 µM) was added. Compound **4.36** (5 µL of a 1 µM solution in 50 % DMSO) and the

respective 100-fold concentrated BIBP 3226 solution (5 µL) were premixed in 1.5 mL Eppendorf reaction vessels. Incubation of the cells was started by addition of cell suspension (490 µL) to the tubes to yield a final volume of 500 µL containing 10 nM of compound **4.36** and the respective concentration of BIBP 3226. After 15 min of incubation at room temperature the samples were measured by flow cytometry, recording the fluorescence signals in channels Fl-2 (585 ± 21 nm) and Fl-3 (> 670 nm) using an excitation wavelength of 488 nm (argon laser). From every sample 15,000 – 18,000 cells were gated.

Saturation binding experiment with 4.31 and displacement of 4.31 by BIBP 3226. MCF-7-Y_1 cells[a] were seeded in a 175-cm^2 culture flask 5-6 days prior to the experiment. 17-β-Estradiol (1 nM) was added 3 days prior to the experiment using a 1 µM solution in ethanol. Cells were treated with trypsin, suspended in culture medium and centrifuged. The cell pellet was re-suspended in buffer (see general experimental conditions), cells were centrifuged and re-suspended in buffer to a density of $0.5 \cdot 10^6$ - $1 \cdot 10^6$ cells/mL. **4.31** was used at final concentrations between 1 and 50 nM for saturation binding experiments. Non-specific binding was determined in the presence of BIBP 3226 (500-fold concentrated). The fluorescent ligand (**4.31**) was used at a concentration of 10 nM for displacement with BIBP 3226 (final concentrations: 0.03 – 3,000 nM).
For total binding 50 % DMSO in water (5 µL) and 50 % DMSO in water (5 µL) with **4.31** (100-fold concentrated) was added to the cell suspension (490 µL). For displacement of **4.31** with BIBP 3226 50 % DMSO in water (5 µL) with **4.31** (100-fold concentrated) and 50 % DMSO in water (5 µL) with BIBP 3226 (100-fold concentrated) was added to the cell suspension (490 µL). After 15 min of incubation in darkness at room temperature the samples were measured by flow cytometry using an excitation wavelength of 633 nm (red diode laser) and recording the fluorescence signals in channel Fl-4 (661 ± 18 nm). Measurements were stopped when 20,000 gated events had been counted (highest flow rate).

4.3.6 Confocal Microscopy

Two days prior to the experiment MCF-7-Y_1 cells (between 175th and 190th passage) were trypsinized and seeded in Nunc LabTek^{TM} II chambered coverglasses with 8 chambers (Nunc, Wiesbaden, Germany) in MEM containing 1 nM 17β-estradiol and 5 % FCS. On the day of the experiment confluency of the cells was 30 - 60 %. The culture medium was removed, the cells were washed twice with Leibowitz L15 culture medium (400 µL) and covered with L15 medium (240 µL). L15 medium (80 µL) and L15 medium (80 µL) with the fluorescent probe (five fold concentrated) was added for total binding. For non-specific binding L15 medium (80 µL) with the competing agent BIBP 3226 (five fold concentrated) and L15 medium (80 µL) with the

fluorescent probe (five fold concentrated) was added. Images of total and non-specific binding were acquired after an incubation period of 6 -24 min.

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 immersion. Table 4 shows the most important settings for the detection of the investigated fluorescent ligands.

Table 4. Conditions for the detection of the fluorescent ligands **4.36**, **4.38**, **4.34**, **4.32**, **4.35**, **4.30**, **4.31**, **4.45**, **4.47** and **4.29-i2** with the Zeiss Axiovert 200 M microscope.

Compd.	Excitation (laser transmission)	Filter	Pinhole [µm]
4.36	488 nm (5.1 %)	LP 505	71
4.38	488 nm (5.1 %)	LP 505	71
4.34	488 nm (5.1 %)	LP 505	71
4.32	488 nm (5.1 %)	LP 505	71
4.35	488 nm (5.1 %)	LP 560	396
4.30	488 nm (5.1 %)	LP 650	896
4.31	488 nm (5.1 %)	LP 650	896
4.45	488 nm (5.1 %)	LP 560	240
4.47	488 nm (5.1 %)	LP 505	71
4.29-i2	633 nm (5.1 %)	LP 650	80

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Chapter 5

Y_1 Receptor Antagonistic PET Ligands

5.1 Introduction

Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) are powerful medical (molecular) imaging techniques in diagnosis. Whereas SPECT uses gamma ray emitting radionuclides such as ^{99m}Tc (decay: isomeric transition) or ^{123}I (decay: electron capture), PET is based on the use of positron emitting isotopes such as ^{18}F ($t_{1/2}$ = 109.7 min), ^{11}C ($t_{1/2}$ = 20.4 min), ^{64}Cu ($t_{1/2}$ = 12.7 h) or ^{124}I ($t_{1/2}$ = 4.18 d). During positron decay a proton is transmuted to a neutron, and a positron as well as a neutrino are emitted. Depending on the kinetic energy of the positron it can travel up to a few millimeters in water or tissue. When the positron decelerates it combines with an electron in the surrounding medium. Annihilation results in the back-to-back emission of a pair of photons (gamma, 511 keV) which are captured by the detector. This means, not the source of positron emission but the location of positron annihilation is detected. Thus, resolution in PET imaging depends not only on the quality of the detector but is also limited by the energy of the emitted positrons. For instance, ^{18}F, the most frequently used radionuclide in PET applications, emits low energy positrons (compared to other positron emitters) which travel in water a distance of less than 1 mm before annihilation. The interaction of the 511-keV gamma photons with tissue, and the implicated scattering, is less pronounced compared to gamma photons (energy: 100 - 200 keV) emitted by nuclides used for SPECT. Radioactive decay is detected in PET by coincidence registration of the gamma quanta, when they reach a scintillator material in a scanning device. For the detection of the 511-keV gamma photons, scintillation crystals are used, which are read out by photomultiplier tubes. Bismuth germanate (BGO) has been the most commonly used crystals in PET, but gadolinium oxyorthosilicate (GSO) and lutetium oxyorthosilicate (LSO) crystals are increasingly applied for the assembly of PET cameras. Compared to SPECT, positron emission tomography provides a higher resolution of acquired 3D images. The clinical adoption of PET imaging was extensively promoted in the last decade with the introduction of integrated PET/CT scanners as the combination of PET and CT shortens the duration of the scan and provides additional valuable diagnostic information for improved accuracy.

The important radionuclide ^{18}F is typically produced by proton bombardment (10 - 20 MeV) of an ^{18}O enriched water target through the ^{18}O(p,n)^{18}F nuclear reaction. It is recovered as an aqueous solution of fluoride-18 and can be easily extracted by ion exchange chromatography.

Today, PET is an important technique in nuclear medicine for diagnoses in the field of oncology, neurology and cardiology. Several powerful imaging probes are already established in clinical routine, and the development of new tracers is on-going. Most efforts were spent in the development of PET and SPECT tracers for tumor imaging. For instance, 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) constitutes a large part of the success story of PET and is extensively used for diagnosis and therapy control of cancer. [^{18}F]FDG is a substrate for glucose transporters and hexokinase and allows the measurement of glucose consumption. A number of biological characteristics of tumors such as high proliferation, angiogenesis, apoptosis, metastasis and hypoxia have been tried to be exploited by the application of appropriate nuclear probes[1]. Since a variety of receptors such as epidermal growth factor receptor[2] and receptors for regulatory peptides (e.g. somatostatin, gastrin, bombesin, gastrin-releasing peptide, neurotensin, neuropeptide Y)[3] are overexpressed on tumors, radiolabeled selective high-affinity ligands for these receptors are regarded as promising tools for cancer diagnosis. The success of these nuclear probes strictly depends on its pharmacokinetic properties, which are determined by the physicochemical profile (size, chemical structure, polarity, etc.) of the radiotracers. Rapid clearance from blood and non-target tissues, a high tumor-to-background ratio, high tumor penetration, penetration across the blood brain barrier (if required), metabolic stability and low immunogenicity are characteristics of an ideal nuclear probe. Generally, macromolecules such as proteins as well as compounds with logP values > 0 cannot fulfill these criteria. Proteins show a slow clearance from blood and non-target organs and consequently a low tumor-to-background ratio. Furthermore, they can be easily degraded by proteases / peptidases, and their immunogenic potential is high. The development of low molecular weight (< 1000 Da) PET tracers is mainly challenged by the difficulty to find the balance between required physicochemical properties (logP < 0) and sufficiently high affinity (K_i < 10 nM).
Therefore, the development of adequate radiotracers for *in vivo* imaging is a highly interdisciplinary and challenging process. A straight forward method is the optimization of endogenous compounds (small receptor ligands, enzyme substrates, peptides, antibodies) with respect to their *in vivo* stability, affinity, high specificity of binding, low unspecific uptake, fast renal excretion, etc.. Toxicity of radiotracers is often a minor problem due to the very low dosage of radiopharmaceuticals. Another issue, which has to be addressed, is the type of action of radioligands used for PET imaging. While receptor agonists induce internalization of the receptor-agonist complex, receptor antagonists are supposed to lack this ability and have not been regarded as promising tracers in oncology. However, very recently a high tumor uptake was reported for radiolabeled peptidic somatostatin receptor antagonists.[4] *In vitro* investigations revealed that the antagonists labeled a higher number of binding sites than the agonists tested.

Recently, the neuropeptide Y Y_1 receptor gained new interest since it has been reported to be expressed in different malignancies such as breast cancer[5], prostate cancer[6] and adrenal

tumors[7], and therefore proposed as a potential tumor marker.[8] Previous studies on N^{ω}-substituted derivatives of the Y_1R antagonist BIBP 3226 revealed a preference for electron-withdrawing substituents in terms of retaining or even increasing the binding affinity[9]. These findings gave reason to prepare Y_1R antagonistic, fluorinated potential PET ligands, which can, labeled with ^{18}F, potentially serve as tools for *in vivo* imaging of Y_1 receptors. Since 4-nitrophenyl 2-[^{18}F]fluoropropanoate **(**[^{18}F]NPFP**)** and *N*-succinimidyl 4-[^{18}F]fluorobenzoate **(**[^{18}F]SFB) are standard reagents for a ^{18}F-labeling, ω-amino functionalized acyl and carbamoyl linkers were introduced into the N^{ω}-position of the (*R*)-argininamide BIBP 3226, the first highly potent and selective NPY Y_1 receptor antagonist.[10] These amine precursors were amidated with 2-fluoropropionic acid and 4-fluorobenzoic acid to obtain the potential Y_1R antagonistic PET ligands, which were evaluated in terms of Y_1R antagonistic activity and affinity. Since the basicity of acylguanidines ($pK_a \approx 7 – 8$) is decreased by 4 - 5 orders of magnitude compared to guanidines ($pK_a \approx 12.5$) these compounds are anticipated to penetrate across the blood brain barrier as a considerable fraction remains uncharged under physiological conditions. This could be shown for arpromidine-derived histamine H_2 receptor agonists which include an acylguanidine moiety.[11]

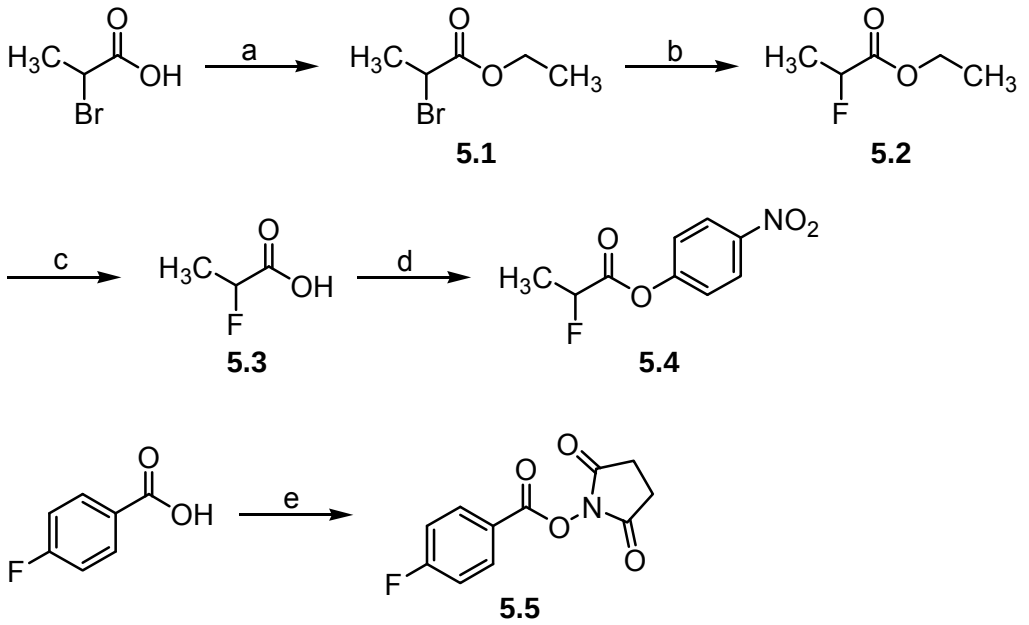
A first set of six BIBP 3226-derived Y_1R antagonistic potential PET ligands (“cold” form), was prepared by A. Brennauer.[12] In continuation of this project, further potential PET ligands with varying structures of the linkers as well as two compounds labeled with ^{18}F are presented here.

5.2 Results and Discussion

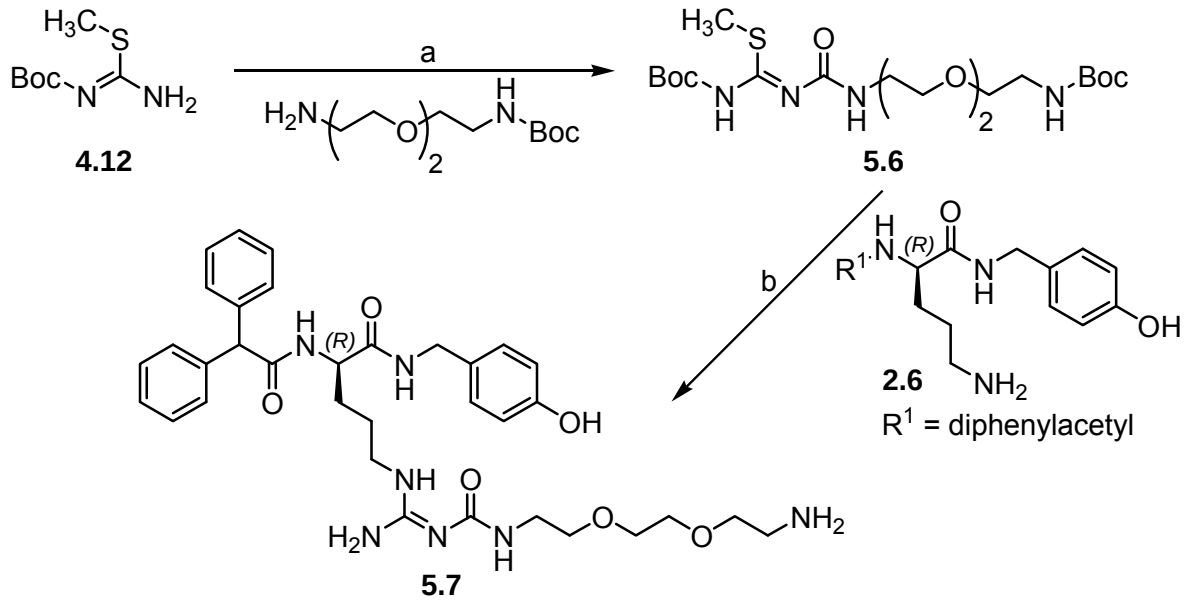
5.2.1 Chemistry

The Y_1R antagonistic potential PET ligands were prepared through 4-fluorobenzoylation and 2-fluoropropionylation of amine precursors consisting of BIBP 3226 and N^{ω}-attached amino-functionalized acyl or carbamoyl linkers (Scheme 3). The synthesis of the amine precursors, except precursor **5.7,** is described in chapter 4. Amine precursor **5.7** was prepared by analogy with the procedure described for compound **4.27** via attachment of the *N*-Boc protected linker to *S*-methylisothiourea and subsequent guanidinylation of amine **2.6** (Scheme 2). In the potential PET ligand **5.8** a linker between the guanidine group and the fluorinated carboxylic acid was omitted. Instead, the latter was directly attached to the guanidine of N^{ω}-Boc, *O-tert*-butyl protected BIBP 3226 (Scheme 3). 2-Fluoropropionic acid was prepared from 2-bromopropionic acid following cited protocols[13, 14] and was activated as 4-nitrophenyl ester[15] (Scheme 1). 4-Fluorobenzoic acid was activated as succinimidyl ester (Scheme 1). As an exception the potential PET ligand **5.21** was prepared via monoamidation of octanedioic acid with 4-

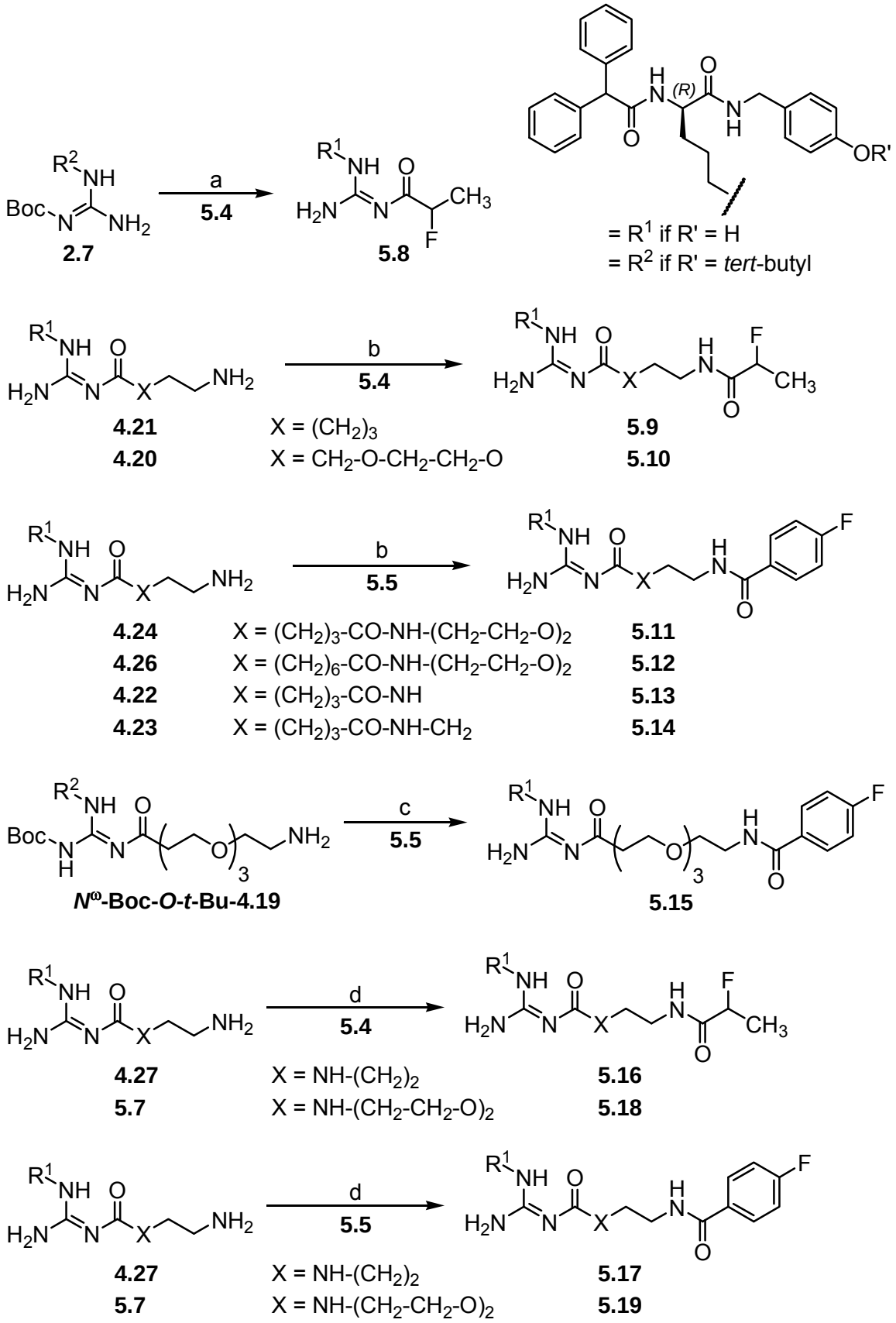
fluorobenzylamine and coupling of the resulting carboxylic acid (**5.20**) to the guanidine group of the argininamide derivative **2.7** (Scheme 4).



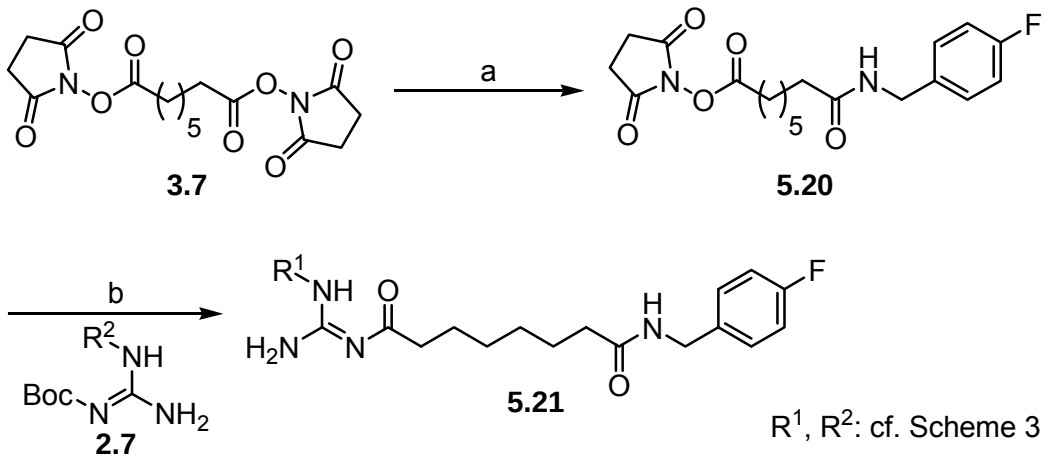
Scheme 1. Synthesis of active esters **5.4** and **5.5**.
Reagents and conditions: (a) EtOH, toluene, reflux, 5.5 h, 76 %; (b) potassium fluoride, acetamide, 125 °C, 3 h, 36 %; (c) NaOH, H_2O, EtOH, reflux, 60 min, 91 %; (d) 4-nitrophenol, dicyclohexylcarbodiimide, THF, 0 °C to rt, 20 h, 27 %; (e) *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, THF, 0 °C to rt, 20 h, 92 %.



Scheme 2. Synthesis of amine precursor **5.7**.
Reagents and conditions: (a) triphosgene, diisopropylethylamine, CH_2Cl_2, rt, 2 h, 75 %; (b) (1) $HgCl_2$, diisopropylethylamine, DMF, rt, 20 h; (2) CH_2Cl_2/TFA/H_2O 10/10/1, rt, 2 h, 50 %.

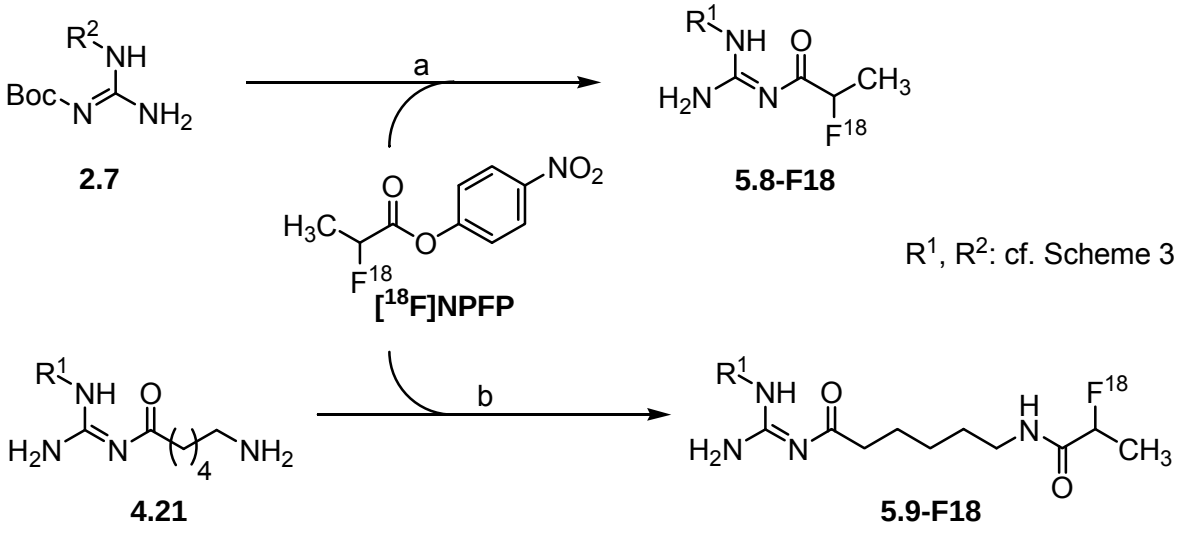


Scheme 3. Synthesis of potential PET ligands **5.8** – **5.19**.
Reagents and conditions: (a) (1) NEt_3, acetonitrile, rt, 16 h, (2) acetonitrile/TFA 1/1, 50 °C, 60 min, 84 %; (b) (1) NEt_3, acetonitrile, rt, 1 - 5 h, 48 – 81 %; (c) (1) NEt_3, CH_2Cl_2, rt, 5 h; (2) CH_2Cl_2, TFA, rt, 2 h, 50 %; (d) NEt_3, DMF, rt, 1 - 2 h, 67 – 91 %.



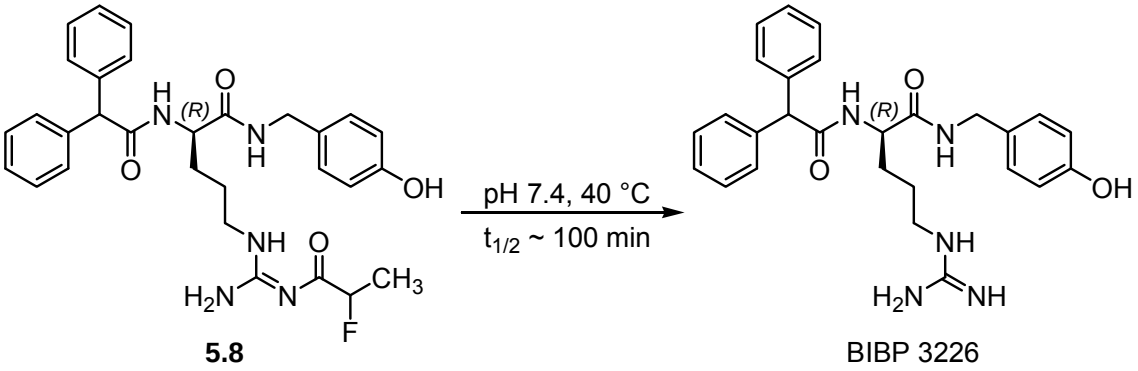
Scheme 4. Synthesis of the potential PET ligand **5.21**.
Reagents and conditions: (a) 4-fluorobenzylamine, NEt_3, CH_2Cl_2, rt, 6 h, 16 %; (b) (1) NEt_3, CH_2Cl_2, rt, 20 h, (2) CH_2Cl_2/TFA 1/1, rt, 2.5 h, 71 %.

The ^{18}F-labeled compounds **5.8-F18** and **5.9-F18** were prepared in the laboratory of Prof. Dr. H.J. Wester (Nuclear Medicine Department of the Klinikum rechts der Isar, TU München) from 4-nitrophenyl 2-[^{18}F]fluoropropionate as well as N^{ω}-Boc, *O-tert*-butyl protected BIBP 3226 (**2.7**) and amine precursor **4.21**, respectively (Scheme 5). 4-Nitrophenyl-2-[^{18}F]fluoropropionate was synthesized as described elsewhere[16] from bis-(4-nitrophenyl) carbonate and 2-fluoropropionic acid. The latter was obtained through hydrolysis of 9-anthrylmethyl 2-[^{18}F]fluoropropionate which was prepared by the treatment of 9-anthrylmethyl 2-bromopropionate with the cryptate ([K/2.2.2]$^+$/^{18}F$^-$ in acetonitrile at 95 °C for 5 min. After its production via the ^{18}O(p,n)^{18}F nuclear reaction using a cyclotron, fluoride-18 was purified by ion exchange chromatography and recovered as potassium cryptate salt to enable the nucleophilic substitution in an organic solvent (acetonitrile).



Scheme 5. Synthesis of PET ligands **5.8-F18** and **5.9-F18**.
Reagents and conditions: (a) (1) NEt_3, acetonitrile, 65 °C, 20 min, (2) acetonitrile/TFA 1/1, 70 °C, 40 min, 70 %; (b) NEt_3, acetonitrile, DMSO, 50 °C, 15 min, 60 %.

While amidation of 2-fluoropropionic acid with primary amines results in stable compounds, the 2-fluoropropionyl-guanidine entity, which is present in compound **5.8**, turned out to be chemically unstable. At pH 7.4 and 40 °C this molecule is degraded to BIBP 3226 with a half-life of about 100 min (Scheme 6, Figure 1). The decomposition takes also place in acidic media but at a considerable lower rate (Figure 1).



Scheme 6. Degradation of **5.8** to BIBP 3226. The half-life at pH 7.4 and 40 °C is about 100 min.

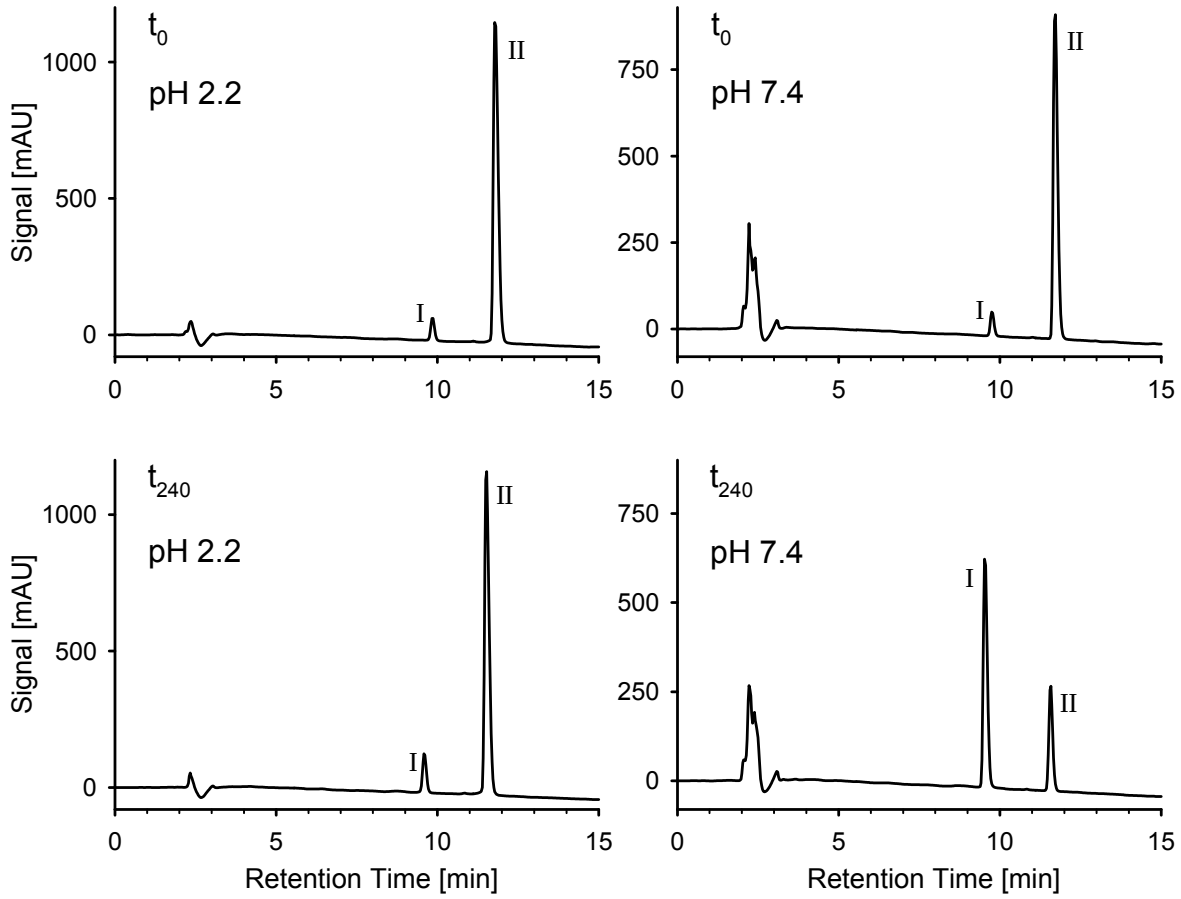


Figure 1. Degradation of compound **5.8** (II) to BIBP 3226 (I) at pH 2.2 (0.05 % aq. TFA) and pH 7.4 (phosphate buffer) at 40 °C monitored by HPLC analysis (column: Nucleodur 100-5 C18, 250 × 4 mm, 5 µm; detection: 210 nm). Depicted are the chromatograms for time points t = 0 min (t_0) and t = 240 min (t_{240}). The presence of about 6 % of BIBP 3226 at the beginning is due to a partial degradation during the work-up of **5.8**. The decomposition under acidic conditions is considerably slower compared to neutral conditions. The half-life in phosphate buffer (pH 7.4) was determined to be about 100 min (40 °C). The identity of BIBP 3226 was determined by analysis of a spiked sample.

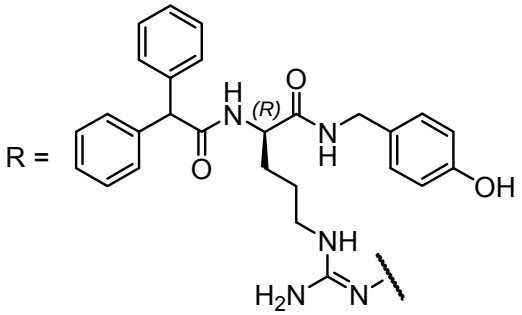
5.2.2 Y_1 Receptor Antagonism, Affinity and Selectivity

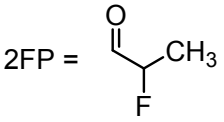
All potential Y_1R antagonistic PET ligands antagonized the NPY induced intracellular Ca^{2+}-mobilization in human erythroleukemia (HEL) cells in the nanomolar range (Table 1). Binding constants were determined by displacement of [^{3}H]-UR-MK114 (cf. chapter 2) on SK-N-MC neuroblastoma cells as well as for the three most potent compounds (**5.10**, **5.16**, **5.17**) on MCF-7 breast cancer cells. Affinities of less than 10 nM, commonly regarded as prerequisite for a successful PET imaging, were not be achieved by 4-fluorobenzoylation and 2-fluoropropionylation of various ω-aminoacylated BIBP 3226 derivatives (Table 1).

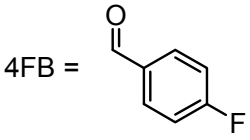
The comparison of Y_1R binding data of *N*$^{\omega}$-butanoylated BIBP 3226 (**6.2**) with its "aza analog", *N*$^{\omega}$-ethylaminocarbonyl BIBP 3226[9] (Figure 1), gave reason to introduce ω-aminocarbamoyl linkers which finally paved the way to a potential PET ligand with an affinity of about 1 nM (**5.16**, Table 1). Compound **5.16** differs from **5.9** only in the α-position of the spacer (Figure 2). The exchange of the α-methylene group by "NH" results in an increase in affinity by a factor of about 20, suggesting a binding enhancing effect of the "carbamoyl-NH". Unfortunately, the gain in affinity is mitigated with increasing size of the residues attached to the carbamoyl nitrogen as becomes obvious from compounds **5.17** – **5.19** (Table 1).

The chemically unstable Y_1R antagonist **5.8** had to be analyzed in the presence of about 8 % BIBP 3226 due to a partial degradation during the preparative work-up. Therefore, the real binding constant of *N*$^{\omega}$-2-fluoropropionylated BIBP 3226 (**5.8**) is probably above the value (K_i = 10 nM) determined in the presence of the high affinity ligand BIBP 3226 (K_i = 1.3 nM, Table 1). Nevertheless, comparing the fluoropropionyl derivative **5.8** and the corresponding non-fluorinated analog **2.8** (K_D = 1.2 nM, cf. chapter 2) the decrease in affinity induced by fluorination is remarkable. A plausible reason for this phenomenon is the lower basicity of the 2-fluoro-propionyl-guanidine moiety compared to a propionylated guanidine (calculated (ACDLABS 9.0) pK_a values: 8.1 and 6.6, resp.). Therefore the degree of protonation of compound **5.8** at physiological pH is considerably decreased and results in an attenuated key interaction of the guanidine function with Asp^{287} of the receptor protein[17].

Table 1. Structures, Y_1 receptor antagonism and binding data of BIBP 3226, amine precursors **4.21** and **4.27** as well as of potential PET ligands **5.8** - **5.19** and **5.21**.







Compd.	Structure	IC_{50} (K_b) [nM][a]	K_i[b] (K_i[c]) [nM]
BIBP 3226	R-H	10±1 (1.5)	1.3±0.2
4.21	R-CO-$(CH_2)_5$-NH_2	620±130 (95)	19±5
4.27	R-CONH-$(CH_2)_4$-NH_2	110±3 (16)	4.3±0.5
5.8	R-2FP	75±11* (12)	10±3*
5.9	R-CO-$(CH_2)_5$-NH-2FP	53±7 (8)	25±4
5.10	R-CO-CH_2-$(O$-CH_2-$CH_2)_2$-NH-2FP	510±51 (79)	15±1 (31±11)
5.11	R-CO-$(CH_2)_3$-CONH-$(CH_2)_2$-$(O$-CH_2-$CH_2)_2$-NH-4FB	930±330 (140)	37±10
5.12	R-CO-$(CH_2)_6$-CONH-$(CH_2)_2$-$(O$-CH_2-$CH_2)_2$-NH-4FB	190±10 (30)	51±11
5.13	R-CO-$(CH_2)_3$-CONH-$(CH_2)_2$-4FB	230±24 (35)	24±2
5.14	R-CO-$(CH_2)_3$-CONH-$(CH_2)_3$-4FB	220±14 (34)	64±3
5.15	R-CO-$(CH_2$-CH_2-$O)_3$-CH_2-CH_2-NH-4FB	140±5 (22)	51±5
5.16	R-CONH-$(CH_2)_4$-NH-2FP	4.3±0.2 (0.7)	1.3±0.4 (3.3±0.1)
5.17	R-CONH-$(CH_2)_4$-NH-4FB	3±0.1 (0.5)	7±2 (6.8±0.5)
5.18	R-CONH-$(CH_2)_2$-$(O$-CH_2-$CH_2)_2$-NH-2FP	130±18 (19)	19±4
5.19	R-CONH-$(CH_2)_2$-$(O$-CH_2-$CH_2)_2$-NH-4FB	23±1 (3.5)	16±5
5.21	R-CO-$(CH_2)_6$-CONH-(4-fluorobenzyl)	46±3 (7.1)	30±5

[a]Inhibition of 10 nM NPY induced $[Ca^{2+}]_i$ mobilization in HEL cells; mean values ± SEM from two independent experiments. [b/c]Dissociation constant determined from the displacement of [^{3}H]-UR-MK114 (SK-N-MC cells: K_D = 1.2 nM, MCF-7 cells: K_D = 2.9 nM) on [b]SK-N-MC and [c]MCF-7-Y_1 cells; mean values ± SEM from two independent experiments performed in duplicate or triplicate. *Determined in the presence of about 8 % BIBP 3226 (analyzed by HPLC).

The potential PET ligand **5.21** was prepared in dependence on the N^{ω}-(7-methoxycarbonyl-heptanoyl)-substituted argininamide **6.4** (Figure 2, cf. also chapter 6), a highly potent Y_1R antagonist (K_i = 0.9 nM) suggesting an affinity-enhancing effect of the methoxycarbonyl group. With respect to the preparation of a PET ligand the methoxy group in **6.4** was replaced by 4-fluorobenzylamine which is also a standard reagent for the introduction of ^{18}F. Unfortunately, this structural variation (**5.21**) resulted in a remarkable decrease in affinity by a factor of about 30 (Figure 2). It might be possible that the 4-fluorobenzyl entity hampers an interaction (hydrogen bonding) of the carbonyl oxygen with the receptor protein as it could be the case for **6.4**.

The K_b (functional assay) and K_i (binding assay) values for the Y_1R antagonists in Table 1 are generally in better accordance than the data for the series of fluorescent Y_1R ligands presented in chapter 4. Possible reasons for discrepancies between K_b and K_i values were discussed in chapter 4.

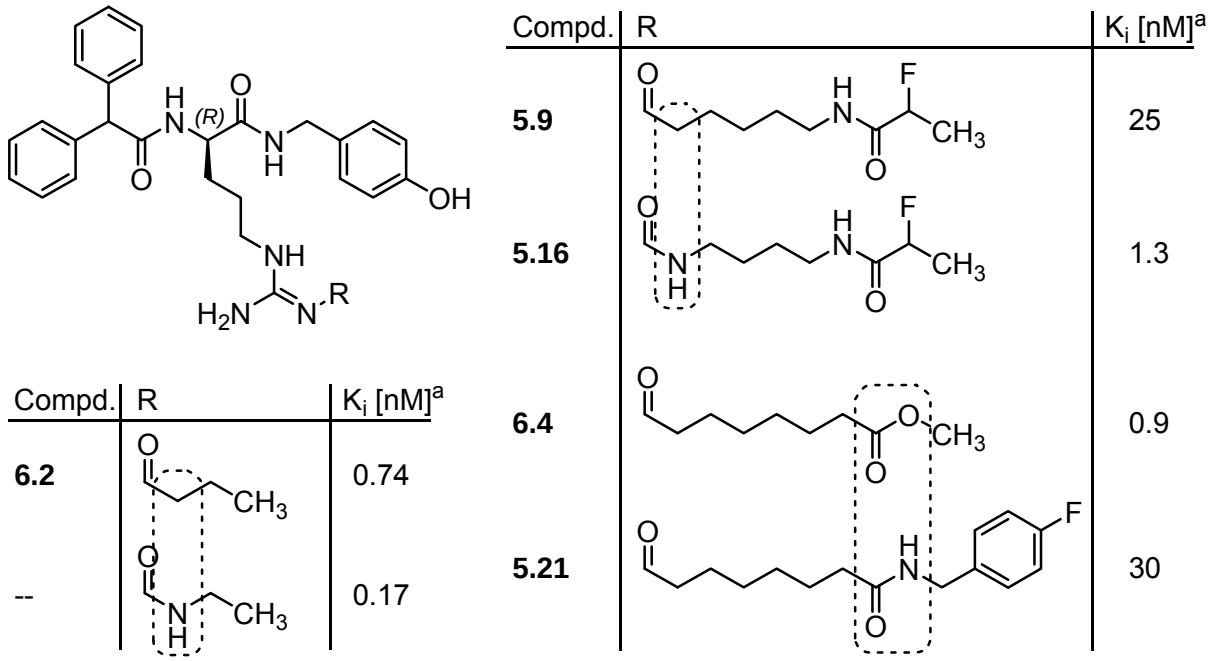


Figure 2. Structures and Y_1R affinities (SK-N-MC cells) of a selection of Y_1R antagonists derived from BIBP 3226. [a]Dissociation constant determinded with [^{3}H]-UR-MK114 as radioligand (c = 1.5 nM).

The Y_1 receptor selectivity was exemplary confirmed for antagonists **5.16** and **5.17**, the most potent potential PET ligands, using established flow cytometric binding assays based on fluorescence labeled pNPY and PP (Table 2).

Table 2. NPY receptor subtype selectivity of the potential PET ligands **5.16** and **5.17**.

Compd.	Y_1 K_i [nM][a]	Y_2 K_i [nM][b]	Y_4 K_i [nM][c]	Y_5 K_i [nM][b]
5.16	1.3	> 2,000	> 10,000	> 5,000
5.17	7	> 2,000	> 5,000	> 5,000

[a]Dissociation constant from radioligand competition assay with [^{3}H]-UR-MK114 (c = 1.5 nM) on SK-N-MC neuroblastoma cells. [b]Flow cytometric binding assay on CHO-Y_2 and HEC-1B-Y_5 cells using Dy-635-pNPY as labeled ligand (10 nM). [c]Flow cytometric binding assay on CHO-Y_4 cells with Cy5-[K^4]-hPP (5 nM) as fluorescent ligand.

5.2.3 PET and Biodistribution Experiments

Fluorine-18 labeling of precursors **2.7** and **4.21** to obtain the Y_1R antagonistic PET ligands **5.8-F18** and **5.9-F18** (Scheme 5) was performed in the laboratory of Prof. Dr. H.J. Wester at the Nuclear Medicine Department of the Klinikum rechts der Isar, TU München. Both PET ligands were administered intravenously to male NMRI (nu/nu) mice with SK-N-MC xenografts in the flank to perform biodistribution experiments as well as 60-min PET scans with small animal PET cameras. The chemical instability of compound **5.8** (Scheme 6, Figure 1) was known at the time

of the preparation of the hot analog **5.8-F18**, but an alternative PET ligand was not yet available, when this proof-of-concept study was started.

After injection of PET ligand **5.8-F18** most of the radioactivity was detected over the whole scan (60 min) in a part of the intestines, probably the cecum (Figure 3, A + B).

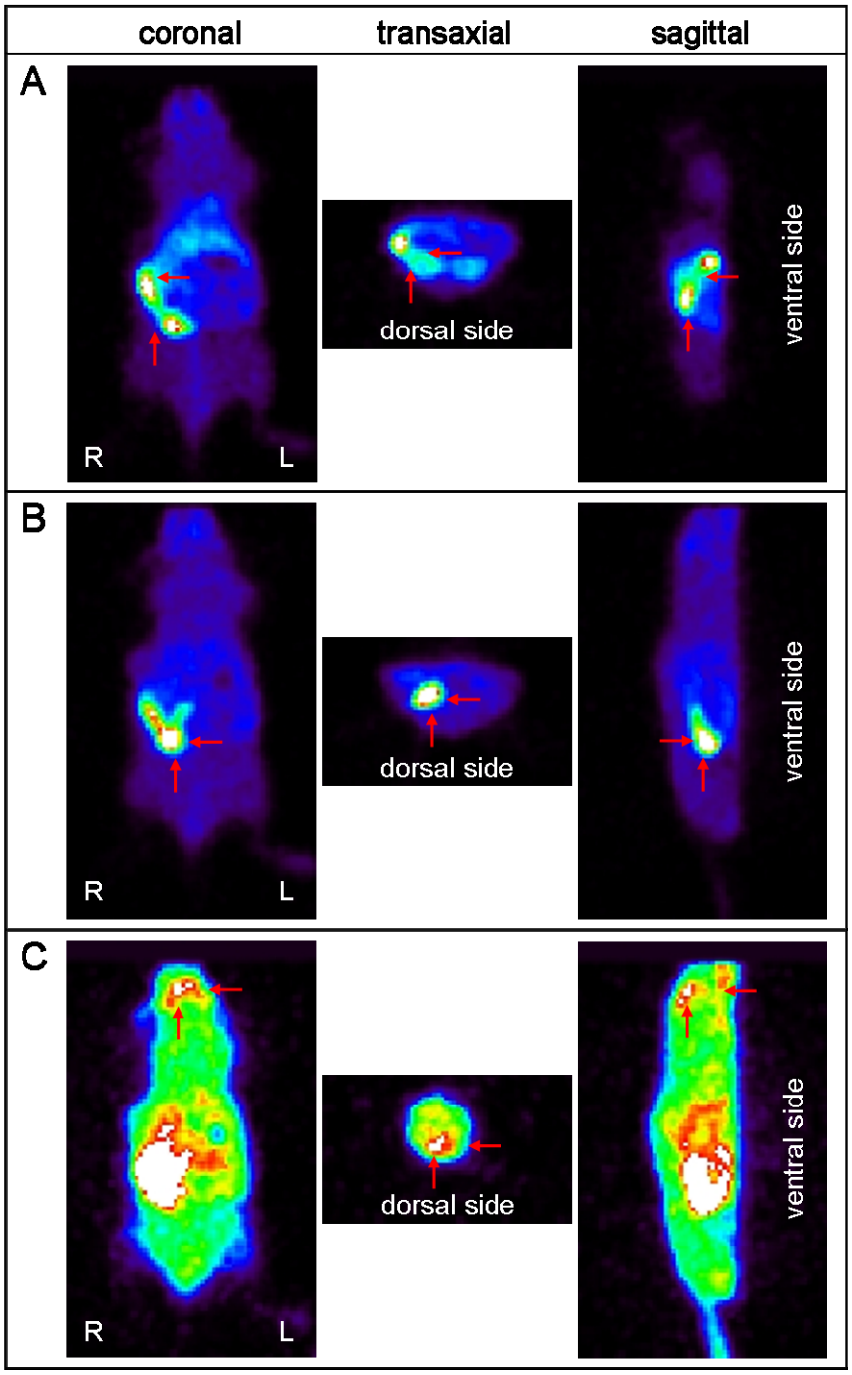


Figure 3. PET images (color table: NIH + white) of a male NMRI (nu/nu) mouse bearing a subcutanous SK-N-MC xenograft in the right flank after intravenous (tail vein) injection of the ^{18}F-labeled Y_1R antagonist **5.8-F18**. The PET scan was started 5 min after injection and images were acquired as six 5-min frames and three 10-min frames. Sets of corresponding coronal, sagittal and transaxial sections (planes are indicated by red arrows) are shown for the first (A) and last (B + C) frame of the scan. Already 5 min after injection most radioactivity was detected in a part of the gastrointestinal tract, probably in the cecum, where it was retained for more than 60 min. An accumulation of radioactivity in the brain was clearly detectable in the last frame (55 – 65 min after injection) (C, image depicted with lower threshold). An accumulation of radioactivity in the SK-N-MC xenograft in the right flank was not observed.

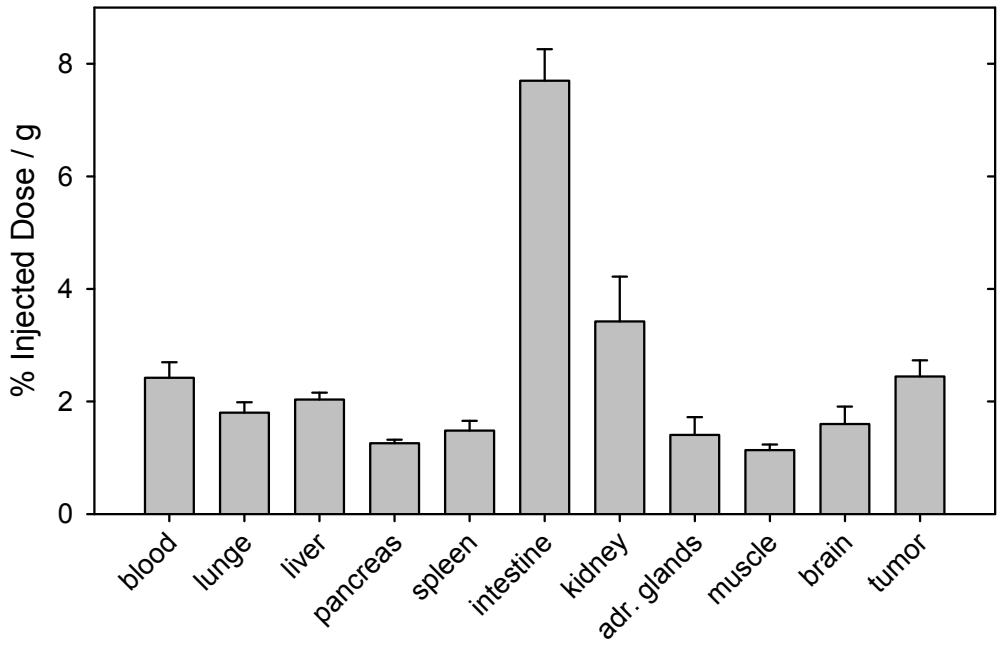


Figure 4. Biodistribution of **5.8-F18** in NMRI (nu/nu) mice bearing subcutaneous SK-N-MC xenografts 30 min after injection of the PET ligand (n = 3).

Compound **5.9-F18** and potential metabolites, respectively, were excreted either to the gall and urinary bladder as becomes obvious from Figure 5A and Figure 6. In both cases the administration of the PET ligand resulted in a moderate accumulation of radioactivity in the brain (examplary shown for **5.8-F18** in Figure 1C).

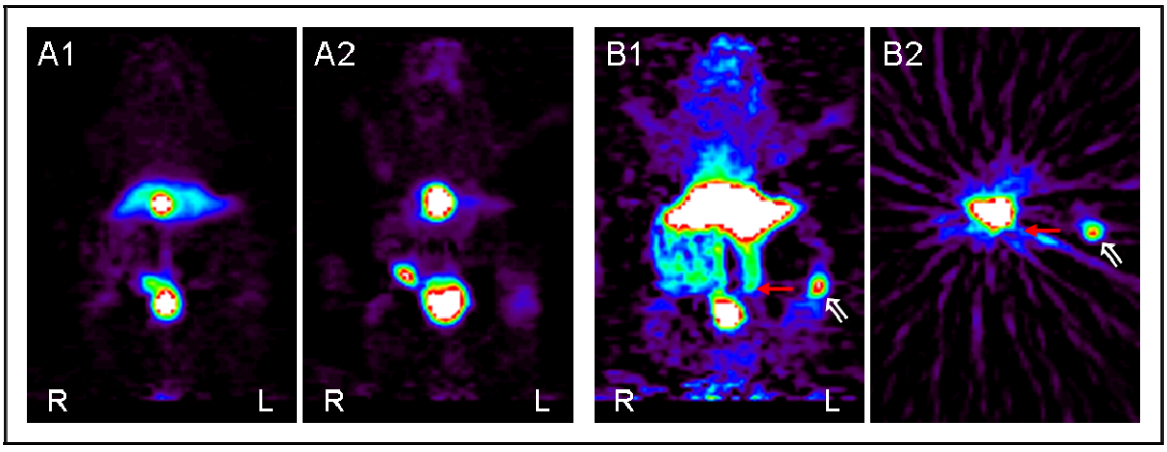


Figure 5. PET images (color table: NIH + white) of a male NMRI (nu/nu) mouse bearing a subcutanous SK-N-MC xenograft in the left flank after intravenous (tail vein) injection of the ^{18}F-labeled Y_1R antagonist **5.9-F18**. A 60-min PET scan was started 10 min after injection and images were acquired as 5-min frames. A1: Coronal PET image of frame 2 (15 – 20 min after injection). A2: Coronal PET image of frame 12 (65 – 70 min after injection). B1: Coronal PET image of frame 2 at lower threshold; B2: Corresponding transaxial image (planes are indicated by red arrows). The biliary and renal excretion of **5.9-F18** and metabolites, respectively, becomes obvious from images A1 and A2 (highest intensity in the gall and urinary bladder). The SK-N-MC tumor (open arrow) could be visualized only in the first three frames (B1 and B2 show images of frame 2).

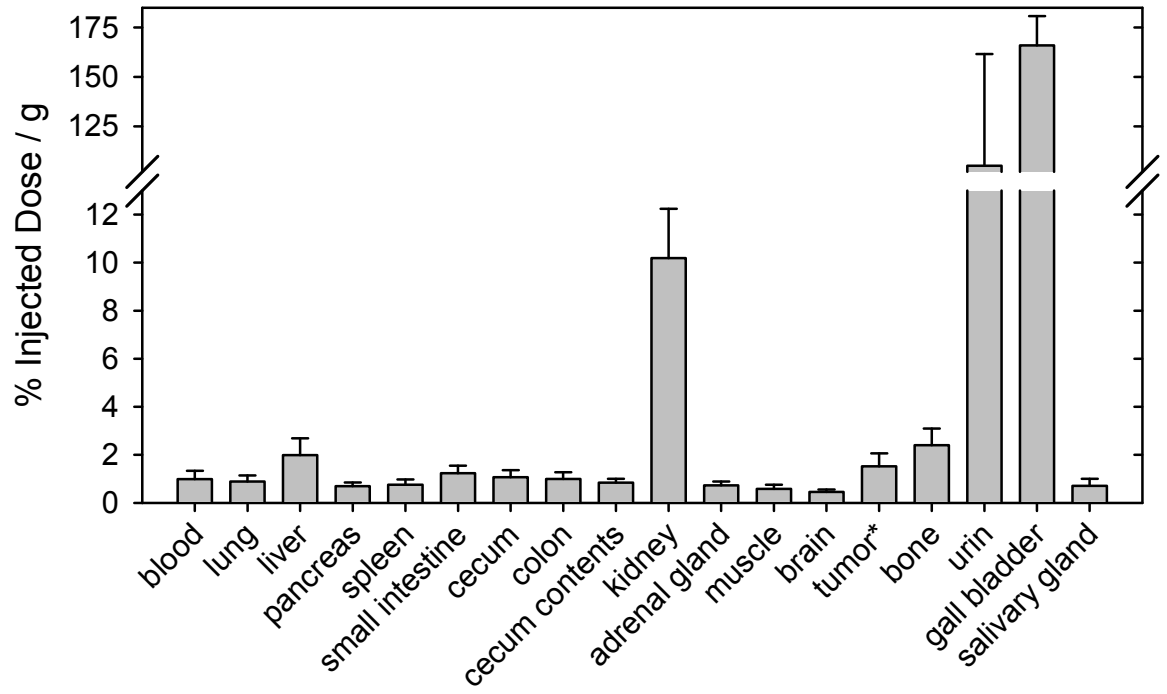


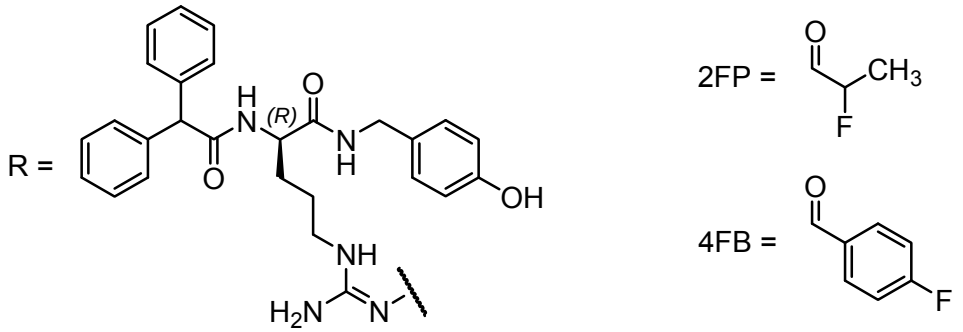
Figure 6. Biodistribution of **5.9-F18** in NMRI (nu/nu) mice with and without subcutaneous SK-N-MC xenografts 90 min after injection of the PET ligand (n = 4, *n = 2).

Whereas imaging of the Y_1R expressing SK-N-MC tumor was sucessful in the early phase of the PET scan after injection of **5.9-F18** (Figure 5B), the xenograft could not be detected after administration of **5.8-F18**.

While biodistribution data correlate well with the PET images in case of the experiments with PET ligand **5.9-F18** (Figure 6), the amount of radioactivity in the intestine after administration of **5.8-F18** appears to be too low compared to the PET images (Figures 3 and 4). Unfortunately, the radioactivity was determined in the whole gastrointestinal (GI) tract and not in individual organ segments. As the activity was normalized by the weight of the sample, potential local enrichment of activity inside the GI tract was not resolved in biodistribution studies with **5.8-F18** (Figure 4).

As already mentioned in the introduction, most PET ligands, which enable tumor imaging, have logP values < 0. Calculated logP values of the presented potential PET ligands are in the range of 1 - 4 indicating a too low water solubility of these compounds (Table 3). 4-Fluorobenzoylation leads to a considerable higher lipophilicity compared to 2-fluoropropionylation as becomes obvious from the logP values computed for compounds which differ only in the fluorinated acyl moiety (**5.16** and **5.17** as well as **5.18** and **5.19**, Table 3).

Table 3. Structures and computed logP values of BIBP 3226 and the potential PET ligands **5.8** - **5.19** as well as **5.21** for the depicted tautomeric forms.



Comp.	Structure	clogP[a]	logP[b]
BIBP 3226	R-H	1.3	1.9±0.9
5.8	R-2FP	1.8	2.8±0.8
5.9	R-CO-$(CH_2)_5$-NH-2FP	2.1	2.5±0.9
5.10	R-CO-CH_2-(O-CH_2-CH_2)$_2$-NH-2FP	1.6	1.6±1
5.11	R-CO-$(CH_2)_3$-CONH-$(CH_2)_2$-(O-CH_2-CH_2)$_2$-NH-4FB	--	3.1±1
5.12	R-CO-$(CH_2)_6$-CONH-$(CH_2)_2$-(O-CH_2-CH_2)$_2$-NH-4FB	--	4.2±1
5.13	R-CO-$(CH_2)_3$-CONH-$(CH_2)_2$-4FB	--	3.3±0.9
5.14	R-CO-$(CH_2)_3$-CONH-$(CH_2)_3$-4FB	--	3.5±0.9
5.15	R-CO-(CH_2-CH_2-O)$_3$-CH_2-CH_2-NH-4FB	--	3.3±1
5.16	R-CONH-$(CH_2)_4$-NH-2FP	1.4	2.0±0.9
5.17	R-CONH-$(CH_2)_4$-NH-4FB	3.0	4.4±0.9
5.18	R-CONH-$(CH_2)_2$-(O-CH_2-CH_2)$_2$-NH-2FP	--	1.3±1
5.19	R-CONH-$(CH_2)_2$-(O-CH_2-CH_2)$_2$-NH-4FB	--	3.5±0.9
5.21	R-CO-$(CH_2)_6$-CONH-(4-fluorobenzyl)	--	4.5±0.8

[a]calculated with ChemDraw Ultra 11.0; [b]calculated with ACDLABS 9.0; no entry: calculation was not possible with the respective software

Protonation of the basic guanidine group under physiological conditions leads to an increase in water solubility, but this is insufficient to prevent a biliary excretion of the compounds presented in this chapter. The partial renal excretion of PET ligand **5.9-F18** can be attributed to a partial metabolic conversion of this compound, which should be investigated e.g. by thin layer chromatography analysis of the urine of the mouse in future studies.

As these PET and biodistribution experiments are preliminary studies, co-administration of a non-labeled Y_1R antagonist to prevent specific binding of the tracer was not performed. This kind of experiment was envisaged in the case of successful tumor imaging. Therefore, it is not possible to state that the accumulation in the brain (Figure 3C) or the visualization of the tumor (Figure 5B) was Y_1 receptor mediated.

The ^{18}F-labeled analog of compound **5.17** (K_i = 7 nM) was synthesized as the third PET ligand of this series in the research laboratories of Bayer Schering Pharma AG (Department of Oncology Imaging Research, Berlin) by acylation of amine precursor **4.27** with *N*-succinimidyl 4-[^{18}F]fluorobenzoate. Unfortunately, the preparation of the hot analog of the 2-fluoropropionylated

high affinity ligand **5.16** (K_i = 1.3 nM) was impossible since 4-nitrophenyl-2-[^{18}F]fluoropropionate is only routinely used in Munich. PET ligand **5.17-F18** was prepared and administered intravenously to estrogen substituted nude mice with subcutaneous Y_1R-expressing MCF-7-Y_1 tumors (for tumor induction same MCF-7-Y_1 cells as used in previous studies in our laboratory were provided, cf. chapter 2). The experiment revealed a strong hepatobiliary excretion pattern of this compound, and an accumulation in the tumor was not observed (20-min PET scans were started 40 min after injection, n = 3). This can be explained by the unfavorable physicochemical properties of this compound, which is even more lipophilic than the PET ligand **5.9-F18** (ACDLABS 9.0 computed logP values: 2.5 and 4.4, resp.).

5.2.4 Summary and Outlook

Based on the (*R*)-argininamide BIBP 3226 a series of potential antagonistic Y_1R PET ligands with affinities in the lower nanomolar range was synthesized ("cold ligands"). Two compounds were prepared as prototype ^{18}F-labeled PET ligands (Scheme 5) and administered intravenously to nude mice bearing SK-N-MC xenografts in the flank. One of these PET ligands (**5.9-F18**) with an affinity of 25 nM enabled the imaging of the subcutaneous SK-N-MC tumor (Figure 5B) in the early stage (up to 25 min) after administration, but a persistent tumor uptake enabling a late imaging after systemic clearance of the tracer was not observed.

A major drawback of the presented potential PET ligands is the low water solubility (calculated logP values in the range of 1 – 4, cf. Table 3) which is the reason for a rapid biliary excretion *in-vivo*. Compound **5.16** is the most promising potential PET ligand. The Y_1R affinity of **5.16** is about 15 times higher and the compound is moderately more polar compared to PET ligand **5.9-F18**, which already enabled a tumor imaging in a preliminary PET experiment (Figure 5B).

Using MCF-7 breast cancer cells instead of SK-N-MC xenografts, a refinened tumor model for Y_1R PET imaging, was developed. MCF-7 cells show a higher Y_1 receptor expression than SK-N-MC cells (cf. chapter 2). Subcutaneous MCF-7 xenografts can be established in estrogen substituted NMRI (nu/nu) mice (cf. appendix). In addition to these achievements in terms of an appropriate in vivo model, PET ligands with improved pharmacokinetic properties are required with respect to the continuation of this project. As such compounds should be more polar than those used in the preliminary studies, the incorporation of hydroxyl groups, sugar moieties, or basic centers (e.g. secondary amines) into the linker seems a promising strategy, whereby the latter structural variation may be best to retain receptor affinity (cf. chapter 6). Such potential PET ligands with improved physicochemical properties and retained affinity (single-digit nM range) will be useful molecular tools – in particular to answer the question if GPCR antagonists in general can serve as PET ligands for an (improved) tumor imaging.

5.3 Experimental Section

5.3.1 General Experimental Conditions

Unless otherwise noted, chemicals and solvents were purchased from commercial suppliers and used without further purification. Dy-635-pNPY was synthesized as described previously.[18] [K^4]-hPP was synthesized in the laboratory of Prof. Beck-Sickinger (University of Leipzig, Germany) and labeled with the cyanine dye Cy5 (FEW chemicals (Bitterfeld-Wolfen, Germany) in our laboratory.[19] Porcine NPY (pNPY) was prepared in-house in the laboratory of Dr. C. Cabrele. Millipore water was used throughout for the preparation of buffers and HPLC eluents. Petroleum ether (40-60 °C) was distilled before use. DMF was stored over molecular sieves (3 Å). Anhydrous reactions were run under an atmosphere of dry nitrogen or argon.

Thin layer chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates. For column chromatography silica gel Geduran 60 (Merck, 0.063-0.200 mm) was used. NMR spectra were recorded on a Bruker Avance 300 (1H: 300 MHz), a Bruker Avance 600 (1H: 600 MHz) and a Bruker Avance III 600 with cryogenic probehead (1H: 600 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. Mass spectrometry analysis (MS) was performed in-house on a Finnigan ThermoQuest TSQ 7000 (ES-MS), a Finnigan SSQ 710A (EI-MS 70 eV, CI-MS) and a Finnigan MAT 95 (LSIMS, HRMS). Melting points were determined with a Büchi 510 melting point apparatus and are uncorrected. Lyophilization was done with a Christ alpha 2-4 LD equipped with a vacuubrand RZ 6 rotary vane vacuum pump.

Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18 (20 × 21 mm, 5 µm; Macherey-Nagel, Germany) and a Eurospher-100 C18 (250 × 32 mm, 5 µm; Knauer, Germany) served as RP-columns at flow rates of 24 and 38 mL/min, respectively. Mixtures of acetonitrile and 0.1 % aq. TFA were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure (final pressure: 60 mbar) at 40 °C prior to lyophilization. Analytical HPLC analysis was performed on a system from Thermo Separation Products (composed of a SN400 controller, a P4000 pump, a degasser (Degassex DG-4400, phenomenex), an AS3000 autosampler and a Spectra Focus UV-VIS detector). An Eurospher-100 C18 (250 × 4 mm, 5 µm, Knauer, Germany) or a Nucleodur 100-5 C18 ec (250 × 4 mm, 5 µm, Macherey-Nagel, Germany) served as RP-columns. Mixtures of acetonitrile (A) and 0.05 % aq. TFA (B) were used as mobile phase. Helium degassing, an oven temperature of 30 °C and a flow rate of 0.8 mL/min were used throughout. Solutions for injection (concentrations in the two-digit µM range) were prepared in a mixture of A and B corresponding to the composition at the beginning of the gradient. The following gradients were applied for analytical HPLC analysis:

Gradient 1: 0 to 25 min: A/B 25/75 to 65/35, 25 to 27 min: 65/35 to 95/5, 27 to 31 min: 95/5

Gradient 2: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5

Purification and purity controls of F-18 labeled compounds was done on a HPLC system from Sykam GmbH (Gilching, Germany) consisting of a gradient pump S 1021, a mixer S 8111 and a controller S 2000. UV detection was performed with a UVIS detector 200 photometer (Linear TM. Instruments Corporation, Reno, USA) and radiometric detection was done with a Wallac EGεG Ortec Ace Mate 925-Scint. Data were analyzed with Winnie-Software (Raytest, Straubenhardt, Germany).

The preparation of the buffer and the lysis solution for Y_1R binding studies on SK-N-MC cells and MCF-7 cells as well as the loading buffer for the determination of the mobilization of intracellular Ca^{2+} in HEL cells are described in chapter 3.
5 mM stock solutions for HPLC purity controls were prepared in acetonitrile with an additive (5 or 10 %) of 0.1 % aq. TFA. Stock solutions for pharmacological studies were prepared in DMSO at concentrations of 10 mM and stored at –78 °C. Tritium Counting was done in 3 mL of a liquid scintillator (Rothiszint^{TM} eco plus) with a Beckman LS 6500 beta-counter.

5.3.2 Chemistry: Experimental Protocols and Analytical Data

Ethyl 2-bromo-propanoate (5.1).[13] 2-Bromopropanoic acid (28.9 g, 17 mL, 189 mmol) and 2.5 g of a cation exchanger (Dowex 50 X8, Fluka) were added to a mixture of toluene (100 mL) and ethanol (25 mL). Azeotropic esterification was performed under reflux using a Dean-Stark distilling trap. After 1 h of reflux about 10 mL of the lower layer were removed from the trap. Reflux was continued for 1 h and cation exchanger (2.5 g) and ethanol (25 mL) were added. After 1.5 h of reflux again 10 mL of the lower layer were drained off. The reaction was finished after 5.5 h (total time) of reflux. The ion exchanger was removed by filtration and the volume of the filtrate was reduced to about 30 mL under reduced pressure. The product was isolated as a colorless liquid (bp 66 °C, 30 mbar) using vacuum distillation (26.1 g, 144 mmol, 76 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.3 (t, 3H, ^3J = 7.13 Hz, C<u>H</u>$_3$-CH$_2$), 1.82 (d, 3H, ^3J = 6.94 Hz, C<u>H</u>$_3$-CHBr), 4.23 (dq, 2H, ^3J = 7.11 Hz, ^2J = 1.01 Hz, CH$_3$-C<u>H</u>$_2$), 4.35 (q, 1H, ^3J = 6.84 Hz, C<u>H</u>Br); $C_5H_9BrO_2$ (181.0)

Ethyl 2-fluoropropanoate (5.2).[20] Dry potassium fluoride (45 g) and dry acetamide (40 g) were mixed in a 250-mL three necked round bottom flask equipped with a mechanical stirrer. The mixture was warmed up to 125 °C (bath temperature), **5.1** (25 g, 138 mmol) was added and stirring was continued at 125 °C for 3 h. The mixture was cooled down to room temperature and water (75 mL) was added yielding a suspension (consisting of 2 liquid phases) which was treated with ultrasound. The liquids were separated from the solid by decantation and the solid was washed with water (25 mL). The washing was combined with the liquid phases and the

product was extracted with ethyl ether (4 × 50 mL). The extracts were pooled, dried over sodium sulfate and filtered. Ethyl ether was removed under reduced pressure prior to vacuum distillation which afforded the product (bp 57 °C at 100 mbar) as a colorless liquid (6 g, 50 mmol, 36 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.31 (t, 3H, ^{3}J = 7.14 Hz, C<u>H</u>$_3$-CH_2), 1.57 (dd, 3H, ^{3}J = 23.62 Hz, ^{3}J = 6.87 Hz, C<u>H</u>$_3$-CHF), 4.25 (q, 2H, ^{3}J = 7.14 Hz, CH_3-C<u>H</u>$_2$), 4.99 (dq, 1H, ^{2}J = 46.68 Hz, ^{3}J = 6.87 Hz, C<u>H</u>F); $C_5H_9FO_2$ (120.1)

2-Fluoropropanoic acid (5.3).[14, 21] Sodium hydroxide (4.5 g, 112.5 mmol, 2.3 eq) was dissolved in water (13 mL) and **5.2** (5.9 g, 49.1 mmol, 1 eq) was added together with ethanol (13 mL). The mixture was heated under reflux for 60 min, cooled down to room temperature, and concentrated to a volume of about 10 mL under reduced pressure. Water (4.5 mL) and concentrated hydrochloric acid (9 mL) were added and the product was extracted with ethyl ether (6 × 15 mL). The extracts were pooled, dried over sodium sulfate and filtered. Ethyl ether was removed under reduced pressure prior to vacuum distillation which afforded the product (bp 86 °C at 40 mbar) as a colorless liquid (4.1 g, 44.5 mmol, 91 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.64 (dd, 3H, ^{3}J = 23.45 Hz, ^{3}J = 6.96 Hz, CH_3), 5.07 (dq, 1H, ^{2}J = 48.51 Hz, ^{3}J = 6.96 Hz, CHF); $C_3H_5FO_2$ (92.1)

4-Nitrophenyl 2-fluoropropanoate (5.4).[22] DCC (2.3 g, 10.8 mmol, 1 eq) was added to a stirred ice-cold solution of **5.3** (1 g, 10.8 mmol, 1 eq) and 4-nitrophenol (1.65 g, 11.9 mmol, 1.1 eq) in anhydrous THF (40 mL). The ice-bath was removed after 30 min and stirring was continued at rt overnight. The white solid (DCU) was removed by filtration and the solvent was removed under reduced pressure. Purification with column chromatography (eluent: PE/EtOAc 9/1 to 3/1) and subsequent crystallization from ethanol yielded the product as a pale yellow crystalline solid (0.62 g, 2.9 mmol, 27 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.78 (dd, 3H, ^{3}J = 23.41 Hz, ^{3}J = 6.91 Hz, CH_3), 5.28 (dq, 1H, ^{2}J = 48.12 Hz, ^{3}J = 6.91 Hz, CHF), 7.39 (m, 2H, Ar), 8.31 (m, 2H, Ar); MS (EI): *m/z* 213 ($M^{\cdot+}$); $C_9H_8FNO_4$ (213.2)

Succinimidyl 4-fluorobenzoate (5.5).[23] DCC (4.54 g, 22 mmol, 1.1 eq) was added to a stirred ice-cold solution of 4-fluorobenzoic acid (2.8 g, 20 mmol, 1 eq) and *N*-hydroxysuccinimide (2.53 g, 22 mmol, 1.1 eq) in anhydrous THF (60 mL). The ice-bath was removed after 60 min and stirring was continued at rt overnight. The white solid (DCU) was removed by filtration and the solvent was removed under reduced pressure. Purification with column chromatography (eluent: PE/EtOAc 5/1 to 1/1) yielded the product as white solid (4.37 g, 18.4 mmol, 92 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 2.9 (s, 4H, CH_2), 7.19 (m, 2H, Ar), 8.16 (m, 2H, Ar); MS (EI): *m/z* 237 ($M^{\cdot+}$); $C_{11}H_8FNO_4$ (237.2)

***N*-*tert*-Butoxycarbonyl-*N'*-[8-(*tert*-butoxycarbonylamino)-3,6-dioxaoctyl]aminocarbonyl-*S*-methylisothiourea (5.6).** The reaction was carried out in an argon purged 50 mL two-necked round bottom flask equipped with a pressure equalizing addition funnel. The flask and the funnel were baked out in a nitrogen atmosphere prior to the reaction. A solution of *N*-Boc-3,6-dioxaoctyl-1,8-diamine (500 mg, 2.01 mmol, 1 eq) and diisopropylethylamine (729 mg, 5.64 mmol, 2.8 eq) in anhydrous CH_2Cl_2 (10 mL) was added dropwise to a solution of triphosgene (299 mg, 1.0 mmol, 0.5 eq) in anhydrous CH_2Cl_2 (10 mL) over a period of 30 min. *N*-Boc-*S*-methylisothiourea (575 mg, 3.02 mmol, 1.5 eq) was added and stirring was continued for 1.5 h. The volume was reduced under reduced pressure to about 6 mL and the mixture was directly subjected to column chromatography (CH_2Cl_2/EtOAc 10/1 to 3/1). Highly viscous yellow oil (671 mg, 1.44 mmol, 72 %); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.36 (m, 9H, *tert*-Bu, 1.44 (m, 9H, *tert*-Bu), 2.28 (s, 3H, C$\underline{H}_3$), 3.04 (m, 2H, C$\underline{H}_2$-NH), 3.19 (m, 2H, C$\underline{H}_2$-NH), 3.36 (t, 2H, ^{3}J = 6.91 Hz, O-C$\underline{H}_2$-CH$_2$-NH), 3.44 (t, 2H, ^{3}J = 5.95 Hz, O-C$\underline{H}_2$-CH$_2$-NH), 3.49 (s, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 6.76 (t, 1H, ^{3}J = 5.42 Hz, CH$_2$-N$\underline{H}$), 7.77 (t, 1H, ^{3}J = 5.86 Hz, CH$_2$-N$\underline{H}$), 12.34 (s, 1H, N$\underline{H}$); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 465 $[M + H]^+$; $C_{19}H_{36}N_4O_7S$ (464.6)

(*R*)-N^{ω}-(8-Amino-3,6-dioxaoctyl)aminocarbonyl-N^{α}-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide (5.6). Compound **5.5** (250 mg, 0.54 mmol, 1.1 eq) and **2.6** (239 mg, 0.49 mmol, 1 eq, cf. chapter 2) were dissolved in anhydrous DMF (12 mL). $HgCl_2$ (200 mg, 0.73 mmol, 1.5 eq) and diisopropylethylamine (127 mg, 0.98 mmol, 2 eq) were added and the mixture was stirred at rt overnight. The solvent was removed under reduced pressure as far as possible (final pressure: 1 mbar, 43 °C) and the intermediate was purified by column chromatography (CH_2Cl_2/EtOAc 2/1 to 1/2) prior to deprotection with CH_2Cl_2/TFA/H_2O 10/10/1 (4 mL) for 2 h. CH_2Cl_2 (20 mL) was added three times, each time followed by evaporation under reduced pressure. Uptake of the green oily residue in water (20 mL) and lyophilization afforded the product as a heterogeneously colored (white/green) fluffy solid which turned into a green oil about 1 min after contact with air. The product was purified with preparative HPLC yielding a white fluffy solid (212 mg, 0.24 mmol, 50 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.32-1.6 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.67 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 2.97 (m, 2H, C$\underline{H}_2$-NH$_2$), 3.2 (m, 2H, C$\underline{H}_2$-NH), 3.28 (m, 2H, C$\underline{H}_2$-NH), 3.48 (t, 2H, ^{3}J = 5.55 Hz, O-C$\underline{H}_2$-CH$_2$-NH), 3.57 (m, 6H, C$\underline{H}_2$-O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.15 (m, 2H, C$\underline{H}_2$-ArOH), 4.33 (m, 1H, CH$^{\alpha}$), 5.13 (s, 1H, C$\underline{H}$-(Ph)$_2$), 6.66 (d, 2H, ^{3}J = 8.51 Hz, AA'BB'), 7.0 (d, 2H, ^{3}J = 8.52 Hz, AA'BB'), 7.18-7.33 (m, 10H, Ph), 7.51 (t, 1H, ^{3}J = 5.31 Hz, CO-N$\underline{H}$-CH$_2$), 7.79 (bs, 3H, N$\underline{H}_3^+$), 8.38 (t, 1H, ^{3}J = 5.77 Hz, CO-N$\underline{H}$-CH$_2$), 8.43 (bs, 1H, N$\underline{H}$), 8.5 (d, 1H, ^{3}J = 8.08 Hz, CO-N$\underline{H}$-CH), 8.96 (s, 1H, N$\underline{H}$), 9.31 (s, 1H, N$\underline{H}$), 10.15 (s, 1H, ArO$\underline{H}$); MS (ES, MeOH + 10 mM NH_4OAc): *m/z* 648 $[M + H]^+$, 324.5 $[M + 2H]^{2+}$; $C_{34}H_{45}N_7O_6 \times C_4H_2F_6O_4$ (875.8)

(*R*)-N^{α}-(2,2-Diphenylacetyl)-N^{ω}-(2-fluoropropanoyl)-*N*-(4-hydroxybenzyl)argininamide (5.8). Compound **2.7** (90 mg, 143 µmol, 1.05 eq, cf. chapter 2) was added to a solution of **5.4** (29 mg, 136 µmol, 1 eq) in dry acetonitrile (3 mL). NEt_3 (29.2 mg, 40 µL, 290 µmol, 2 eq) was added and the mixture was stirred at rt for 16 h. TFA (3 mL) was added and stirring was continued at 50 °C for 1 h. MeOH (5 mL) was added twice, each time followed by evaporation under reduced pressure. Purification with preparative HPLC (column: Nucleodur 250 × 21 mm) and lyophilization afforded the product as a white fluffy solid (75.6 mg, 114 µmol, 84 %). Mp 126-128 °C; ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) 1.34-1.61 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.53 (dd, 3H, ^3J = 24.7 Hz, ^3J = 6.74 Hz, CH_3), 1.68 (m, 1H, CH-C$\underline{H}_2$-CH_2), 3.25 (m, 2H, CH_2-C$\underline{H}_2$-NH), 4.12 (dd, 1H, ^2J = 14.96 Hz, ^3J = 5.73 Hz, C$\underline{H}_2$-ArOH), 4.18 (dd, 1H, ^2J = 15.17 Hz, ^3J = 5.79 Hz, C$\underline{H}_2$-ArOH), 4.34 (m, 1H, CH^{α}), 5.13 (s, 1H, C$\underline{H}$-$(Ph)_2$), 5.33 (m, 1H, CHF), 6.67 (d, 2H, ^3J = 8.53 Hz, AA'BB'), 7.01 (d, 2H, ^3J = 8.5 Hz, AA'BB'), 7.24 (m, 2H, Ph), 7.28 (m, 8H, Ph), 8.4 (t, 1H, ^3J = 5.69 Hz, CH-CO-N$\underline{H}$-CH_2), 8.51 (d, 1H, ^3J = 8.18 Hz, CO-N$\underline{H}$-CH), 8.58 (bs, 1H, N$\underline{H}$), 8.84 (bs, 2H, N$\underline{H}_2$), 9.3 (s, 1H, N$\underline{H}$), 10.98 (s, 1H, ArO$\underline{H}$); RP-HPLC (Nucleodur 250 × 4 mm, 210 nm, gradient 1): 95 % (t_R = 11.8 min, k = 4.1); HRMS: (FAB$^+$, CH_2Cl_2/n-butylamine): *m/z* calcd. for $[C_{30}H_{34}FN_5O_4 + H]^+$ 548.2673, found: 548.2684; $C_{30}H_{34}FN_5O_4 \times C_2HF_3O_2$ (661.6)

(*R*)-N^{α}-(2,2-Diphenylacetyl)-N^{ω}-[6-(2-fluoropropanoylamino)hexanoyl]-*N*-(4-hydroxybenzyl)argininamide (5.9). Amine precursor **4.21** (78 mg, 95.7 µmol, 1.3 eq; cf. chapter 4) was dissolved in acetonitrile (5 mL). NEt_3 (18.6 mg, 25.5 µL, 184 µmol, 2.5 eq) and active ester **5.4** (15.7 mg, 73.6 µmol, 1 eq) were added and the mixture was stirred at rt for 2 h prior to the addition of a few drops of TFA. Purification with preparative HPLC (column: Nucleodur 250 × 21 mm) and lyophilization afforded the product as a white fluffy solid (30.4 mg, 39.2 µmol, 53 %). Mp > 86 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.27 (m, 2H, CH_2-CH_2-C$\underline{H}_2$-CH_2-CH_2), 1.4 (dd, 3H, ^3J = 24.49 Hz, ^3J = 6.69 Hz, CH_3), 1.4-1.49 (m, 4H, 2 × C$\underline{H}_2$-CH_2-NH), 1.56 (m, 3H, CH-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$), 1.68 (m, 1H, CH-C$\underline{H}_2$), 2.41 (t, 2H, ^3J = 7.30 Hz, CO-CH_2), 3.08 (m, 2H, C$\underline{H}_2$-NH-CO-CHF), 3.23 (m, 2H, CH-CH_2-CH_2-C$\underline{H}_2$-NH), 4.12 (dd, 1H, ^2J = 14.79 Hz, ^3J = 5.71 Hz, C$\underline{H}_2$-ArOH), 4.17 (dd, 1H, ^2J = 14.79 Hz, ^3J = 5.84 Hz, C$\underline{H}_2$-ArOH), 4.34 (m, 1H, CH^{α}), 4.96 (dq, 1H, ^2J = 49.0 Hz, ^3J = 6.69 Hz, CHF), 5.13 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.67 (d, 2H, ^3J = 8.5 Hz, AA'BB'), 7.0 (d, 2H, ^3J = 8.5 Hz, AA'BB'), 7.22 (m, 2H, Ph), 7.29 (m, 8H, Ph), 8.11 (t, 1H, ^3J = 5.01 Hz, N$\underline{H}$-CO-CHF), 8.35 (t, 1H, ^3J = 5.8 Hz, CH-CO-N$\underline{H}$-CH_2), 8.47 (d, 1H, ^3J = 8.09 Hz, CO-N$\underline{H}$-CH), 8.7 (bs, 2H, N$\underline{H}_2$), 9.17 (t, 1H, ^3J = 5.01 Hz, CH-CH_2-CH_2-CH_2-N$\underline{H}$), 9.29 (s, 1H, N$\underline{H}$), 11.73 (s, 1H, ArO$\underline{H}$); RP-HPLC (Nucleodur 250 × 4 mm, 210 nm, gradient 1): > 99 % (t_R = 12.8 min, k = 4.6); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{36}H_{45}FN_6O_5 + H]^+$ 661.3514, found: 661.3496; $C_{36}H_{45}FN_6O_5 \times C_2HF_3O_2$ (774.8)

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*$^{\omega}$-[8-(2-fluoropropanoylamino)-3,6-dioxaoctanoyl]-*N*-(4-hydroxybenzyl)argininamide (5.10). Amine precursor **4.20** (110 mg, 130 µmol, 1 eq; cf. chapter 4) was dissolved in acetonitrile (8 mL). NEt_3 (32.9 mg, 45 µL, 325 µmol, 2.5 eq) and active ester **5.4** (27.7 mg, 130 µmol, 1 eq) were added and the mixture was stirred at rt for 1 h prior to the addition of a few drops of TFA. Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as a yellowish fluffy solid which turned into a resin after contact with air (79.1 mg, 98 µmol, 75 %). ^{1}H-NMR (300 MHz, CD_3OD): δ (ppm) 1.49 (dd, 3H, ^3J = 24.33 Hz, ^3J = 6.77 Hz, CH_3), 1.51-1.78 (bm, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.85 (m, 1H, CH-C$\underline{H}_2$-CH_2), 3.26 (m, 2H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.43 (m, 2H, O-CH_2-C$\underline{H}_2$-NH), 3.58 (t, 2H, ^3J = 5.51 Hz, O-C$\underline{H}_2$-CH_2-NH), 3.72 (m, 4H, O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.22 (d, 1H, ^2J = 14.79 Hz, C$\underline{H}_2$-ArOH), 4.23 (s, 2H, CH_2-CO), 4.28 (d, 1H, ^2J = 14.79 Hz, C$\underline{H}_2$-ArOH), 4.43 (m, 1H, CH$^{\alpha}$), 4.97 (dq, 1H, ^2J = 48.84 Hz, ^3J = 6.83 Hz, CHF), 5.06 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 2H, ^3J = 8.57 Hz, AA'BB'), 7.06 (d, 2H, ^3J = 8.58 Hz, AA'BB'), 7.21-7.33 (m, 10H, Ph); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 98 % (t_R = 13.9 min, k = 4.1); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{36}H_{45}FN_6O_7 + H]^+$ 693.3412, found: 693.3395; $C_{36}H_{45}FN_6O_7 \times C_2HF_3O_2$ (806.8)

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*$^{\omega}$-{5-[8-(4-fluorobenzoylamino)-3,6-dioxaoctyl]amino-5-oxo-pentanoyl}-*N*-(4-hydroxybenzyl)argininamide (5.11). Amine precursor **4.24** (62 mg, 65.5 µmol, 1 eq; cf. chapter 4) was dissolved in acetonitrile (6 mL). NEt_3 (16.6 mg, 23 µL, 164 µmol, 2.5 eq) and active ester **5.5** (15.5 mg, 65.5 µmol, 1 eq) were added and the mixture was stirred at rt for 5 h prior to the addition of 10 % aq. TFA (corresponding to 2.5 eq of TFA). Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as a white fluffy solid (50.8 mg, 53.2 µmol, 81 %). Mp 52-54 °C; ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.42 (m, 2H, CH_2-C$\underline{H}_2$-CH_2-NH), 1.53 (m, 1H, CH-C$\underline{H}_2$), 1.66 (m, 1H, CH-C$\underline{H}_2$), 1.75 (p, 2H, ^3J = 7.38 Hz, CO-CH_2-C$\underline{H}_2$-CH_2-CO), 2.12 (t, 2H, ^3J = 7.29 Hz, CO-CH_2), 2.41 (t, 2H, ^3J = 7.39 Hz, CO-CH_2), 3.17 (m, 2H, O-CH_2-C$\underline{H}_2$-NH), 3.22 (m, 2H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.39 (m, 4H, C$\underline{H}_2$-O-CH_2-CH_2-O, O-CH_2-C$\underline{H}_2$-NH), 3.51 (m, 6H, C$\underline{H}_2$-O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.1 (dd, 1H, ^2J = 14.78 Hz, ^3J = 5.65 Hz, C$\underline{H}_2$-ArOH), 4.16 (dd, 1H, ^2J = 14.78 Hz, ^3J = 5.86 Hz, C$\underline{H}_2$-ArOH), 4.33 (m, 1H, CH$^{\alpha}$), 5.11 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.66 (d, 2H, ^3J = 8.5 Hz, AA'BB' (Ar-OH)), 6.99 (d, 2H, ^3J = 8.5 Hz, AA'BB' (Ar-OH)), 7.21 (m, 2H, Ph), 7.28 (m, 10H, Ph, CC'DD' (Ar-F)), 7.86 (t, 1H, ^3J = 5.59 Hz, O-CH_2-CH_2-N$\underline{H}$), 7.9 (m, 2H, CC'DD' (Ar-F)), 8.33 (t, 1H, ^3J = 5.83 Hz, CH-CO-N$\underline{H}$-CH_2), 8.45 (d, 1H, ^3J = 8.11 Hz, CO-N$\underline{H}$-CH), 8.51 (t, 1H, ^3J = 5.51 Hz, O-CH_2-CH_2-N$\underline{H}$), 8.53 (bs, 2H, N$\underline{H}_2$), 8.85 (s, 1H, CH_2-CH_2-CH_2-N$\underline{H}$), 9.26 (s, 1H, N$\underline{H}$), 11.26 (s, 1H, ArO$\underline{H}$); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): > 99 % (t_R = 14.7 min, k = 4.4); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{45}H_{54}FN_7O_8 + H]^+$ 840.4069, found: 840.4087; $C_{45}H_{54}FN_7O_8 \times C_2HF_3O_2$ (954.0)

(*R*)-N^{α}-(2,2-Diphenylacetyl)-N^{ω}-{8-[8-(4-fluorobenzoylamino)-3,6-dioxaoctyl]amino-8-oxo-octanoyl}-*N*-(4-hydroxybenzyl)argininamide (5.12). Amine precursor **4.26** (40 mg, 40.5 µmol, 1.1 eq; cf. chapter 4) was dissolved in acetonitrile (3 mL). NEt_3 (10.2 mg, 14 µL, 101 µmol, 2.5 eq) and active ester **5.5** (8.6 mg, 36.4 µmol, 1 eq) were added and the mixture was stirred at rt for 4 h prior to the addition of 10 % aq. TFA (corresponding to 2.5 eq of TFA) Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as a white fluffy solid which turned into a resin after contact with air (17.6 mg, 17.6 µmol, 48 %). ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.24 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-CH_2), 1.35-1.48 (m, 4H, CH_2-C$\underline{H}_2$-CH_2-NH, CO-CH_2-C$\underline{H}_2$), 1.53 (m, 3H, CH-C$\underline{H}_2$, CO-CH_2-C$\underline{H}_2$), 1.66 (m, 1H, CH-C$\underline{H}_2$), 2.03 (t, 2H, ^{3}J = 7.41 Hz, CO-CH_2), 2.38 (t, 2H, ^{3}J = 7.3 Hz, CO-CH_2), 3.16 (m, 2H, O-CH_2-C$\underline{H}_2$-NH), 3.22 (m, 2H, CH_2-CH_2-C$\underline{H}_2$-NH), 3.38 (m, 4H, C$\underline{H}_2$-O-CH_2-CH_2-O, O-CH_2-C$\underline{H}_2$-NH), 3.51 (m, 6H, C$\underline{H}_2$-O-C$\underline{H}_2$-C$\underline{H}_2$-O), 4.1 (dd, 1H, ^{2}J = 14.77 Hz, ^{3}J = 5.67 Hz, C$\underline{H}_2$-ArOH), 4.16 (dd, 1H, ^{2}J = 14.75 Hz, ^{3}J = 5.84 Hz, C$\underline{H}_2$-ArOH), 4.33 (m, 1H, CH^{α}), 5.11 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.66 (d, 2H, ^{3}J = 8.49 Hz, AA'BB' (Ar-OH)), 6.99 (d, 2H, ^{3}J = 8.49 Hz, AA'BB' (Ar-OH)), 7.22 (m, 2H, Ph), 7.28 (m, 10H, Ph, CC'DD' (Ar-F)), 7.77 (t, 1H, ^{3}J = 5.59 Hz, O-CH_2-CH_2-N$\underline{H}$), 7.9 (m, 2H, CC'DD' (Ar-F)), 8.33 (t, 1H, ^{3}J = 5.83 Hz, CH-CO-N$\underline{H}$-CH_2), 8.45 (d, 1H, ^{3}J = 8.1 Hz, CO-N$\underline{H}$-CH), 8.48 (bs, 2H, N$\underline{H}_2$), 8.51 (t, 1H, ^{3}J = 5.52 Hz, O-CH_2-CH_2-N$\underline{H}$), 8.77 (s, 1H, CH_2-CH_2-CH_2-N$\underline{H}$), 9.25 (s, 1H, N$\underline{H}$), 11.08 (s, 1H, ArO$\underline{H}$); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 90 % (t_R = 15.9 min, k = 4.9); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{48}H_{60}FN_7O_8 + H]^+$ 882.4566, found: 882.4558; $C_{48}H_{60}FN_7O_8 \times C_2HF_3O_2$ (996.1)

General procedure for the synthesis of the potential PET-ligands 5.13 and 5.14

Amine precursor **4.22** or **4.23** (1 eq; cf. chapter 4) was dissolved in acetonitrile (4 - 6 mL). NEt_3 (2.5 eq) and active ester **5.5** (1 eq) were added and the mixture was stirred at rt for 5 h prior to the addition of 10 % aq. TFA (corresponding to 2.5 eq of TFA). Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as a white fluffy solid.

(*R*)-N^{α}-(2,2-Diphenylacetyl)-N^{ω}-{5-[2-(4-fluorobenzoylamino)ethyl]amino-5-oxopentanoyl}-*N*-(4-hydroxybenzyl)argininamide (5.13). Yield: 52 % (52.9 mg); mp 201-203 °C; ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.42 (m, 2H, CH_2-C$\underline{H}_2$-CH_2-NH), 1.53 (m, 1H, CH-C$\underline{H}_2$), 1.66 (m, 1H, CH-C$\underline{H}_2$), 1.77 (p, 2H, ^{3}J = 7.35 Hz, CO-CH_2-C$\underline{H}_2$-CH_2-CO), 2.12 (t, 2H, ^{3}J = 7.28 Hz, CO-CH_2), 2.42 (t, 2H, ^{3}J = 7.33 Hz, CO-CH_2), 3.21 (m, 4H, NH-CH_2-C$\underline{H}_2$-NH, CH_2-CH_2-C$\underline{H}_2$-NH), 3.3 (m, 2H, NH-CH_2-C$\underline{H}_2$-NH), 4.1 (dd, 1H, ^{2}J = 14.78 Hz, ^{3}J = 5.64 Hz, C$\underline{H}_2$-ArOH), 4.16 (dd, 1H, ^{2}J = 14.78 Hz, ^{3}J = 5.86 Hz, C$\underline{H}_2$-ArOH), 4.33 (m, 1H, CH^{α}), 5.11 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.66 (d, 2H, ^{3}J = 8.46 Hz, AA'BB' (Ar-OH)), 6.99 (d, 2H, ^{3}J = 8.45 Hz, AA'BB' (Ar-OH)), 7.21 (m, 2H, Ph), 7.28 (m, 10H, Ph, CC'DD' (Ar-F)), 7.89 (m, 2H, CC'DD' (Ar-F)), 7.95 (t, 1H, ^{3}J = 5.71

Hz, NH-CH_2-CH_2-NH), 8.33 (t, 1H, ^{3}J = 5.81 Hz, CH-CO-NH-CH_2), 8.45 (d, 1H, ^{3}J = 8.1 Hz, CO-NH-CH), 8.49 (t, 1H, ^{3}J = 5.55 Hz, NH-CH_2-CH_2-NH), 8.52 (bs, 2H, NH_2), 8.83 (s, 1H, CH_2-CH_2-CH_2-NH), 9.26 (s, 1H, NH), 11.22 (s, 1H, ArOH); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 92 % (t_R = 14.4 min, k = 5.9); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{41}H_{46}FN_7O_6 + H]^+$ 752.3572, found: 752.3552; $C_{41}H_{46}FN_7O_6 \times C_2HF_3O_2$ (865.9)

(*R*)-*N*$^\alpha$-(2,2-Diphenylacetyl)-*N*$^\omega$-{5-[3-(4-fluorobenzoylamino)propyl]amino-5-oxo-pentanoyl}-*N*-(4-hydroxybenzyl)argininamide (5.14). Yield: 65 % (66.2 mg); mp > 86 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.36-1.48 (m, 2H, CH-CH_2-CH_2), 1.53 (m, 1H, CH-CH_2), 1.64 (m, 3H, CH-CH_2, NH-CH_2-CH_2-CH_2-NH), 1.77 (p, 2H, ^{3}J = 7.37 Hz, CO-CH_2-CH_2-CH_2-CO), 2.12 (t, 2H, ^{3}J = 7.28 Hz, CO-CH_2), 2.42 (t, 2H, ^{3}J = 7.36 Hz, CO-CH_2), 3.09 (m, 2H, NH-CH_2-CH_2-CH_2-NH), 3.23 (m, 4H, NH-CH_2-CH_2-CH_2-NH, CH-CH_2-CH_2-CH_2-NH), 4.1 (dd, 1H, ^{2}J = 14.78 Hz, ^{3}J = 5.68 Hz, CH_2-ArOH), 4.16 (dd, 1H, ^{2}J = 14.77 Hz, ^{3}J = 5.87 Hz, CH_2-ArOH), 4.33 (m, 1H, CH$^\alpha$), 5.11 (s, 1H, CH-$(Ph)_2$), 6.66 (d, 2H, ^{3}J = 8.52 Hz, AA'BB' (Ar-OH)), 6.99 (d, 2H, ^{3}J = 8.53 Hz, AA'BB' (Ar-OH)), 7.21 (m, 2H, Ph), 7.28 (m, 10H, Ph, CC'DD' (Ar-F)), 7.85 (t, 1H, ^{3}J = 5.65 Hz, NH-CH_2-CH_2-CH_2-NH), 7.89 (m, 2H, CC'DD' (Ar-F)), 8.33 (t, 1H, ^{3}J = 5.82 Hz, CH-CO-NH-CH_2), 8.45 (m, 2H, CO-NH-CH, NH-CH_2-CH_2-CH_2-NH), 8.53 (bs, 2H, NH_2), 8.85 (s, 1H, CH-CH_2-CH_2-CH_2-NH), 9.25 (bs, 1H, NH), 11.25 (s, 1H, ArOH); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 97 % (t_R = 14.8 min, k = 4.5); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{42}H_{48}FN_7O_6 + H]^+$ 766.3728, found: 766.3733; $C_{42}H_{48}FN_7O_6 \times C_2HF_3O_2$ (879.9)

(*R*)-*N*$^\alpha$-(2,2-Diphenylacetyl)-*N*$^\omega$-[*N*-(4-fluorobenzoyl)-12-Amino-4,7,10-trioxa-dodecanoyl]-*N*-(4-hydroxybenzyl)argininamide (5.15). This compound was prepared from *N*$^\omega$-Boc, *O*-*t*-butyl protected amine precursor **4.19** (170 mg, 190.4 µmol, 1 eq; cf. chapter 4) which was dissolved in CH_2Cl_2 (3 mL) followed by the addition of NEt_3 (58 mg, 79 µL, 571 µmol, 3 eq) and active ester **5.5** (50 mg, 209.4 µmol, 1.1 eq). The mixture was stirred at rt for 5 h. TFA (2.5 mL) was added and stirring was continued for 2 h. CH_2Cl_2 (25 mL) was added three times, each time followed by evaporation under reduced pressure. Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as a white fluffy solid which turned into a resin after contact with air (86.9 mg, 95.2 µmol, 50 %). ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.42 (m, 2H, CH-CH_2-CH_2), 1.53 (m, 1H, CH-CH_2), 1.66 (m, 1H, CH-CH_2), 2.64 (t, 2H, ^{3}J = 5.92 Hz, CH_2-CO), 3.22 (m, 2H, CH_2-CH_2-CH_2-NH), 3.4 (m, 2H, O-CH_2-CH_2-NH), 3.5 (m, 10H, O-CH_2-CH_2-O, O-CH_2-CH_2-NH), 3.65 (t, 2H, ^{3}J = 6.02 Hz, CH_2-CH_2-CO), 4.1 (dd, 1H, ^{2}J = 14.78 Hz, ^{3}J = 5.65 Hz, CH_2-ArOH), 4.16 (dd, 1H, ^{2}J = 14.77 Hz, ^{3}J = 5.86 Hz, CH_2-ArOH), 4.33 (m, 1H, CH$^\alpha$), 5.11 (s, 1H, CH-$(Ph)_2$), 6.66 (d, 2H, ^{3}J = 8.49 Hz, AA'BB' (Ar-OH)), 6.99 (d, 2H, ^{3}J = 8.49 Hz, AA'BB' (Ar-OH)), 7.21 (m, 2H, Ph), 7.27 (m, 10H, Ph, CC'DD'

(Ar-F)), 7.9 (m, 2H, CC'DD' (Ar-F)), 8.33 (t, 1H, ^{3}J = 5.82 Hz, CH-CO-NH-CH$_2$), 8.45 (d, 1H, ^{3}J = 8.12 Hz, CO-NH-CH), 8.5 (t, 1H, ^{3}J = 5.48 Hz, O-CH$_2$-CH$_2$-NH), 8.58 (bs, 2H, NH$_2$), 8.91 (s, 1H, CH$_2$-CH$_2$-CH$_2$-NH), 9.26 (s, 1H, NH), 11.34 (s, 1H, ArOH); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 91 % (t_R = 15.9 min, k = 4.9); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{43}H_{51}FN_6O_8 + H]^+$ 799.3831, found: 799.3835; $C_{43}H_{51}FN_6O_8 \times C_2HF_3O_2$ (912.9)

General procedure for the synthesis of the potential PET-ligands 5.16 - 5.19

The pertinent amine precursor (1.05 eq) and NEt_3 (3 eq) were dissolved in DMF (ca. 15 µmol/100 µL) followed by the addition of active ester **5.4** or **5.5** (1 eq) in DMF (ca. 100 µL/15 µmol). The reaction was stopped by addition of 10 % aq. TFA (corresponding to 2 - 3 eq of TFA) after an incubation period of 1-2 h at rt. Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the products as white fluffy solids.

(*R*)-*N*$^\alpha$-(2,2-Diphenylacetyl)-*N*$^\omega$-[4-(2-fluoropropanoylamino)butyl]aminocarbonyl-*N*-(4-hydroxybenzyl)argininamide (5.16). From **4.27** and **5.4**; yield: 76 % (10.9 mg); mp > 65 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d$_6$, COSY): δ (ppm) 1.4 (dd, 3H, ^{3}J = 24.52 Hz, ^{3}J = 6.7 Hz, CH$_3$), 1.43 (m, 6H, CH$_2$-CH$_2$-CH$_2$-CH$_2$, CH-CH$_2$-CH$_2$), 1.53 (m, 1H, CH-CH$_2$), 1.66 (m, 1H, CH-CH$_2$), 3.09 (m, 4H, 2 × CH$_2$-CH$_2$-NH), 3.18 (m, 2H, CH$_2$-CH$_2$-NH), 4.1 (dd, 1H, ^{2}J = 14.78 Hz, ^{3}J = 5.63 Hz, CH$_2$-ArOH), 4.16 (dd, 1H, ^{2}J = 14.76 Hz, ^{3}J = 5.86 Hz, CH$_2$-ArOH), 4.33 (m, 1H, CH$^\alpha$), 4.95 (dq, 1H, ^{2}J = 48.98 Hz, ^{3}J = 6.69 Hz, CHF), 5.11 (s, 1H, CH-(Ph)$_2$), 6.66 (d, 2H, ^{3}J = 8.49 Hz, AA'BB'), 6.99 (d, 2H, ^{3}J = 8.49 Hz, AA'BB'), 7.22 (m, 2H, Ph), 7.28 (m, 8H, Ph), 7.46 (s, 1H, CH$_2$-CH$_2$-NH), 8.12 (t, 1H, ^{3}J = 5.16 Hz, CH$_2$-CH$_2$-NH), 8.34 (t, 1H, ^{3}J = 5.82 Hz, CH-CO-NH-CH$_2$), 8.36 (bs, 2H, NH$_2$), 8.45 (d, 1H, ^{3}J = 8.13 Hz, CO-NH-CH), 8.86 (s, 1H, CH$_2$-CH$_2$-NH), 9.25 (s, 1H, NH), 9.51 (s, 1H, ArOH); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 97 % (t_R = 13.9 min, k = 4.1); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{35}H_{44}FN_7O_5 + H]^+$ 662.3466, found: 662.3472; $C_{35}H_{44}FN_7O_5 \times C_2HF_3O_2$ (775.8)

(*R*)-*N*$^\alpha$-(2,2-Diphenylacetyl)-*N*$^\omega$-[4-(4-fluorobenzoylamino)butyl]aminocarbonyl-*N*-(4-hydroxybenzyl)argininamide (5.17). From **4.27** and **5.5**; yield: 91 % (34.9 mg); mp > 66 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d$_6$, COSY): δ (ppm) 1.41 (m, 2H, CH-CH$_2$-CH$_2$), 1.51 (m, 5H, CH$_2$-CH$_2$-CH$_2$-CH$_2$, CH-CH$_2$), 1.66 (m, 1H, CH-CH$_2$), 3.12 (m, 2H, CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH), 3.18 (m, 2H, CH-CH$_2$-CH$_2$-CH$_2$-NH), 3.26 (m, 2H, CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH), 4.1 (dd, 1H, ^{2}J = 14.78 Hz, ^{3}J = 5.67 Hz, CH$_2$-ArOH), 4.16 (dd, 1H, ^{2}J = 14.78 Hz, ^{3}J = 5.88 Hz, CH$_2$-ArOH), 4.33 (m, 1H, CH$^\alpha$), 5.11 (s, 1H, CH-(Ph)$_2$), 6.66 (d, 2H, ^{3}J = 8.5 Hz, AA'BB' (Ar-OH)), 6.99 (d, 2H, ^{3}J = 8.51 Hz, AA'BB' (Ar-OH)), 7.22 (m, 2H, Ph), 7.28 (m, 10H, Ph, CC'DD' (Ar-F)), 7.48 (s, 1H, CH$_2$-CH$_2$-CH$_2$-CH$_2$-NH), 7.89 (m, 2H, CC'DD' (Ar-F)), 8.33 (t, 1H, ^{3}J = 5.82 Hz, CH-CO-NH-CH$_2$), 8.36 (bs, 2H, NH$_2$), 8.45 (d, 1H, ^{3}J = 8.17 Hz, CO-NH-CH), 8.47 (t, 1H, ^{3}J = 5.7 Hz, CH$_2$-

CH_2-CH_2-CH_2-N<u>H</u>), 8.85 (s, 1H, CH-CH_2-CH_2-CH_2-N<u>H</u>), 9.25 (s, 1H, N<u>H</u>), 9.51 (s, 1H, ArO<u>H</u>); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 98 % (t_R = 16.0 min, k = 4.9); HRMS: (FAB^+, MeOH/glycerin): *m/z* calcd. for $[C_{39}H_{44}FN_7O_5 + H]^+$ 710.3466, found: 710.3460; $C_{39}H_{44}FN_7O_5 \times C_2HF_3O_2$ (823.8)

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*$^{\omega}$-[8-(2-fluoropropanoyl)amino-3,6-dioxaoctyl]amino-carbonyl-*N*-(4-hydroxybenzyl)argininamide (5.18). From **5.7** and **5.4**; yield: 67 % (12.1 mg); mp > 44 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.4 (dd, 3H, ^3J = 24.54 Hz, ^3J = 6.7 Hz, CH_3), 1.43 (m, 2H, CH-CH_2-C<u>H</u>$_2$), 1.54 (m, 1H, CH-C<u>H</u>$_2$), 1.67 (m, 1H, CH-C<u>H</u>$_2$), 3.19 (m, 2H, CH_2-CH_2-C<u>H</u>$_2$-NH), 3.27 (m, 4H, 2 × O-CH_2-C<u>H</u>$_2$-NH), 3.44 (t, 2H, ^3J = 6.13 Hz, O-C<u>H</u>$_2$-CH_2-NH), 3.48 (t, 2H, ^3J = 5.47 Hz, O-C<u>H</u>$_2$-CH_2-NH), 3.53 (s, 4H, O-C<u>H</u>$_2$-C<u>H</u>$_2$-O), 4.12 (dd, 1H, ^2J = 14.79 Hz, ^3J = 5.65 Hz, C<u>H</u>$_2$-ArOH), 4.17 (dd, 1H, ^2J = 14.78 Hz, ^3J = 5.84 Hz, C<u>H</u>$_2$-ArOH), 4.34 (m, 1H, CH$^{\alpha}$), 4.98 (dq, 1H, ^2J = 48.93 Hz, ^3J = 6.69 Hz, CHF), 5.12 (s, 1H, C<u>H</u>-$(Ph)_2$), 6.67 (d, 2H, ^3J = 8.49 Hz, AA'BB'), 7.0 (d, 2H, ^3J = 8.49 Hz, AA'BB'), 7.23 (m, 2H, Ph), 7.29 (m, 8H, Ph), 7.51 (s, 1H, O-CH_2-CH_2-N<u>H</u>), 8.07 (s, 1H, O-CH_2-CH_2-N<u>H</u>), 8.34 (t, 1H, ^3J = 5.82 Hz, CH-CO-N<u>H</u>-CH_2), 8.39 (bs, 2H, N<u>H</u>$_2$), 8.46 (d, 1H, ^3J = 8.12 Hz, CO-N<u>H</u>-CH), 8.89 (s, 1H, CH_2-CH_2-CH_2-N<u>H</u>), 9.26 (s, 1H, N<u>H</u>), 9.67 (s, 1H, ArO<u>H</u>); RP-HPLC (Eurospher 250 × 4 mm, 220 nm, gradient 2): 99 % (t_R = 13.7 min, k = 4.1); HRMS: (FAB^+, MeOH/glycerin): *m/z* calcd. for $[C_{37}H_{48}FN_7O_7 + H]^+$ 722.3677, found: 722.3687; $C_{37}H_{48}FN_7O_7 \times C_2HF_3O_2$ (835.8)

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*$^{\omega}$-[8-(4-fluorobenzoylamino)-3,6-dioxaoctyl]aminocarbonyl-*N*-(4-hydroxybenzyl)argininamide (5.19). From **5.7** and **5.5**; yield: 73 % (14 mg); mp > 58 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.41 (m, 2H, CH-CH_2-C<u>H</u>$_2$), 1.53 (m, 1H, CH-C<u>H</u>$_2$), 1.66 (m, 1H, CH-C<u>H</u>$_2$), 3.18 (m, 2H, CH_2-CH_2-C<u>H</u>$_2$-NH), 3.24 (m, 2H, O-CH_2-C<u>H</u>$_2$-NH), 3.4 (m, 2H, O-CH_2-C<u>H</u>$_2$-NH), 3.46 (t, 2H, ^3J = 5.46 Hz, O-C<u>H</u>$_2$-CH_2-NH), 3.53 (m, 6H, C<u>H</u>$_2$-O-C<u>H</u>$_2$-C<u>H</u>$_2$-O), 4.11 (dd, 1H, ^2J = 14.78 Hz, ^3J = 5.68 Hz, C<u>H</u>$_2$-ArOH), 4.16 (dd, 1H, ^2J = 14.78 Hz, ^3J = 5.89 Hz, C<u>H</u>$_2$-ArOH), 4.33 (m, 1H, CH$^{\alpha}$), 5.11 (s, 1H, C<u>H</u>-$(Ph)_2$), 6.66 (d, 2H, ^3J = 8.49 Hz, AA'BB' (Ar-OH)), 6.99 (d, 2H, ^3J = 8.5 Hz, AA'BB' (Ar-OH)), 7.21 (m, 2H, Ph), 7.27 (m, 10H, Ph, CC'DD' (Ar-F)), 7.49 (s, 1H, O-CH_2-CH_2-N<u>H</u>), 7.9 (m, 2H, CC'DD' (Ar-F)), 8.33 (t, 1H, ^3J = 5.82 Hz, CH-CO-N<u>H</u>-CH_2), 8.38 (bs, 2H, N<u>H</u>$_2$), 8.45 (d, 1H, ^3J = 8.12 Hz, CO-N<u>H</u>-CH), 8.51 (t, 1H, ^3J = 5.54 Hz, O-CH_2-CH_2-N<u>H</u>), 8.87 (s, 1H, CH_2-CH_2-CH_2-N<u>H</u>), 9.25 (s, 1H, N<u>H</u>), 9.59 (s, 1H, ArO<u>H</u>); RP-HPLC (Eurospher 250 × 4 mm, 220 nm, gradient 2): 97 % (t_R = 15.7 min, k = 4.8); HRMS: (FAB^+, MeOH/glycerin): *m/z* calcd. for $[C_{41}H_{48}FN_7O_7 + H]^+$ 770.3677, found: 770.3691; $C_{41}H_{48}FN_7O_7 \times C_2HF_3O_2$ (883.9)

Succinimidyl 8-(4-fluorobenzylamino)-8-oxooctanoate (5.20).[24] Octanedioic acid disuccinimidyl ester **3.7** (220 mg, 0.6 mmol, 1.4 eq; cf. chapter 3) and NEt_3 (8.5 mg, 11.6 µl, 83.6 µmol, 0.2 eq) were dissolved in CH_2Cl_2 (15 mL). 4-Fluorobenzylamine (52.3 mg, 47.5 µL,

0.42 mmol, 1 eq) in CH_2Cl_2 (5 mL) was added dropwise over a period of 2 h and stirring was continued for 4 h. Purification with column chromatography (CH_2Cl_2/EtOAc 3/1 to 1/2) afforded the product as crystalline solid (25 mg, 66.1 µmol, 16 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.4 (m, 4H, CH_2-CH_2-C<u>H</u>$_2$-C<u>H</u>$_2$-CH_2-CH_2), 1.73 (m, 4H, CH_2-C<u>H</u>$_2$-CH_2-CH_2-C<u>H</u>$_2$-CH_2), 2.23 (t, 2H, ^{3}J = 7.39 Hz, CO-CH_2), 2.6 (t, 2H, ^{3}J = 7.13 Hz, CO-CH_2), 2.79 (s, 4H, CO-C<u>H</u>$_2$-C<u>H</u>$_2$-CO), 4.4 (d, 2H, ^{3}J = 5.7 Hz, C<u>H</u>$_2$-Ar), 5.91 (bs, 1H, N<u>H</u>-CH_2-Ar), 7.01 (pseudo-t, 2H, ^{3}J = 8.71 Hz, Ar), 7.25 (m, 2H, Ar); MS (CI, NH_3): *m/z* 379 $[M + H]^+$; $C_{19}H_{23}FN_2O_5$ (378.4)

(*R*)-*N*$^\alpha$-(2,2-Diphenylacetyl)-*N*$^\omega$-[8-(4-fluorobenzylamino)-8-oxooctanoyl]-*N*-(4-hydroxybenzyl)argininamide (5.21). 5.20 (25 mg, 66.1 µmol, 1 eq) and **2.7** (104 mg, 165 µmol, 2.5 eq) were dissolved in CH_2Cl_2 (4 mL). NEt_3 (6.7 mg, 9.2 µl, 66.1 µmol, 1 eq) was added and the mixture was kept under stirring at rt for 20 h. TFA (4 mL) was added and stirring was continued for 2.5 h. The solvent was removed under reduced pressure, and MeOH (25 mL) was added followed by evaporation under reduced pressure. Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as white solid (39.9 mg, 46.9 µmol, 71 %). Mp 163-166 °C; ^{1}H-NMR (600 MHz, CD_3OD, COSY): δ (ppm) 1.36 (m, 4H, CH_2-CH_2-C<u>H</u>$_2$-C<u>H</u>$_2$-CH_2-CH_2), 1.49-1.74 (m, 7H, CH-C<u>H</u>$_2$-C<u>H</u>$_2$, CH_2-C<u>H</u>$_2$-CH_2-CH_2-C<u>H</u>$_2$-CH_2), 1.83 (m, 1H, CH-C<u>H</u>$_2$), 2.22 (t, 2H, ^{3}J = 7.42 Hz, CO-CH_2), 2.42 (t, 2H, ^{3}J = 7.39 Hz, CO-CH_2), 3.25 (m, 2H, CH_2-C<u>H</u>$_2$-NH), 4.22 (d, 1H, ^{2}J = 14.55 Hz, C<u>H</u>$_2$-ArOH), 4.26 (d, 1H, ^{2}J = 14.56 Hz, C<u>H</u>$_2$-ArOH), 4.32 (s, 2H, CH_2-Ar-F), 4.42 (m, 1H, CH^α), 5.06 (s, 1H, C<u>H</u>-$(Ph)_2$), 6.7 (d, 2H, ^{3}J = 8.54 Hz, AA'BB' (ArOH)), 7.02 (pseudo-t, 2H, ^{3}J = 8.8 Hz, CC'DD' (Ar-F)), 7.05 (d, 2H, ^{3}J = 8.54 Hz, AA'BB' (ArOH)), 7.2-7.31 (m, 12H, Ph, CC'DD' (Ar-F)); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 99 % (t_R = 18.0 min, k = 5.7); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{42}H_{49}FN_6O_5 + H]^+$ 737.3827, found: 737.3839; $C_{42}H_{49}FN_6O_5 \times C_2HF_3O_2$ (850.9)

4-Nitrophenyl 2-[^{18}F]fluoropropanoate ([^{18}F]NPFP). [^{18}F]NPFP was synthesized at the Nuclear Medicine Department of the Klinikum rechts der Isar at TU München according to a previously described protocol[16].

(*R*)-*N*$^\alpha$-(2,2-Diphenylacetyl)-*N*$^\omega$-(2-[^{18}F]fluoropropanoyl)-*N*-(4-hydroxybenzyl)argininamide (5.8-F18). The synthesis was carried out in a 15-ml Falcone tube under an atmosphere of argon. 18 µL of a 1:9 mixture (v/v) of triethylamine (1.31 mg, 1.8 µL, 13 µmol) in anhydrous acetonitrile were added to a solution of **2.7** (3 mg, cf. chapter 2) in anhydrous acetonitrile (600 µL). 250 µL of this solution (**2.7**: 1.21 mg, 1.9 µmol; NEt_3: 0.53 mg, 5.2 µmol) were added to [^{18}F]-NPFP (**5.4-F18**) (10.9 mCi at the time of addition, provided in a 15-ml Falcone tube) using a 1-mL-syringe. The mixture was briefly vortexed, then stirred (micro stirrer) at 65 °C for 20 min. TFA (250 µL, 3.25 mmol) was added and stirring was continued for 40 min at 70 °C. 5 M aq. NaOH (250 µL, 1.25 mmol) was added and a white precipitate occurred. Acetonitrile (150 µL)

was added prior to removal of the precipitate using a 4-mm syringe filter (0.2 µm). The activity retained in the filter was negligible. 0.1 % aq. TFA (50 µL) was added to the filtrate (ca. 900 µL) yielding the solution for injection into the HPLC system. The product was purified by reversed-phase HPLC on a semi-preparative column (YMC RP 18 S, 4 µm, 80 Å, 150 x 20 mm; YMC Europe GmbH), using mixtures of 0.1 % TFA in acetonitrile (A) and 0.1 % aq. TFA (B) as eluent and a flow rate of 5 mL/min (gradient: 0-20 min: A/B 20/80 to 60/40). The eluate containing the product (t_R ≈ 15 min) was transferred into a 20-mL-syringe, diluted with water (16 mL) and charged to a conditioned (MeOH, water) C18 SPE-cartridge. The passed liquid contained no measurable activity. The SPE cartridge was washed with water (5 mL) and the liquid was removed as far as possible by air flow. The product was eluted with ethanol (4 mL) into a 10-mL round bottom flask and the solvent was removed under reduced pressure at 35 °C (final pressure: 50 mbar). The residue (ca. 1.2 mL, 2.8 mCi, 2 h 40 min after start of synthesis) was transferred into a 2-mL Eppendorf reaction vessel and 20 µL (40 µCi) were mixed with 80 µL of 0.1 % aq. TFA. 25 µL of this solution were analyzed by HPLC as purity control using a reversed-phase column (Nucleosil 100 C18, 125 x 4 mm, 5 µm; CS - Chromatographie Service GmbH, Langerwehe, Germany) and a flow rate of 1 mL/min. Mixtures of 0.1 % TFA in acetonitrile (A) and 0.1 % aq. TFA (B) were used as eluent (gradient: 0-20 min: A/B 20/80 to 60/40). The purity was determined to be > 98 % (radiometric detection, t_R = 14.8 min). A little impurity (t_R = 15.8 min) was detected at 220 nm. Decay corrected yield: 70 %.

Preparation of injection solutions for *in-vivo* studies:

DMSO (20 µL) was added to 1000 µL of the aqueous residue remained after evaporation of ethanol. To obtain the injection solution for biodistribution and PET studies 200 µL of this solution were diluted with 700 µL of PBS and 20 µL of 10-fold concentrated PBS, respectively.

(*R*)-N^{α}-(2,2-Diphenylacetyl)-N^{ω}-[6-(2-[^{18}F]fluoropropanoylamino)hexanoyl]-*N*-(4-hydroxybenzyl)argininamide (5.9-F18). The synthesis was carried out in a 15-ml Falcone tube under an atmosphere of argon. DMSO (25 µL) and 17 µL of a 1:9 mixture (v/v) of triethylamine (1.24 mg, 1.7 µL, 12.3 µmol) in anhydrous acetonitrile were added to a solution of **4.21** (1 mg, 1.23 µmol, cf. chapter 4) in anhydrous acetonitrile (250 µL). This clear solution was added to [^{18}F]-NPFP **5.4-F18** (13.53 mCi at the time of addition, provided in a 15-ml Falcone tube) using a 1-mL-syringe. The mixture was briefly vortexed, then stirred (micro stirrer) at 50 °C for 15 min and additionally 10 min at rt. 1.1 mL of acetonitrile/0.1 % aq. TFA (47/53 v/v) was added to acidify the mixture and to obtain a solution for injection into the HPLC system. The product was purified by reversed-phase HPLC on a semi-preparative column (YMC RP 18 S, 4 µm, 80 Å, 150 x 20 mm; YMC Europe GmbH) using mixtures of 0.1 % TFA in acetonitrile (A) and 0.1 % aq. TFA (B) as eluent and a flow rate of 5 mL/min (gradient: 0-20 min: A/B 30/70 to 70/30). The eluate containing the product (t_R ≈ 11 min, vol.: 4.5 – 5 mL) was transferred into a 20-mL-syringe, diluted with water (15 mL) and charged to a conditioned (MeOH, water) C18 SPE-cartridge. The

passed liquid contained no measurable activity. The SPE cartridge was washed with water (5 mL) and the liquid was removed as far as possible by air flow. The product was eluted with ethanol (4 mL) into a 10-mL round bottom flask and the solvent was removed under reduced pressure at 40 °C (final pressure: 90 mbar). The residue (ca. 200 µL) was filtered with a 4-mm syringe filter (0.2 µm) and transferred into a 1.5-mL Eppendorf reaction vessel. The flask and the filter were washed with PBS (800 µL) and the filtrates were combined to give the injection solution for *in-vivo* PET and biodistribution experiments (3.84 mCi, 1h 56 min after start of synthesis). 10 µL (30 µCi) of this solution were analyzed by HPLC as purity control using a reversed-phase column (Nucleosil 100 C18, 125 x 4 mm, 5 µm; CS - Chromatographie Service GmbH, Langerwehe, Germany) and a flow rate of 1 mL/min. Mixtures of acetonitrile (A) and 0.1 % aq. TFA (B) were used as eluent (gradient: 0-20 min: A/B 30/70 to 75/25). The purity was determined to be > 98 % (radiometric detection, t_R = 9.8 min). Decay corrected yield: 60 %.

5.3.3 Pharmacology: Cell Culture, Fura-2 Assay and Competition Binding Assay

Cell culture. HEL and SK-N-MC cells were cultured as described elsewhere.[25, 26] HEL cells were subcultured by 1:6-dilution with fresh culture medium 24 h prior to the fura Ca^{2+}-assay. MCF-7-Y_1[a] cells were maintained in MEM (Sigma), supplemented with 5 % FCS (Biochrom AG, Berlin, Germany). The CHO cells, transfected with the Y_2R, $G_{\alpha qi5}$ and aequorin, were cultured as previously described.[18] CHO cells, transfected with the Y_4R, $G_{\alpha qi5}$ and aequorin, were cultured under the same conditions. HEC-1B cells, transfected with the Y_5R, were cultured as previously described.[27]

([a]This cell line was established by subcloning from MCF-7 cells (ATCC number HTB 22) in the 157th passage and shows a 2 - 3-fold higher Y_1R expression than the original MCF-7 cells.)

Fura-2 assay on HEL cells and radioligand competition binding assay. The Fura assay was performed with HEL cells as previously described[28] using a Perkin-Elmer LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany). The fura-2 AM (Calbiochem/Merck Biosciences, Beeston, UK) stock solution (1 mM) was prepared in anhydrous DMSO. Pluronic F-127 (Calbiochem/Merck Biosciences, Beeston, UK) was dissolved in DMSO to a concentration of 20 %.

Radioligand competition experiments with [^{3}H]-UR-MK114 (cf. chapter 2) were performed as described in chapter 3.

5.3.4 Pharmacology: Flow Cytometric Binding Experiments

Flow cytometric binding studies at the Y_2R (CHO cells transfected with the Y_2R, $G\alpha_{qi5}$ and aequorin)[29] and at the Y_5R (expressed by HEC-1B cells transfected with the Y_5R)[18, 30] were performed as previously described. The cell density in loading buffer was 10^6 cells/mL (Y_2R) or $0.5 \cdot 10^6$ cells/mL (Y_5R). For Y_2R binding studies Dy-635-pNPY (10 nM) was used instead of Cy5-pNPY (10 nM).

Flow cytometric binding studies at the Y_4R (expressed by CHO cells transfected with the Y_4R, $G\alpha_{qi5}$ and aequorin)[19] were performed as reported[31] with the following variations: Cy5-[K^4]-hPP[19] (5 nM) was used instead of S0586-[K^4]-hPP (10 nM). The cell density in loading buffer was 10^6 cells/mL. Non-specific binding was determined in the presence of hPP (100 nM). The stock solution of Cy5-[K^4]-hPP was prepared in 10 mM HCl + 0.1 % BSA (500 nM).

All flow cytometric assays were performed in triplicate on a FACSCalibur™ flow cytometer (Becton Dickinson, Heidelberg, Germany), equipped with an argon laser (488 nm) and a red diode laser (635 nm). **5.16** and **5.17** were tested at a concentration of 1 µM and 10 µM. No displacement of the fluorescent ligand by **5.16** and **5.17** (10 µM) was observed in case of the Y_5R, and at the Y_2R both compounds (10 µM) displaced around 50 % of the fluorescent ligand. Whereas no displacement of fluorescent ligand by **5.16** (10 µM) was found at the Y_4R, **5.17** displaced about 30 % of Cy5-[K^4]-hPP (5 nM) at a concentration of 10 µM.

5.3.5 Biodistribution and PET Experiments

Biodistribution of the PET ligands **5.8-F18** and **5.9-F18** was investigated in NMRI (nu/nu) mice (male, 4 - 5 month) bearing subcutaneous SK-N-MC xenografts, which were established by subcutaneous injection of a cell suspension in culture medium without FCS ($3 \cdot 10^6$ cells in 50 µL, between 50^{th} and 60^{th} in vitro passage) into the flank. At the day of experiment the size of the tumors ranged from 5 × 5 mm to 11 × 10 mm.

Biodistribution studies with 5.8-F18. 45 – 65 µCi (180 – 200 µL of the above described injection solution) of the PET ligand **5.8-F18** were injected intravenously into the tail vein of SK-N-MC tumor-bearing (right flank) NMRI (nu/nu) mice. The animals were killed (CO_2) 30 min after injection and the radioactivity of weighted tissue or secretion samples as well as of a standard with known radioactivity was measured using a γ-counter. Results are expressed as the percentage injected dose per gram (%ID/g).

Biodistribution studies with 5.9-F18. 108 and 54 µCi (50 – 100 µL of the above described injection solution) of the PET ligand **5.9-F18** were injected intravenously into the tail vein of SK-N-MC tumor-bearing (left flank) NMRI (nu/nu) mice, and 120 as well as 84 µCi (100 – 150 µL of the above described injection solution) of **5.9-F18** were injected intravenously into the tail vein of two NMRI (nu/nu) mice without SK-N-MC tumor. The animals were killed by cardiac puncture 90 min after injection, and the radioactivity of weighted tissue or secretion samples as well as of a standard with known radioactivity was measured using a γ-counter. Results are expressed as the percentage injected dose per gram (%ID/g).

PET imaging with 5.8-F18. For PET imaging with the PET ligand **5.8-F18**, 280 µCi (ca. 200 µL of the above described injection solution) of the ^{18}F-labeled compound was injected intravenously into the tail vein of a SK-N-MC tumor-bearing (right flank) NMRI (nu/nu) mouse. 5 min after injection a dynamic PET scan (60 min, 6 5-min frames, 3 10-min frames) was performed using a Philips MOSAIC small animal PET scanner (Philips, Hamburg, Germany). The mouse was anesthetized using a combination of xylazine and ketamine.

PET imaging with 5.9-F18. For PET imaging with the PET ligand **5.9-F18**, a dynamic PET scan (60 min, 5-min frames) was performed with two NMRI (nu/nu) mice (anesthetized with isofluorane) of the biodistribution experiment (mice with 108 and 120 µCi injection dose) using a microPET Focus 120 small animal PET scanner (Siemens Healthcare, Erlangen, Germany). Scans were started 10 min after injection.

5.4 References

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Chapter 6

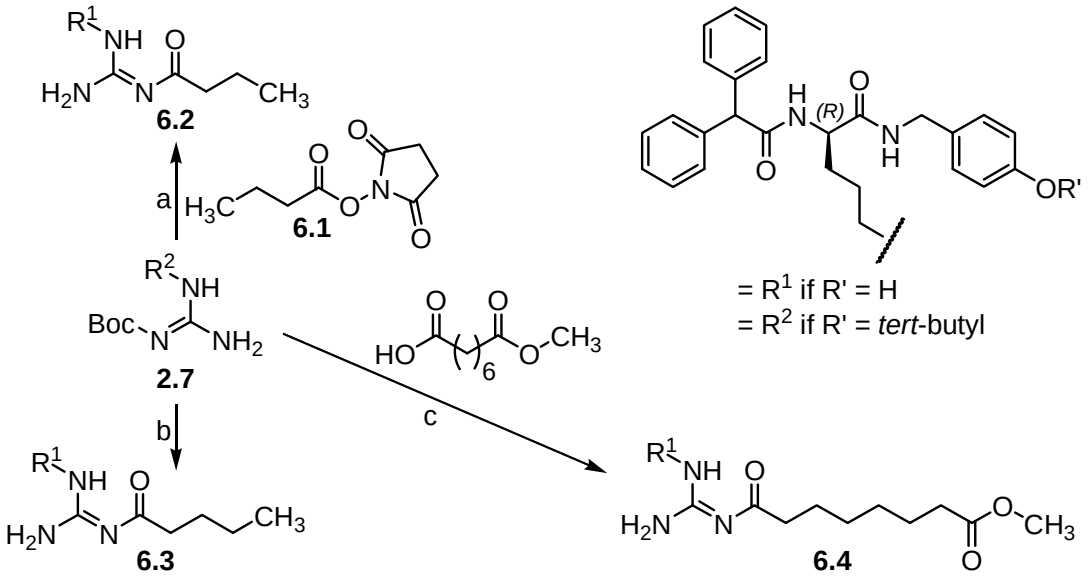
Miscellaneous Y_1 Receptor Antagonists

6.1 Introduction

This chapter comprises BIBP 3226 or BIBO 3304 derived Y_1R antagonists, which are neither bivalent Y_1R ligands (chapter 3) nor fluorescent (chapter 4) nor potential PET ligands (chapter 5). The Y_1R antagonists, presented in this chapter, are either "non-functional" or represent potential monovalent tritiated Y_1R ligands. Bivalent potential tritiated ligands for the Y_1R were shown in chapter 3. Finally, proposals (in addition to suggestions given in chapters 4 and 5) are made for the preparation of other BIBP 3226 or BIBO 3304 analogs, which could not be realized anymore.

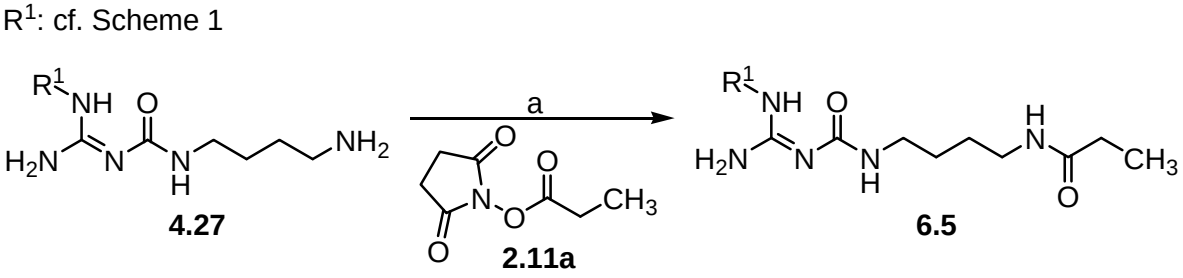
6.2 Chemistry

N$^{\omega}$-Butanoyl-BIBP 3226 (**6.2**) and *N*$^{\omega}$-pentanoyl-BIBP 3226 (**6.3**) were obtained through treatment of the argininamide building block **2.7** (cf. chapter 2) with succinimidyl butanoate (**6.1**) and pentanoic anhydride, respectively, followed by deprotection with TFA (Scheme 1). Y_1R antagonist **6.4** was synthesized through the coupling of suberic acid monomethylester to building block **2.7** (Scheme 1).



Scheme 1. Synthesis of *N*$^{\omega}$-butanoyl-BIBP 3226 (**6.2**), *N*$^{\omega}$-pentanoyl-BIBP 3226 (**6.3**) and *N*$^{\omega}$-(8-methoxy-8-oxooctanoyl)-BIBP 3226 (**6.4**).
Reagents and conditions: (a) (1) NEt_3, acetonitrile, rt, 20 h; (2) TFA, acetonitrile, 40 °C, 1.5 h, 55 %; (b) (1) pentanoic acid anhydride, NEt_3, CH_2Cl_2, rt, 5 h; (2) TFA, CH_2Cl_2, rt, 2 h, 59 %; (c) (1) EDAC, DMAP, CH_2Cl_2, rt, 20 h; (2) TFA, CH_2Cl_2, rt, 4 h, 82 %.

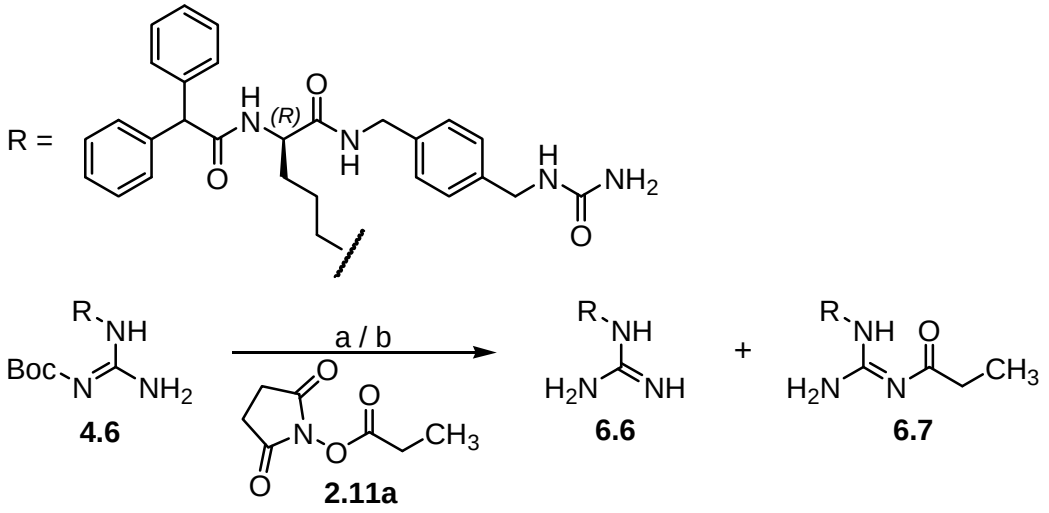
The potential radioligand **6.5** was prepared from succinimidyl propanoate (**2.11a**, cf. chapter 2) and amine precursor **4.27** (synthesis see chapter 4) in very good yield (Scheme 2).



Scheme 2. Synthesis of the potential radioligand **6.5**.
Reagents and conditions: (a) (1) NEt_3, DMF, rt, 2.5 h, 86 %.

In an analogous way, the BIBO 3304 building block **4.6** (cf. chapter 4) was treated with succinimidyl propanoate (**2.11a**, cf. chapter 2) to obtain N^G-propanoyl-BIBO 3304 (**6.7**). Non-converted building block **4.6** was recovered as BIBO 3304 (**6.6**) after deprotection with TFA (Scheme 3). A major drawback of this reaction is the low solubility of building block **4.6** in a broad variety of solvents. When MeOH was used as solvent (**4.6** is better soluble in MeOH than e.g. in acetonitrile and CH_2Cl_2), the yield of N^G-propanoyl-BIBO 3304 (**6.7**) was very low (ca. 4 %). As an alternative the synthesis of **6.7** was explored using a microwave synthesizer (Biotage Initiator 8): 300 mg of **4.6** could be dissolved in acetonitrile (3 mL) at temperatures > 80 °C. A reaction time of 10 min and a temperature of 120 °C as well as subsequent deprotection with TFA afforded compound **6.7** with 30 % yield.

Since building block **4.6** is well soluble in DMF (30 mg in 1 mL of DMF at 20 °C), acylation at the guanidine group should be performed in DMF. The removal of the solvent (DMF) prior to the deprotection with TFA is considered an acceptable compromise.

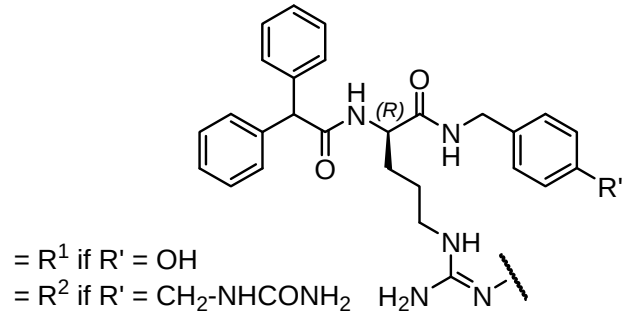


Scheme 3. Synthesis of BIBO 3304 (**6.6**) and N^G-propanoyl-BIBO 3304 (**6.7**).
Reagents and conditions: (a) (1) NEt_3, MeOH, rt/50 °C, 24 h; (2) TFA, MeOH, rt, 4 h, 66 % (**6.6**), 4 % (**6.7**); (b) (1) NEt_3, acetonitrile, 120 °C, 10 min (microwave); (2) acetonitrile/TFA/H_2O 10/1/1, 80 °C, 10 min (microwave), 30 % (**6.7**).

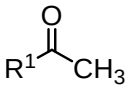
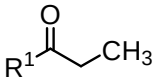
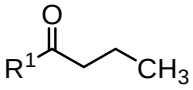
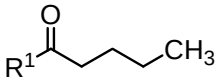
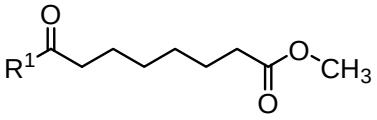
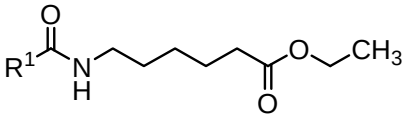
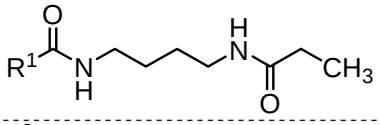
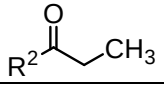
6.3 Y_1 Receptor Antagonism, Affinity and Selectivity

The N^{ω}-butanoyl (**6.2**) and the N^{ω}-pentanoyl derivative of BIBP 3226 (**6.3**) were synthesized for comparison with the lower homologues N^{ω}-propanoyl- (**2.8**) and N^{ω}-acetyl-BIBP 3226[1] (**6.8**) (Table 1). While the extension of the acetyl residue to a propanoyl substituent leads to a considerable increase in Y_1R affinity (from 12 nM to 1.0 nM, Table 1), the affinity remains almost unaffected by a further homologization to butanoyl (**6.2**) and pentanoyl (**6.3**) entities (Table 1).

Table 1. Structures, Y_1 receptor antagonism and binding data of BIBP 3226, Y_1R antagonists **6.2** – **6.7** and a selection of reported BIBP 3226 derivatives.



= R^1 if R' = OH
= R^2 if R' = CH_2-$NHCONH_2$

Compd.	Structure	IC_{50} (K_b) [nM][a]		K_i [nM]
BIBP 3226	R^1-H	10±1	(1.5)	1.3±0.2[b]
6.8		14[1]	(2.2)	12[1, c]
2.8a		2.3±0.7	(0.35)	1.0±0.1[b]
6.2		0.44±0.01	(0.07)	0.74±0.01[b]
6.3		1.0±0.02	(0.15)	1.6±0.3[b]
6.4		0.4±0.08	(0.06)	0.94±0.06[b]
6.9		0.64[1]	(0.1)	0.72[1, c]
6.5		3.8±0.2	(0.6)	1.2±0.2[b]
6.6 (BIBO 3304)	R^2-H	4.6±1	(0.7)	0.25±0.01[b]
6.7		230±4	(36)	7.8±2.6[b]

[a]Inhibition of 10 nM NPY induced $[Ca^{2+}]_i$ mobilization in HEL cells, mean values ± SEM from two independent experiments. [b]Dissociation constant determined from the displacement of [^{3}H]-UR-MK114 (K_D = 1.2 nM, c = 1.5 nM) on SK-N-MC cells, mean values ± SEM from two independent experiments performed in triplicate. [c]Dissociation constant determined from the displacement of ([2,3-^{3}H]propionyl)-pNPY on SK-N-MC cells.[1]

The high affinity (K_i = 0.94 nM, Table 1) of N^{ω}-(8-methoxy-8-oxo-octanoyl)-BIBP 3226 (**6.4**) suggests an affinity enhancing effect of the ester carbonyl group. However, the increase in binding could also arise from hydrophobic interactions of the alkyl chain as this is the case for compounds **2.8a**, **6.2** and **6.3**. Y_1R ligand **6.4** was isolated serendipitously as a by-product during the work-up of bivalent ligand **3.8** (cf. chapter 3) which is composed of two BIBP 3226 entities linked through octanedioic acid. As described in chapter 3 compound **3.8** was prepared from octanedioic acid disuccinimidylester (**3.7**) and BIBP 3226 building block **2.7** (for structure of **2.7** see Scheme 1). Since the formation of the bivalent ligand was incomplete, a part of the semi-reacted octandioic acid was esterified with MeOH at the carboxylic group to give **6.4** during the removal of TFA (for Boc and *O-tert*-butyl deprotection) with the aid of MeOH. Compound **6.4** was isolated during the purification of bivalent ligand **3.8** with preparative HPLC and turned out to be an Y_1 receptor antagonist with an affinity in the very low nanomolar range (K_i = 0.94, Table 1). NMR analysis enabled the elucidation of the structure and consequently the synthesis of compound **6.4**. Interestingly, the N^{ω}-carbamoylated BIBP 3226 derivative **6.9**, bearing an ethylester group in the same distance from the guanidine as the methyl ester moiety in compound **6.4**, was formerly reported to bind with a comparable high affinity (K_i = 0.74, Table 1).[1, 2]

BIBO 3304 (**6.6**), reported to be about ten times more potent than BIBP 3226[3], was prepared with the intension to obtain functionalized (fluorescence and radiolabeled) Y_1R ligands with increased affinities over analogous BIBP 3226 derivatives (described in chapters 4 and 5). Unfortunately N^{G}-propanoyl-BIBO 3304 (**6.7**) proved to be about 30 times less potent than the parent compound (K_i = 7.8 and 0.25 nM, resp., Table 1), whereas N^{ω}-propanoyl-BIBP 3226 (**2.8**) exhibits the same affinity as unsubstituted BIBP 3226 (K_i = 1.0 and 1.3 nM, resp., Table 1). This suggests different binding modes of these two argininamides in the binding pocket of the Y_1 receptor. Though the preparation of tritiated N^{G}-propanoyl-BIBO 3304 (**6.7**) may be taken into account, the corresponding tritiated BIBP 3226 derivative, [^{3}H]-UR-MK114 (**2.8b**, cf. chapter 2), is more attractive in terms of affinity and synthetic availability.

A much more attractive alternative to [^{3}H]-UR-MK114 (**2.8b**) is the potential radioligand **6.5** (Table 1), which is as potent as **2.8b** (K_i = 1.2 nM and K_D = 1.2 nM, resp.) and can be easily prepared as a tritiated ligand from amine precursor **4.27** and succinimidyl [2,3-^{3}H]propanoate (cf. Scheme 2). Since tritiated propanoic acid is attached to a primary amine in case of **6.5**, and not to the guanidine group as in **2.8b**, the labeling reaction is anticipated to be superior to the preparation of radioligand **2.8b** with respect to yield, time and deprotection after radiolabeling. In contrast to the preparation of [^{3}H]-UR-MK114 (**2.8b**) final deprotection is not required for a synthesis of the tritiated analog of **6.5**.

The K_i and K_b values from binding and functional assays are in good accordance for Y_1R antagonists such as BIBP 3226, BIBO 3304 (**6.6**) and compound **6.5**. For other argininamide-type Y_1R antagonists there was a discrepancy between K_i and K_b value of about a factor of ten (Table 1), suggesting the correlation between K_b and K_i value to depend on the chemical structure of the Y_1R antagonists. Further reasons for a misrepresentation of the observed antagonistic effect in the fura-2 Ca^{2+}-assay were discussed in chapter 4 (4.2.2).

The Y_1 receptor subtype selectivity was confirmed for the potential radioligand **6.5**, which is an attractive alternative to the tritium ligand [^{3}H]-UR-MK114 (cf. chapter 2). Selectivity data are shown in Table 2.

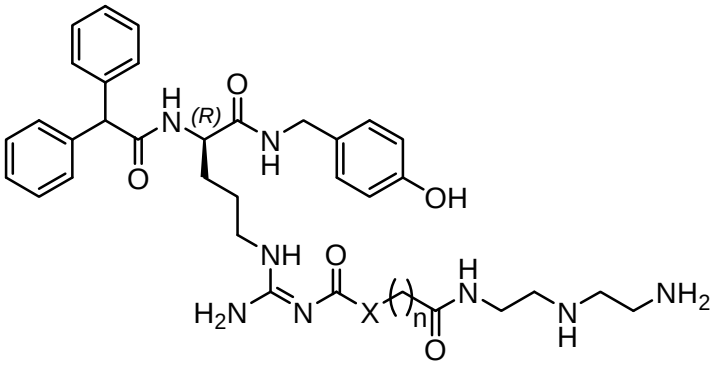
Table 2. NPY receptor subtype selectivity of the potential radioligand **6.5**.

Y_1 K_i [nM][a]	Y_2 K_i [nM][b]	Y_4 K_i [nM][c]	Y_5 K_i [nM][b]
1.3	> 2,000	> 5,000	> 5,000

[a]Dissociation constant from radioligand competition binding assay with [^{3}H]-UR-MK114 (cf. chapter 2, c = 1.5 nM) on SK-N-MC neuroblastoma cells. [b]Flow cytometric binding assay on CHO-Y_2 and HEC-1B-Y_5 cells using Dy-635-pNPY as labeled ligand (10 nM). [c]Flow cytometric binding assay on CHO-Y_4 cells with Cy5-[K^4]-hPP (3 nM) as fluorescent ligand.

6.4 Perspectives

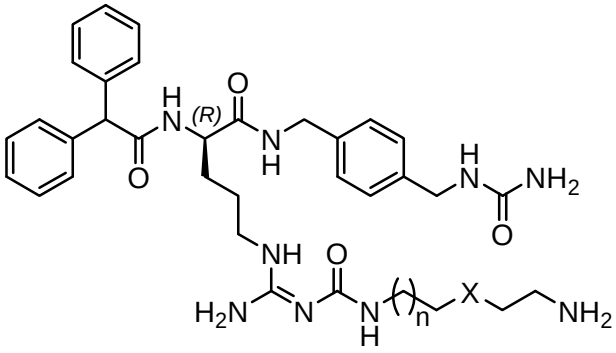
As discussed in chapter 5, the lipophilicity of the potential PET ligands is a major drawback of these BIBP 3226 derivatives, probably being the reason for the failure to image Y_1 receptors *in vivo*. The calculated logP values of most of the BIBP 3226 derived Y_1R antagonists, presented in this thesis, are in the range of 1 - 5, for some fluorescent ligands (chapter 4) even higher. Although the actual lipophilicity of the compounds at pH 7.4 is lower due to one or more positive charges (guanidine, fluorophore), a further decrease in lipophilicity is preferable. Compared to the introduction of glycol ether moieties, which leads only to a moderate gain in polarity, the integration of secondary amines into the linker (Figure 1) should have a stronger impact on the polarity. Such a decrease in lipophilicity is supposed to reduce interferences with membrane binding and cell penetration. Acylation of such amine precursors at the primary amino group in terms of functionalization should give only a low portion of side products as the primary amine is sterically preferred, and an excess of the precursor can be used.



X = CH_2, NH

Figure 1. N^{ω}-Acylated BIBP 3226 derivatives with terminal amino group and secondary basic nitrogen.

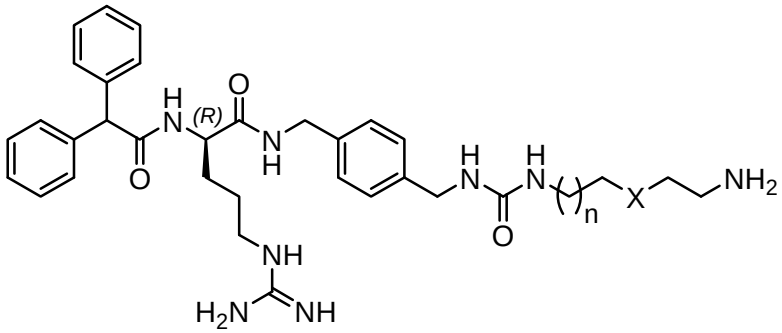
Different Y_1 receptor binding modes for the (*R*)-argininamides BIBP 3226 and BIBO 3304 were concluded from the binding data of these Y_1R antagonists and their N^G-propionylated derivatives (discussed above, cf. Tabe 1). Additionally, N^{ω}-acylated and N^{ω}-carbamoylated BIBP 3226 analogs seem to bind in slightly different modes (compare chapter 4). Therefore, the introduction of carbamoyl substituents into BIBO 3304 (cf. Figure 2) should be considered with respect to further investigation of the structure-activity relationships and binding modes of argininamide-type Y_1R antagonists.



X = $(CH_2)_n$, O-CH_2-CH_2-O, CONH, HNCO, NH, etc.

Figure 2. N^G-Carbamoylated BIBO 3304 derivatives with terminal amino group.

Assumed that the second polar moiety of BIBO 3304, the ureido group, is oriented to the extracellular space, an attachment of amino-functionalized linkers to the ureido function could be tolerated without a substantial loss of affinity (cf. Figure 3). The synthesis of such BIBO 3304 derivatives seems feasible according to the synthetic strategy described in this thesis (cf. synthesis of **4.6** in chapter 4).



X = $(CH_2)_n$, O-CH_2-CH_2-O, CONH, HNCO, NH, etc.

Figure 3. N^U-Substituted BIBO 3304 derivatives with terminal amino group.

6.5 Experimental Section

6.5.1 General Experimental Conditions

Unless otherwise noted, chemicals and solvents were purchased from commercial suppliers and used without further purification. Dy-635-pNPY was synthesized as described previously.[4] [K[4]]-hPP was synthesized in the laboratory of Prof. Beck-Sickinger (University of Leipzig, Germany) and labeled with the cyanine dye Cy5 (FEW chemicals (Bitterfeld-Wolfen, Germany) in our laboratory.[5] Porcine NPY (pNPY) was prepared in-house in the laboratory of Dr. C. Cabrele. Millipore water was used throughout for the preparation of buffers and HPLC eluents. Petroleum ether (40-60 °C) was distilled before use. DMF was stored over a molecular sieves (3 Å). Anhydrous reactions were run under an atmosphere of dry nitrogen or argon.
Thin layer chromatography was performed on Merck silica gel 60 F_{254} TLC aluminium plates. For column chromatography silica gel Geduran 60 (Merck, 0.063-0.200 mm) was used. NMR spectra were recorded on a Bruker Avance 300 (1H: 300 MHz), a Bruker Avance 600 (1H: 600 MHz) and a Bruker Avance III 600 with cryogenic probehead (1H: 600 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. Mass spectrometry analysis (MS) was performed in-house on a Finnigan SSQ 710A (EI-MS 70 eV) and a Finnigan MAT 95 (LSIMS, HRMS). Melting points were determined with a Büchi 510 melting point apparatus and are uncorrected.

Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Eurospher-100 C18 (250 × 32 mm, 5 µm; Knauer, Germany) served as RP-column at a flow rate of 38 mL/min. Mixtures of acetonitrile (in case of **6.2** MeOH) and 0.1 % aq. TFA were used as mobile phase. Acetonitrile or MeOH was removed from the eluates under reduced pressure (final pressure: 60 mbar) at 40 °C prior to lyophilization. Analytical HPLC analysis was performed on a system from Thermo Separation Products (composed of a SN400 controller, a P4000 pump, a degasser (Degassex DG-4400, phenomenex), an AS3000 autosampler and a Spectra Focus UV-VIS detector). An Eurospher-100 C18 (250 × 4 mm, 5 µ, Knauer, Germany) or a Nucleodur 100-5 C18 ec (250 × 4 mm, 5 µm, Macherey-Nagel, Germany) served as RP-columns. Acetonitrile (A) and 0.05 % aq. TFA (B) were used as mobile phase components. Helium degassing, an oven temperature of 30 °C and a flow rate of 0.8 mL/min were used throughout. Solutions for injection (concentrations in the two-digit µM range) were prepared in a mixture of A and B corresponding to the composition at the beginning of the gradient. The following gradients were applied for analytical HPLC analysis:
Gradient 1: 0 to 25 min: A/B 25/75 to 65/35, 25 to 27 min: 65/35 to 95/5, 27 to 31 min: 95/5
Gradient 2: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5

The preparation of the buffer and the lysis solution for Y_1R binding studies on SK-N-MC cells as well as the loading buffer for the determination of intracellular Ca^{2+} mobilization in HEL cells are described in chapter 3.

Stock solutions were prepared in DMSO at concentrations of 10 mM and stored at –78 °C.

6.5.2 Chemistry: Experimental Protocols and Analytical Data

Succinimidyl butanoate (6.1).[6] Butyric chloride (1.02 g, 9.6 mmol, 1.2 eq) was added dropwise to a stirred ice-cold solution of *N*-hydroxysuccinimide (0.92 g, 8 mmol, 1 eq) and NEt_3 (1.2 g, 1.7 mL, 12 mmol, 1.5 eq) in anhydrous THF (40 mL) over a period of 5 min. The ice-bath was removed and stirring was continued at rt for 5 h. The white solid (triethylammonium chloride) was removed by filtration and the solvent was removed under reduced pressure. Purification with column chromatography (eluent: PE/EtOAc 2/1 to 1/1) yielded the product as white solid (1.41 g, 7.61 mmol, 95 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.03 (t, 3H, ^3J = 7.42 Hz, CH_3), 1.78 (m, 2H, $C\underline{H}_2$-CH_3), 2.58 (t, 2H, ^3J = 7.33 Hz, CO-$C\underline{H}_2$-CH_2-CH_3), 2.83 (s, 4H, CO-$C\underline{H}_2$-$C\underline{H}_2$-CO); MS (EI): *m/z* 185 ($M\cdot^+$); $C_8H_{11}NO_4$ (185.2)

(*R*)-*N*$^{\omega}$-Butanoyl-*N*$^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide (6.2). 2.7 (32.7 mg, 51.9 µmol, 1 eq, cf. chapter 2) in dry acetonitrile (1.5 mL) was added to a solution of **6.1** (9.6 mg, 51.9 µmol, 1 eq) in anhydrous acetonitrile (0.2 mL). NEt_3 (5.3 g, 7.2 µL, 51.9 µmol, 1 eq) was added and the mixture was stirred at rt for 20 h. TFA (2 mL) was added and stirring was continued at 40 °C for 1.5 h. MeOH (6 mL) was added followed by evaporation under reduced pressure. Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as a white fluffy solid (18.9 mg, 28.7 µmol, 55 %). Mp > 164 °C (decomp.); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 0.91 (t, 3H, ^3J = 7.39 Hz, CH_3), 1.44 (m, 2H, CH-CH_2-$C\underline{H}_2$), 1.5-1.74 (m, 4H, CH-$C\underline{H}_2$, CO-CH_2-$C\underline{H}_2$), 2.39 (t, 2H, ^3J = 7.23 Hz, CO-CH_2), 3.23 (m, 2H, CH_2-$C\underline{H}_2$-NH), 4.14 (m, 2H, $C\underline{H}_2$-ArOH), 4.34 (m, 1H, $CH^{(\alpha)}$), 5.12 (s, 1H, $C\underline{H}$-$(Ph)_2$), 6.67 (d, 2H, ^3J = 8.5 Hz, AA'BB'), 7.0 (d, 2H, ^3J = 8.49 Hz, AA'BB'), 7.18-7.31 (m, 10H, Ph), 8.38 (t, 1H, ^3J = 5.72 Hz, CH-CO-N$\underline{H}$-CH_2), 8.49 (d, 1H, ^3J = 8.07 Hz, CO-N$\underline{H}$-CH), 8.56 (bs, 2H, $N\underline{H}_2$), 8.88 (s, 1H, N$\underline{H}$), 9.29 (s, 1H, N$\underline{H}$), 11.26 (s, 1H, ArO$\underline{H}$); RP-HPLC (Nucleodur 250 × 4 mm, 210 nm, gradient 1): > 99 % (t_R = 13.3 min, k = 4.8); HRMS: (FAB^+, MeOH/glycerin): *m/z* calcd. for $[C_{31}H_{37}N_5O_4 + H]^+$ 544.2924, found: 544.2909; $C_{31}H_{37}N_5O_4 \times C_2HF_3O_2$ (657.7)

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-hydroxybenzyl)-*N*$^{\omega}$-pentanoylargininamide (6.3). Pentanoic anhydride (97.6 mg, 0.52 mmol, 1.1 eq) was added to a solution of **2.7** (300 mg, 0.48 mmol, 1 eq, cf. chapter 2) and NEt_3 (48 mg, 66 µL, 0.48 mmol, 1 eq) in CH_2Cl_2 (10 mL). The mixture was stirred at rt for 5 h. TFA (10 mL) was added and stirring was continued at rt for 2 h.

MeOH (30 mL) was added followed by evaporation under reduced pressure. Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as a white fluffy solid (190 mg, 0.28 mmol, 59 %). Mp > 118 °C (decomp.); ^{1}H-NMR (400 MHz, CD_3OD, COSY): δ (ppm) 0.93 (t, 3H, ^3J = 7.35 Hz, CH_3), 1.37 (m, 2H, C$\underline{H}_2$-CH_3), 1.48-1.76 (bm, 5H, CH-C$\underline{H}_2$-C$\underline{H}_2$, C$\underline{H}_2$-CH_2-CH_3), 1.83 (m, 1H, CH-C$\underline{H}_2$-CH_2), 2.45 (t, 2H, ^3J = 7.44 Hz, CH_2-CO), 3.23 (m, 2H, CH_2-C$\underline{H}_2$-NH), 4.2 (d, 1H, ^2J = 14.6 Hz, C$\underline{H}_2$-ArOH), 4.26 (d, 1H, ^2J = 14.59 Hz, C$\underline{H}_2$-ArOH), 4.43 (m, 1H, CH$^{\alpha}$), 5.07 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 2H, ^3J = 8.6 Hz, AA'BB'), 7.04 (d, 2H, ^3J = 8.62 Hz, AA'BB'), 7.16-7.31 (m, 10H, Ph); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 99 % (t_R = 16.6 min, k = 5.1); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{32}H_{39}N_5O_4 + H]^+$ 558.3080, found: 558.3080; $C_{32}H_{39}N_5O_4 \times C_2HF_3O_2$ (671.7)

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-hydroxybenzyl)-*N*$^{\omega}$-(8-methoxy-8-oxooctanoyl)arginin-amide (6.4). EDAC (21.3 mg, 111 µmol, 1.4 eq) was added to a solution of **2.7** (50 mg, 79 µmol, 1 eq, cf. chapter 2), octanedioic acid monomethylester (18 mg, 95 µmol, 1.2 eq) and DMAP (3 mg) in CH_2Cl_2 (2 mL). The mixture was stirred at rt overnight. TFA (1.5 mL) and two drops of water were added and stirring was continued at rt for 4 h. CH_2Cl_2 (20 mL) was added three times, each time followed by evaporation under reduced pressure. Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as a white fluffy solid (49.3 mg, 65 µmol, 82 %). Mp > 106 °C (decomp.); ^{1}H-NMR (600 MHz, CD_3OD, COSY): δ (ppm) 1.36 (m, 4H, CH_2-CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-CH_2), 1.5-1.74 (m, 7H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CH_2-C$\underline{H}_2$-CH_2-CH_2-C$\underline{H}_2$-CH_2), 1.83 (m, 1H, CH-C$\underline{H}_2$-CH_2), 2.32 (t, 2H, ^3J = 7.39 Hz, CO-CH_2), 2.44 (t, 2H, ^3J = 7.37 Hz, CO-CH_2), 3.25 (m, 2H, CH_2-C$\underline{H}_2$-NH), 3.64 (s, 3H, CH_3), 4.22 (d, 1H, ^2J = 14.56 Hz, C$\underline{H}_2$-ArOH), 4.26 (d, 1H, ^2J = 14.56 Hz, C$\underline{H}_2$-ArOH), 4.42 (m, 1H, CH$^{\alpha}$), 5.06 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.7 (d, 2H, ^3J = 8.56 Hz, AA'BB'), 7.05 (d, 2H, ^3J = 8.57 Hz, AA'BB'), 7.21-7.32 (m, 10H, Ph); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 99 % (t_R = 17.3 min, k = 5.4); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{36}H_{45}N_5O_6 + H]^+$ 644.3448, found: 644.3432; $C_{36}H_{45}N_5O_6 \times C_2HF_3O_2$ (757.8)

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-hydroxybenzyl)-*N*$^{\omega}$-[4-(propanoylamino)butyl]amino-carbonyl-argininamide (6.5). Amine precursor **4.27** (20 mg, 24.5 µmol, 1.05 eq) and NEt_3 (7.1 mg, 9.7 µL, 70 µmol, 3 eq) were dissolved in DMF (150 µL) followed by the addition of active ester **2.11a** (4 mg, 23.4 µmol, 1 eq) in DMF (150 µL). The reaction was stopped by addition of 10 % aq. TFA (corresponding to 2 - 3 eq of TFA) after an incubation period of 2.5 h at rt. Purification with preparative HPLC (column: Eurospher 250 × 32 mm) and lyophilization afforded the product as a white fluffy solid (15.2 mg, 20.1 µmol, 86 %). Mp > 65 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 0.97 (t, 3H, ^3J = 7.61 Hz, CH_3), 1.4 (m, 6H, CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2, CH-CH_2-C$\underline{H}_2$), 1.53 (m, 1H, CH-C$\underline{H}_2$), 1.66 (m, 1H, CH-C$\underline{H}_2$), 2.04 (q, 2H, ^3J =

7.6 Hz, CO-CH_2), 3.02 (m, 2H, CH_2-CH_2-CH_2-C$\underline{H}_2$-NH), 3.09 (m, 2H, CH_2-CH_2-CH_2-C$\underline{H}_2$-NH), 3.18 (m, 2H, CH-CH_2-CH_2-C$\underline{H}_2$-NH), 4.11 (dd, 1H, ^{2}J = 14.78 Hz, ^{3}J = 5.69 Hz, C$\underline{H}_2$-ArOH), 4.16 (dd, 1H, ^{2}J = 14.79 Hz, ^{3}J = 5.87 Hz, C$\underline{H}_2$-ArOH), 4.33 (m, 1H, CH$^{\alpha}$), 5.11 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.66 (d, 2H, ^{3}J = 8.49 Hz, AA'BB'), 6.99 (d, 2H, ^{3}J = 8.49 Hz, AA'BB'), 7.22 (m, 2H, Ph), 7.28 (m, 8H, Ph), 7.47 (s, 1H, CH_2-CH_2-CH_2-CH_2-N$\underline{H}$), 7.71 (t, 1H, ^{3}J = 5.17 Hz, CH_2-CH_2-CH_2-CH_2-N$\underline{H}$), 8.34 (t, 1H, ^{3}J = 5.82 Hz, CH-CO-N$\underline{H}$-CH_2), 8.37 (bs, 2H, N$\underline{H}_2$), 8.45 (d, 1H, ^{3}J = 8.12 Hz, CO-N$\underline{H}$-CH), 8.89 (s, 1H, CH-CH_2-CH_2-CH_2-N$\underline{H}$), 9.26 (s, 1H, N$\underline{H}$), 9.68 (s, 1H, ArO$\underline{H}$); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 99 % (t_R = 13.1 min, k = 3.9); HRMS: (FAB^+, MeOH/glycerin): *m/z* calcd. for $[C_{35}H_{45}N_7O_5 + H]^+$ 644.3555, found: 644.3572; $C_{35}H_{45}N_7O_5 \times C_2HF_3O_2$ (757.8)

Synthesis of BIBO 3304 (6.6) and *N*$^{\omega}$-propanoyl-BIBO 3304 (6.7). Succinimidyl propanoate (**2.11a**, cf. chapter 2) (47.6 mg, 278 µmol, 0.8 eq) and NEt_3 (141 mg, 193 µL, 139 µmol, 4 eq) were added to a cloudy solution of BIBO 3304 building block **4.6** (240 mg, 348 µmol, 1 eq, cf. chapter 4) in MeOH (5 mL). The mixture was stirred at rt overnight. HPLC analysis revealed that almost no product was formed. Therefore succinimidyl propanoate (95.2 mg, 556 µmol, 1.6 eq) and NEt_3 (141 mg, 193 µL, 139 µmol, 4 eq) were added and stirring was continued at 50 °C for 2.5 h. TFA (15 mL) was added and stirring was continued at rt for 4 h. MeOH (25 mL) was added three times, each time followed by evaporation under reduced pressure. Purification with preparative HPLC (column: Eurospher, 250 × 32 mm) and lyophilization afforded the products as white fluffy solids.

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-ureidomethylbenzyl)argininamide (6.6). 146.6 mg, 228 µmol, 66 %; mp > 60 °C (decomp.); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.4 (m, 2H, CH-CH_2-C$\underline{H}_2$), 1.53 (m, 1H, CH-C$\underline{H}_2$), 1.66 (m, 1H, CH-C$\underline{H}_2$), 3.06 (m, 2H, CH_2-C$\underline{H}_2$-NH), 4.15 (s, 2H, C$\underline{H}_2$-Ar), 4.23 (d, 2H, ^{3}J = 5.71 Hz, C$\underline{H}_2$-Ar), 4.34 (m, 1H, C$\underline{H}^{\alpha}$), 5.13 (s, 1H, C$\underline{H}$-$(Ph)_2$), 6.47 (s, 1H, NH), 7.05 (bs, 2H, NH_2), 7.15 (m, 4H, AA'BB'), 7.26 (m, 10H, Ph), 7.59 (t, 1H, ^{3}J = 5.63 Hz, NH), 8.52 (m, 2H, 2 × NH); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): > 99 % (t_R = 10.8 min, k = 3.0); HRMS: (FAB^+, MeOH/glycerin): *m/z* calcd. for $[C_{29}H_{35}N_7O_3 + H]^+$ 530.2880, found: 530.2867; $C_{29}H_{35}N_7O_3 \times C_2HF_3O_2$ (643.7)

(*R*)-*N*$^{\alpha}$-(2,2-Diphenylacetyl)-*N*$^{\omega}$-propanoyl-*N*-(4-ureidomethylbenzyl)argininamide (6.7). 9.4 mg, 13.4 µmol, 4 %; mp > 192 °C (decomp.); ^{1}H-NMR (600 MHz, DMSO-d_6, COSY): δ (ppm) 1.04 (t, 3H, ^{3}J = 7.41 Hz, CH_3), 1.42 (m, 2H, CH-CH_2-C$\underline{H}_2$), 1.54 (m, 1H, CH-C$\underline{H}_2$), 1.68 (m, 1H, CH-C$\underline{H}_2$), 2.46 (q, 2H, ^{3}J = 7.39 Hz, CO-CH_2), 3.22 (m, 2H, CH_2-C$\underline{H}_2$-NH), 4.13 (d, 2H, ^{3}J = 5.85 Hz, C$\underline{H}_2$-Ar), 4.22 (m, 2H, C$\underline{H}_2$-Ar), 4.34 (m, 1H, C$\underline{H}^{\alpha}$), 5.11 (s, 1H, C$\underline{H}$-$(Ph)_2$), 5.5 (bs, 2H, NH_2), 6.37 (t, 1H, ^{3}J = 5.95 Hz, N$\underline{H}$-CH_2-Ar), 7.12 (d, 2H, ^{3}J = 8.48 Hz, AA'BB'), 7.14 (d, 2H, ^{3}J = 8.46

Hz, AA'BB'), 7.22 (m, 2H, Ph), 7.28 (m, 8H, Ph), 8.45 (t, 1H, ^3J = 5.96 Hz, N<u>H</u>-CH_2-Ar), 8.48 (d, 1H, ^3J = 8.1 Hz, CO-N<u>H</u>-CH), 8.53 (bs, 2H, N<u>H_2</u>), 8.87 (s, 1H, CH_2-CH_2-N<u>H</u>), 11.22 (s, 1H, NH); RP-HPLC (Eurospher 250 × 4 mm, 210 nm, gradient 2): 98 % (t_R = 12.3 min, k = 3.6); HRMS: (FAB$^+$, MeOH/glycerin): *m/z* calcd. for $[C_{32}H_{39}N_7O_4 + H]^+$ 586.3142, found: 586.3100; $C_{32}H_{39}N_7O_4 \times C_2HF_3O_2$ (699.7)

6.5.3 Pharmacology: Cell Culture, Fura-2 Assay and Competition Binding Assay

Cell culture. HEL and SK-N-MC cells were cultured as described elsewhere.[7, 8] HEL cells were subcultured by 1:6-dilution with fresh culture medium 24 h prior to the fura Ca^{2+}-assay. The CHO cells transfected with the Y_2R, $G_{\alpha qi5}$ and aequorin were cultured as previously described.[4] CHO cells transfected with the Y_4R, $G_{\alpha qi5}$ and aequorin were cultured under the same conditions. HEC-1B cells were cultured as previously described.[9]

Fura-2 assay on HEL cells and radioligand competition binding assay. The Fura assay was performed with HEL cells as previously described[10] using a Perkin-Elmer LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany). The fura-2 AM (Calbiochem/Merck Biosciences, Beeston, UK) stock solution (1 mM) was prepared in anhydrous DMSO. Pluronic F-127 (Calbiochem/Merck Biosciences, Beeston, UK) was dissolved in DMSO to a concentration of 20 %.
Radioligand competition experiments with [^{3}H]-UR-MK114 (cf. Chapter 2) were performed as described in chapter 3.

6.5.4 Pharmacology: Flow Cytometric Experiments with the Potential Radioligand 6.5

Flow cytometric binding studies at the Y_2R (CHO cells transfected with the Y_2R, $G\alpha_{qi5}$ and aequorin)[11] and at the Y_5R (expressed by HEC-1B cells transfected with the Y_5R)[4, 12] were performed as previously described. The cell density in loading buffer was 10^6 cells/mL (Y_2R) or $0.5 \cdot 10^6$ cells/mL (Y_5R). For Y_2R binding studies Dy-635-pNPY (10 nM) was used instead of Cy5-pNPY (10 nM).
Flow cytometric binding studies at the Y_4R (expressed by CHO cells transfected with the Y_4R, $G\alpha_{qi5}$ and aequorin)[5] were performed as reported[13] with the following variations: Cy5-[K^4]-hPP[5] (3 nM) was used instead of S0586-[K^4]-hPP (10 nM). The cell density in loading buffer was 10^6 cells/mL. Non-specific binding was determined in the presence of hPP (100 nM). The stock solution of Cy5-[K^4]-hPP was prepared in 10 mM HCl + 0.1 % BSA (500 nM).

All flow cytometric assays were performed in triplicate on a FACSCalibur™ flow cytometer (Becton Dickinson, Heidelberg, Germany), equipped with an argon laser (488 nm) and a red diode laser (635 nm). **6.5** was tested at a concentration of 1 µM and 10 µM. Whereas no displacement of the fluorescent ligand by **6.5** (10 µM) was observed in case of the Y_5R, displacement at the Y_2R was around 50 % and at the Y_4R around 30 %.

6.6 References

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Chapter 7

Functionalized Arginine Building Blocks

7.1 Introduction

Radio- and fluorescence labeled analogs of endogenous oligopeptides addressing membrane receptors (e.g. GPCRs) are valuable pharmacological tools for the identification and pharmacological characterization of these receptors and their ligands. Moreover, labeling of peptides is a promising strategy to develop radiopharmaceuticals. Since a wide range of peptide receptors is over-expressed on tumor cells or matrix components, chemical strategies have been developed that allow radiolabeling of the corresponding peptidic ligands in order to obtain tracers for *in vivo* imaging techniques such as PET and SPECT.

As only a few natural amino acids (e.g. Lys, Tyr, Cys) enable fluorescence or radiolabeling of peptides, the introduction of such amino acids, i.e. structural variation of the amino acid sequence, is often required. Generally, such structural variations may be associated with a decrease in affinity. However, there are examples of retained affinity after replacement of amino acids with Lys for introduction of a label, for instance [Lys^4]hPP as a Y_4R agonist.[1]

In contrast to labeling strategies targeting Lys, Tyr or Cys, structural modifications of arginine are not routine, since appropriate building blocks are not available. Moreover, it may be speculated that such derivatization approaches have little prospect of success since the label has to be attached to the guanidino group in Arg which is a key amino acid for receptor binding of many peptides. Thus, N^{ω}-substitution in Arg can negatively affect receptor affinity.

Recently, a successful guanidine-acylguanidine bioisosteric approach was reported by our research group for NPY Y_1R[2] (cf. also chapters 2, 4 - 6), Y_2R[3] and histamine H_2 receptor[4] ligands. Acylation of the guanidine or alkyl-guanidine group leads to a decrease in the strong basicity of the guanidine (pK_a = 12 – 13) to pK_a values of about 8. On one hand such acylguanidines are still sufficiently basic to interact with acid key residues of the receptor protein, on the other hand the lower basicity leads to more favorable pharmacokinetics[5]. This bioisosteric approach proved to be of special value in the development of radio- and fluorescence labeled argininamide-type Y_1R antagonists as described in chapters 2 – 6.

The achievements in the NPY antagonist field prompted to prepare N^{α}-Fmoc-N^{ω}-Boc-arginine bearing an appropriate $N^{\omega'}$-acyl residue as a versatile building block for solid phase synthesis of modified peptides. In principle, such arginine derivatives could be universally applied to prepare ligands for any peptidergic receptor. With respect to radio- and fluorescence labeling the $N^{\omega'}$-

substituent should be functionalized, preferably with an ω-amino group (protected during peptide synthesis), to enable labeling.

Target peptides for the integration of the modified arginine have to contain at least one arginine residue, preferably in the N-terminal region, and should not exceed 10 – 15 amino acids, since the presented arginine building blocks are less stable (discussed below) than the protected amino acids routinely used in solid phase synthesis. Some physiologically relevant peptides containing at least one arginine residue are listed in Table 1. As arginine-containing receptor ligands, such as the RGD peptides as well as neurotensin, bombesin, substance P (Table 1) and analogs thereof, are investigational diagnostic radiopharmaceuticals,[6] the novel arginine building blocks described in this chapter could promote the development and broaden the spectrum of molecular imaging tools.

Table 1. Sequences and targets of a selection of endogenous arginine-containing oligopeptides.

Peptide	Sequence	Receptor
RGD peptides	small peptides containing the *Arg*-Gly-Asp motif	$\alpha_V\beta_3$ integrin
angiotensin II	Asp-*Arg*-Val-Tyr-Ile-His-Pro-Phe-OH	AT_1
bradykinin	*Arg*–Pro–Pro–Gly–Phe–Ser–Pro–Phe–*Arg*-OH	B_1, B_2
neurotensin	pyroGlu-Leu-Tyr-Glu-Asn-Lys-Pro-*Arg*-*Arg*-Pro-Tyr-Ile-Leu-OH	NT receptors
bombesin	pyroGlu-Gln-*Arg*-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	GRP[a], NMB[b], BB_3[c]
substance P	*Arg*−Pro−Lys−Pro−Gln−Gln−Phe−Phe−Gly−Leu−Met−NH_2	NK1, NK2, NK3[d]

[a]gastrin releasing peptide, [b]neuromedin B, [c]bombesin subtype-3, [d]neurokinin 1, 2 and 3

7.2 Results and Discussion

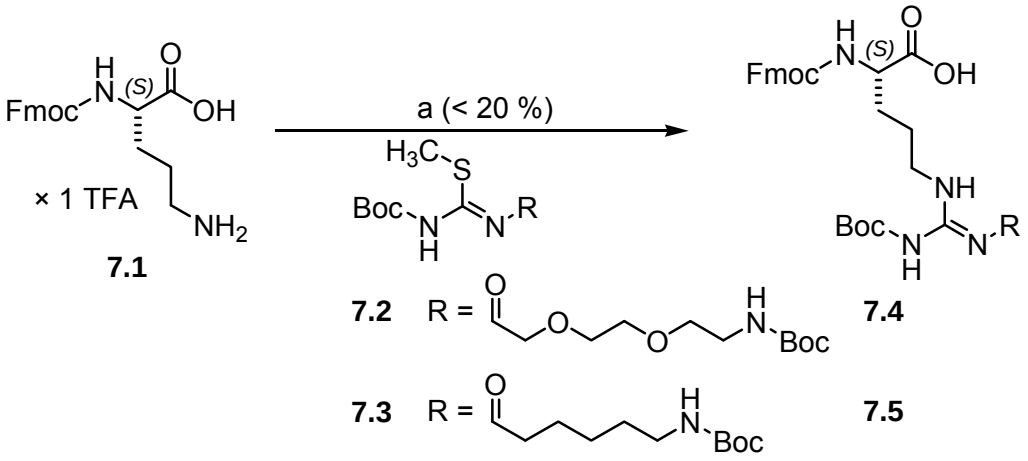
7.2.1 Preparation of Modified Arginine Building Blocks

The synthesis of N^{α}-Fmoc, N^{ω}-Boc, $N^{\omega'}$-acylated arginine derivatives is impeded in terms of efficiency (yield), purification and stability of building blocks and target compounds. Guanidine moieties bearing two electron-withdrawing substituents (e.g. Cbz and Boc or acyl and carbamoyl residues) are less stable than the corresponding mono-substituted guanidines. This could be clearly demonstrated by the mono-Boc deprotection of arginine derivative **7.11** which contains a N^{G},$N^{G'}$-diBoc protected guanidine entity. One of the Boc groups can be selectively removed at pH 2 – 3 (Scheme 3, Figure 2) resulting in a mono-Boc-substituted guanidine, which is more resistant under the same acidic conditions, i. e. the retained Boc group gains stability upon cleavage of the first one. Moreover, for N^{G}-Boc, $N^{G'}$-acylated guanidines the sensitivity of

the acyl residue was higher than that of the Boc group at pH > 5. The impact of the electron-withdrawing groups on the stability of the linker has to be considered as long as the N^{ω}-Boc (or Cbz) group is present: as soon as this protecting group is removed (e.g. after completion of the peptide synthesis), the N^{ω}-acylated arginine derivatives turn into relatively stable chemical structures.

Here some efforts are presented to cope with the aforementioned problems, to economically prepare a few arginine building blocks in mg scale (up to 200 mg) as well as to demonstrate that these building blocks can be used for solid phase peptide synthesis. In view of a proof of concept racemization (enantiomeric purities of the presented arginine building blocks) was not taken into account.

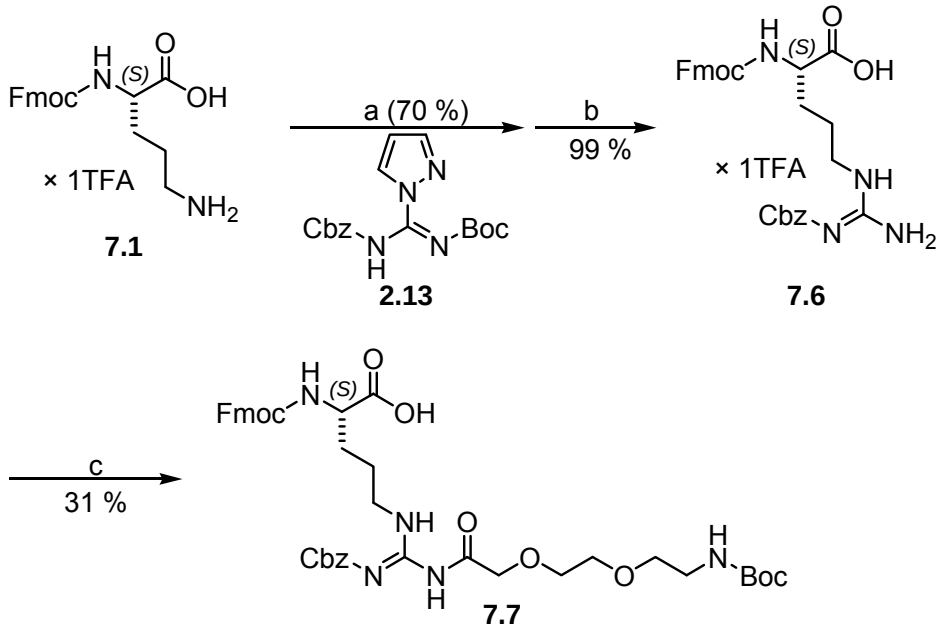
Initial attempts to prepare the arginine building blocks **7.4** and **7.5** through guanidinylation of N^{α}-Fmoc-*L*-ornithine (**7.1**) using *S*-methylisothiourea derivatives (**7.2**, **7.3**) failed due to very low yields and numerous by-products (Scheme 1). The guanidinylating reagent **7.3** was synthesized by A. Brennauer in our research group[3] and the analog **7.2** was synthesized using a similar protocol. N^{α}-Fmoc-*L*-ornithine (**7.1**) was obtained through Cbz-deprotection of N^{δ}-Cbz-N^{α}-Fmoc-*L*-ornithine **3.1** (cf. chapter 3). As the Fmoc group is cleaved to some extent during hydrogenolysis (Pd/C, H_2, rt, atmospheric pressure), traces of ornithine had to be removed with preparative reversed-phase HPLC.



Scheme 1. Synthesis of the arginine building blocks **7.4** and **7.5** via guanidinylation of N^{α}-Fmoc-ornithine. Reagents and conditions: (a) $HgCl_2$, NEt_3, CH_2Cl_2/DMF, rt, 24-72 h, 10-20 %.

Guanidinylation of N^{α}-Fmoc-*L*-ornithine (**7.1**) using the *N*,*N*'-diurethane-protected 1*H*-pyrazole-1-carboxamidine **2.13** (cf. chapter 2) afforded the corresponding arginine derivative in good yield (Scheme 2) but the purification with preparative HPLC using acetonitrile and 0.02 % aq. TFA as eluent resulted in partial cleavage of the N^{ω}-Boc group. This gave reason to prepare N^{α}-Fmoc, N^{ω}-Cbz protected *L*-arginine (**7.6**) instead of the N^{ω}-Boc analog as a precursor for the introduction of ω-(Boc-amino)alkanoyl substituents at the guanidine group (Scheme 2). The coupling of 8-(Boc-amino)-3,6-dioxaoctanoic acid to **7.6** resulted in the N^{ω}-Cbz protected

arginine building block **7.7** (Scheme 2). This compound was obtained with about 30 % yield after purification with preparative HPLC using acetonitrile and 0.05 % aq. TFA as eluent. A minor fraction was degraded to **7.6** during evaporation of acetonitrile at 37 °C (analyzed with ES-MS and HPLC, cf. Figure 1).



Scheme 2. Synthesis of the arginine building block **7.7**.
Reagents and conditions: (a) NEt_3, CH_2Cl_2, acetonitrile, rt, 20 h, 70 %; (b) acetonitrile, 0.05 % aq. TFA, 40 °C, 4 h, 99 %; (c) 8-(Boc-amino)-3,6-dioxaoctanoic acid, CDI, NEt_3, CH_2Cl_2, rt, 20 h, 31 %.

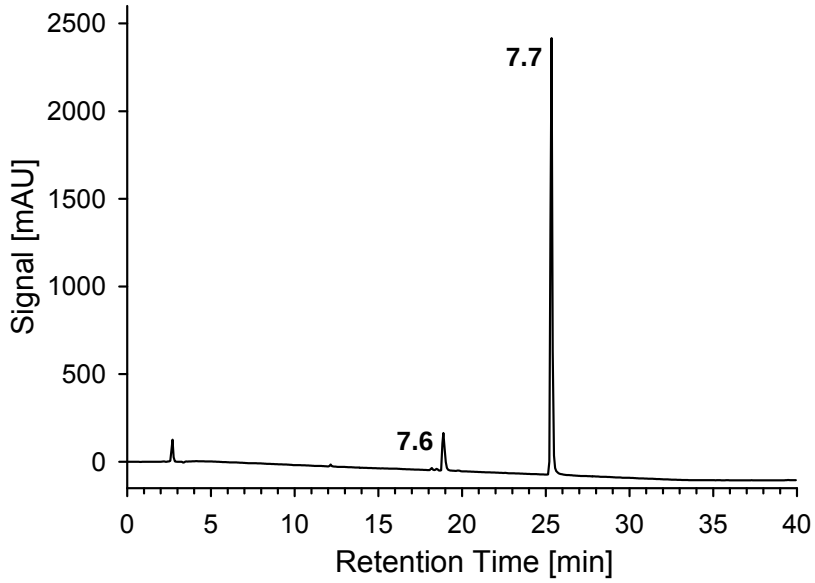
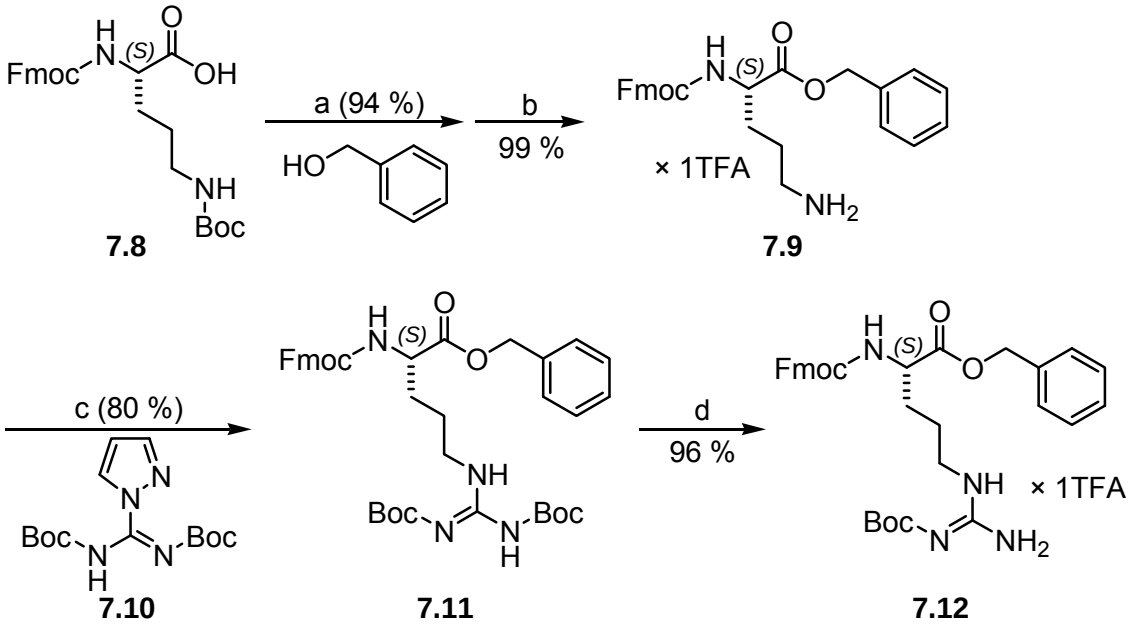


Figure 1. HPLC purity control of the arginine building block **7.7**. Conditions: column: Eurospher-100 C18 (250 × 4 mm, 5 µm), eluent: mixtures of acetonitrile (A) and 0.025 % aq. TFA (B), gradient: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5, flow rate: 0.8 mL/min, UV-detection: 210 nm. **7.7**: t_R = 25.3 min, 89 %; **7.6**: t_R = 18.9 min, 10 %. The partial degradation to building block **7.6** occurred during evaporation of acetonitrile at 37 °C from the eluate of preparative HPLC (eluent: acetonitrile and 0.05 % aq. TFA).

With the intension to introduce the $N^{\omega'}$-acyl substituent with higher efficiency N^{ω}-Boc-N^{α}-Fmoc-arginine benzyl ester (**7.12**) was prepared as a universal precursor for a direct attachment of various $N^{\omega'}$-acyl substituents (Scheme 3). The synthesis of compound **7.12** was accomplished with excellent yield starting from commercially available N^{δ}-Boc-N^{α}-Fmoc-*L*-ornithine which was successively converted to the benzyl ester, Boc-deprotected and guanidinylated using diBoc-protected 1*H*-pyrazole-carboxamidine **7.10** (provided by A. Brennauer[3]) to give the N^{ω},$N^{\omega'}$-diBoc protected arginine derivative **7.11** (Scheme 3).



Scheme 3. Synthesis of building block **7.12**.
Reagents and conditions: (a) DCC, DMAP, CH_2Cl_2, rt, 20 h, 94 %; (b) CH_2Cl_2/TFA (7.5/1), rt, 16 h, 99 %; (c) NEt_3, CH_2Cl_2, rt, 1.5 h, 80 %; (d) acetonitrile/water (3/1, supplemented with ≈ 0.3 % TFA), 24-50 °C, 4.5 h, 96 %.

Building block **7.12** was obtained via mono-Boc-deprotection of compound **7.11** taking advantage of the above described phenomenon that guanidines bearing two electron-withdrawing substituents (urethane, acyl, carbamoyl, etc.) are less stable than the corresponding mono-substituted analogs (Scheme 3, Figure 2) enabling selective mono-deprotection.

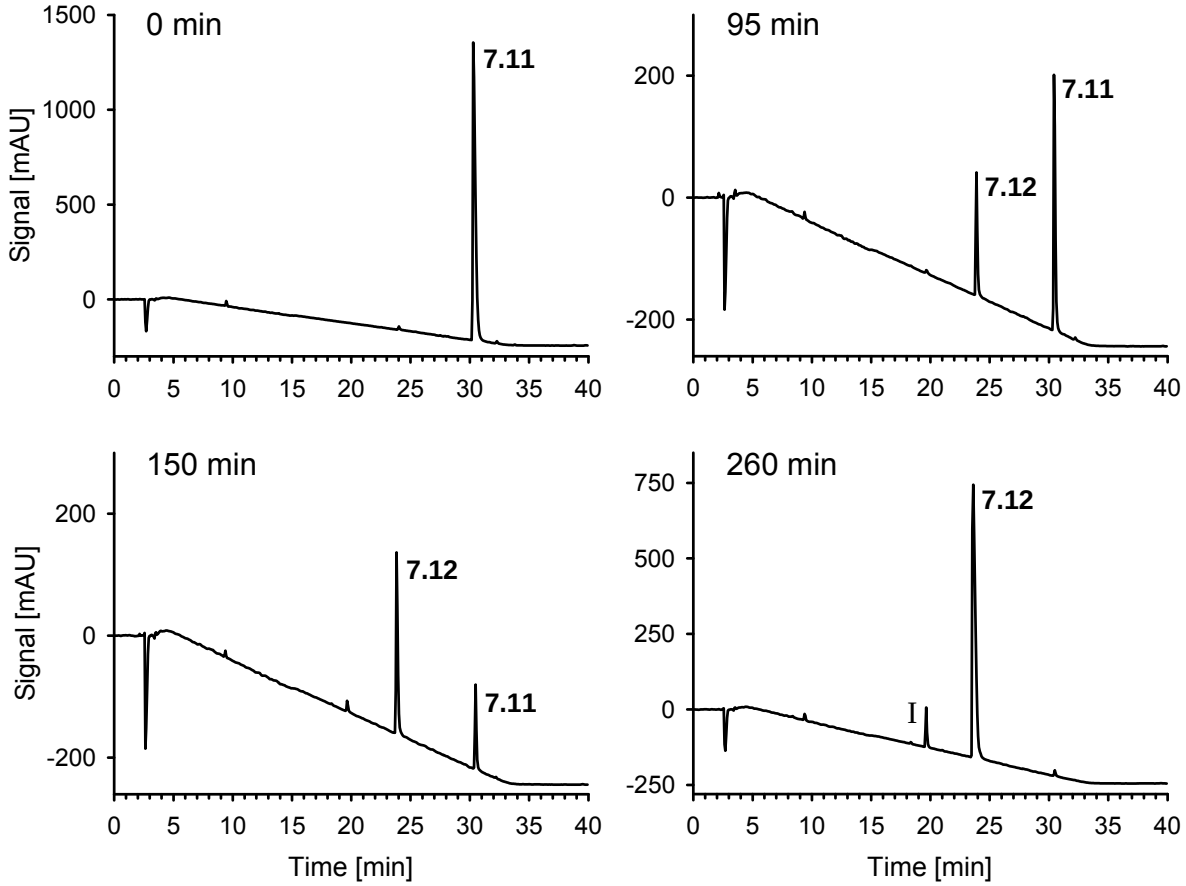
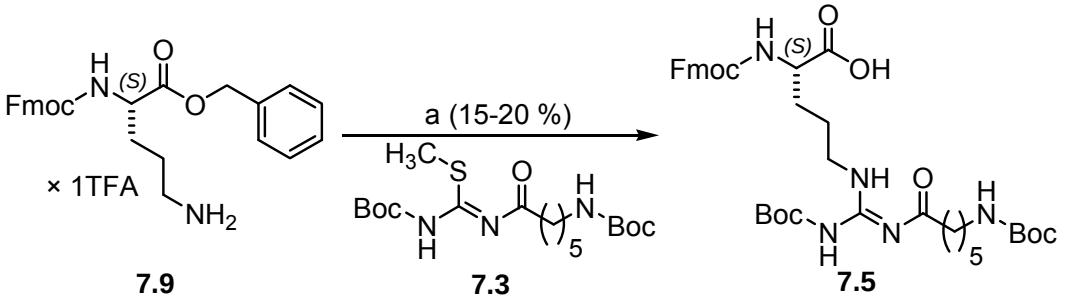


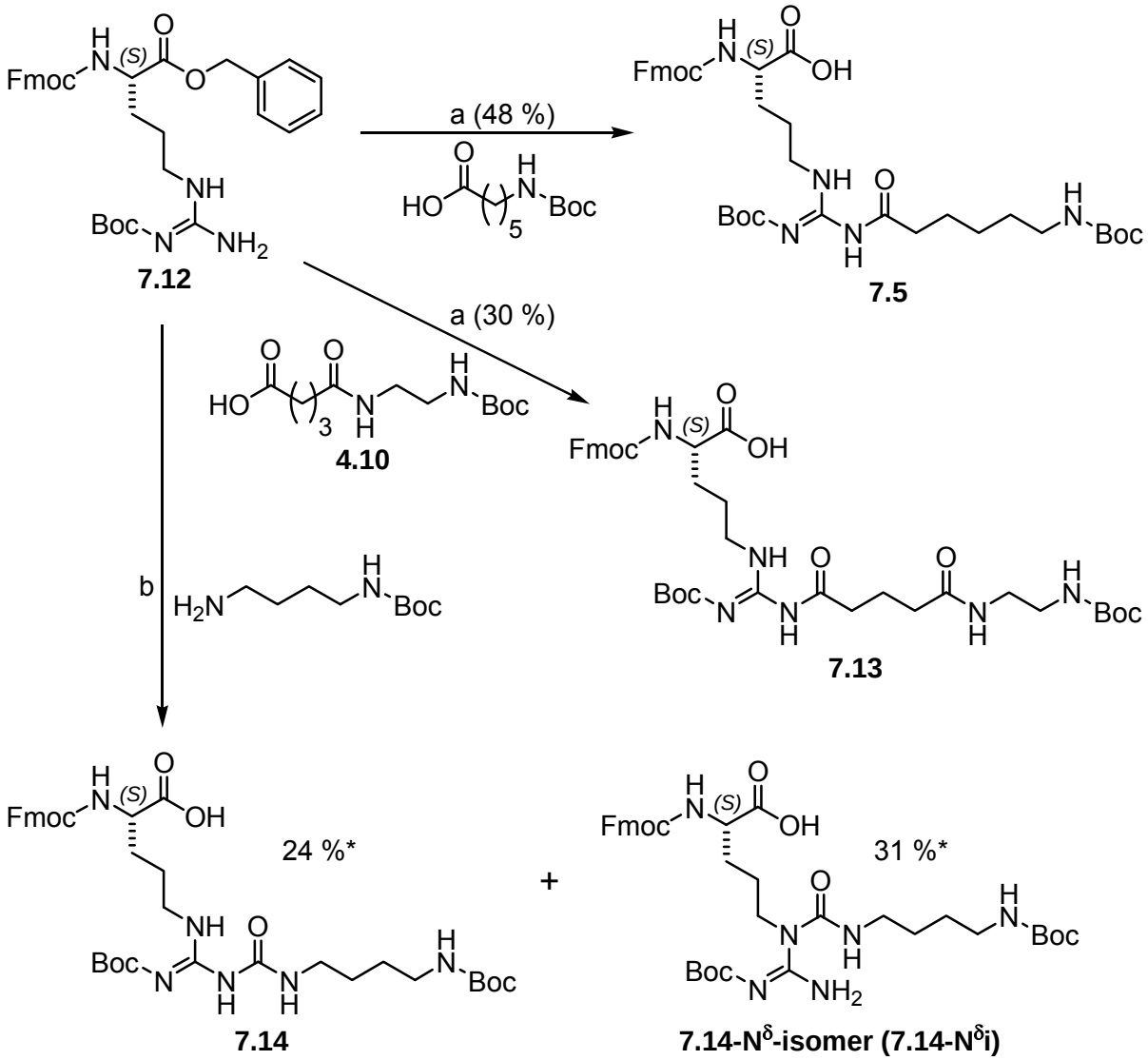
Figure 2. HPLC monitoring of selective mono-deprotection of the guanidine group in **7.11** yielding **7.12** in acetonitrile/water (3/1, supplemented with about 0.3 % TFA) at ≤ 50 °C (cf. exp. section). HPLC conditions: column: Eurospher-100 C18 (250 × 4 mm, 5 µm), eluent: mixtures of acetonitrile (A) and 0.05 % aq. TFA (B), gradient: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5, flow rate: 0.8 mL/min, UV-detection: 210 nm. **7.11**: t_R = 30.5 min, **7.12**: t_R = 23.7 min, I: (*S*)-N^{α}-Fmoc-arginine benzyl ester, t_R = 19.7 min.

In contrast to the efficient guanidinylation of N^{α}-Fmoc-*L*-ornithine benzyl ester (**7.9**) using 1*H*-pyrazole-carboxamidine derivative **7.10** (Scheme 3) the reaction of **7.9** with the *S*-methyl-isothiourea derivative **7.3** resulted in low amounts of product (**7.5**) and numerous by-products (Scheme 4) as also observed for the reaction of N^{α}-Fmoc-*L*-ornithine (**7.1**) with guanidinylating reagent **7.3** (Scheme 1).



Scheme 4. Synthesis of the arginine building block **7.5** via guanidinylation of amine **7.9** and subsequent hydrogenolysis.
Reagents and conditions: (a) (1) $HgCl_2$, NEt_3, CH_2Cl_2, rt, 24 h; (2) 10 % Pd/C, H_2, MeOH, rt, 45 min, 15-20 %.

As intended, acylation of building block **7.12** with ω-(Boc-amino)alkanoic acids was efficiently achieved (≈ 70 %). A partial cleavage of the Fmoc group during subsequent hydrogenolysis of the benzyl ester was expected, but purification, i.e. separation from the N-terminally deprotected by-product and 9-methylene-9*H*-fluorene, turned out to be quite complicated. This resulted again in a considerable decrease in yield (Scheme 5).



Scheme 5. Synthesis of the arginine building blocks **7.5**, **7.13** and **7.14** from building block **7.12**. Reagents and conditions: (a) (1) CDI, NEt_3, CH_2Cl_2, rt, 20 h; (2) 10 % Pd/C, H_2, MeOH, rt, 15 min, 48 % (**7.5**), 30 % (**7.13**); (b) triphosgene, diisopropylethylamine, CH_2Cl_2, rt, 2.5 h; (2) 10 % Pd/C, H_2, MeOH, rt, 30 min, 20 % (**7.14**). *Raw yields.

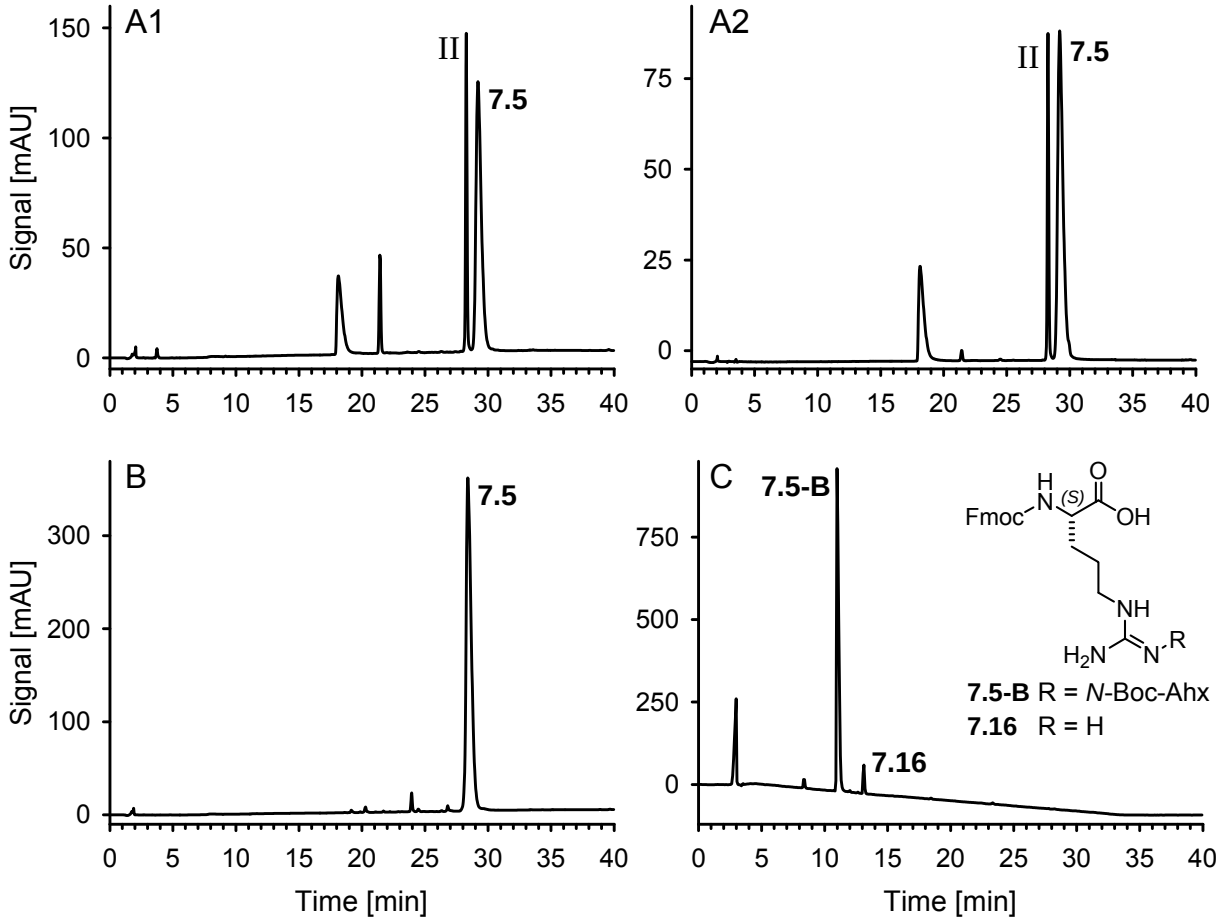


Figure 3. Panel A: HPLC analysis of the crude product **7.5** obtained after cleavage of the benzyl ester (Scheme 5). Conditions: column: Eurospher-100 C18 (250 × 4 mm, 5 µm), eluent: acetonitrile (A) and water (B), gradient: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5, flow rate: 0.8 mL/min, UV-detection: 220 nm (A1) and 254 nm (A2). **7.5**: t_R = 29.2 min, II: 9-methylene-9*H*-fluorene (Cleavage of N^{α}-Fmoc!). B: HPLC analysis of **7.5** purified with preparative HPLC. Conditions: the same as in A, UV-detection: 220 nm. **7.5**: t_R = 28.4 min, 97 %. C: HPLC analysis of Boc-deprotected **7.5** (**7.5-B**). Conditions: the same as in A, but with 0.05 % aq. TFA instead of water, UV-detection: 220 nm. **7.5-B**: t_R = 11.0 min, **7.16 (**N^{α}-Fmoc-arginine): t_R = 13.1 min.

An attempt to purify arginine building block **7.13** with column chromatography using methylene chloride and methanol as eluent (column packed with 1 % triethylamine) resulted in an extensive degradation of the compound. Therefore building blocks **7.5**, **7.13** and **7.14** were isolated with preparative HPLC. Because of the carboxyl group an acidification of the mobile phase is advantageous to improve the separation, but under these conditions a significant cleavage of the N^{ω}-Boc group occurred during removal of acetonitrile from the eluate under reduced pressure (35 °C). Consequently, acetonitrile and pure water were used as eluent components (pH ≈ 5 – 6) to purify compounds **7.5** and **7.13**, although this led to broadening of the peaks, large volumes of product fractions and substantial increase in time necessary (> 60 min) for removal of the organic solvent at 35 °C prior to lyophilization. Under these conditions the N^{ω}-acyl substituents were cleaved off, as analyzed with electrospray mass spectrometry

(ES-MS) and confirmed by HPLC of the Boc-deprotected compounds (Figure 3C and 4B). To exclude fragmentation during MS analysis a small amount of pure product **7.13** (1 mL of the eluate from preparative HPLC) was treated under mild conditions to remove the solvent (temp. < 10 °C); ES-MS analysis of the residue revealed no degradation.

Whereas degradation of compound **7.5** to N^{ω}-Boc-N^{α}-Fmoc-arginine (**7.17**) during evaporation of acetonitrile at 35 °C was moderate (Figure 3B/C), about 40 % of the linker in **7.13** was cleaved off under the same conditions (Figure 4B). The mixture of arginine building block **7.13** and its degradation product N^{ω}-Boc-N^{α}-Fmoc-arginine (**7.17**) was not separated when analyzed with HPLC using acetonitrile and water (both compounds were "co-eluted" with a fronting; Figure 4A). By contrast the Boc-deprotected compounds were readily separated as shown in Figure 4B.

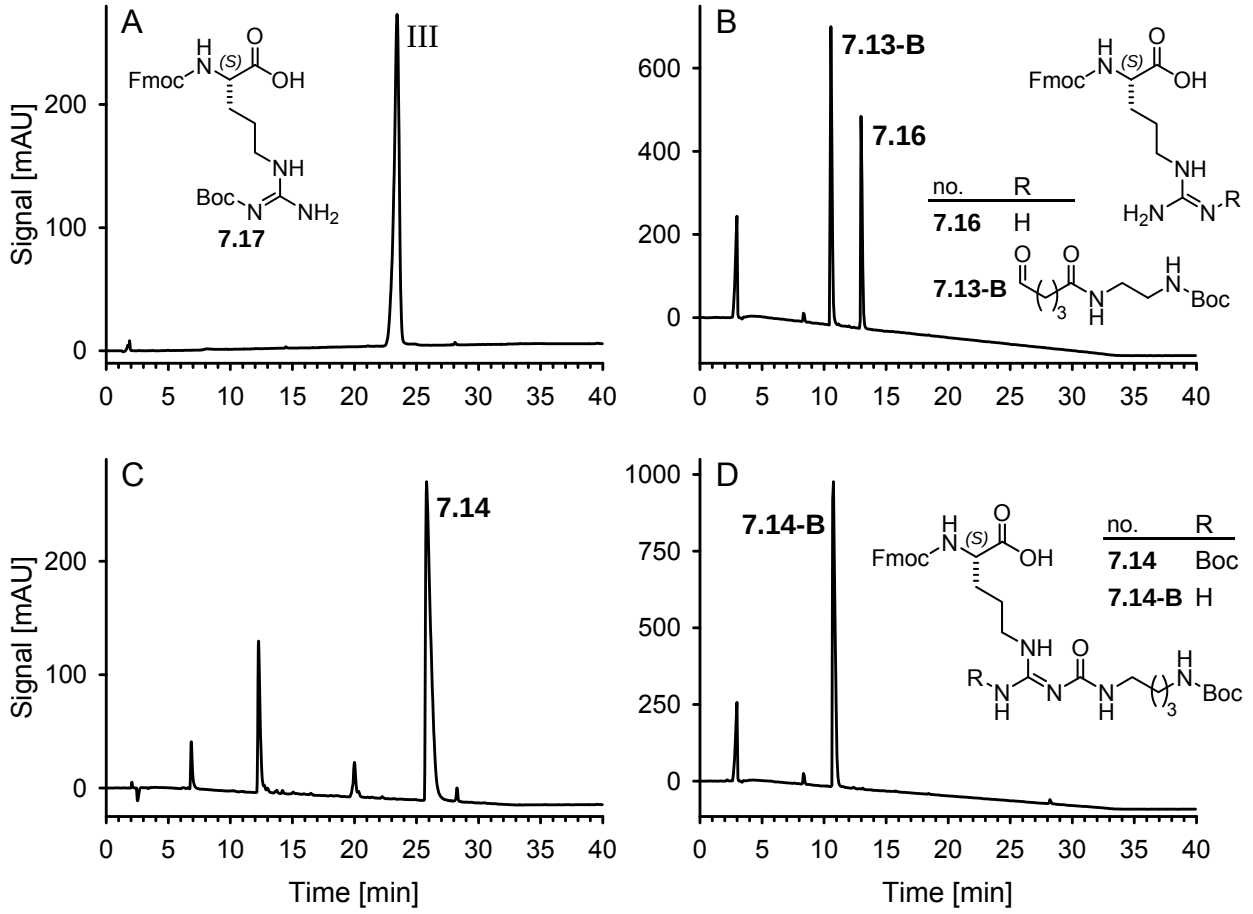


Figure 4. A: HPLC analysis of the arginine building block **7.13** purified with preparative HPLC. Conditions: column: Eurospher-100 C18 (250 × 4 mm, 5 µm), eluent: acetonitrile (A) and water (B), gradient: 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5, flow rate: 0.8 mL/min, UV-detection: 210 nm. III: co-elution of **7.13** and its degradation product N^{ω}-Boc-N^{α}-Fmoc-arginine **7.17**, t_R = 23.4 min (peak-fronting). The coexistence of **7.13** and **7.17** becomes obvious from B and was confirmed with ES-MS. B: HPLC analysis of Boc-deprotected **7.13** (**7.13-B**). Conditions: the same as in A, but with 0.05 % aq. TFA instead of water, UV-detection: 220 nm. **7.13-B**: t_R = 10.5 min, 61 %; **7.16 (**N^{α}-Fmoc-arginine): t_R = 13.0 min, 37 %. C: HPLC analysis of **7.14** purified with preparative HPLC. Conditions: the same as in A, UV-detection: 220 nm. **7.14**: t_R = 25.8 min, 74 %. D: HPLC analysis of Boc-deprotected **7.14** (**7.14-B**). Conditions: the same as in B. **7.14-B**: t_R = 10.7 min. N^{α}-Fmoc-arginine (**7.16**) (t_R ≈ 13 min) was not detected, i.e. the N^{ω}-carbamoyl-substituent was not cleaved during the preparative work-up.

Carbamoylation of precursor **7.12** with the isocyanate generated from *N*-Boc-butane-1,4-diamine gave a considerable amount of the N^{δ}-substituted isomer of **7.14** (Scheme 5). This was also observed for the carbamoylation of N^{ω}-Boc, *O*-*t*-butyl protected BIBP 3226 (**2.7**) using the same isocyanate (discussed in chapter 4). Purification of the arginine building block **7.14** with preparative HPLC using acetonitrile and 0.025 % aqueous TFA obviously resulted in fractional cleavage of the N^{ω}-Boc group whereas the N^{ω}-carbamoyl substituent was retained (Figure 4C/D).

Thus, the synthetic route using building block **7.12** (Scheme 3) is very efficient including the N^{ω}-acylation step, but subsequent hydrogenolytic debenzylation results in a fractional cleavage of the N^{α}-Fmoc group, and due to instability of the desired compounds the separation from by-products is complicated. The N^{ω}-Boc analog of the N^{ω}-Cbz-protected compound **7.6** (Scheme 2) as a universal building block for the introduction of aminoalkanoyl substituents is considered a promising alternative, but preventing decomposition during purification of the products remains challenging anyway. Separation of the products from by-products formed by N^{α}-Fmoc cleavage during hydrogenolytic debenzylation under water free conditions taking advantage of different solubilities may be feasible.

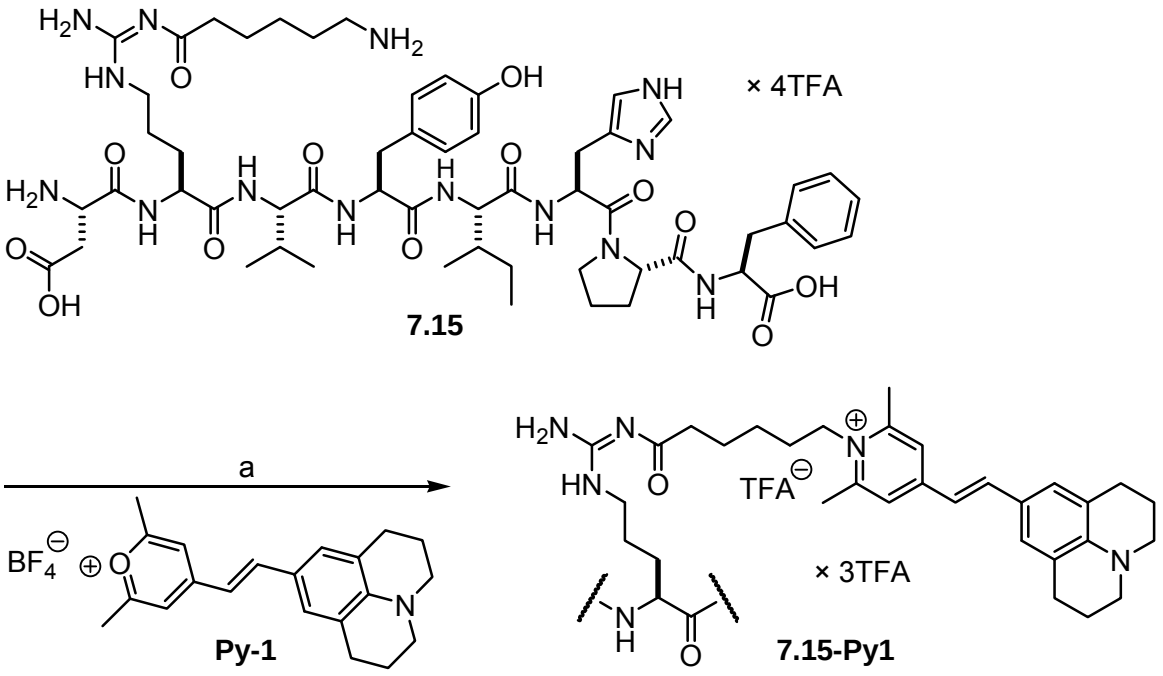
7.2.2 Application of the Modified Arginine Building Blocks in Peptide Synthesis

The octapeptide angiotensin II (Asp-*Arg*-Val-Tyr-Ile-His-Pro-Phe-OH) was selected as a model compound to investigate the suitability of the arginine building blocks for solid phase peptide synthesis. Since the peptide chain is built from C-terminus to N-terminus in the case of angiotensin II only one additional amino acid (Asp) had to be coupled after the introduction of the modified arginine. In a first approach the N^{ω}-Cbz protected building block **7.7** was introduced. Modified angiotensin II was obtained in acceptable yield after cleavage from the resin under neutral conditions. However, hydrogenolytic cleavage of Cbz in MeOH gave considerable amounts of C-terminal methyl ester, presumably due to catalysis by a small amount of acid (TFA, AcOH) in the reaction mixture. After Cbz-deprotection the acid sensitive protecting groups were removed. Unfortunately, purification of the target compound turned out to be too difficult because of nearly identical retention times of both the desired product and the C-terminally esterified peptide. When the peptide mixture was treated with lithium hydroxide extensive cleavage of the N^{ω}-acyl linker occured.

In a second attempt building block **7.5** was employed. For coupling 2.5 equivalents of **7.5** were used as well as 2.5 equivalents of HOBt/HBTU and 5 equivalents of DIPEA (single coupling, 8 h). The resulting angiotensin II derivative **7.15** (Scheme 6) was cleaved from the trityl resin under acidic conditions. Since the N^{ω}-Boc group was simultaneously removed in this step the

N^{ω}-acyl substituent gained better stability (discussed in the beginning). HPLC-MS analysis revealed two main products, the designated one (**7.15**) and the heptapeptide lacking the arginine. Thus, coupling of the solid phase bound angiotensin 1 - 6 with arginine building block **7.5** was incomplete although a Kaiser-test had been negative. Only a very small fraction of peptide was lacking the N^{ω}-acyl linker. The angiotensin II derivative **7.15** was obtained with 95 % purity (210 nm) after isolation with preparative HPLC (Figure 5A).

The aminohexanoyl substituted angiotensin II (**7.15**) was allowed to react with the pyrylium dye Py-1[7, 8] (Scheme 6, cf. also chapter 4) to yield the fluorescence labeled peptide **7.5-Py1** with about 20 % yield and 94 % purity (220 nm, Figure 5B).



Scheme 6. Synthesis of the fluorescence labeled angiotensin II derivative **7.15-Py1**.
Reagents and conditions: (a) NEt_3, acetonitrile, DMF, rt, 4.5 h, 19 %.

With respect to broader application of these arginine building blocks in solid phase synthesis the required number of coupling steps after the integration of the modified arginine has to be taken into account since a fractional cleavage of the N^{ω}-acyl substituent is anticipated. Therefore, N-terminal position of the modified arginine residue is preferable.

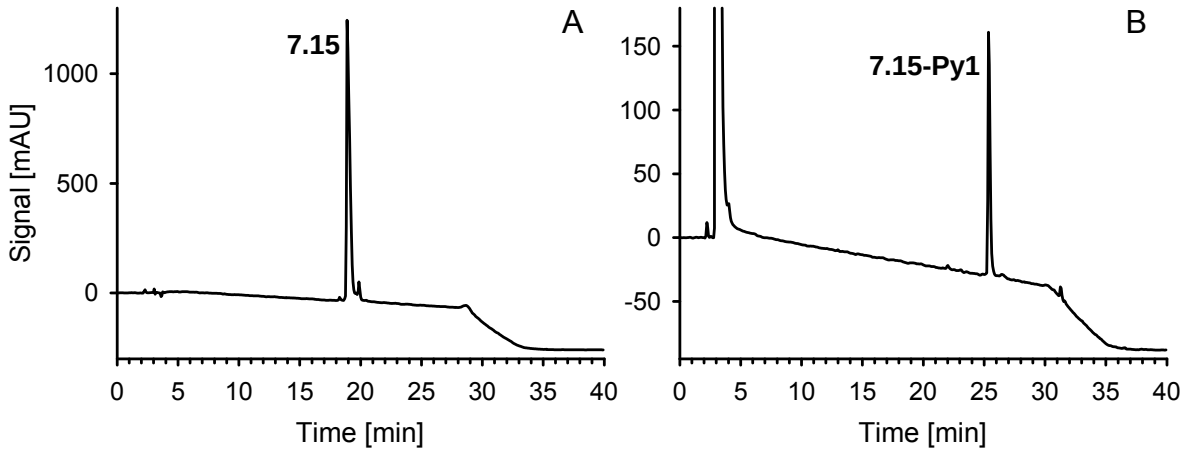


Figure 5. HPLC purity control of the angiotensin II derivative **7.15** (A) and its Py-1 labeled analog **7.15-Py1** (B). A: Conditions: column: Eurospher-100 C18 (250 × 4 mm, 5 µm), eluent: acetonitrile (A) and 0.05 % aq. TFA (B), gradient: 0 to 25 min: A/B 10/90 to 35/65, 25 to 30 min: 35/65 to 90/10, 30 to 40 min: 90/10, flow rate: 0.8 mL/min, UV-detection: 210 nm. **7.15**: t_R = 18.9 min, 95 %. B: Conditions: column, eluent and flow rate as in A, gradient: 0 to 27 min: A/B 15/85 to 50/50, 27 to 32 min: 50/50 to 90/10, 32 to 40 min: 90/10, UV-detection: 220 nm. **7.15-Py1**: t_R = 25.4 min, 94 %.

7.2.3 AT_1 Receptor Agonism

The angiotensin II derivatives **7.15** and **7.15-Py1** were investigated for AT_1 receptor agonism in a fura-2 based Ca^{2+}-assay on rat mesangial cells. As becomes obvious from Figure 6, compounds **7.15** and **7.15-Py1** (EC_{50} = 21 nM and 80 nM, resp.) were about as potent or only slightly less potent than angiotensin II (EC_{50} = 17 nM) and produced nearly maximal Ca^{2+}-response in rat mesangial cells. This result supports the working hypothesis that the guanidine-acylguanidine bioisosteric approach is applicable to arginine residues in endogenous peptides.

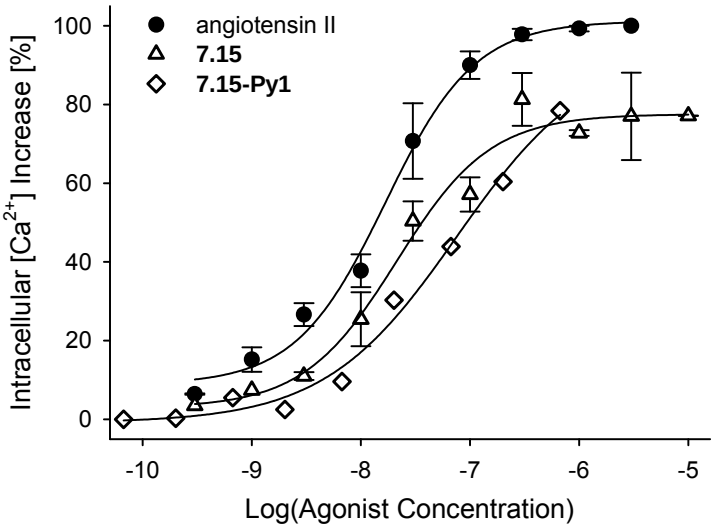


Figure 6. Concentration-response curves (CRCs) of angiotensin II, **7.15** and **7.15-Py1** from a fura-2 assay on rat mesangial cells expressing the AT_1 receptor. EC_{50}: 17 nM (Angiotensin II), 21 nM (**7.15**), ca. 80 nM (**7.15-Py1**). Mean values ± SEM from two independent experiments (angiotensin II and **7.15**).

Unfortunately, several trials to image AT_1 receptor binding of **7.5-Py1** with confocal microscopy using rat mesangial cells were unsuccessful (Leibowitz L15 medium with (1 %) and without fetal calf serum; same settings as for the imaging of Y_1 receptors with Py-1 labeled Y_1R antagonists, cf. chapter 4).

7.2.4 Conclusion

The preparation of the arginine building blocks presented in this chapter was hampered by the fact that guanidines bearing two electron withdrawing substituents such as urethane (Boc, Cbz), acyl and carbamoyl exhibit a definite instability. Consequently, the synthetic protocols and purification procedures for the arginine building blocks need further optimization. Nevertheless, such a building block (**7.5**) was effectually integrated in the octapeptide angiotensin II using automated solid phase synthesis. The resulting angiotensin II derivative **7.15** was equipotent with the parent compound in a calcium assay on AT_1 receptor expressing cells. The functionalized arginine building block used for the preparation of **7.15** may also be used for the derivatization of other arginine-containing peptides and pave the way to radio- or fluorescence labeled receptor ligands.

7.3 Experimental Section

7.3.1 General Experimental Conditions

Unless otherwise noted, chemicals and solvents were purchased from commercial suppliers and used without further purification. Angiotensin II was obtained from Bachem (Weil am Rhein, Germany) The Py-1 pyrylium salt (Py-1) was synthesized in the Institute of Analytical Chemistry, Chemo- and Biosensors at the University of Regensburg.[7, 8] Pyrylium dyes are commercially available from Active Motif Chromeon (www.activemotif.com). Millipore water was used throughout for the preparation of buffers and HPLC eluents. Petroleum ether (40-60 °C) was distilled before use. DMF was stored over a molecular sieves (3 Å). Anhydrous reactions were run under an atmosphere of dry nitrogen or argon.

Thin layer chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates. For column chromatography silica gel Geduran 60 (Merck, 0.063-0.200 mm) was used. NMR spectra were recorded on a Bruker Avance 300 (1H: 300 MHz) and a Bruker Avance 600 (1H: 600 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. Mass spectrometry analysis (MS) was performed in-house on a Finnigan ThermoQuest TSQ 7000 (ES-MS).

Lyophilization was done with a Christ alpha 2-4 LD equipped with a vacuubrand RZ 6 rotary vane vacuum pump.

Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. An Eurospher-100 C18 (250 × 32 mm, 5 µm; Knauer, Germany) served as RP-column at a flow rate of 38 mL/min. Analytical HPLC analysis was performed on a system from Thermo Separation Products (composed of a SN400 controller, a P4000 pump, a degasser (Degassex DG-4400, phenomenex), an AS3000 autosampler and a Spectra Focus UV-VIS detector). An Eurospher-100 C18 (250 × 4 mm, 5 µm, Knauer, Germany) served as RP-column. Helium degassing, an oven temperature of 30 °C and a flow rate of 0.8 mL/min were used throughout. Solutions for injection (concentrations in the two-digit µM range) were prepared in mobile phase corresponding to the composition at the beginning of the gradient. The following gradients were applied for analytical HPLC analysis of **7.12**, **7.15** and **7.15-Py1** (mobile phase composed of acetonitrile (A) and 0.05 % aq. TFA (B):

Gradient 1 (**7.12**): 0 to 30 min: A/B 20/80 to 95/5, 30 to 40 min: 95/5

Gradient 2 (**7.15**): 0 to 25 min: A/B 10/90 to 35/65, 25 to 30 min: 35/65 to 90/10, 30 to 40 min: 90/10

Gradient 3 (**7.15-Py1**): 0 to 27 min: A/B 15/95 to 50/50, 27 to 32 min: 50/50 to 90/10, 32 to 40 min: 90/10

A stock solutions of **7.15** (ca. 5 mM) for HPLC purity control was prepared in acetonitrile/0.05 % aq. TFA (1/1). Stock solutions for pharmacological studies as well as for the purity control (**7.15-Py1**) were prepared in DMSO (Angiotensin II: 1 mM, **7.15**: 5 mM, **7.15-Py1**: 1 mM) and stored at –78 °C.

7.3.2 Chemistry: Experimental Protocols and Analytical Data

(*S*)-*N*$^{\alpha}$-(9-Fluorenylmethoxycarbonyl)ornithine (7.1). (*S*)-*N*-Benzyloxycarbonyl-*N*$^{\alpha}$-(9-fluorenylmethoxycarbonyl)ornithine (**2.2**) (2.25 g, 4.61 mmol, 1 eq, cf. chapter 2) was dissolved in MeOH (110 mL) and a 10 % Pd/C catalyst (225 mg) was added under an atmosphere of nitrogen. Ammonium formate (5.8 g, 92.1 mmol, 20 eq) was added and the suspension was kept under vigorous stirring for 3 h. Glacial acetic acid (4.5 mL) was added and stirring was continued for 2 h. Glacial acetic acid (45 mL) was added prior to removal of the catalyst by filtration. Volatiles were removed under reduced pressure as far as possible (45 °C, 10 mbar final pressure), water (60 mL) was added twice, each time followed by lyophilization. Purification with preparative HPLC using mixtures of acetonitrile (32 %) and 0.1 % aq. TFA (68 %) as eluent afforded the product as a white solid (0.99 g, 2.11 mmol, 46 %); ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.63 (m, 3H, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.79 (m, 1H, CH-C$\underline{H}_2$-CH$_2$), 2.79 (m, 2H, C$\underline{H}_2$-NH$_2$), 3.96 (m, 1H, CH$^{\alpha}$), 4.18-4.36 (m, 3H, C$\underline{H}$-C$\underline{H}_2$ Fmoc), 7.38 (m, 4H, Fmoc), 7.72 (m, 3H, Fmoc, NH),

7.81 (bs, 2H, NH_2), 7.90 (d, 2H, ^3J = 7.43 Hz, Fmoc), 12.79 (bs, 1H, COOH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 355 $[M + H]^+$; $C_{20}H_{22}N_2O_4 \times C_2HF_3O_2$ (468.4)

(*S*)-N^{ω}-*tert*-Butoxycarbonyl-$N^{\omega'}$-(6-*tert*-butoxycarbonylaminohexanoyl)-N^{α}-(9-fluorenylmethoxycarbonyl)arginine (7.5). A solution of 6-*tert*-butoxycarbonylaminohexanoic acid (172 mg, 0.74 mmol, 1.3 eq) and CDI (130 mg, 0.8 mmol, 1.4 eq) in CH_2Cl_2 (8 mL) was stirred at rt for 30 min. **7.12** (400 mg, 0.57 mmol, 1 eq) and NEt_3 (116 mg, 158 µL, 1.14 mmol, 2 eq) were added and the mixture was kept under stirring at rt for 20 h. The volume of the mixture was reduced under reduced pressure to about 3 mL and the intermediate was purified by column chromatography (PE/EtOAc 3/1 to 1/1) prior to hydrogenation at rt and air pressure in MeOH (10 mL) using hydrogen and a 10 % Pd/C catalyst (60 mg). The flow of hydrogen was stopped after 15 min when starting material was no longer detectable by TLC. The catalyst was removed by filtration and removal of the solvent under reduced pressure yielded a highly viscous colorless oil which hardened to a solid during drying *in vacuo*. Purification with preparative HPLC using mixtures of acetonitrile (A) and water (B) as eluent (gradient: 0 – 25 min: A/B 30/70 to 90/10, $t_R \approx$ 19 – 25 min) afforded the product as a white fluffy solid (193 mg, 0.27 µmol, 48 %). For HPLC and NMR analysis a small amount (6.8 mg) of **7.5** was Boc-deprotected through the treatment with acetonitrile/TFA (1:2, 300 µL) at 50 °C for 30 min. The mixture was analyzed by HPLC (Figure 3C) prior to the isolation of Boc-deprotected **7.5** (**7.5-B**) with preparative HPLC (eluent: mixtures of acetonitrile (A) and 0.05 % aq. TFA (B), gradient: 0 – 20 min: A/B 20/80 to 60/40, $t_R \approx$ 13 min). ^{1}H-NMR (600 MHz, DMSO-d_6, COSY) **7.5-B**: δ (ppm) 1.31 (m, 2H, CH_2-CH_2-C$\underline{H}_2$-CH_2-CH_2), 1.47-1.65 (m, 7H, CH_2-C$\underline{H}_2$-CH_2-C$\underline{H}_2$-CH_2, CH-C$\underline{H}_2$-C$\underline{H}_2$), 1.75 (m, 1H, CH-C$\underline{H}_2$-CH_2), 2.41 (t, 2H, ^3J = 7.30 Hz, CO-CH_2), 2.76 (m, 2H, C$\underline{H}_2$-NH_2), 3.26 (m, 2H, C$\underline{H}_2$-NH), 3.96 (m, 1H, CH^{α}), 4.22 (m, 1H, CH-CH_2, Fmoc), 4.29 (m, 2H, CH-CH_2, Fmoc), 7.32 (t, 2H, ^3J = 7.41 Hz, Fmoc), 7.41 (t, 2H, ^3J = 7.45 Hz, Fmoc), 7.66 (m (bs + d), 3H, CH_2-N$\underline{H}_2$, CH^{α}-N$\underline{H}$), 7.71 (m, 2H, Fmoc), 7.89 (d, 2H, ^3J = 7.54 Hz, Fmoc), 8.77 (bs, 2H, NH_2), 9.24 (s, 1H, CH_2-N$\underline{H}$), 11.8 (s, 1H, NH), 12.65 (bs, 1H, COOH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc) **7.5**: *m/z* 710 $[M + H]^+$, 497 [M - (N^{ω}-acyl substituent) + $H]^+$; MS (ES, MeCN/TFA) **7.5-B**: *m/z* 510 $[M + H]^+$; $C_{37}H_{51}N_5O_9$ (709.8)

(*S*)-N^{ω}-Benzyloxycarbonyl-N^{α}-(9-fluorenylmethoxycarbonyl)arginine (7.6). **7.1** (500 mg, 1.07 mmol, 1 eq) and **2.13** (551 mg, 1.6 mmol, 1.5 eq, cf. chapter 2) were dissolved in CH_2Cl_2 (15 mL) yielding a clear solution which turned into a white, highly viscous suspension after addition of NEt_3 (216 mg, 296 µL, 2.14 mmol, 0.5 eq). The viscosity of the suspension decreased with the addition of CH_2Cl_2 (20 mL) and acetonitrile (10 mL). The amount of the white precipitate decreased during stirring overnight at rt. Volatiles were removed under reduced pressure (40 °C) and the intermediate was isolated with preparative HPLC using mixtures of acetonitrile and 0.02 % aq. TFA as eluent. Removal of the acetonitrile (40 °C, ~ 1.5 h) from the

fraction containing the intermediate resulted already in a partial cleavage of the N^{ω}-Boc group (30 – 50 %). Boc-deprotection was completed through the treatment of the white solid (obtained after lyophilization) with a mixture of acetonitrile and 0.05 % aq. TFA (1:1, 100 mL) at 40 °C for 4 h. Removal of acetonitrile under reduced pressure and subsequent lyophilization afforded the product as a white solid (480 mg, 0.75 mmol, 70 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.59 (m, 3H, CH-C$\underline{H_2}$-C$\underline{H_2}$), 1.76 (m, 1H, CH-C$\underline{H_2}$-CH$_2$), 3.28 (m, 2H, C$\underline{H_2}$-NH), 3.96 (m, 1H, CH$^{\alpha}$), 4.18-4.34 (m, 3H, C$\underline{H}$-C$\underline{H_2}$ Fmoc), 5.24 (s, 2H, C$\underline{H_2}$-Ph), 7.28-7.45 (m, 10H, Ph, Fmoc, NH), 7.66 (d, 1H, ^{3}J = 8.05 Hz, CO-N$\underline{H}$-CH), 7.72 (d, 2H, ^{3}J = 7.41 Hz, Fmoc), 7.89 (d, 2H, ^{3}J = 7.40 Hz, Fmoc), 8.59 (bs, 2H, NH$_2$), 8.8 (bs, 1H, NH) 12.15 (bd, 1H, COOH); MS (ES, MeCN/MeOH + 10 mM NH_4OAc): *m/z* 531 $[M + H]^+$; $C_{29}H_{30}N_4O_6 \times C_2HF_3O_2$ (644.6).

(*S*)-N^{ω}-Benzyloxycarbonyl-$N^{\omega'}$-(8-*tert*-butoxycarbonylamino-3,6-dioxaoctanoyl)-N^{α}-(9-fluorenylmethoxycarbonyl)arginine (7.7). 8-*tert*-butoxycarbonylamino-3,6-dioxaoctanoic acid × dicyclohexylamine (160 mg) was dissolved in dimethoxyethane (10 mL). The amine was precipitated by the addition of 37 % hydrochloric acid (~ 60 µL) and removed by filtration. Removal of the solvent under reduced pressure and drying *in vacuo* yielded a yellow oil (105 mg, 0.4 mmol, 1.3 eq) which was dissolved in anhydrous CH_2Cl_2 (3 mL). Carbonyldiimidazole (69.6 mg, 0.43 mmol, 1.4 eq) was added and the mixture was allowed to stand at rt for 40 min. **7.6** (198 mg, 0.31 mmol, 1 eq) and NEt_3 (62 mg, 85 µL, 0.6 mmol, 2 eq) were added and the mixture was kept under stirring at rt for 20 h. Volatiles were removed under reduced pressure and purification with preparative HPLC (eluent: mixtures of acetonitrile and 0.05 % aq. TFA) afforded the product as a white fluffy solid (74.4 mg, 0.096 mmol, 31 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.35 (s, 9H, *tert*-butyl), 1.58 (m, 3H, CH-C$\underline{H_2}$-C$\underline{H_2}$), 1.72 (m, 1H, CH-C$\underline{H_2}$-CH$_2$), 3.07 (m, 2H, C$\underline{H_2}$-NH), 3.33 (m, 2H, C$\underline{H_2}$-NH), 3.39 (t, 2H, ^{3}J = 6.05 Hz, C$\underline{H_2}$-CH$_2$), 3.59 (m, 2H, O-CH$_2$), 3.67 (m, 2H, O-CH$_2$), 3.96 (m, 1H, CH$^{\alpha}$), 4.18 (s, 2H, CO-CH$_2$-O), 4.26 (m, 3H, CH-CH$_2$, Fmoc), 5.05 (s, 2H, C$\underline{H_2}$-Ph), 6.76 (t, 1H, ^{3}J = 4.7 Hz, CH$_2$-N$\underline{H}$), 7.35 (m, 9H, Ph, Fmoc), 7.7 (m, 3H, Fmoc, NH), 7.89 (d, 2H, ^{3}J = 7.43 Hz, Fmoc), 9.01 (t, 1H, ^{3}J = 5.50 Hz, CH$_2$-N$\underline{H}$), 12.54 (s, 1H, NH), 12.57 (bs, 1H, COOH); MS (ES, MeOH + 10 mM NH_4OAc): *m/z* 776 $[M + H]^+$, 531 [M - (N^{ω}-acyl substituent) + H]$^+$; $C_{40}H_{49}N_5O_{11}$ (775.8)

(*S*)-N^{α}-(9-Fluorenylmethoxycarbonyl)ornithine benzyl ester (7.9).[9] (*S*)-N^{δ}-Boc-N^{α}-Fmoc-ornithine (**7.8**) (5 g, 11 mmol, 1 eq) was suspended in CH_2Cl_2 (200 mL). Benzyl alcohol (1.55 g, 14.3 mmol, 1.3 eq) was added resulting in a decrease of the amount of white precipitate. The mixture cleared up completely when dicyclohexylcarbodiimide (2.5 g, 12.1 mmol, 1.1 eq) was added. DMAP (40 mg, 0.33 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 removed under reduced pressure. The intermediate was purified with column chromatography (eluent: CH_2Cl_2 to

CH_2Cl_2/EtOAc 8/1) yielding a white solid (5.6 g, 94 %) which was dissolved in CH_2Cl_2 (150 mL). TFA (20 mL) was added, the mixture was stirred at rt for 16 h, and then it was concentrated under reduced pressure. CH_2Cl_2 (100 mL) was added and evaporated under reduced pressure. This was repeated twice. The residue, a yellow oil, was suspended in water (70 mL) and lyophilization afforded the product as a white solid (5.72 g, 10.24 mmol, 93 %). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.68 (bs, 3H, CH-C$\underline{H_2}$-C$\underline{H_2}$), 1.89 (bs, 1H, CH-C$\underline{H_2}$-CH_2), 2.94 (bs, 2H), 4.11 (m, 1H, CH^{α}), 4.29 (m, 3H, C$\underline{H}$-C$\underline{H_2}$, Fmoc), 5.1 (s, 2H, C$\underline{H_2}$-Ph), 5.74 (m, 1H, NH), 7.24 (m, 7H, Ph, Fmoc), 7.34 (m, 2H, Fmoc), 7.51 (m, 2H, Fmoc), 7.7 (d, 2H, ^{3}J = 7.46 Hz, Fmoc), 7.77 (bs, 3H, NH_3^+); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 445 $[M + H]^+$; $C_{27}H_{28}N_2O_4 \times C_2HF_3O_2$ (558.5)

(*S*)-*N*$^{\omega}$,*N*$^{\omega'}$-Bis(*tert*-butoxycarbonyl)-*N*$^{\alpha}$-(9-fluorenylmethoxycarbonyl)arginine benzyl ester (7.11). 7.9 (3.85 g, 6.89 mmol, 1 eq) and **7.10** (2.3 g, 8.6 mmol, 1.07 eq) were dissolved in CH_2Cl_2 (16 mL) and NEt_3 (0.79 g, 1.08 mL, 7.8 mmol, 1.13 eq) was added. The reaction mixture was stirred at rt for 1.5 h and the product was isolated using column chromatography (eluent: PE/EE 5/1 to 3/1, column was packed with 0.5 % NEt_3). The eluate containing the product was evaporated to dryness *in vacuo* yielding a hardened foam (3.8 g, 5.53 mmol, 80 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.38 (s, 9H, *tert*-butyl), 1.47 (s, 9H, *tert*-butyl), 1.54 (m, 2H, CH-CH_2-C$\underline{H_2}$), 1.68 (bm, 2H, CH-C$\underline{H_2}$-CH_2), 3.28 (m, 2H, C$\underline{H_2}$-NH), 4.12 (m, 1H, CH), 4.22 (m, 1H, CH), 4.31 (m, 2H, CH-CH_2, Fmoc), 5.12 (s, 2H, C$\underline{H_2}$-Ph), 7.33 (m, 7H, Ph, Fmoc), 7.41 (m, 2H, Fmoc), 7.7 (d, 2H, ^{3}J = 7.4 Hz, Fmoc), 7.88 (m, 3H, Fmoc, NH), 8.29 (t, 1H, ^{3}J = 5.49 Hz, CH_2-N$\underline{H}$), 11.51 (s, 1H, NH); $C_{38}H_{46}N_4O_8$ (686.8)

(*S*)-*N*$^{\omega}$-*tert*-Butoxycarbonyl-*N*$^{\alpha}$-(9-fluorenylmethoxycarbonyl)arginine benzyl ester (7.12). This reaction was monitored with analytical HPLC (cf. Figure 2). Sample drawing was performed at the following points: 0 min, 50 min, 1 h 35 min, 2 h 30 min, 3 h 20 min and 4 h 20 min. **7.11** (3.75 g, 5.46 mmol) was dissolved in acetonitrile (100 mL). Water (30 mL) was added and a sample was drawn for HPLC analysis (point 0 min) prior to the addition of TFA (300 µL) and heating to 40 °C. The mixture was stirred at 40 °C for 1 h and 20 min. TFA (150 µL) was added and stirring was continued at 50 °C for 30 min. Then the following incubation periods and temperatures were applied: 1 h 30 min at 45 °C, 30 min at 24 °C, 30 min at 45 °C and 30 min at 5 °C (total incubation time: 4 h and 50 min). The addition of water (200 mL) resulted in the formation of a suspension. Acetonitrile was removed under reduced pressure at 35 °C (40 mbar final pressure). Lyophilization afforded the product as a white solid (3.69 g, 5.27 mmol, 96 %). ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 1.48 (s, 9H, *tert*-butyl), 1.55 (m, 2H, CH-CH_2-C$\underline{H_2}$), 1.65 (m, 1H, CH-C$\underline{H_2}$-CH_2), 1.76 (m, 1H, CH-C$\underline{H_2}$-CH_2), 3.25 (m, 2H, C$\underline{H_2}$-NH), 4.12 (m, 1H, CH), 4.24 (m, 1H, CH), 4.33 (m, 2H, CH-CH_2, Fmoc), 5.13 (s, 2H, C$\underline{H_2}$-Ph), 7.34 (m, 7H, Ph, Fmoc), 7.42 (m, 2H, Fmoc), 7.7 (d, 2H, ^{3}J = 7.7 Hz, Fmoc), 7.88 (m, 3H, Fmoc, NH), 8.41 (m, 3H, NH,

NH_2), 10.84 (s, 1H, NH); reversed-phase HPLC (gradient 1, cf. Figure 2, t_{end}): 92 % (t_R = 23.6 min, k = 7.7); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 587 $[M + H]^+$; $C_{33}H_{38}N_4O_6 \times C_2HF_3O_2$ (700.7)

(*S*)-N^{ω}-*tert*-Butoxycarbonyl-$N^{\omega'}$-[4-(2-*tert*-butoxycarbonylaminoethyl)aminocarbonyl-butanoyl]-N^{α}-(9-fluorenylmethoxycarbonyl)arginine (7.13). A solution of 4-(2-*tert*-butoxycarbonylaminoethyl)aminocarbonylbutanoic acid (**4.10**, cf. chapter 4) (141 mg, 0.51 mmol, 1.2 eq) and CDI (90 mg, 0.56 mmol, 1.3 eq) in CH_2Cl_2 (2 mL) was stirred at rt for 30 min. **7.12** (300 mg, 0.43 mmol, 1 eq) was added in CH_2Cl_2 (2 mL). NEt_3 (56 mg, 77 µL, 0.56 mmol, 1.3 eq) was added and the mixture was kept under stirring at rt for 20 h. Volatiles were removed under reduced pressure and the intermediate was purified with column chromatography (eluent: CH_2Cl_2/EtOAc 2/1 to EtOAc, column was packed with 0.5 % NEt_3) yielding a highly viscous colorless oil (260 mg, 72 %) which was dissolved in MeOH (8 mL). A 10 % Pd/C catalyst (25 mg) was added under an atmosphere of nitrogen and hydrogen was conducted through the vigorously stirred suspension. The flow of hydrogen was stopped after 15 min and no starting material could be detected with TLC analysis. The catalyst was removed by filtration and removal of the solvent under reduced pressure yielded a highly viscous colorless oil which hardened to a solid during drying *in vacuo*. Purification with preparative HPLC using mixtures of acetonitrile (A) and water (B) as eluent (gradient: 0 – 35 min: A/B 30/70 to 90/10, $t_R \approx$ 20 – 27 min) afforded the product as a white fluffy solid (96 mg, 0.13 mmol, 30 %). For HPLC and NMR analysis 6.2 mg of **7.13** were Boc-deprotected through the treatment with acetonitrile/TFA (1:2, 300 µL) at 50 °C for 30 min. The mixture was analyzed by HPLC (Figure 4B) prior to the isolation of Boc-deprotected **7.13** (**7.13-B**) with preparative HPLC (eluent: mixtures of acetonitrile (A) and 0.05 % aq. TFA (B), gradient: 0 – 20 min: A/B 20/80 to 60/40, $t_R \approx$ 13 min). ^{1}H-NMR (600 MHz, DMSO-d_6, COSY) **7.13-B**: δ (ppm) 1.59 (m, 3H, CH-C<u>H</u>$_2$-C<u>H</u>$_2$), 1.77 (m, 3H, CH-C<u>H</u>$_2$-CH_2, CO-CH_2-C<u>H</u>$_2$-CH_2-CO), 2.14 (t, 2H, ^{3}J = 7.42 Hz, CO-CH_2), 2.43 (t, 2H, ^{3}J = 7.29 Hz, CO-CH_2), 2.83 (m, 2H, C<u>H</u>$_2$-NH_2), 3.26 (m, 4H, C<u>H</u>$_2$-NH), 3.96 (m, 1H, CH^{α}), 4.22 (m, 1H, CH-CH_2, Fmoc), 4.29 (m, 2H, CH-CH_2, Fmoc), 7.32 (t, 2H, ^{3}J = 7.42 Hz, Fmoc), 7.41 (t, 2H, ^{3}J = 7.45 Hz, Fmoc), 7.65 (d, 1H, ^{3}J = 8.14 Hz, CH^{α}-N<u>H</u>), 7.71 (m, 4H, Fmoc, CH_2-N<u>H</u>$_2$), 7.89 (d, 2H, ^{3}J = 7.48 Hz, Fmoc), 7.99 (t, 1H, ^{3}J = 5.54 Hz, CH_2-N<u>H</u>), 8.66 (s, 2H, NH_2), 9.04 (s, 1H, CH_2-N<u>H</u>), 11.51 (s, 1H, NH), 12.65 (bs, 1H, COOH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc) **7.13**: *m/z* 497 [M - (N^{ω}-acyl substituent) + H]$^+$, 753 $[M + H]^+$; MS (ES, MeCN/TFA) **7.13-B**: *m/z* 553 $[M + H]^+$; $C_{38}H_{52}N_6O_{10}$ (752.9)

(*S*)-N^{ω}-*tert*-Butoxycarbonyl-$N^{\omega'}$-[(4-*tert*-Butoxycarbonylaminobutyl)aminocarbonyl]-N^{α}-(9-fluorenylmethoxycarbonyl)arginine (7.14). The reaction was carried out in an argon purged 50 mL two-necked round bottom flask equipped with a pressure equalizing addition funnel. The flask and the funnel were baked out in a nitrogen atmosphere prior to the reaction. A solution of

N-Boc-butane-1,4-diamine (281 mg, 1.49 mmol, 1.1 eq) and diisopropylethylamine (491 mg, 3.8 mmol, 2.8 eq) in anhydrous CH_2Cl_2 (8 mL) was added dropwise to a solution of triphosgene (221 mg, 0.75 mmol, 0.55 eq) in anhydrous CH_2Cl_2 (4 mL) over a period of 30 min. This solution, containing the isocyanate, was added dropwise to a solution of **7.12** (950 mg, 1.36 mmol, 1 eq) over a period of 30 min and stirring was continued for 1.5 h. The volatiles were removed under reduced pressure and both intermediates (the N^{ω}- as well as the N^{δ}-carbamoylated arginine derivative, cf. Scheme 5) were isolated using column chromatography (CH_2Cl_2/EtOAc 5/1 to 3/1, column was packed with 0.5 % NEt_3). Each intermediate (yellowish oils, N^{ω}-derivative: 282 mg, N^{δ}-derivative: 359 mg) was dissolved in MeOH (10 mL). A 10 % Pd/C catalyst (45 mg and 55 mg, resp.) was added under an atmosphere of nitrogen and hydrogen was conducted through the vigorously stirred suspension. The flow of hydrogen was stopped after 30 min and depletion of starting material was nearly complete (TLC analysis). The catalyst was removed by filtration and removal of the solvent under reduced pressure yielded a highly viscous colorless oil which hardned to a solid during drying *in vacuo*. Purification with preparative HPLC (only N^{ω}-derivative) using mixtures of acetonitrile (A) and 0.025 % aq. acetic acid (B) as eluent (gradient: 0 – 20 min: A/B 50/50 to 95/5, $t_R \approx 18 – 22$ min) afforded the product as a white fluffy solid (188 mg, 0.26 mmol, 19.5 %). For NMR analysis 7.5 mg of **7.14** were Boc-deprotected through the treatment with acetonitrile/TFA (1:2, 300 µL) at 50 °C for 30 min. The mixture was analyzed by HPLC (Figure 4D) prior to the isolation of Boc-deprotected **7.14** (**7.14-B**) with preparative HPLC (eluent: mixtures of acetonitrile (A) and 0.05 % aq. TFA (B), gradient: 0 – 20 min: A/B 20/80 to 60/40, $t_R \approx 13$ min). ^{1}H-NMR (600 MHz, DMSO-d_6, COSY) **7.14-B**: δ (ppm) 1.43-1.65 (m, 7H, CH-C$\underline{H}_2$-C$\underline{H}_2$, CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2), 1.75 (m, 1H, CH-C$\underline{H}_2$-CH_2), 2.78 (m, 2H, C$\underline{H}_2$-NH_2), 3.1 (m, 2H, C$\underline{H}_2$-NH), 3.23 (m, 2H, C$\underline{H}_2$-NH), 3.96 (m, 1H, CH^{α}), 4.22 (m, 1H, CH-CH_2, Fmoc), 4.29 (m, 2H, CH-CH_2, Fmoc), 7.32 (dt, 2H, ^{3}J = 7.42 Hz, ^{4}J = 0.83 Hz, Fmoc), 7.41 (t, 2H, ^{3}J = 7.45 Hz, Fmoc), 7.56 (s, 1H, CH_2-N$\underline{H}$), 7.66 (d, 1H, ^{3}J = 8.11 Hz, CH^{α}-N$\underline{H}$), 7.71 (m, 4H, Fmoc, CH_2-N$\underline{H}_2$), 7.89 (d, 2H, ^{3}J = 7.54 Hz, Fmoc), 8.46 (s, 2H, NH_2), 8.97 (s, 1H, CH_2-N$\underline{H}$), 10.22 (s, 1H, NH), 12.67 (bs, 1H, COOH); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc) **7.14**: *m/z* 711 $[M + H]^+$; MS (ES, MeCN/TFA) **7.14-B**: *m/z* 511 $[M + H]^+$; $C_{36}H_{50}N_6O_9$ (710.8)

H_2N-Asp-Arg(N^{ω}-Ahx)-Val-Tyr-Ile-His-Pro-Phe-OH (7.15). The angiotensin II derivate **7.15**, containing an Ahx-substituted arginine (derived from the arginine building block **7.5**), was synthesized on a Fmoc-Phe-trityl resin (200-400 mesh; loading 0.6 mmol/g) using a standard Fmoc protocol for solid-phase peptide synthesis with *N*-hydroxybenzotriazole (HOBt) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), respectively, as coupling reagents and *N*-diisopropylethylamine (DIPEA) as a base. The synthesis was performed in a mixture of DMF and *N*-methyl-2-pyrrolidon (NMP) (80/20). Natural Fmoc-

protected amino acids were coupled using 5 eq. of the pertinent amino acid, 5 eq. of HOBt/HBTU and 10 eq. of DIPEA (double coupling, 2 × 40 min). For coupling of **7.5** 2.5 eq. were used as well as 2.5 eq. of HOBt/HBTU and 5 eq. of DIPEA (single coupling, 8 h). Cleavage from the resin was achieved with CH_2Cl_2/hexafluoroisopropanol (3:1) and subsequent side-chain deprotection was performed using TFA/H_2O/CH_2Cl_2 (5:1:4). The octapeptide was purified with preparative HPLC using mixtures of acetonitrile (A) and 0.1 % aq. TFA (B) as eluent (gradient: 0 – 30 min: A/B 10/90 to 35/65, $t_R \approx 32.5$ min). Acetonitrile was removed under reduced pressure at 37 °C (final pressure: 50 mbar) prior to lyophilization which afforded the product as a white fluffy solid (3.3 mg, 2.04 µmol). Reversed-phase HPLC (gradient 2, cf. Figure 5A): 95 % (t_R = 18.9 min, k = 6.0); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 580.4 $[M + 2H]^{2+}$, 1159.8 $[M + H]^+$; $C_{56}H_{82}N_{14}O_{13} \times C_8H_4F_{12}O_8$ (1615.4)

Peptide 7.15 labaled with the fluorescent dye Py-1 (7.15-Py1). This reaction was monitored with analytical HPLC. Therefore the pyrylium dye Py-1 was added in dependence on the amount of starting material **7.15** detected with HPLC.

H_2N-Asp-Arg(N^{ω}-Ahx)-Val-Tyr-Ile-His-Pro-Phe-OH (**7.15**) (0.8 mg, 0.53 µmol, 1 eq) and NEt_3 (1.08 mg, 1.4 µL, 10.6 µmol, 20 eq) were dissolved in a mixture of acetonitrile (50 µL) and DMF (75 µL) followed by the addition of the pyrylium dye Py-1 × 1 BF_4^- (0.21 mg, 0.53 µmol, 1 eq) in DMF (25 µL). The mixture was stirred at rt. Additional portions of Py-1 × 1 BF_4^- (each 0.21 mg, 0.53 µmol, 1 eq) were added 70 min, 2 h, 3 h and 4 h after beginning of the reaction. The reaction was stopped by addition of 10 % aq. TFA (15 µL) after an incubation period of 4.5 h. The product was purified with preparative HPLC using mixtures of acetonitrile (A) and 0.1 % aq. TFA (B) as eluent (gradient: 0 – 30 min: A/B 15/85 to 52/48, t_R = 28.7 min). Acetonitrile was removed under reduced pressure at 35 °C (final pressure: 50 mbar) and lyophilization afforded the product as a colored solid which was transferred into a 1.5-mL eppendorf reaction vessel (screw top) using mixtures of acetonitrile and 0.1 % aq. TFA. Evaporation in a vacuum concentrator and lyophilization yielded 0.19 mg (0.1 µmol, 19 %) of the product as fourfold TFA salt. Reversed-phase HPLC (gradient 3, cf. Figure 5B): 94 % (t_R = 25.4 min, k = 8.4); MS (ES, CH_2Cl_2/MeOH + 10 mM NH_4OAc): *m/z* 724.1 $[M + 2H]^{2+}$, 483.1 $[M + 3H]^{3+}$, 1447.1 $[M + H]^+$; $C_{77}H_{104}N_{15}O_{13} \times C_8H_3F_{12}O_8$ (1902.8)

7.3.3 Fura-2 Ca^{2+}-Assay on Rat Mesangial Cells

The rat mesangial cell line was kindly provided by Prof. Dr. A. Kurtz, Department of Physiology, University of Regensburg. Cells were maintained in antibiotic-free RPMI 1640 medium (Sigma, Deisenhofen, Germany) containing 10 % fetal calf serum (FCS) and 0.24 mg/L insulin in a water saturated atmosphere (95 % water, 5 % CO_2) at 37 °C. 4 - 5 days before testing, cells from a

confluent 25-cm^2 flask (Nunc GmbH & Co. KG, Langenselbold, Germany) were transferred to four 175-cm^2 flasks (Nunc GmbH & Co. KG, Langenselbold, Germany). On the day of the experiment cells were trypsinized, suspended in medium and centrifuged for 7 min at 200 g and room temperature. Cells were resuspended in 40 mL of loading buffer (25 mM HEPES (Sigma, Deisenhofen, Germany), 120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose), pH 7.4, and 30 µL of fura-2/AM (1 mM in DMSO, Calbiochem/Merck Biosciences, Beeston, UK), 37.5 µL of pluronic F-127 (20 % in DMSO, Calbiochem/Merck Biosciences, Beeston, UK) as well as BSA (200 mg) were added. The cell suspension was incubated in the dark at room temperature for 30 min and then centrifuged. Cells were resuspended in fresh loading buffer and allowed to stand for 60 min at room temperature in the dark. After two washing/centrifugation cycles (loading buffer, 200 g, 7 min) and resuspension in 25 mL of loading buffer, cells were incubated for at least 15 min at 20 °C in the dark. Measurements were performed as described for a fura-2 assay on human erythroleukemia (HEL) cells[10] using a Perkin-Elmer LS 50 B Luminescence Spectrometer (Perkin Elmer, Überlingen, Germany).

7.4 References

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Chapter 8

Summary

Neuropeptide Y (NPY) is one of the most abundant neuropeptides in the central and peripheral nervous system. This 36-amino acid peptide is involved in the regulation of numerous physiological functions such as food intake, blood pressure, stress, pain and hormone secretion. In humans, the action of NPY is mediated by four receptor subtypes (Y_1, Y_2, Y_4 and Y_5), members of the superfamily of G-protein coupled receptors (GPCRs). The Y_1 receptor (Y_1R) is expressed e.g. in vascular smooth muscle, adipose tissue, in the gastrointestinal tract and in distinct brain regions such as the cerebral cortex and the hypothalamus. Recently, the Y_1R gained new interest as it was reported to be expressed in malignancies (e.g. breast and prostate cancer, ovarian and adrenal tumors). The first highly potent and selective Y_1R antagonist was the (*R*)-argininamide BIBP 3226, a C-terminal mimic of NPY. N^{ω}-Acylation of BIBP 3226 was demonstrated to retain or even increase the Y_1R affinity, thereby improving the pharmacokinetic properties. Taking advantage of these phenomena led to selective radio- and fluorescence labeled argininamide-type Y_1R antagonists described in this thesis.

N^{ω}-substitution with [2,3-^{3}H]-propanoic acid yielded the easily accessible, highly potent and selective tritiated Y_1R antagonist [^{3}H]-UR-MK114 (K_B: 0.8 nM, Ca^{2+}-assay, HEL cells; K_D, saturation: 1.2 nM, SK-N-MC cells), which was shown to be a useful pharmacological tool for the radiochemical determination of Y_1R ligand affinities, the quantification of Y_1R binding sites and for autoradiography. Another high affinity potential tritium ligand (K_i = 1.3 nM), containing an ω-aminocarbamoyl linker between the BIBP 3226 pharmacophore and tritiated propionic acid, was prepared and investigated pharmacologically.

Aiming at molecular probes for *in vivo* imaging of Y_1Rs, several potential Y_1R antagonistic PET ligands were prepared and characterized. For this purpose, ω-aminocarboxylic acids of different length and chemical structure were attached to the guanidine of BIBP 3226, yielding amine precursors for coupling with small fluorinated carboxylic acids (2-fluoropropanoic and 4-fluorobenzoic acid). Two Y_1R antagonists with K_i values in the low two-digit nM range were prepared as prototype ^{18}F-labeled PET ligands and administered intravenously to nude mice bearing Y_1R expressing subcutaneous SK-N-MC xenografts. One of these PET ligands enabled the detection of the tumor during an early period (< 25 min) after administration, but late imaging of the tumor after systemic clearance of the tracer failed. The relatively high lipophilicity of the PET ligands (logP in the range of 1 – 4) led to rapid biliary excretion, requiring optimization of the physicochemical properties.

Diverse BIBP 3226 derived amine precursors were also used for the preparation of a series of Y_1R selective fluorescent ligands. In terms of retaining affinity, low molecular weight pyrylium dyes as well as a newly prepared benzothiazolium derivative proved to be superior to cyanine dyes such as S0436. The majority of the most potent fluorescent Y_1R antagonists (K_i = 10 - 50 nM) was successfully applied in confocal microscopy and flow cytometric equilibrium binding studies.

As bivalent ligands are considered as potential tools to study the dimerization of GPCRs, a series of bivalent Y_1R antagonists was prepared through the connection of two BIBP 3226 pharmacophoric entities by dicarboxylic acids of different chain length (8 – 36 atoms) and structure. To exploit the fact that the distomer of BIBP 3226, the (*S*)-enantiomer, is more than 1000 times less potent, sets of (*R*,*R*)-, (*R*,*S*)- and (*S*,*S*)-configured bivalent ligands were synthesized. The most potent bivalent ligand (K_i = 9 nM), consisting of a 20 atom spacer, revealed a moderately lower Y_1R affinity for the (*R*,*S*)-stereoisomer compared to the (*R*,*R*)-enantiomer (factor of 5), which was not observed for other pairs of (*R*,*R*)- and (*R*,*S*)-configured bivalent ligands. Interestingly, among the (*R,R*)-configured ligands only minor differences in affinities (K_i values 9 - 63 nM), irrespective of spacer length and chemical diversity, were observed. A potential bivalent radioligand (K_i = 18 nM) is considered as a potential tool to perform investigations on the stoichiometry of binding sites in combination with monovalent radioligands.

The achievements in the Y_1R antagonists motivated the preparation of N^{ω}-Boc-N^{α}-Fmoc-arginine bearing an appropriate $N^{\omega'}$-acyl residue as a versatile building block for solid phase synthesis of modified peptides. The incorporation of such an arginine substitute into angiotensin II (AT) resulted in amino-functionalized and fluorescence-labeled AT derivatives with retained agonistic potency at the AT_1 receptor (EC_{50}: 21 and 80 nM, AT: 17 nM). As these arginine derivatives could be universally applied to prepare ligands for any peptidergic receptor, the proof of concept described in this thesis could promote the development and broaden the spectrum of molecular imaging tools.

Appendix

A.1 Growth of Subcutaneous SK-N-MC and MCF-7-Y_1 Tumors in Nude Mice

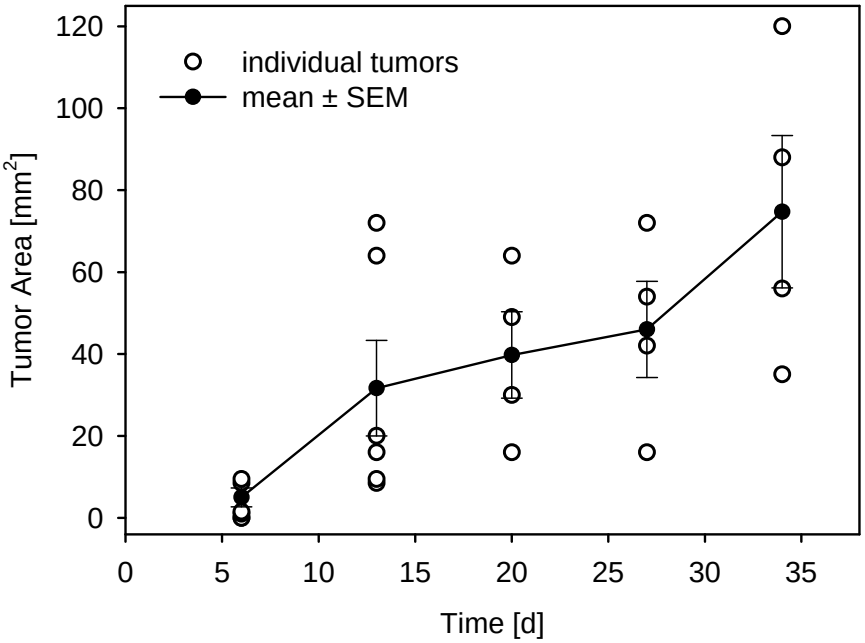

Figure 1. Growth of subcutaneous SK-N-MC tumors (0^{th} passage) in male NMRI (nu/nu) mice after injection of 3 million cells (50^{th} in vitro passage). Number of animals: 6, take rate: 6/6.

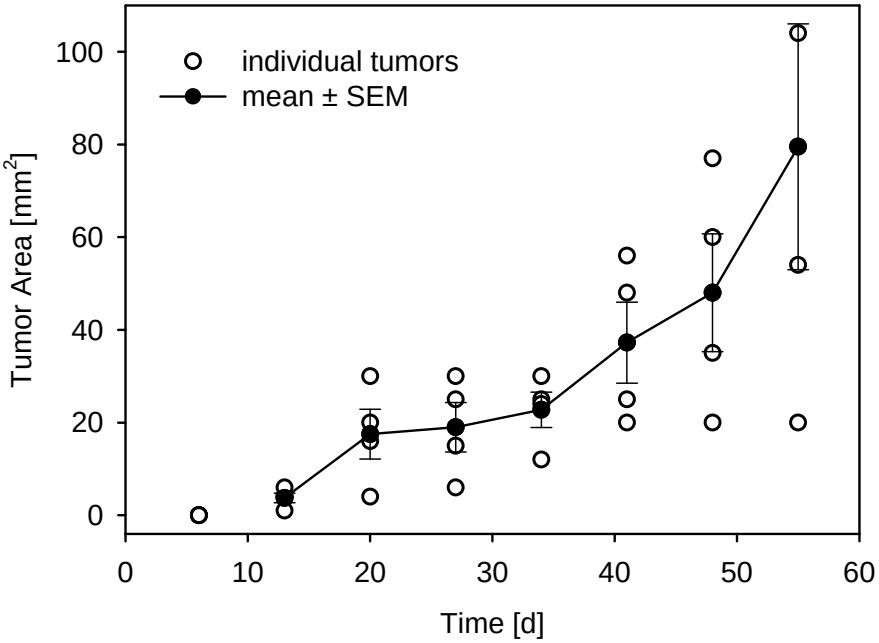

Figure 2. Growth of subcutaneous SK-N-MC tumors (1^{st} passage) in male NMRI (nu/nu) mice after transplantation of freshly dissected pieces (ca. 2 × 2 mm) of a s.c. tumor (0^{th} passage). Number of animals: 6, take rate: 4/6.

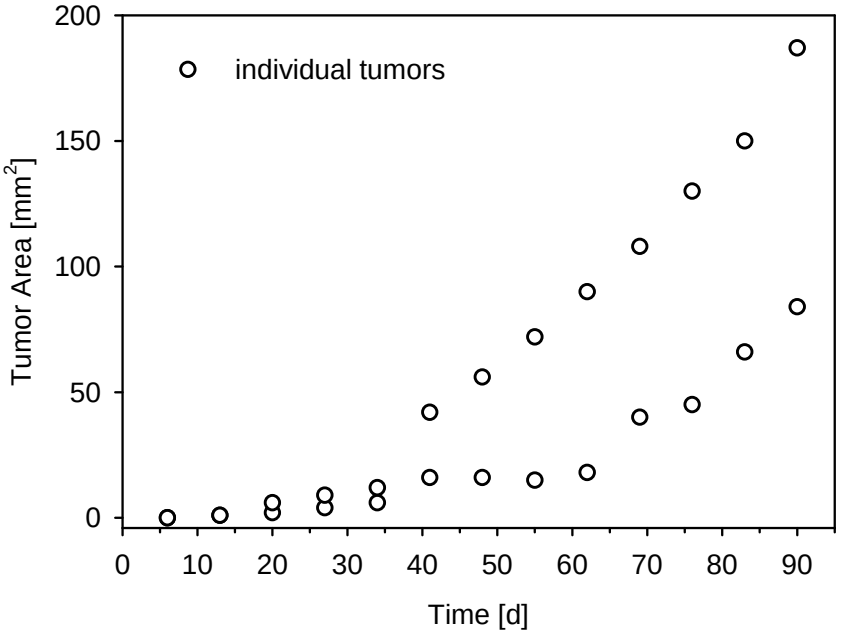

Figure 3. Growth of subcutaneous SK-N-MC tumors (2nd passage) in male NMRI (nu/nu) mice after transplantation of freshly dissected pieces (ca. 2 × 2 mm) of a s.c. tumor (1st passage). Number of animals: 4, take rate: 2/4.

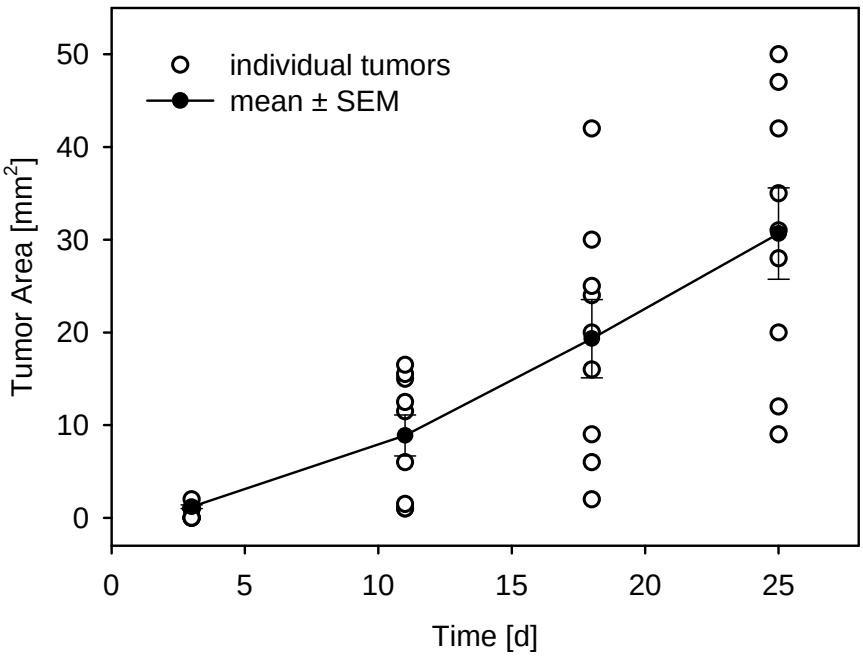

Figure 4. Growth of subcutaneous MCF-7-Y_1 tumors (0th passage) in female estrogen-substituted NMRI (nu/nu) mice after injection of 4 million cells (171st in vitro passage). Number of animals: 12, take rate: 9/12. Subcutaneous estrogen depots were implanted 10 – 12 days prior to tumor implantation.

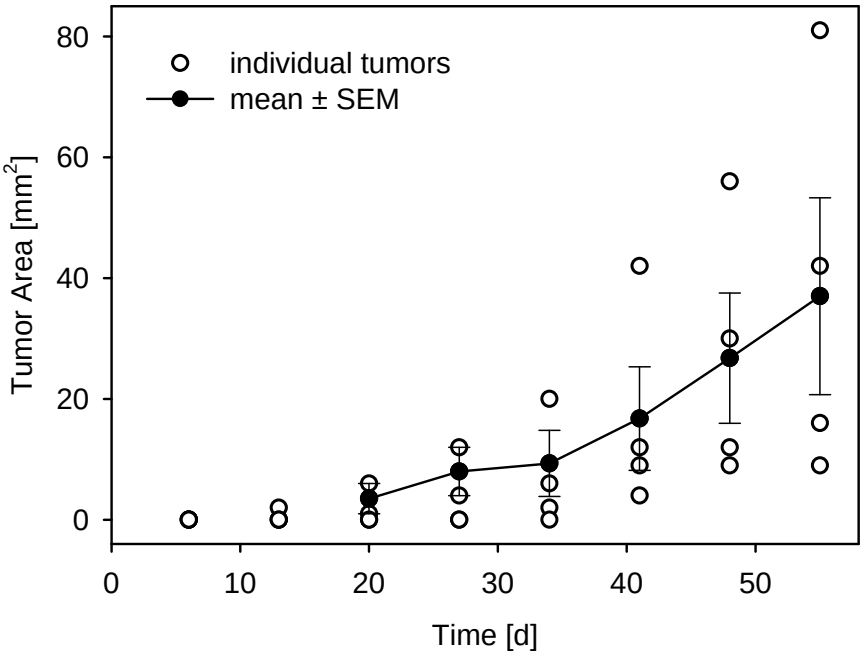

Figure 5. Growth of subcutaneous MCF-7-Y_1 tumors (1st passage) in female estrogen-substituted NMRI (nu/nu) mice after transplantation of pieces (ca. 2 × 2 mm) of a s.c. tumor (0th passage), stored in liquid nitrogen in culture medium without FCS and supplemented with 10 % DMSO. Number of animals: 9, take rate: 4/9. Subcutaneous estrogen depots were implanted 10 – 12 days prior to tumor implantation.

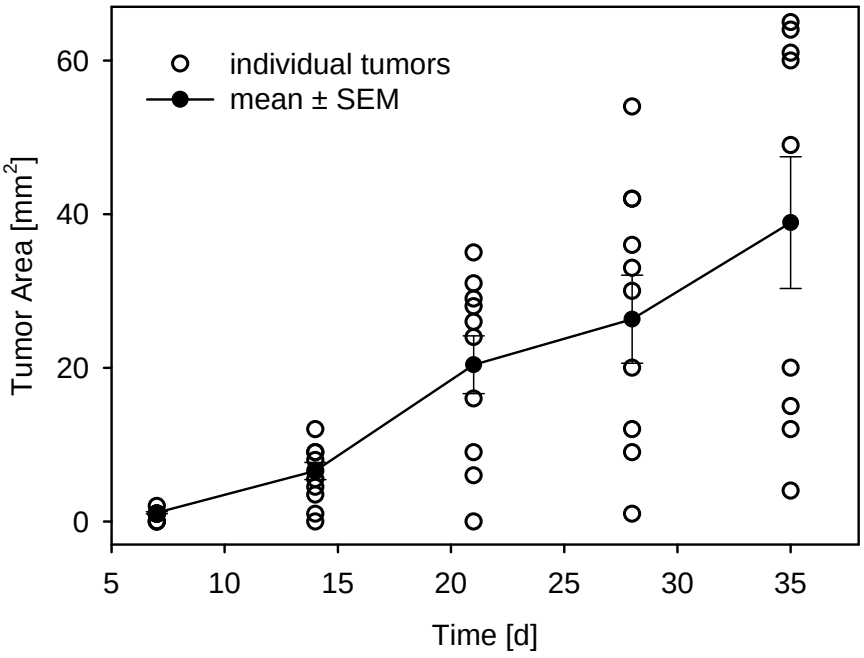

Figure 6. Growth of subcutaneous MCF-7-Y_1 tumors (2nd passage) in female estrogen-substituted NMRI (nu/nu) mice after transplantation of freshly dissected pieces (ca. 2 × 2 mm) of a s.c. tumor (1st passage). Number of animals: 12, take rate: 10/12. Subcutaneous estrogen depots were implanted 10 – 12 days prior to tumor implantation.

A.2 Data Processing

All data to be fitted were processed with SigmaPlot 9.0. Data from radioligand competition experiments and concentration response curves (fura-2 assays) were analyzed by 4 parameter sigmoidal fits. IC_{50} values from radioligand competition studies were converted to K_i values according to the Cheng-Prusoff equation[1] using the respective K_D value of the radioligand. Three data points (between 20 and 80 % inhibition, from fura-2 assays) served for the calculation of IC_{50} values after logit-log transformation. IC_{50} values were converted to K_b values according to the Cheng-Prusoff equation[1] using an EC_{50} value of 1.8 nM for pNPY (mean value from 4 independently determined concentration-effect curves on HEL cells).

Data from saturation experiments (chapter 2 and 4) were evaluated by one-site saturation fits (K_D, B_{max}). Rate constants (k_{ob}, k_{off}, chapter 2) were analyzed using linear plots and simple linear regressions. The association rate constant (k_{on}) in chapter 2 was calculated from k_{ob}, k_{off} and the radioligand concentration according to the correlation: $k_{on} = (k_{ob} – k_{off})/[\text{radioligand}]$. For Schild analysis in chapter 2 $\log_{10}(r - 1)$ was plotted against $\log_{10}[\text{antagonist}]$ with $r = 10^{\Delta pEC50}$.

Raw data from flow cytometric experiments were processed with the aid of the WinMDI 2.7 software. The confocal images were processed with the Carl Zeiss LSM image browser 4.2^{TM}. The retention (capacity) factor was calculated according to $k = (t_R – t_0)/t_0$. All error bars in the diagrams represent the standard errors of the means or errors calculated according to the law of error propagation.

A.3 Abbreviations

AA	amino acid(s)
Ahx	6-aminohexanoic acid (6-aminohexanoyl)
aq.	aqueous
Ar	aryl
a_s	specific activity
BBB	blood brain barrier
B_{max}	maximum number of binding sites
Boc	*tert*-butoxycarbonyl
Bq	becquerel
BRET	bioluminescence resonance energy transfer
bs	broad singulet
BSA	bovine serum albumin
^{t}Bu	*tert*-butyl
c	concentration
$[Ca^{2+}]_i$	intracellular calcium ion concentration
Cbz	benzyloxycarbonyl
CDI	carbonyldiimidazole
CH_2Cl_2	methylene chloride
CHO cells	chinese hamster ovary cells
Ci	curie
CI	chemical ionization
CNS	central nervous system
COSY	correlated spectroscopy
d	doublet
δ	chemical shift
DCC	*N*,*N*'-dicyclohexylcarbodiimide
DCU	*N*,*N*'-dicyclohexylurea
decomp.	decomposition
$CDCl_3$	deuterated chloroform
DIPEA	*N*,*N*-diisopropyl-ethylamine
DMAP	4-(dimethylamino)-pyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
DMSO-d_6	per-deuterated dimethylsulfoxide
EC_{50}	agonist concentration which induces 50 % of the maximum response
EtOAc	ethyl acetate

EDAC	*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (hydrochloride)
EI	electron impact ionization
eq.	equivalent(s)
FAB	fast atom bombardment
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
Fmoc	9-fluorenylmethoxycarbonyl
FmocOSu	9-fluorenylmethyl-*N*-succinimidylcarbonate
FRET	fluorescence resonance energy transfer
GPCR	G-protein coupled receptor
h	hour(s)
HBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
H&E	hematoxylin and eosin
HEK-1B cells	human endometrial carcinoma cells
HEL cells	human erythroleukemia cells
HEPES	2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid
HMBC	heteronuclear multiple bond correlation
hPP	human pancreatic polypeptide
HOBt	1-hydroxybenzotriazole (monohydrate)
HR-MS	high resolution mass spectrometry
HSQC	heteronuclear single quantum coherence
IC_{50}	inhibitor/antagonist concentration which suppresses 50 % of an agonist induced effect or displaces 50 % of a labeled ligand from the binding site
^{n}J	coupling constant for geminal (n = 2), vicinal (n = 3), etc. coupling
k	retention (capacity) factor
K_b	dissociation constant derived from a functional assay
K_D	dissociation constant derived from a saturation experiment
K_i	dissociation constant derived from a competition binding assay
$LiAlH_4$	Lithiumaluminiumhydrid
k_{ob}	observed/macroscopic association rate constant
k_{off}	dissociation rate constant
k_{on}	association rate constant
logP	common logarithm of the n-octanol/water partition coefficient
m	multiplet
M	molar (mol/L)
MCF-7 cells	human breast adenocarcinoma cells
MEM	minimum essential medium eagle
MeOH-d_4	per-deuterated methanol

mol	mole(s)
mp	melting point
mRNA	messenger RNA
NEt_3	triethylamine
N^G	guanidine nitrogen (cp. N^ω nomenclature for arginines)
NHS	*N*-hydroxysuccinimide
NIH	national (US) institutes of health
PBS	phosphate buffered saline
PE	petroleum ether
PET	positron emission tomography
Ph	phenyl
pNPY	porcine neuropeptide Y
ppm	parts per million
q	quartet
RP	reversed-phase
rt	room temperature
s	(1) singulet, (2) second(s)
SEM	standard error of the mean
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	(1) time, (2) triplet
t_0	hold-up time (also designated as t_M)
TFA	trifluoroacetic acid
THF	tetrahydrofurane
t_R	retention time
UV	ultraviolet
Y_n	NPY receptor subtypes, n = 1, 2, 4, 5, 6, 7

A.4 References

1. Cheng, Y.; Prusoff, W. H., Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973,** 22, (23), 3099-3108.

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Max Keller