Molecular Tools for the NPY Y_4 Receptor Derived from the C-Terminus of hPP and from Argininamide-type Y_1R Antagonists

Dissertation

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Mimicking of arginine by functionalized *N*(omega)-carbamoylated arginine as a new broadly applicable approach to labeled bioactive peptides: high affinity angiotensin, neuropeptide Y, neuropeptide FF and neurotensin receptor ligands as examples. *Journal of Medicinal Chemistry* 59 (5), S. 1925-1945.

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Abbreviations

AA	amino acids(s)
aib	2-aminoisobutyric acid
aq.	aqueous
Ar	aryl
βcpe	cis-cyclopentane β-amino acid
B_{max}	maximum number of binding sites
Boc	*tert*-butoxycarbonyl
Bq	becquerel
bs	broad singulet
BSA	bovine serum albumin
c	concentration
cAMP	cyclic adenosine monophosphate
Cbz	benzyloxycarbonyl
CD	circular dichroism
CDI	carbonyldiimidazole
CHO cells	Chinese hamster ovary cells
COSY	orrelated spectroscopy
d	doublet
δ	chemical shift
DCC	*N*,*N*'-dicyclohexylcarbodiimide
DCM	dichloromethane
DIPEA	*N*,*N*-diisopropyl-ethylamine
DMAP	4-(dimethylamino)-pyridine
DMEM	dulbecco's modified eagle medium
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
DPM	disintegrations per minute
EC_{50}	agonist concentration which induces 50% of the maximum response
EtOAc	ethyl acetate
EtOH	ethanol
eq.	equivalent(s)
ESI	electrospray ionization
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
Fmoc	9-fluorenylmethoxycarbonyl
FRET	Förster resonance energy transfer
GI	gastro intestinal
GPCR	G-protein coupled receptor
h	hour(s)

HBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEC-1b	human endometrial carcinoma cells
HEK293	human embryonic kidney cells
HEL cells	human erythroleukemia cells
HEPES	2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid
HMBC	heteronuclear multiple bond correlation
HPLC	high-performance liquid chromatography
hPP	human pancreatic polypeptide
HOBt	1-hydroxybenzotriazole (monohydrate)
HR-MS	high resolution mass spectrometry
HSQC	heteronuclear single quantum coherence
IC_{50}	inhibitor concentration which suppresses 50% of an agonist induced effect or displaces 50% of a labeled ligand from the binding site
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
k_{obs}	observed/macroscopic association rate constant
k_{off}	dissociation rate constant
k_{on}	association rate constant
m	multiplet
M	molar (mol/L)
MCF-7 cells	human breast adenocarcinoma cells
MeCN	acetonitrile
MeOH	methanol
mol	mole(s)
mp	melting point
N^G	guanidine nitrogen
NHS	*N*-hydroxysuccinimide
NMP	*N*-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
NPY	neuropeptide Y
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PBS	phosphate buffered saline
PE	petroleum ether
PYY	peptide YY
RP	reversed phase
rt	room temperature
s	(1) singulet, (2) second(s)
SEM	standard error of the mean
t	(1) time, (2) triplet
*t*Bu	*tert*-butyl

TLC	thin layer chromatography
TFA	trifluoroacetic acid
THF	tetrahydrofurane
t_R	retention time
UV	ultraviolet
Y_n	NPY receptor subtypes, n = 1, 2, 4, 5, 6

Contents

Chapter 1

General Introduction

1.1 The Neuropeptide Y (NPY) Family

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) belong to a peptide family of neuroendocrine hormones, referred to as the neuropeptide Y family (Figure 1). All three peptides consist of 36 amino acids with C-terminal amidation and share considerable sequence homologies.[1-3]

NPY YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2

PYY YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2

PP APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH_2

Figure 1: Amino acid sequences of human NPY, PYY and PP

NPY, PYY and PP interact with the same family of rhodopsin-like (class 1) G-protein coupled receptors (GPCRs), referred to as NPY receptors (NPYR). Until now five different subtypes have been identified (Y_1R, Y_2R, Y_4R, Y_5R, y_6R).[4-12] The y_6R is a functional receptor in rabbit and mouse, but non-functional in most mammalian species. It is an inactive pseudogene in human and pig and is absent in rat.[13] NPY receptors act via $G_{i/o}$-proteins mediating the inhibition of cAMP accumulation.[14,15] Furthermore, NPYRs were reported to activate phospholipase C resulting in subsequent elevation of intracellular Ca^{2+} levels.[6,7,16-21] The intensity of this effect is highly dependent on the investigated cell type.[14] NPY and PYY bind with high affinity to Y_1, Y_2 and Y_5 receptors, but exhibit only low affinity to the Y_4R. The latter is addressed by PP. Therefore, the Y_4R was originally designated as PP_1R.[8]

The tertiary structure of the three peptide hormones was studied by several spectroscopic methods. The crystal structure of chicken (avian) pancreatic polypeptide was resolved by x-ray analysis.[22] PP comprises an extended polyproline-like helix (residues 1-8), followed by a β-turn (9-13) and an α-helix (14-31) that runs roughly antiparallel to the polyproline-like helix, bringing N-terminus and C-terminus into close proximity. The helices are stabilized by hydrophobic interactions between prolines 2, 5 and 8 and hydrophobic side chains of the α-helix. The amidated C-terminus (32-36), which comprises the most crucial residues for receptor binding, was reported to be rather flexible. The hairpin-like conformation which is formed between the polyproline-like helix and the α-helix, the so called PP-fold, was later also proposed for porcine NPY (Figure 2) since the homology between aPP and NPY

preserves all of the residues which are essential for the maintenance of the tertiary structure.[23]

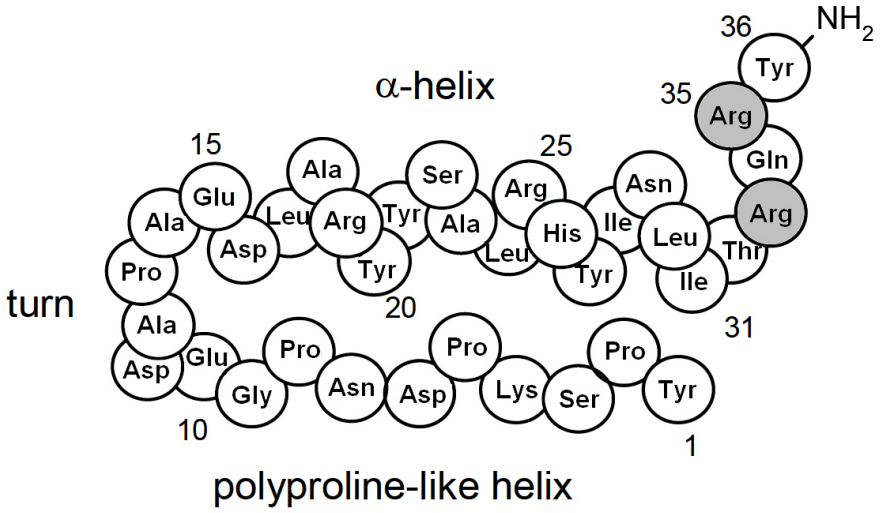


Figure 2: tertiary structure of porcine NPY according to Allen et al.[23]

The tertiary structure of NPY in solution was intensively investigated using NMR and CD spectroscopy as well as FRET-based approaches with contradictory results. Some of these studies confirmed the PP-fold structure.[24,25] Other groups reported on α-helical structures of the C-terminus, rather flexible N-termini and the formation of dimers through hydrophobic contacts of the reported α-helices.[26] Major disadvantages of conformational studies using NMR spectroscopy are the required high concentrations of the protein, which are physiologically irrelevant, and the pH values considerably lower than 7.4 to increase the solubility of the peptide. CD spectroscopy revealed that NPY exists in different states, depending on the concentration, pH value and the temperature. Under physiological conditions, the monomeric form with the PP-fold seems to be the most favored one.[27] A comparable tertiary structure in solution could later be verified for PYY too.[28]

Over the last years methods to determine the tertiary structure of GPCRs including x-ray analysis of GPCR crystals, NMR spectroscopy, mutagenesis based approaches and molecular modeling have clearly advanced. Several groups described structures of NPY receptors in complex with the respective endogenous ligands using NMR spectroscopy, NPY receptor mutants and homology models based on crystal structures of related GPCRs. Merten et al. published a model outlining the importance of $Asp^{6.59}$, an amino acid that is highly conserved throughout all NPY receptors. In Y_1R and Y_4R, $Asp^{6.59}$ was identified to interact with Arg^{35} of

PP or NPY. However, in Y_2R and Y_5R, $Asp^{6.59}$ interacts with Arg^{33}.[29] Xu et al. reported on a alternative model of the Y_2R. The two C-terminal arginines in NPY and PYY, Arg^{33} and Arg^{35} were found to form a double salt bridge with both $Glu^{5.24}$ and $Asp^{6.59}$ on the Y_2R. Several additional amino acids, e.g. $Gln^{3.32}$, $Leu^{6.51}$ and $His^{7.39}$ were identified to contribute to the orthosteric binding site.[30] This model was basically verified very recently by NMR measurements of specific isotope-labeled NPY in complex with the Y_2R.[31] Based on all available class A GPCR crystal structures and data from mutagenesis studies, a model of the Y_4R in complex with PP was constructed.[32] In addition to the above-mentioned interaction between $Asp^{6.59}$ and Arg^{35}, several residues were found to contribute to a complex network of ionic or hydrophobic interactions and hydrogen binding. Specifically, $Tyr^{2.64}$ undergoes hydrophobic interactions with Tyr^{27}, and $Asn^{7.32}$ interacts with Arg^{33} via hydrogen bonding. Requiring rather artificial conditions, every method for the structure determination of GPCRs has its own limitations. However, three-dimensional models of NPY receptors might still facilitate the rational design of NPY receptor ligands, agonists and antagonists, with increased affinity and selectivity.

The neuropeptide Y receptor family is involved in the regulation of numerous physiological and pathophysiological processes[33] including energy metabolism and the development of obesity,[34-39] mental and psychiatric disorders,[40-44] neurodegeneration[44-47] or the regulation of blood pressure.[48,49] A summary of important biological functions is shown in Table 1.

Table 1. Overview of biological effects mediated by the stimulation of NPY receptors.

Y_1R	Food intake (↑) and energy metabolism (↓), depression, anxiolysis, alcohol consumption (↓), seizures (↑), pain sensitivity (↑), regulation of blood pressure (vasoconstriction)
Y_2R	Food intake (↓) and energy metabolism (↑), anxiety (due to reduced NPY release), schizophrenia-relevant symptoms (↑), seizures (↓), neuroprotection in patients with Huntingtons' disease, bone formation, hypoxia-induced tumor growth, regulation of blood pressure (slowing of the heart rate), NPY release (↓, presynaptic autoreceptor)
Y_4R	Food intake (↓), gastrointestinal motility (↑)
Y_5R	Food intake and obesity (↑), hypoxia-induced tumor growth

1.2 NPY Y_1, Y_2 and Y_5 Receptor Ligands

Modifications of the flexible C-terminal part resulted in Y_1R agonists with selectivity over the Y_2R, e.g. [Leu^{31}, Pro^{34}]NPY.[50] Mullins et al. reported on a series of D-peptides with increased Y_1R selectivity. [D-Arg^{25}, D-His^{26}]NPY displayed a 40-fold selectivity over the Y_2R and a 300-fold selectivity over the Y_5R, respectively.[51] A further increase in selectivity and affinity was achieved with centrally truncated analogs such as Des-AA$^{11-18}$[Cys^{7,21}, D-Lys^{9} (Ac), D-His^{26}, Pro^{34}]NPY. The incorporation of artificial amino acids such as norleucine or L-4-benzoylphenylalanine (Bpa) into the C-terminal part of NPY led to the identification of the first selective Y_1R agonists with reduced size. The nonapeptide [Pro^{30}, Nle^{31}, Bpa^{32}, Leu^{34}]NPY(28-36) binds to the Y_1R with an affinity in the two-digit nanomolar range and was proposed as a precursor for diagnostic agents to specifically label Y_1R expressing tumors.[52] The first selective non-peptide Y_1R antagonist, the (*R*)-argininamide BIBP 3226, was described in 1994.[53] The structure was designed as a mimic of the C-terminal moiety in NPY since an alanine scan for NPY revealed arginine residues 33 and 35 to be essential for Y_1R binding.[54] The affinity of the (*S*)-configured enantiomer, BIBP 3435, was lower by a factor of 1000.[55] Replacement of the phenolic hydroxyl group with an ureidomethyl moiety gave another potent Y_1R antagonist, BIBO 3304 (K_i = 0.4 nM).[56] Even though BIBP 3226 was never considered as an appropriate candidate for clinical trials due to the lack of drug like properties, it was used as a starting point in the development of numerous pharmacological tools, e.g. radiolabelled and fluorescent ligands.[57-60] Very recently, [^{3}H]UR-MK299, a Y_1R radioligand with a K_d value in the two-digit picomolar range was described.[60] Apart from superior Y_1R affinity, a considerably increased target-residence time was characteristic of [^{3}H]UR-MK299. An overview about further structurally diverse Y_1R antagonists is given in Figure 3.

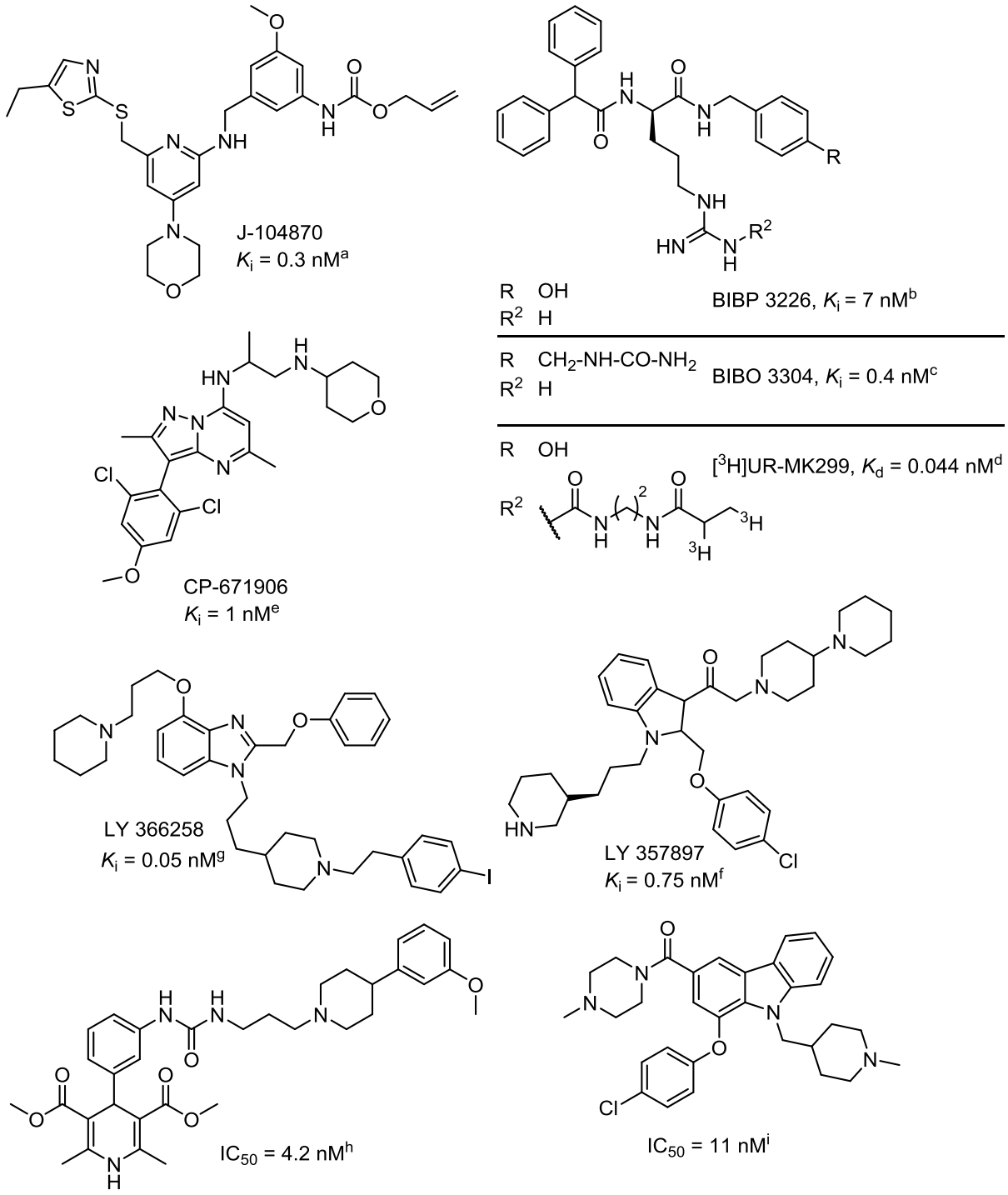


Figure 3: Structures of several selective Y_1R antagonists: a: Kanatani et al[61]; b: Rudolf et al[53]; c: Wieland et al[56]; d: Griffith et al[62] ; f: Hipskind et al[63]; g: Zarrinmayeh et al[64]; h: Sit et al[65]; I: Leslie et al[66].

In contrast to the Y_1R, N-terminal truncations of NPY are well tolerated with respect to Y_2R affinity. For example, NPY(18-36) showed an only 6-fold lower affinity to the Y_2R than NPY, but the affinity to the Y_1R was reduced by the factor of 70.[67] A peptidomimetic comprising four C-terminal NPY fragments (33-36) linked to a cyclic template molecule mimicking two β-turns was identified as a selective Y_2R antagonist[68] illustrating that the C-terminus is the

most crucial part for interaction with the Y_2R, whereas the N-terminus seems to be less important. In 1999, the (*S*)-argininamide BIIE 0246 was described as the first non-peptide Y_2R antagonist with a K_i value in the lower nanomolar range.[69] Growing interest in the Y_2R as a potential target for the treatment of obesity led to the identification of several structurally diverse Y_2R antagonists. A selection of different, more drug-like compounds is shown in Figure 4. Recently, [^{3}H]UR-PLN196 was reported as the first non-peptide radioligand for the Y_2R.[70] By analogy with the development of BIBP 3226 derived pharmacological tools, electron-withdrawing residues such as acyl or carbamoyl moieties carrying a terminal amino group were attached to the guanidine group of BIIE 0246.[70]

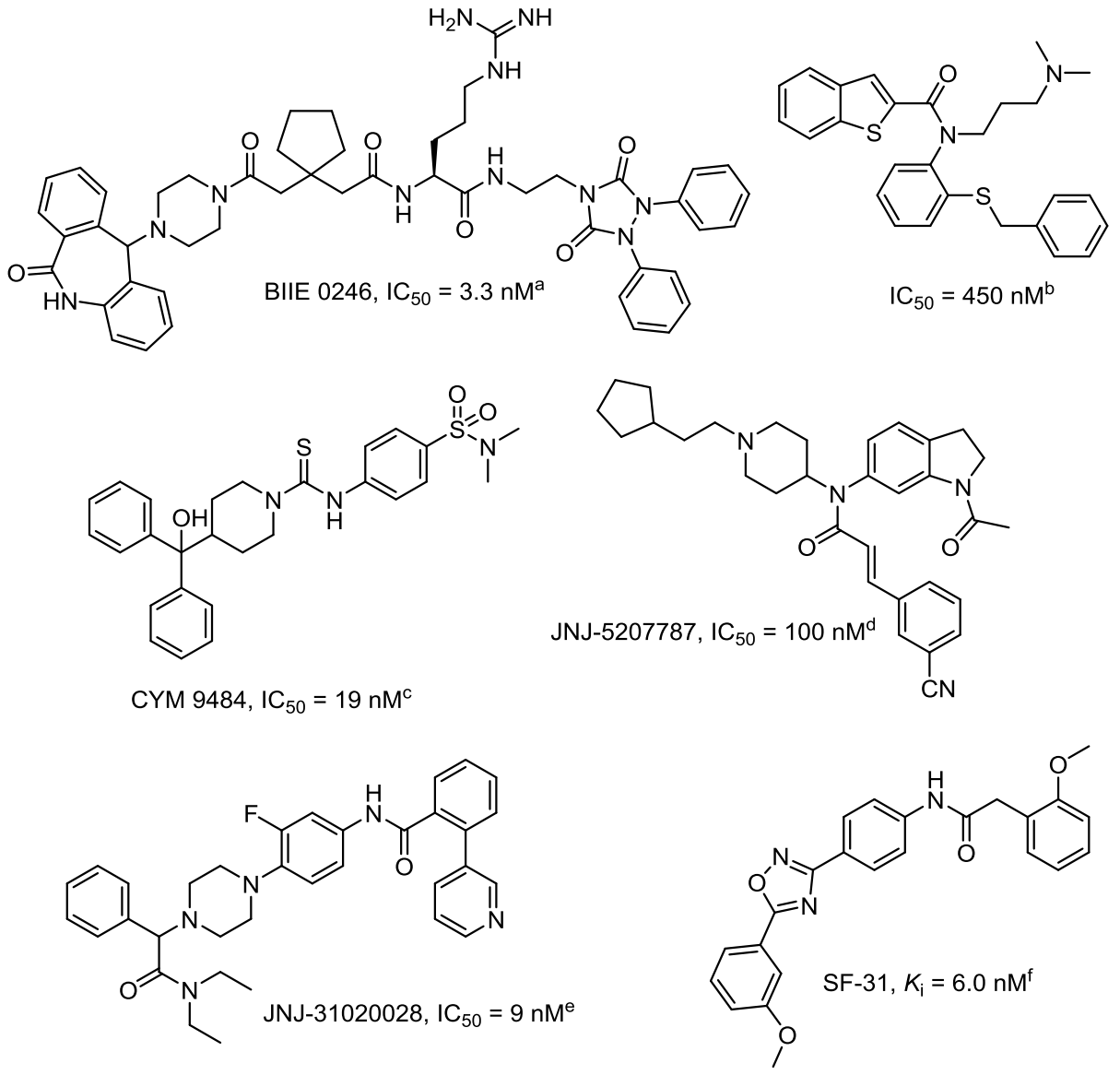


Figure 4: Structures of selective Y_2R antagonists: a: Doods et al[69]; b: Andres et al[71]; c: Mittapalli et al[72]; d: Bonaventure et al[73]; e: Shoblock et al[74]; f: Brothers et al[75].

A selective Y_5R agonist was obtained by incorporationg the turn inducing sequence Ala-Aib into positions 31 and 32 of porcine NPY. Y_5R affinity could be further improved by introducing the Ala-Aib motif into NPY/PP chimeras.[76] The search for new anti-obesity drugs led to the identification of numerous small-molecule Y_5R antagonists (Figure 5). The first Y_5R antagonist, CGP 71683A, was reported by Novartis in 1997.[19] Some compounds entered clinical trials, e.g. MK-0557 (Merck & Co., Inc.),[77] but none of these drug candidates reached the market.

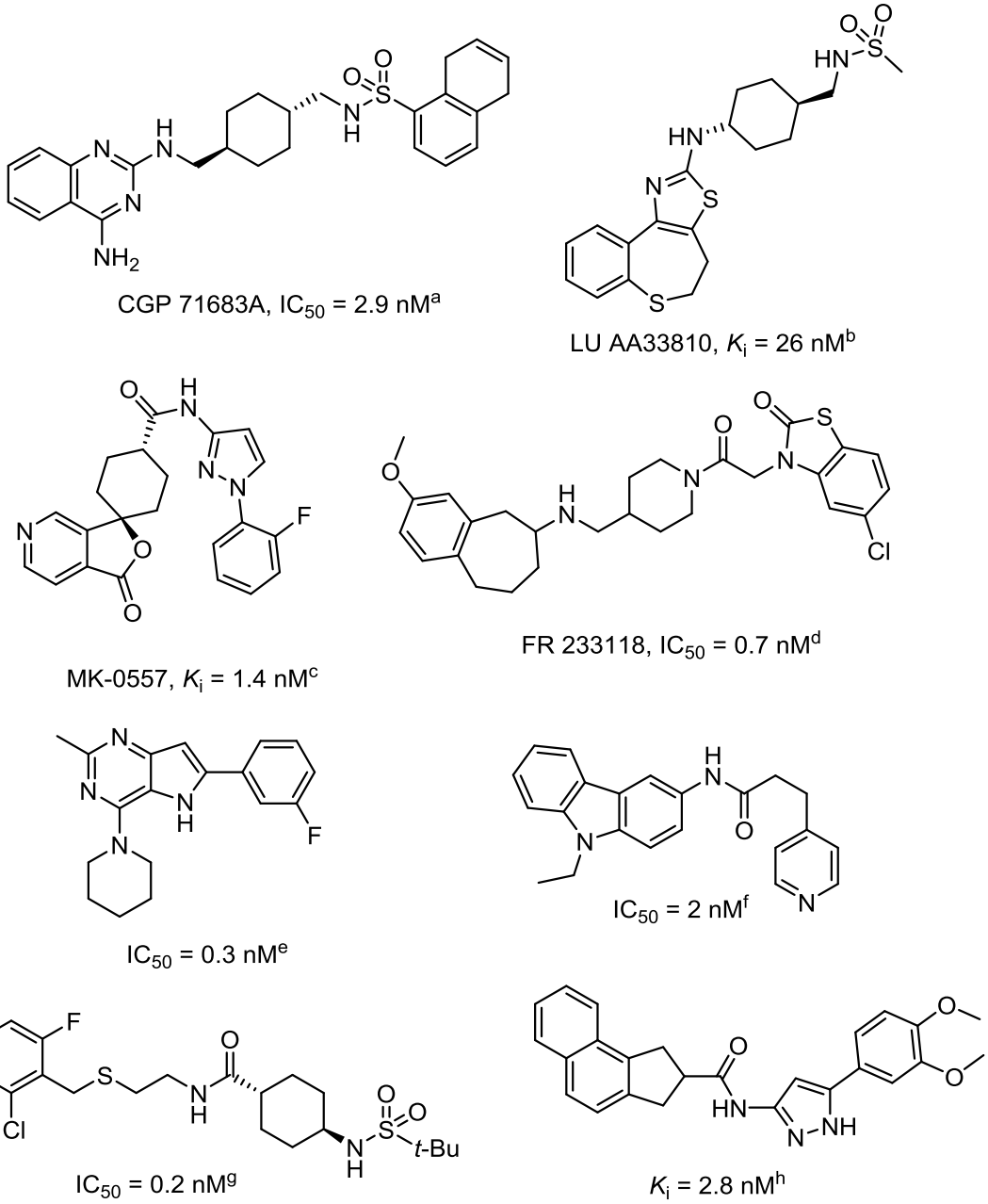


Figure 5: Structures of selective Y_5R antagonists: a: Criscione et al[19]; b: Walker et al[78]; c: Erondu et al[77]; d: Itani et al[79]; e: Norman et al[80]; f: Block et al[81]; g: Kawanishi et al[82]; h: Sato et al[83].

1.3 Y_4R Ligands

In contrast to the other NPY receptors, only few selective Y_4R ligands are known so far (Figures 6+7). Surprisingly, among a series of dimeric Y_1R peptide antagonists, some compounds turned out to act as Y_4R agonists.[84-86] The compound with the highest affinity, GW1229 (also designated as GR231118 and 1229U91) (K_i (Y_1R) = 0.51 nM), binds to the Y_4R with picomolar affinity (K_i (Y_4R) = 0.36 nM). In 2006 Balasubramaniam et al. prepared a series of dimeric peptides with improved Y_4R selectivity by connecting the C-terminal pentapeptides of GW1229 (Tyr-Arg-Leu-Arg-Tyr-NH_2) via aliphatic dicarboxylic acids with different chain lengths.[87] Highest activity was achieved with 2,7-*D*/*L*-diaminosuberic acid as a linker (BVD74-D). In vivo studies describe a regulatory effect of BVD74-D on food intake in mice[35] suggesting Y_4R agonists as potential agents for the treatment of obesity. One pentapeptide sequence of BVD-74D instead of the crosslinked compound was reported to bind to the Y_4R as well (**1**: K_i (Y_4R) = 50 nM, EC_{50} = 65 nM). Whereas **1** was a full agonist in a [γ-^{33}P]GTPase assay, analogs containing the cyclic beta amino acid cis-cyclopentane β-amino acid (βcpe) instead of Leu^3 were partial agonists (**2**: K_i (Y_4R) = 105 nM, EC_{50} = 94 nM, E_{max} = 73%).[88]

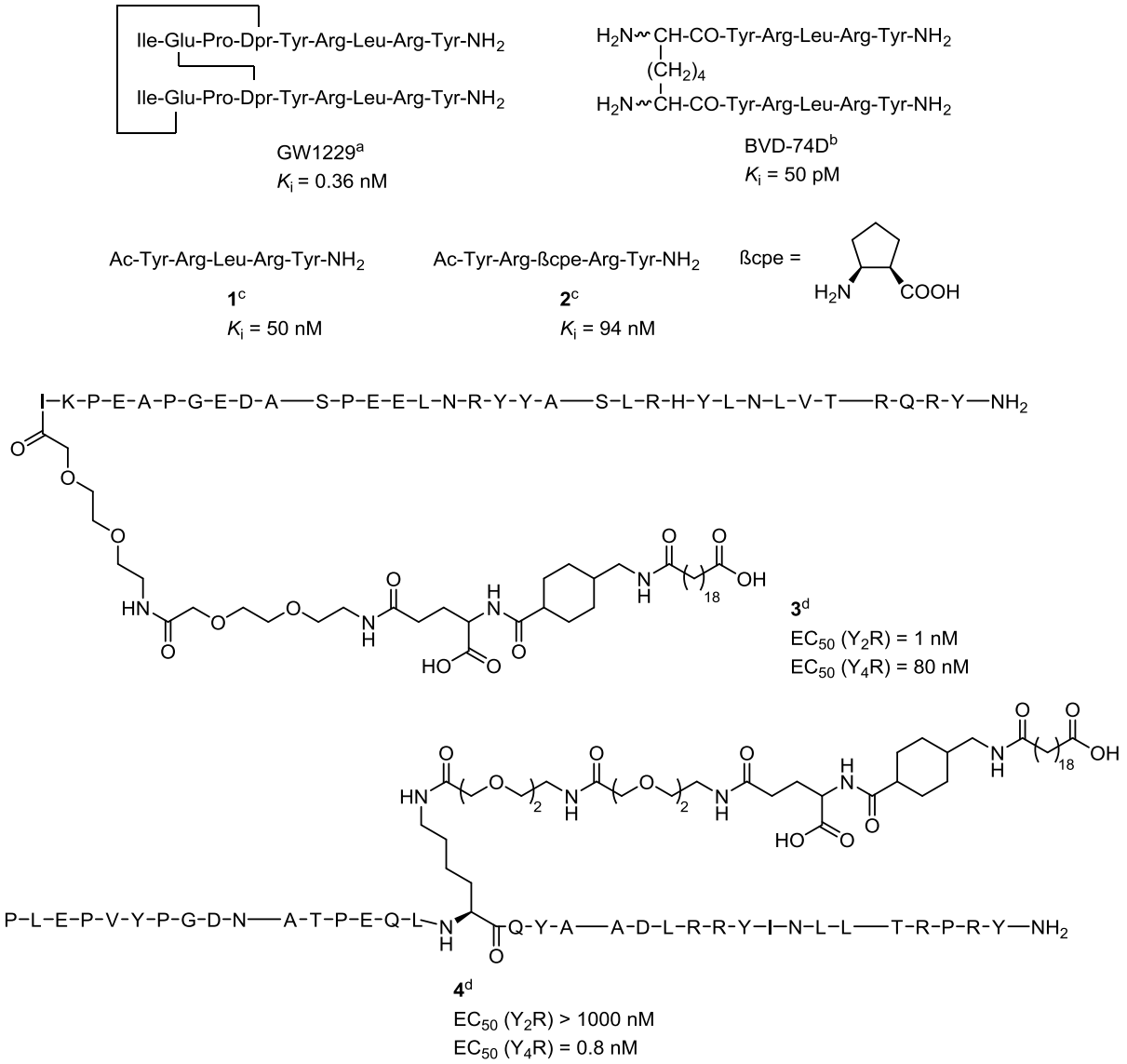
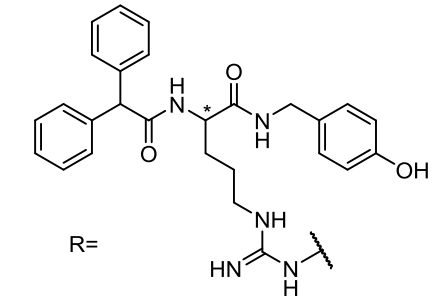
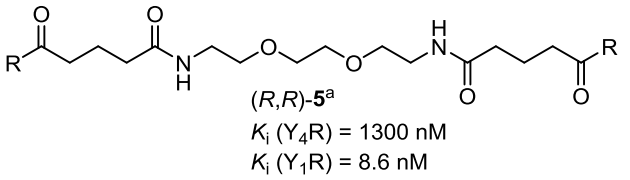


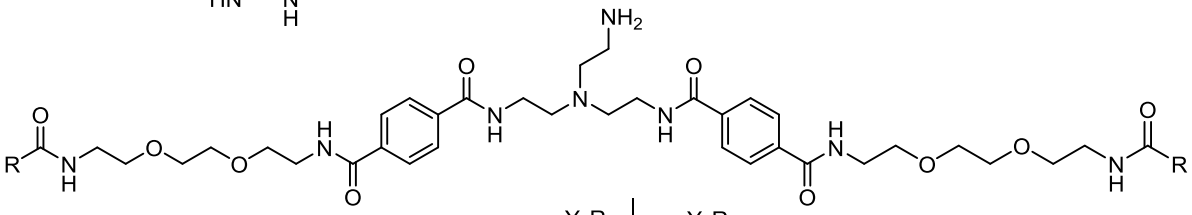
Figure 6: Y_4R agonists and partial agonists: a: Daniels et al[84]; b: Balasubramaniam et al[87]; c: Berlicki et al[88]; d: Oestergaard et al[89].

As the endogenous ligand PP already displays moderate Y_4R selectivity over the other receptor subtypes, approaches to stabilize PP against enzymatic degradation might result in peptides with improved suitability for *in vivo* applications. The lipidated analog [K^{30}(E-Prop)]PP_{2-36} (EC_{50} (Y_4R) = 1.3 nM, EC_{50} (Y_5R) = 937 nM) is equipotent with PP (EC_{50} (Y_4R) = 1.3 nM, EC_{50} (Y_5R) = 11.4 nM) at the Y_4R, but has a better selectivity over the Y_5R. Moreover, [K^{30}(E-Prop)]PP_{2-36} displayed a prolonged half-life in human plasma.[90] Novo Nordisk reported on a series of selective Y_4R or $Y_{2/4}R$ dual agonists (**3**, **4**) that were able to reduce the cumulative food intake in fasted lean mice for 48 h after subcutaneous injection.[89] Prolonged terminal half-lives in plasma were achieved by attaching fatty acids to the peptides enabling reversible binding to albumin. Whereas the half-life of PP was only around 2 min, **4** showed a prolonged half-life of 55 h.

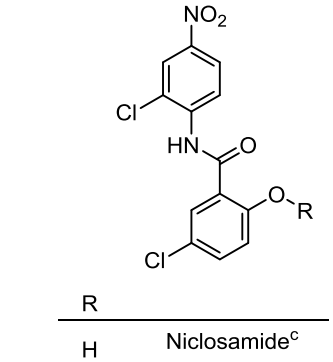
The only Y_4R antagonists with an affinity in the nanomolar range derive from a series of bivalent N^G-acylated BIBP 3226 derivatives, which were initially designed as dimeric Y_1R antagonists (Figure 6).[91] Several structures with linkers modified in chemical nature, length, rigidity and attachment position where synthesized resulting in UR-MK188, the Y_4R antagonist with the highest affinity known so far.[92] The selectivity profile of these dimeric antagonists could be shifted towards the Y_4R by replacement of the *R*-configured argininamide moieties with the *S*-configured enantiomers, that is, UR-MEK388 had about the same affinity and antagonist activity as UR-MK188 at the Y_4R, but was inactive at the Y_1R.[92]



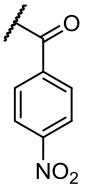




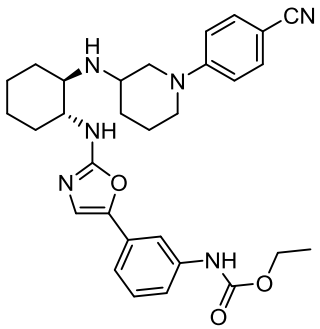
	*	Y_1R K_i [nM]	Y_4R K_i [nM]	Y_4R K_b [nM]
UR-MK188[b]	*R*,*R*	24	130	20
UR-MEK388[b]	*S*,*S*	> 5000	260	27



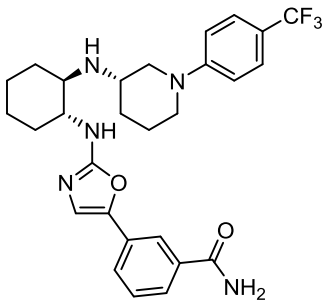
R	
H	Niclosamide[c] EC_{50} = 620 nM



VU0118784[c]
EC_{50} = 473 nM



6[d]
EC_{50} = 0.2 nM



7[d]
EC_{50} = 0.2 nM

Figure 7: Bivalent N^G-carbamoylated BIBP 3226 derived Y_4R antagonists and Y_4R 'modulators'. a: Keller et al[91]; b: Keller et al[92]; c: Sliwoski et al[93]; d: Ewing et al[94].

Very recently, several small molecules were described as Y_4R positive allosteric modulators. The antiparasitic drug niclosamide and structural variations thereof were reported to potentiate the EC_{20} signal of PP with an EC_{50} value in the three-digit nanomolar range in a Ca^{2+} assay and an inositol phosphate accumulation assay.[93] Several compounds within a series of substituted diaminocyclohexanes were described as Y_4R ligands(**6**, **7**).[94] However, the information about the functional assays and the interpretation of the functional data is very sparse. The investigated compounds were declared as agonists, partial agonists or modulators, although the described setup of the functional assay (suppression of forskolin-induced cAMP formation) would have only allowed the identification of agonists. In general, small molecule positive allosteric Y_4R modulators which enhance the biological effect of the endogenous ligand PP represent an interesting new approach to address the Y_4R and harbour a potential for the treatment of metabolic disorders and obesity. However, the clinical relevance of such compounds remains still unclear.

1.4 Scope of the Thesis

The neuropeptide Y family contributes to the regulation of numerous physiological processes. Especially, the involvement in the development of metabolic disorders has attracted the attention of scientists in academia and industry.

Over the last decades, obesity has emerged to a serious global health problem being linked with secondary diseases such as diabetes type II, arthrosis or cardiovascular diseases. Research on the implication of NPY in obesity has led to an amount of information, for example, on the occurrence of the receptor subtypes in brain and periphery, cellular signalling and interactions with numerous other neurotransmitters and hormones. However, the physiological role of both, the different NPY receptors and their endogenous ligands, is still far from being understood due to the complex and in part opposite effects of receptor-subtype stimulation. For instance, even though peripheral administration of PP has been shown to reduce food intake in rodents and humans,[95-97] Y_4R knockout in mice resulted in lean phenotypes.[98]

Whereas many peptide agonists and non-peptide antagonists for Y_1, Y_2 and Y_5 receptors were reported, only few Y_4R ligands are known so far. Even though Y_4R agonists should have

the higher clinical potential, high-affinity antagonists are required as well to investigate the physiological and pathophysiological role of the Y_4R in more detail.

UR-MK188, a bivalent ligand composed of two N^G-carbamoylated BIBP 3226 derived moieties connected via a linker, is the most potent Y_4R antagonist known to date (K_i = 130 nM, K_b = 20 nM).[92] Previous investigations on such dimeric argininamides, initially designed as bivalent Y_1R antagonists, suggest that the linker length should be reduced. Taking UR-MK188 as a lead structure, one objective of this thesis is to optimize the linker of bivalent Y_4R ligands aiming at Y_4R antagonists with increased affinity and reduced size.

Furthermore, the incorporation of cyclic beta amino acids such as cis-cyclopentane β-amino acid into the C-terminal part of Y_4R full agonists resulted in partial agonism.[88] This effect had to be further investigated and complementary possibilities of backbone modifications had to be applied to more suitable agonist precursors. Based on the working hypothesis that the rigidization of the backbone of Y_4R peptide agonists may result in a change of the quality of action, various modifications, e.g. the incorporation of *D*-amino acids or aza-amino acids and backbone cyclization should be applied to Y_4R peptide agonists.

The availability of selective Y_4R radioligands for binding studies is very limited. [^{125}I]-labeled compounds suffer from a short half-life of only 60 days, but have the broadest application.[87,99-101] Due to their longer half-life, tritiated radioligands are highly preferred in our laboratory. To explore the applicability of reported Y_4R ligands as precursors for the attachment of a radioactive (tritiated) moiety was another aim of the thesis. Compounds with an amino group enabling acylation with commercially available *N*-succinimidyl-[2,3-^{3}H]propi onate should be preferred as precursors. A selective Y_4R radioligand is anticipated to be a useful pharmacological tool for the detection of Y_4 receptors, autoradiographic studies or the determination of Y_4R binding data of non-labelled compounds.

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Chapter 2

High Affinity Agonists of the Neuropeptide Y (NPY) Y_4 Receptor Derived from the C-terminal Pentapeptide of Human Pancreatic Polypeptide (hPP): Synthesis, Stereochemical Discrimination and Radiolabeling

Note: Prior to the submission of this thesis, parts of this chapter were published in cooperation with partners:

Kuhn, K.; Ertl, T.; Dukorn, S., Keller, M.; Bernhardt, G.; Reiser, O.; Buschauer, A. High affinity agonists of the neuropeptide Y (NPY) Y4 receptor derived from the C-terminal pentapeptide of human pancreatic polypeptide (hPP): synthesis, stereochemical discrimination and radiolabeling. *J. Med. Chem*. **2016**, 59, 6045-6058.

The following experiments were performed by co-authors:

T.E.: Synthesis of **2.6**, **2.7** and **2.8**
S.D.: Luciferase assay on HEK293-hY_4-CRE Luc cells
M.K.: Synthesis of **2.11** and support in the radio synthesis of [^{3}H]**2.10** and [^{3}H]**2.18**

2.1 Introduction

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) consist of 36 amino acids and share considerable sequence similarities (Figure 1).[1-3] NPY, PYY and PP activate NPY receptors, in humans comprising four functional subtypes of G-protein coupled receptors (Y_1R, Y_2R, Y_4R, Y_5R), which are involved in the regulation of numerous central and peripheral biological processes.[4] The Y_4R shares only low sequence identity with the other subtypes (e.g. 42% sequence identity of hY_4R with hY_1R[5]) and is an exception as it prefers PP over NPY and PYY.[5] The Y_4R is considered to play a role, for example, in feeding behavior and the regulation of energy metabolism.[6,7] Recently, Y_4R agonists were proposed as putative anti-obesity agents.[8,9] Therefore, there is a need for high affinity Y_4R ligands, agonists as well as antagonists, to explore the potential of the Y_4R as a biological target.

Previous work from different groups suggests that connecting two pharmacophoric moieties favors Y_4R binding, in particular, in case of peptidic agonists such as GW1229 and **2.2** (BVD-74D) (Figure 2), which are derived from the C-terminal pentapeptide of hNPY or hPP, respectively. UR-MK188[10] is an example of a Y_4R antagonist, composed of two molecules of an argininamide-type Y_1R antagonist ((*R*)-4-guanidino-1-({[(4-hydroxyphenyl)methyl]amino}-carbonyl)butyl-α-phenylbenzeneacetamide, BIBP3226),[11] which are connected via the guanidine groups. Generally, the arginine residues in positions 33 and 35 of the endogenous peptides are important for NPY receptor binding.[12] Similarly, the arginine moieties are considered crucial structural features of the Y_4R ligands mentioned above. The "dimeric peptide" GW1229 (also known as GR23118 or 1229U91)[13] is both, a high-affinity antagonist at the Y_1R and an agonist at the Y_4R.[14] The pentapeptide sequence Tyr-Arg-Leu-Arg-Tyr-NH_2 in GW1229 is very similar to the C-terminal sequence of hPP (…Thr^{32}-Arg^{33}-Pro^{34}-Arg^{35}-Tyr^{36}-NH_2). *D/L*-2,7-diaminooctanedioyl-bis(YRLRY-NH_2) (BVD-74D, **2.2**),[15] comprising the same pentapeptide moieties as GW1229, but a different linker, is more selective for the Y_4R. Data reported for **2.2** refer to the diastereomeric mixture of (2*R*,7*R*)-**2.2** and (2*S*,7*S*)-**2.2**,[15] not to a single stereoisomer (note: in the following, positions 2 and 7 refer to the stereo centers in the 2,7-diaminosuberic acid moiety).

NPY YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2

PYY YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2

PP APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH_2

Figure 1. Amino acid sequences of human NPY, PYY and PP

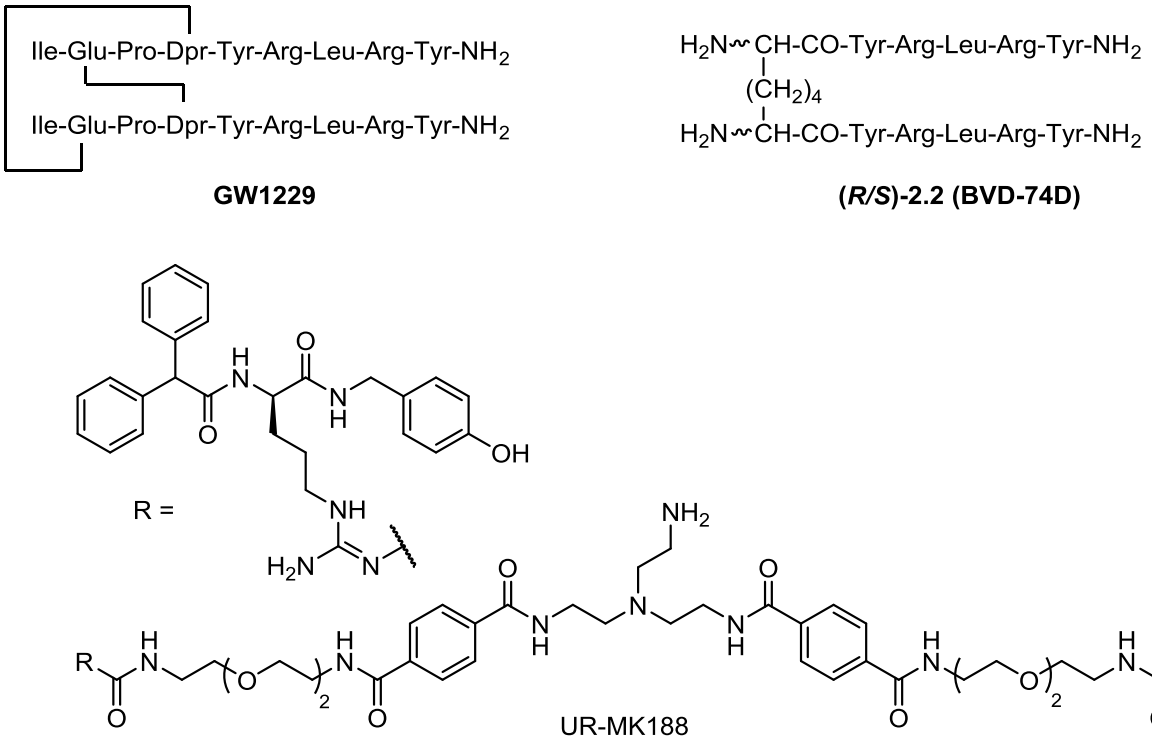


Figure 2. Structures of the described NPY Y_1R antagonist/Y_4R agonist GW1229, the Y_4R agonist **2.2**, and the Y_1R/Y_4R antagonist UR-MK188. Dpr: *L*-2,3-diaminopropionic acid; **2.2** was previously described as a mixture of two diastereomers due to (2*R*,7*R*)- and (2*S*,7*S*)-configuration of the 2,7-diaminosuberic acid linker.

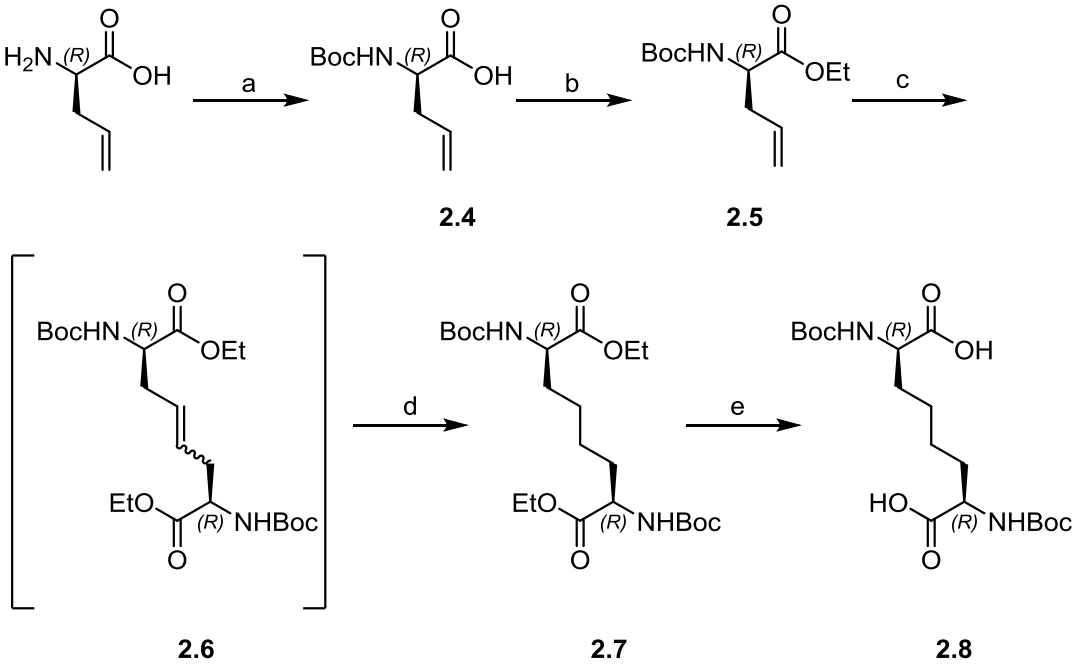
Here we report on the synthesis and pharmacological characterization of the pure stereoisomers (2*R*,7*R*)-**2.2** and (2*S*,7*S*)-**2.2**. Very recently, a pilot study from our laboratory suggested that the amino groups in the linker of **2.2** are dispensable.[16] Therefore, we synthesized a set of analogues using octanedioic acid instead of 2,7-diaminosuberic acid as linker. To investigate the contribution of the individual arginines to the Y_4R affinity of the dimers, we prepared compounds in which one or two Arg moieties in the pentapeptide building block were replaced by Ala. Furthermore, the suitability of the 'dimeric' pentapeptides as precursors for the preparation of radiolabeled compounds was explored, the target compounds were characterized in functional assays, and two tritiated derivatives were synthesized and used to perform Y_4R binding studies.

2.2 Results and Discussion

2.2.1 Chemistry

The enantiopure 2,7-diaminooctanedioic acids were synthesized from (*S*)- and (*R*)-2-allylglycine via the *N*-Boc protected esters (*S*)-**2.5** and (*R*)-**2.5** (Scheme 1) by analogy with procedures from the literature.[17,18] The enantiomers of **2.5** were submitted to a cross-metathesis reaction, employing Grubbs' II catalyst to build up the core structure **2.6**. After exchanging the solvent (DCM) with ethanol, compound **2.6** was not isolated, but hydrogenated at 20 bar in the presence of the same catalyst,[19,20] in contrast to reported procedures requiring workup of **2.6** and Pd/C catalyzed hydrogenation.[21] Besides the convenience of this protocol, overall yields were improved, affording (2*R*,7*R*)-**2.7** and (2*S*,7*S*)-**2.7** in 95% and 74% yield, respectively, over two steps. Hydrolysis of the ethyl ester quantitatively provided the diacids (2*R*,7*R*)-**2.8** and (2*S*,7*S*)-**2.8**.

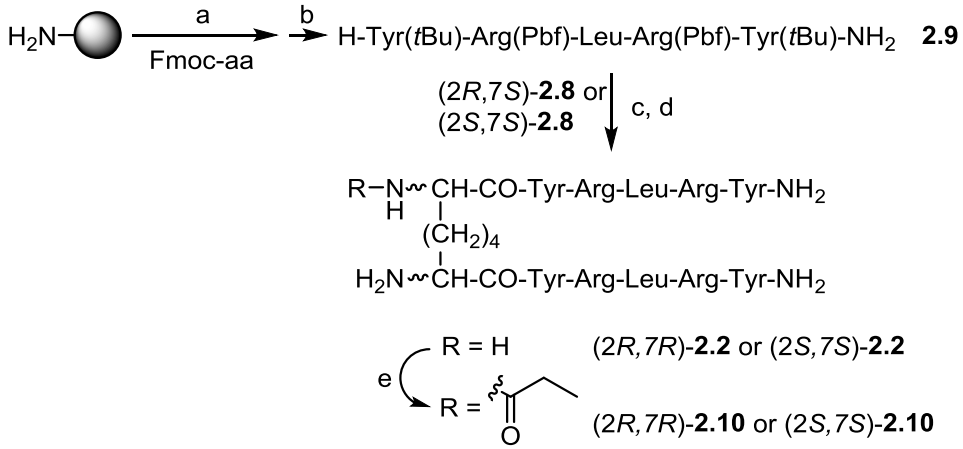
Scheme 1. Enantioselective synthesis of the diacids (2*R,*7*R*)-**2.8** and (2*S,*7*S*)-**2.8**[a]



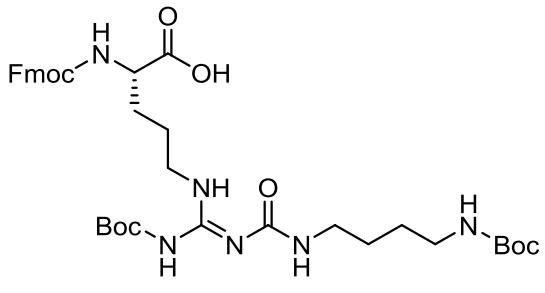
[a](2*S,*7*S*)-**2.8** was prepared according to the same protocol using (*S*)-2-allylglycine as starting material. Reagents and conditions: (a) Boc_2O, 1 M NaOH/dioxane (2:1), 0 °C to rt, 18 h; (b) DCC, 4-DMAP, EtOH, DCM, 0 °C to rt, 12 h; (c) 0.1 equiv Grubbs' II catalyst, anhydrous DCM, N_2 atmosphere, reflux, 12 h; compd. **2.6** was not isolated, but subjected to hydrogenation in the next step; (d) 0.1 equiv Grubbs' II catalyst (from reaction step (c)), ethanol, H_2 (20 bar), rt, 15 h (95% for (*R,R*)-**2.7**, 74% for (*S,S*)-**2.7** over two steps, (c) and (d)); (e) 1 M NaOH, EtOH, rt, 21 h (quant.).

Peptide synthesis was carried out manually on a Sieber-amide resin applying an Fmoc protocol and 'double' coupling procedures with HBTU/HOBt/DIPEA (Scheme 2). After cleavage from the resin, two equivalents of the protected pentapeptide **2.9**[16] were cross-linked with the *in situ* activated (*R*,*R*)- and (*S*,*S*)-configured enantiomers of 2,7-diaminosuberic acid (**2.8**) to obtain the corresponding peptides (2*R,*7*R*)-**2.2** and (2*S,*7*S*)-**2.2** after deprotection. Subsequent acylation (monitored by HPLC) of an excess of precursor with succinimidyl propionate gave the propionamides (2*R,*7*R*)-**2.10** (for preparation of [^{3}H]-labeled form see below) and (2*S,*7*S*)-**2.10**. With respect to labeling of compounds devoid of amino groups in the linker (Scheme 3), we introduced the recently reported arginine derivative **2.11** (Figure 3), in which the guanidine group is replaced by a nonclassical bioisostere, a functionalized carbamoylguanidine moiety.[16]

Scheme 2. Synthesis of (2*R,*7*R*)-**2.2** and (2*S,*7*S*)-**2.2** and the propionylated analogues (2*R,*7*R*)-**2.10** and (2*S,*7*S*)-**2.10**[a]



[a]Reagents and conditions: (a) SPPS (Fmoc strategy), Fmoc-aa/HBTU/HOBt/DIPEA (5/5/5/10 equiv), solvent: DMF/NMP (8:2), 'double' coupling at rt, 60 min; Fmoc deprotection was carried out with 20% piperidine in DMF/NMP (8:2), rt, 2 × 10 min; (b) DCM/TFA (97:3), 10 × 6 min; (c) HBTU, HOBt, DIPEA, anhydrous DMF, 35 °C, 16 h; (d) TFA/H_2O (95:5), rt, 2.5 h; (e) succinimidyl propionate, 3% DIPEA in anhydrous DMF, rt, 2 h.

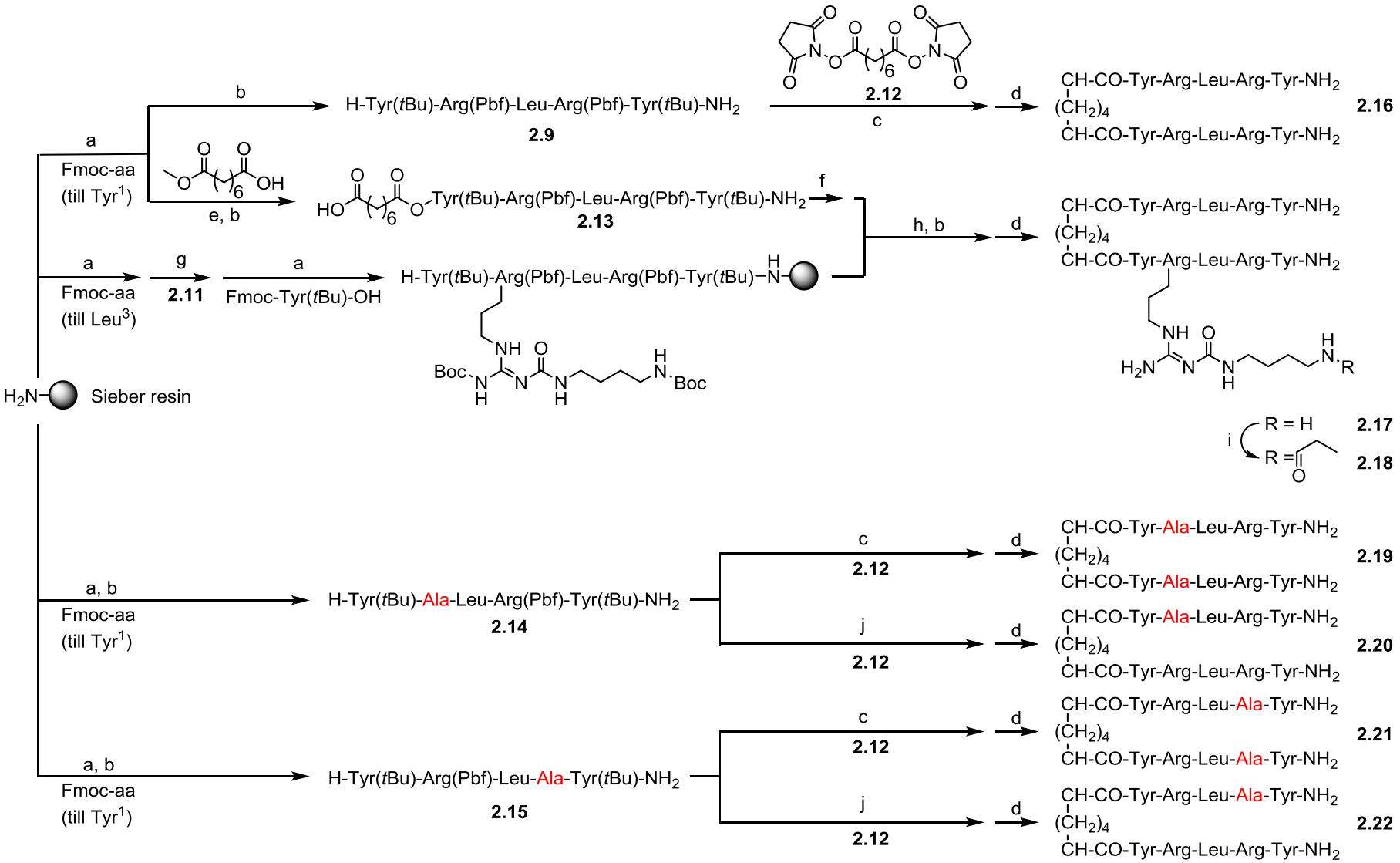


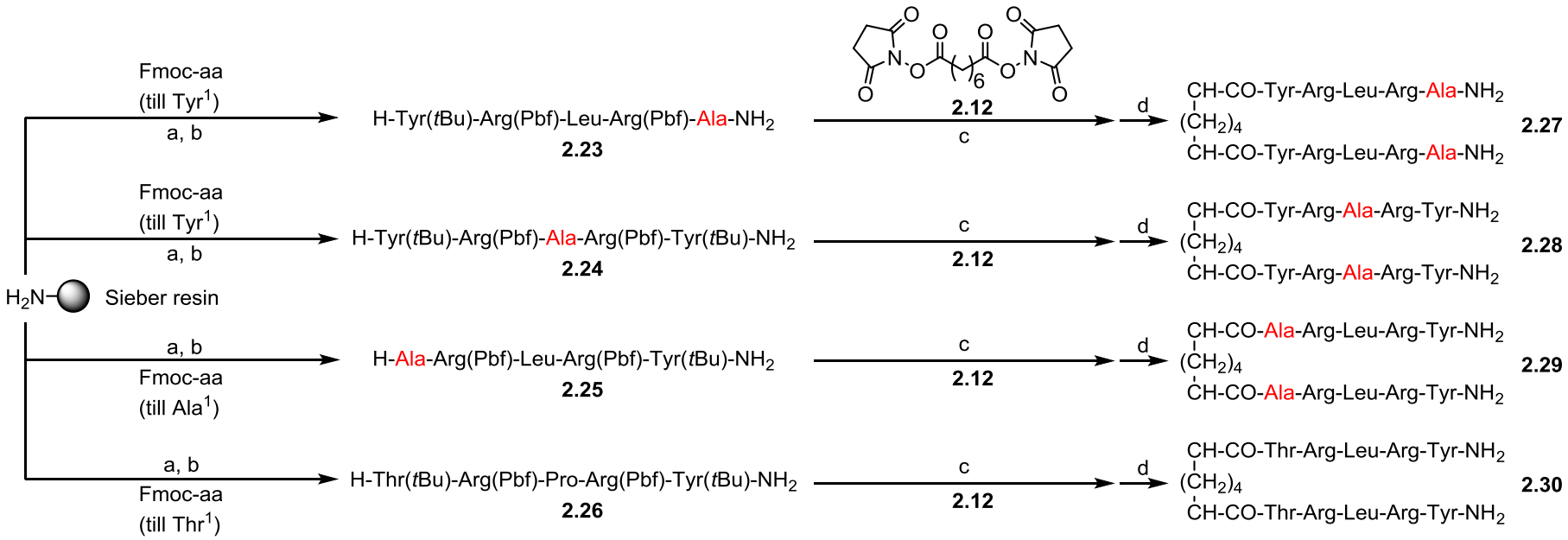
2.11

Figure 3. Structure of the N^{ω}-carbamoylated arginine building block **2.11**.[16]

For the preparation of the homodimeric ligands (**2.16**, **2.19**, **2.21**, **2.27**-**2.30**) shown in Scheme 3, octanedioic acid, activated as di-succinimidyl ester (**2.12**[22]), was used for cross-linking (Scheme 3). By contrast, as a building block for the synthesis of heterodimeric ligands (**2.17**,[16] **2.18**,[16] **2.20**, **2.22**,), compound **2.13**[16] was prepared by coupling suberic acid monomethyl ester to the resin-bound peptide sequence Tyr(*t*Bu)-Arg(Pbf)-Leu-Arg(Pbf)-Tyr(*t*Bu). The side-chain protected peptides **2.14**, **2.15** and **2.23**-**2.26** were treated with **2.12** to give **2.16**,[16] **2.19** and **2.21** and **2.27**-**2.30**), whereas *in situ* activated **2.13** was coupled to the side-chain protected peptides **2.14** and **2.15** for the preparation of the heterodimeric ligands **2.20** and **2.22**, respectively. By analogy with the synthesis of the potential radioligands (2*S,*7*S*)-**2.10** and (2*R,*7*R*)-**2.10**, the precursor **2.17**[16] was treated with succinimidyl propionate to give **2.18** as described very recently.[16]

Scheme 3. Synthesis of homo- and heterodimeric Y_4R ligands[a]



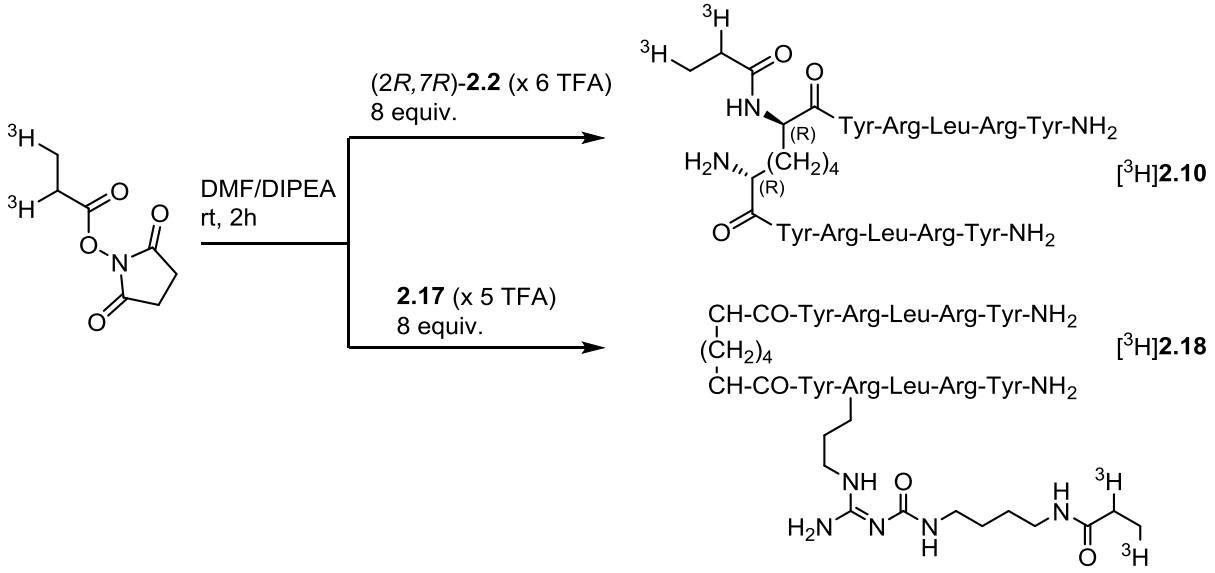


[a]Reagents and conditions: (a) cf. legend to Scheme 2; (b) DCM/TFA (97:3), 10 × 6 min; (c) 1 equiv of **2.12** in 1% DIPEA in anhydrous DMF, followed by 2.5 equiv of **2.9**, **2.14**, **15**, or **2.23**-**2.26**, 35 °C, 16 h; (d) TFA/H_2O (95:5), rt, 2.5 h; (e) suberic acid monomethyl ester/HBTU/HOBt/DIPEA (4/4/4/8 equiv), solvent: DMF/NMP (8:2), single coupling at 30 °C, 14 h; (f) 0.5 M NaOH/MeOH (1:5), reflux, 3 h; (g) **2.11**/HBTU/HOBt/DIPEA (3/3/3/6 equiv), solvent: DMF/NMP (8:2), single coupling at 35 °C, 16 h; (h) **2.13**/HBTU//HOBt/DIPEA (1/1/1/2 equiv), solvent: DMF/NMP (8:2), single coupling at 35 °C, 16 h; (i) succinimidyl propionate, DMF, DIPEA, rt, 2 h; (j) **2.13**/HBTU/HOBt/DIPEA (1/1/1/3.5 equiv), solvent: DMF, rt, 5 min, subsequent addition of 1.1 equiv of **2.14** or **2.15**, rt, 17 h.

2.2.2 Synthesis of the Radiolabeled Ligands [^{3}H]2.10 and [^{3}H]2.18

Based on the results obtained in the functional assays (see below), (2*R*,7*R*)-**2.10** and **2.18** were selected for the synthesis of the corresponding tritiated versions. An 8-fold excess of the precursor peptides (2*R*,7*R*)-**2.2** and **2.17**[16] was treated with commercially available succinimidyl [^{3}H]propionate (Scheme 4). The radioligands were obtained in high radiochemical purities (Figure 4A and Figure 4C). After storage of the radioligands in a mixture of EtOH/H_2O (1:1) at -20 °C over 9 months, approximately 10% were decomposed (Figure 4B and Figure 4D). No decomposition was observed after an incubation period of 48 h, when the 'cold' analogues, (2*R*,7*R*)-**2.10** and **2.18**, were investigated for stability (Figure 5A and Figure 5B) in a HEPES buffer (buffer I, pH 7.4).

Scheme 4. Synthesis of the radioligands [^{3}H]**2.10** and [^{3}H]**2.18** by propionylation of the precursors (2*R,*7*R*)-**2.2** and **2.17**[a]



[a]Radiochemical yield: 34% ([^{3}H]**2.10**), 30% ([^{3}H]**2.18**).

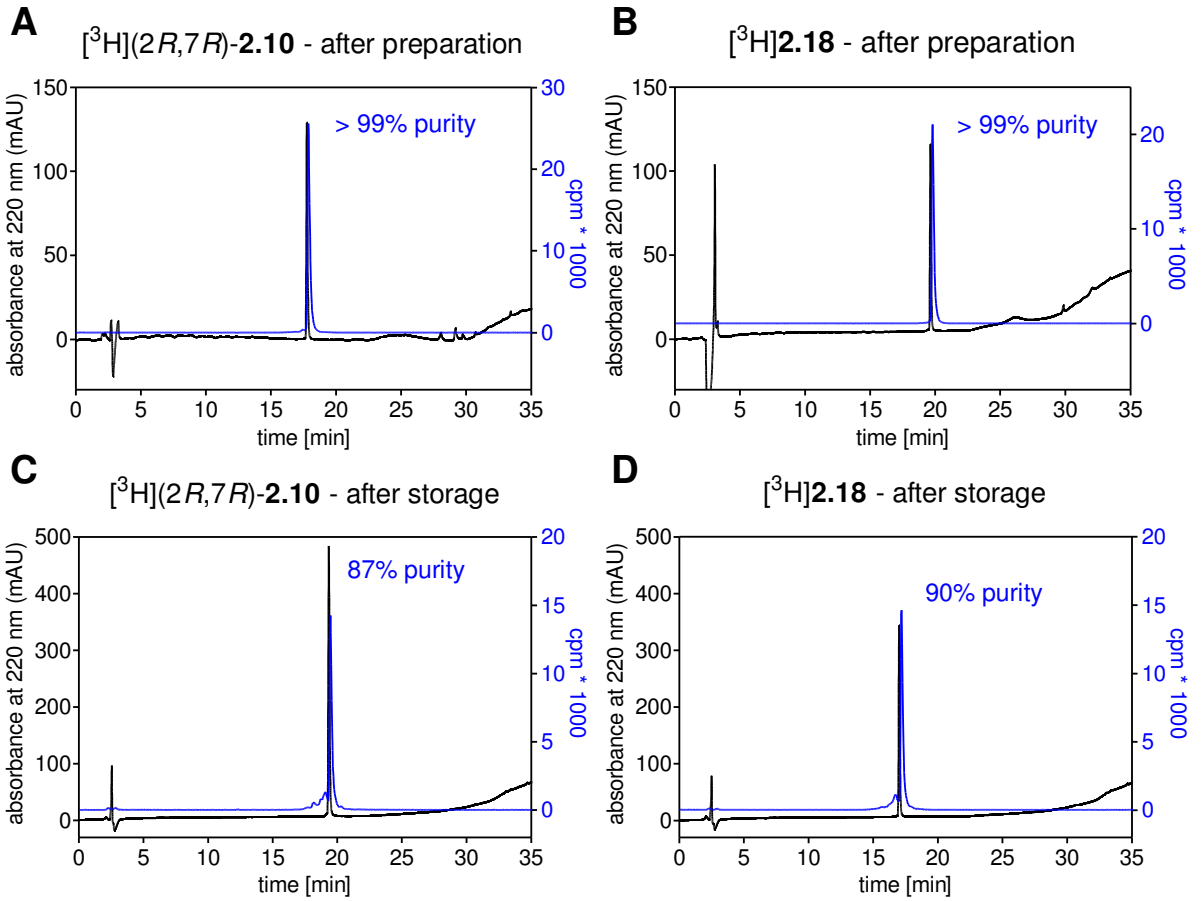


Figure 4. Purity, identity control and long-term stability of the radiolabeled peptides [^{3}H]**2.10** and [^{3}H]**2.18**. (**A**) HPLC analysis of [^{3}H]**2.10** (ca. 0.35 μM) spiked with 'cold' (2*R*,7*R*)-**2.10** (5 μM), analyzed 3 days after synthesis. Radiochemical purity > 99%. (**B**) HPLC analysis of [^{3}H]**2.10** (ca. 0.25 μM) spiked with 'cold' (2*R*,7*R*)-**2.10** (13 μM), analyzed after 9 months of storage at -20 °C in EtOH/H_2O (1:1). Radiochemical purity: 87%. (**C**) HPLC analysis of [^{3}H]**2.18** (ca. 0.35 μM) spiked with 'cold' **2.18** (5 μM), analyzed 10 days after synthesis. Radiochemical purity > 99%. (**D**) HPLC analysis of [^{3}H]**2.18** (ca. 0.25 μM) spiked with 'cold' **2.18** (13 μM), analyzed after 9 months of storage at -20 °C in EtOH/H_2O (1:1). Radiochemical purity: 90%. The minor differences in t_R result from serial detection of the UV and radiometric signals.

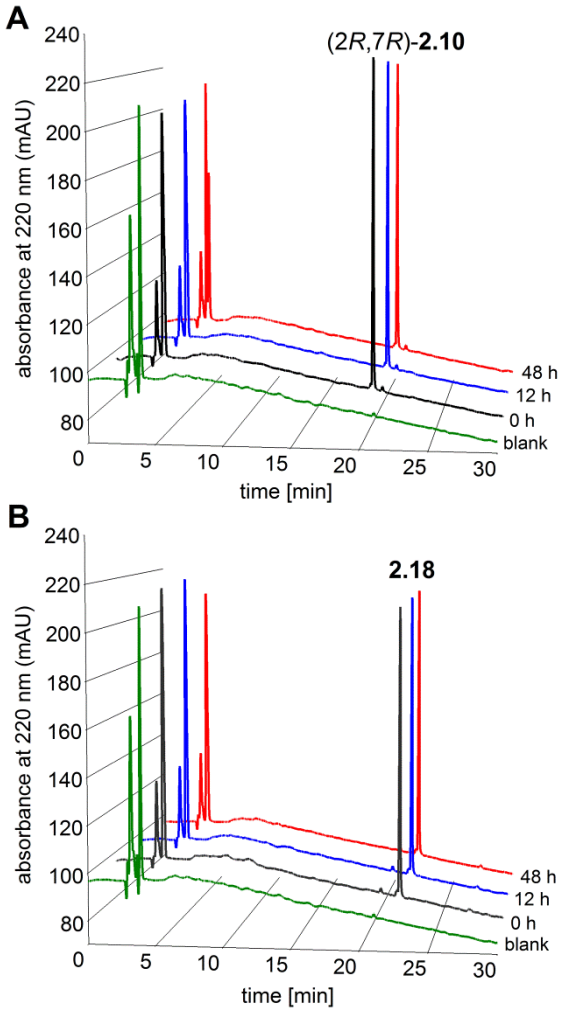


Figure 5. HPLC analysis of 'cold' (2*R*,7*R*)-**2.10** (**A**) and **2.18** (**B**) after incubation in buffer I (HEPES buffer, sodium-free) (pH 7.4) for up to 48 h. Both compounds showed no decomposition. Peaks between 0 and 5 min correspond to buffer components.

2.2.3 Functional Studies at the hY$_4$R

The target compounds were investigated for Y_4R agonism in an aequorin Ca^{2+} assay[23] and a luciferase gene reporter assay on genetically engineered CHO and HEK293 cells, respectively (data cf. Table 1 and Figure 6). The configuration of the stereo centers in the 2,7-diaminosuberic acid linker in **2.2** had an impact on Y_4R potency. In the luciferase assay (2*R*,7*R*)-**2.2** (EC_{50} = 1.6 nM) was 12 times more potent than (2*S*,7*S*)-**2.2** (EC_{50} = 19 nM). In the aequorin assay the difference in potency was less pronounced (EC_{50} values: 6.9 nM ((2*R*,7*R*)-**2.2**) vs. 18 nM ((2*S*,7*S*)-**2.2**)). The same tendency became obvious for the propionylated analogues (2*R*,7*R*)-**2.10** and (2*S*,7*S*)-**2.10** in both assays. Generally, propionylation of an amino group of the linker did only slightly (by a factor of two) affect Y_4R potency in both assays.

Table 1. NPY Y_4R agonist potencies (EC_{50}) and intrinsic activities (α) of 'dimeric' pentapeptides and reference compounds PP and GW1229.

	Aequorin assay [a]		**Luciferase assay** [b]	
Compd.	EC_{50} [nM]	α	EC_{50} [nM]	α
PP	9.7 ± 0.4	1	0.73 ± 0.2	1
GW1229	8.5[c]	0.62	5.5 ± 0.8	1.01
(2*R*,7*R*)-**2.2**	6.9 ± 0.6	0.75	1.6 ± 0.6	1.03
(2*S*,7*S*)-**2.2**	18 ± 1.6	0.78	19 ± 4.8	1.07
(2*R*,7*R*)-**2.10**	14 ± 2.0	0.76	4.0 ± 0.9	1.00
(2*S*,7*S*)-**2.10**	59 ± 5.6	0.70	27 ± 4.6	0.98
2.16	49 ± 13	0.73	86 ± 16	0.93
2.17	6.4 ± 1.3	0.80	1.7 ± 0.2	1.06
2.18	13 ± 1.1	0.67	7.1 ± 1.9	1.05
2.19	>3000	-	>3000	-
2.20	290 ± 33	0.85	160 ± 29	0.98
2.21	>3000	-	>3000	-
2.22	310 ± 38	0.85	150 ± 18	0.99
2.27	>3000	-	-	-
2.28	>3000	-	-	-
2.29	486 ± 45	0.41	-	-
2.30	>3000	-	-	-

[a]Aequorin calcium mobilization assay on CHO-hY_4-G_{qi5}-mtAEQ cells.[23] [b]CRE-luciferase reporter gene assay on HEK293 cells stably co-expressing the hY_4R and the CRE-controlled luciferase gene reporter. Y_4R agonist potency was determined from the inhibition of forskolin (2 μM) stimulated luciferase activity. The maximal response (intrinsic activity, α) is referred to the effect of PP set to α = 1.0. [c]EC_{50} value reported by Ziemek et al.[23]

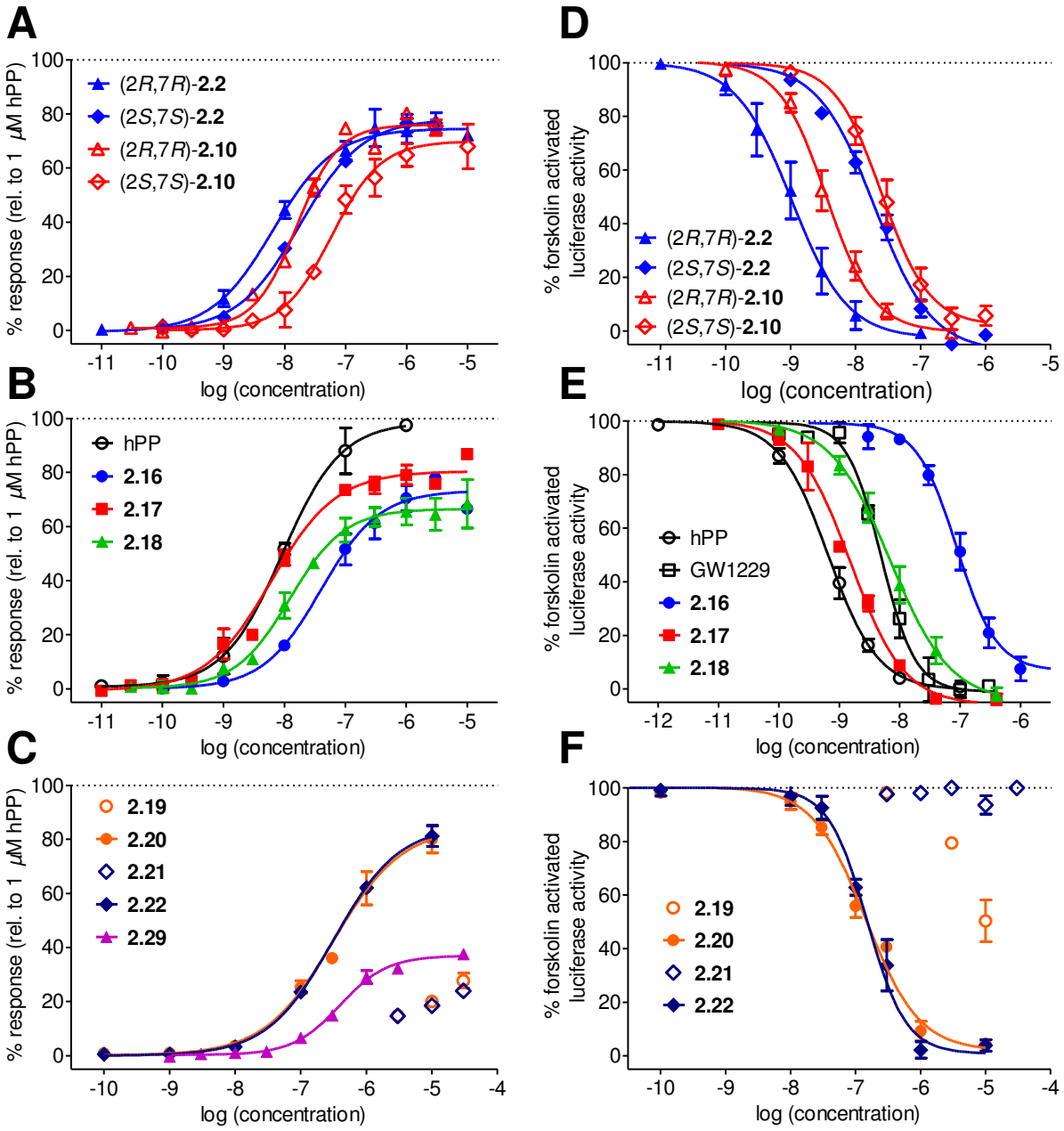


Figure 6. Y_4R agonism of PP, GW1229, **2.2**, **2.10**, **2.16**-**2.22** and **2.29** determined in a calcium (aequorin) assay and a luciferase reporter gene assay. (**A**), (**B**) and (**C**) Concentration-response curves (CRCs) from an aequorin assay on CHO-hY_4R-mtAEQ-G_{qi5} cells. Mean values ± SEM were from at least 3 independent experiments (performed in triplicate). (**D**), (**E**) and (**F**) Inhibition of forskolin-stimulated (2 μM) luciferase activity (corresponding to 100%) in hY_4R expressing HEK293 cells by PP, GW1229, **2.2**, **2.10** and **2.16**-**2.22** with the maximum inhibitory effect of the endogenous ligand PP which is set to 0% luciferase activity and corresponds to full agonism (α = 1.0). Data points shown are the mean ± SEM of at least three independent experiments performed in triplicate.

Except for **2.17** and **2.18**, compounds devoid of an amino group in the linker were significantly less potent Y_4R agonists. Interestingly, replacement of one of the 'inner arginines' in **2.16** by the N^G-carbamoylated arginine **2.11** considerably increased Y_4R agonist potency (**2.17**, EC_{50}: 6.4 nM and 1.7 nM in the aequorin assay and the luciferase assay, respectively), that is, **2.17** was equipotent with (2*R*,7*R*)-**2.2**. As observed for the

stereoisomers of **2.2** and **2.10**, propionylation of **2.17**, resulting in the derivative **2.18**, led only to a minor decrease in Y_4R potency.

The homodimeric compounds **2.19**, **2.21**, **2.27** and **2.28** in which two amino acids were replaced by alanine, proved to be inactive at the Y_4R. Only **2.29** in which the tyrosine adjacent to the linker was replaced with alanine was still active at the Y_4R (EC_{50} = 486 nM in the aequorin assay), suggesting that the presence of at least one unaltered tetrapeptide sequence Arg-Leu-Arg-Tyr-NH_2 is indispensable. Compounds **2.20** and **2.22**, in which only one Arg was replaced by Ala, were less potent than **2.16** by a factor of 5 to 6 in the aequorin assay and by a factor of 2 in the luciferase assay. Considering the high affinity of the endogenous ligand PP, the C-terminal pentapeptide of PP was applied as a putatively superior scaffold for dimeric Y_4R agonists. However, compound **2.30**, a dimer of the C-terminal pentapeptide of PP, was inactive at the Y_4R. Except for the homodimeric compounds that were inactive at the Y_4R, all investigated compounds were partial agonists in the aequorin assay (intrinsic activities between 40% and 85% referred to hPP), but full agonists in the luciferase assay. Moreover, the potencies obtained in the luciferase assay were on average slightly higher than those obtained in the aequorin assay, presumably due to more pronounced signal amplification in the gene reporter assay.[24]

2.2.4 Characterization of [^{3}H]2.10 and [^{3}H]2.18

Saturation binding experiments with the tritiated radioligands (Figure 7A and Figure 8A), performed on intact CHO-hY_4-G_{qi5}-mtAEQ cells in buffer I (HEPES, sodium-free) as used by Balasubramaniam et al.[15] to investigate the diastereomeric mixture **2.2**, revealed a K_d value of 0.67 ± 0.1 nM for both compounds, [^{3}H]**2.10** and [^{3}H]**2.18**. The number of specific binding sites per cell, determined with [^{3}H]**2.10** or [^{3}H]**2.18**, was 320,000 ± 20,000 and 340,000 ± 15,000, respectively. At concentrations around the K_d value, unspecific binding amounted to 10% ([^{3}H]**2.10**) and 20% ([^{3}H]**2.18**), respectively, of the total binding. Association to the receptor was complete after 100 min (Figure 7B and Figure 8B). Dissociation from the receptor was biphasic (Figure 7C and Figure 8C) with a fast k_{off} (0.0329 min^{-1} for [^{3}H]**2.10** and 0.0309 min^{-1} for [^{3}H]**2.18**) and a slow k_{off} (0.0024 min^{-1} for [^{3}H]**2.10** and 0.0026 min^{-1} for [^{3}H]**2.18**).

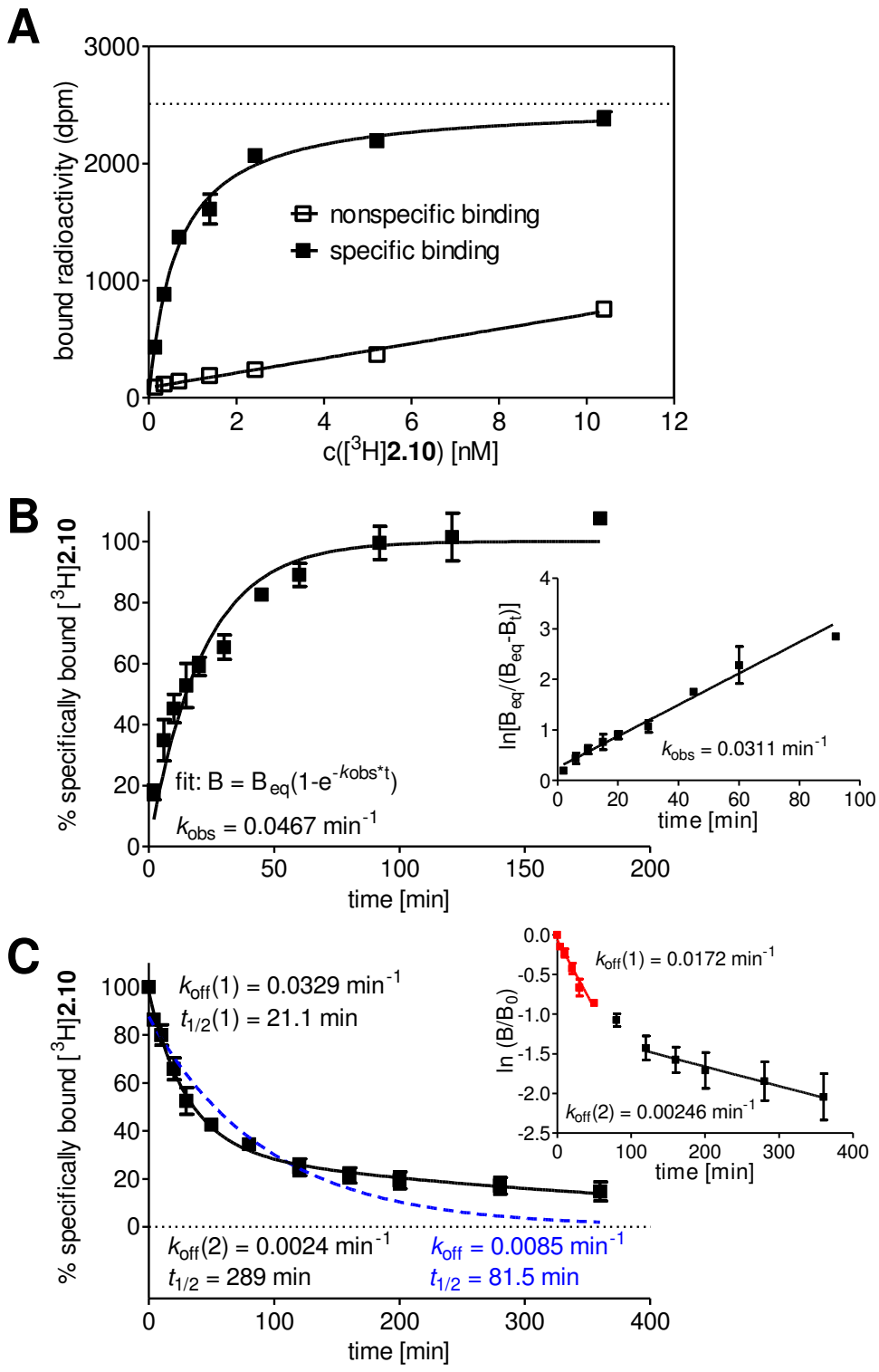


Figure 7. Y_4R binding characteristics of the radioligand [^{3}H]**2.10** determined at live CHO-hY_4-G_{qi5}-mtAEQ cells at 22 °C in buffer I (HEPES, sodium-free). (**A**) Representative saturation binding experiment with [^{3}H]**2.10**. (**B**) Y_4R association kinetics of [^{3}H]**2.10** (c = 1.5 nM). Inset: Linearization $\ln[B_{eq}/(B_{eq}-B_t)]$ versus time. (**C**) Y_4R dissociation kinetics of [^{3}H]**2.10** (c = 1.5 nM, pre-incubation time: 2 h) determined in the presence of a 200-fold excess of (2*R*,7*R*)-**2.2**. Fitting of the data according to a biphasic exponential decay (black curve, $B = B_0(1) \cdot e^{-k_{off(1)}t} + B_0(2) \cdot e^{-k_{off(2)}t}$, $R^2 = 0.9532$) resulted in a much better correlation than an analysis according to a monophasic exponential decay (blue dotted curve, $B = B_0 \cdot e^{-k_{off}t}$, $R^2 = 0.8321$). Inset: Linearization $\ln[B/B_0]$ versus time.

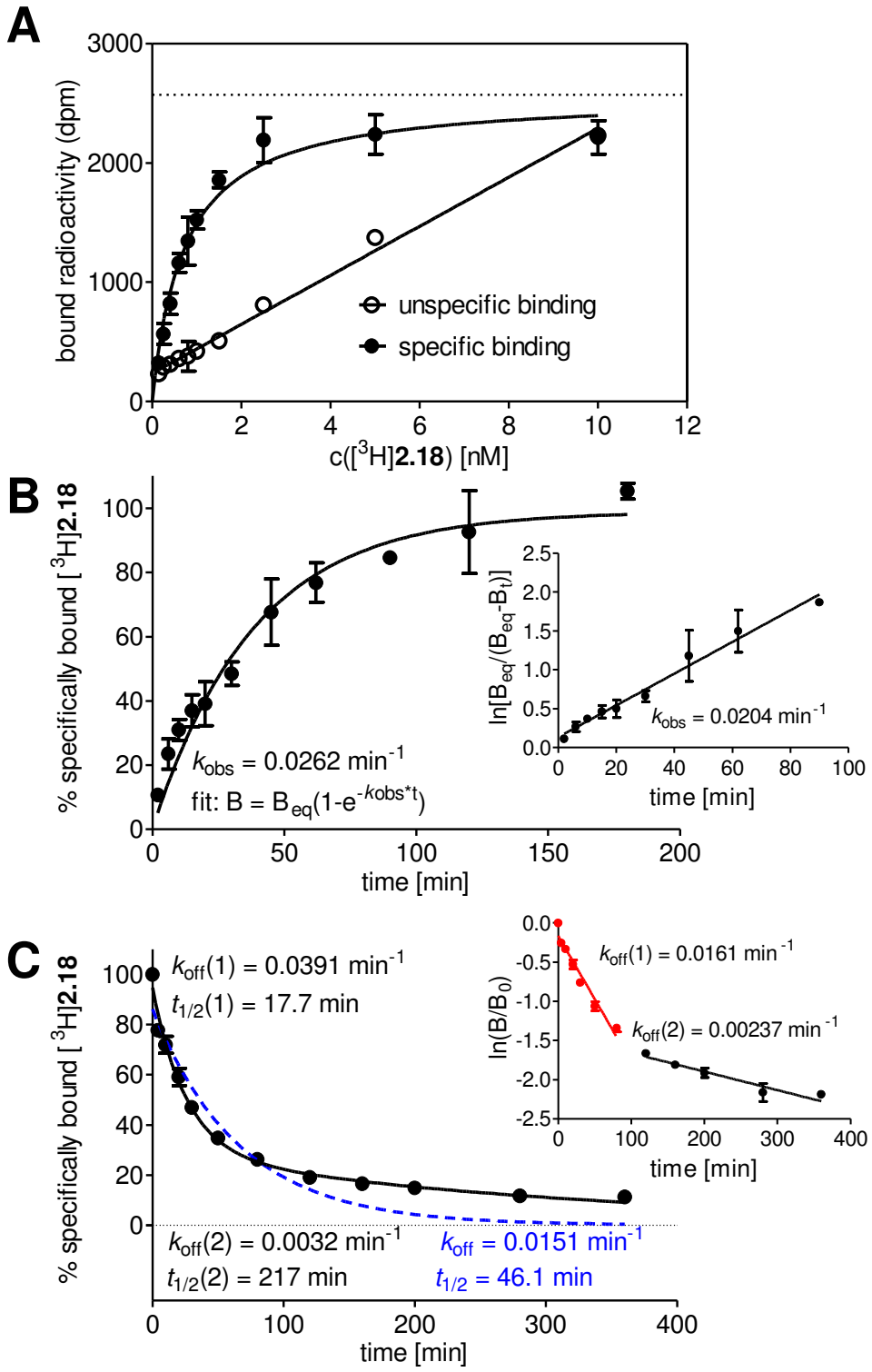


Figure 8. Y_4R binding characteristics of the radioligand [^{3}H]**2.18** determined at live CHO-hY_4-G_{qi5}-mtAEQ cells at 22 °C in buffer I (HEPES, sodium-free). (**A**) Representative saturation binding experiment with [^{3}H]**2.18**. (**B**) Y_4R association kinetics of [^{3}H]**2.18** (c = 1.5 nM). Inset: Linearization $\ln[B_{eq}/(B_{eq}-B_t)]$ versus time. (**C**) Y_4R dissociation kinetics [^{3}H]**2.18** (c = 1.5 nM, pre-incubation time: 2 h) determined in presence of 200-fold excess of **2.17**. Fitting of the data according to a biphasic exponential decay (black curve, $B = B_0(1) \cdot e^{-k_{off(1)}t} + B_0(2) \cdot e^{-k_{off(2)}t}$, $R^2 = 0.9819$) resulted in a much better correlation than an analysis according to a monophasic exponential decay (blue dotted curve, $B = B_0 \cdot e^{-k_{off}t}$, $R^2 = 0.9166$). Inset: Linearization $\ln[B/B_0]$ versus time.

Table 2. Binding data of [^{3}H]**2.10** and [^{3}H]**2.18**.

	Saturation binding		**Binding kinetics**		
radioligand	K_d [nM]		k_{obs} [s^{-1}][c]	k_{off1} [s^{-1}][d]	k_{off2} [s^{-1}][e]
[^{3}H]**2.10**	0.67 ± 0.1[a]	9.8 ± 2.5[b]	0.0467	0.0329	0.0024
[^{3}H]**2.18**	0.67 ± 0.1[a]		0.0262	0.0391	0.0032

[a]Equilibrium dissociation constant determined on CHO-hY_4-G_{qi5}-mtAEQ cells in buffer I; mean ± SEM from at least 4 independent experiments (performed in triplicate). [b]Equilibrium dissociation constant determined on CHO-hY_4-G_{qi5}-mtAEQ cells in Leibovitz' L-15 medium; mean ± SEM from at least 4 independent experiments (performed in triplicate). [c]Apparent association constant, mean from 3 independent experiments (performed in triplicate). [d]Fast dissociation constant, mean from 3 independent experiments (performed in triplicate), calculated according to a four-parameter equation describing a biphasic exponential decay. [e]Slow dissociation constant, mean from 3 independent experiments (performed in triplicate), calculated according to a four-parameter equation describing a biphasic exponential decay. Note: A kinetically derived dissociation constant $K_{d(kin)}$ and the association rate constant k_{on} was not calculated, as k_{off} was ambiguous due to a fast (k_{off1}) and a slow (k_{off2}) component.

2.2.5 Competition Binding Studies at NPY Receptor Subtypes

K_i values of the 'dimeric' pentapeptides were determined in competition binding experiments on live cells expressing human Y_1, Y_2, Y_4 or Y_5 receptors to determine the NPY receptor subtype selectivity. The unspecific binding at the respective NPY receptor expressing cells was low, and there was no hint to an internalization of the used radioligands, which might lead to a misinterpretation of the binding data. Therefore, investigations on a potential internalization, e. g. by acid wash experiments,[16] were not considered necessary.

2.2.5.1 Y_4R Binding

The binding data of the 'dimeric' pentapeptides and the reference compounds PP, GW1229 and UR-MK188, were determined at the Y_4R, using the new radioligands [^{3}H]**2.10** and [^{3}H]**2.18** (Figure 9, Figure 11). The K_i value of PP (0.65 nM) was in good agreement with published data (K_i: 0.50 nM,[16] 0.53 nM[25]), whereas the affinity of UR-MK188 (K_i = 660 nM) was slightly lower than reported (K_i = 130 nM[10]). In case of the 'cold' analogues of the radioligands, (2*R,7R*)-**2.10** (0.87 nM) and **2.18** (1.2 nM), the K_i values were in good agreement with the K_d values (cf. Figure 9, Figure 11, Table 2). Generally, the K_i values of the 'dimeric' pentapeptides at the Y_4R were by a factor of approximately 10 lower than the EC_{50} values determined in the functional assays (aequorin or luciferase assay, Table 1). Nevertheless, the Y_4R affinities correlated with the order of potency.

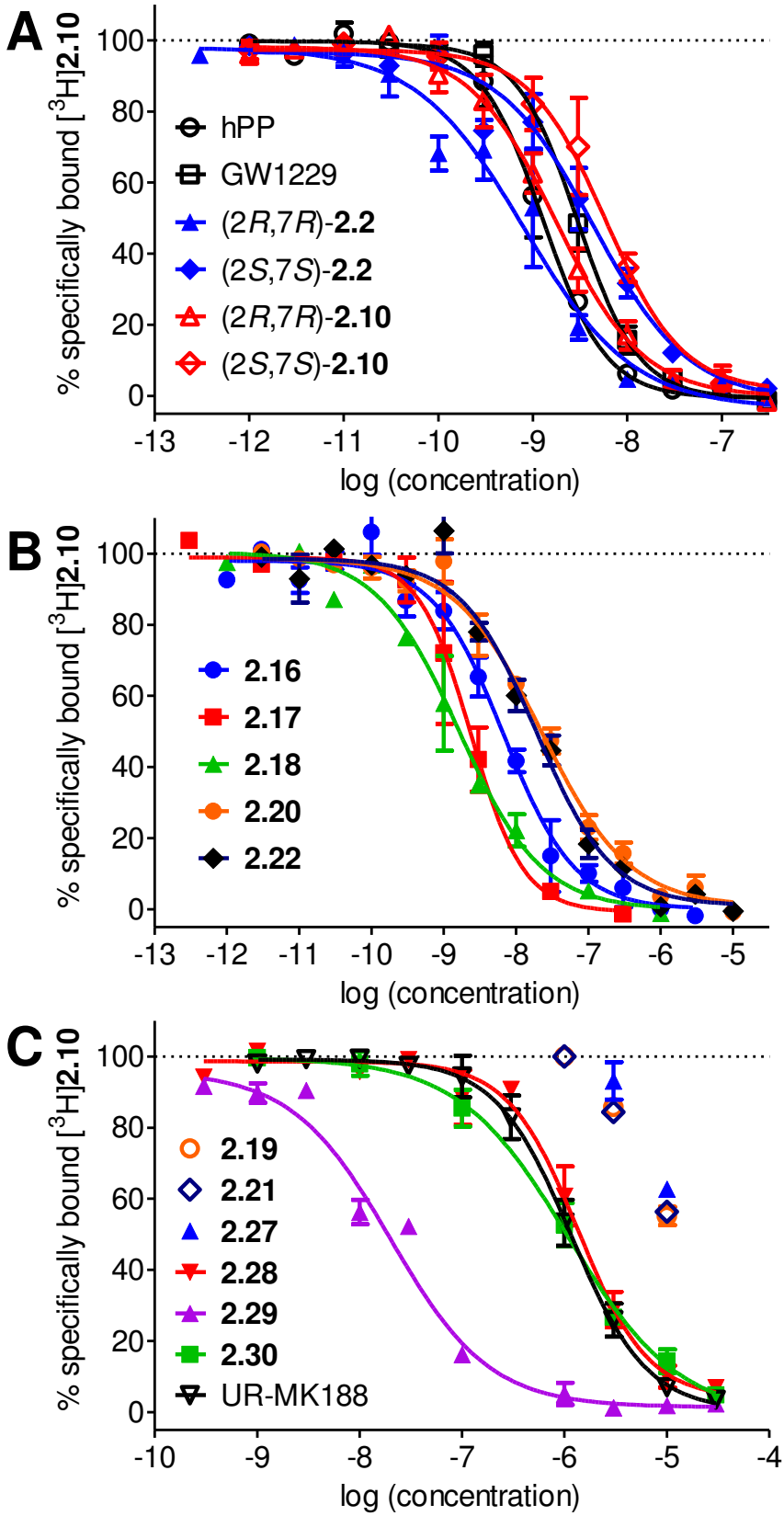


Figure 9. Radioligand displacement curves from competition binding experiments performed with [^{3}H]**2.10** at CHO-hY$_4$R-G$_{qi5}$-mtAEQ cells in buffer I. (**A**), (**B**) and (**C**) Displacement of [^{3}H]**2.10** (K_d = 0.67 nM, c = 0.6 nM). Data represent mean values ± SEM of at least 3 independent experiments, each performed in triplicate.

The diastereomeric mixture of **2.2** was previously reported[15] to show Y_4R agonism in the two-digit nM range (EC_{50} = 14.8 nM, cAMP assay), but much higher affinity (K_i = 0.05 nM, radioligand [^{125}I]PP) determined in sodium-free HEPES buffer (buffer I), which has a broad application in binding experiments in the NPY field. Experiments performed with different buffers in our laboratory revealed that the discrepancies between functional and binding data are, at least in part, caused by the absence (buffer I) or presence (Leibovitz' L-15 medium) of sodium ions. An exchange of the sodium-free buffer I with the Na^+ containing L-15 medium led to an increase in the K_d value of [^{3}H]**2.10** from 0.67 nM to 9.8 nM (cf. Table 2, footnote b). Similarly, K_i values determined in competition binding experiments using [^{3}H]**2.10** in L-15 medium were by a factor of almost 10 higher (K_i values, (2*R*,7*R*)-**2.2**: 2.0 nM; (2*S*,7*S*)-**2.2**: 17 nM; (2*R*,7*R*)-**2.10**: 6.3 nM). It should be stressed that the binding constants determined in L-15 medium were in good agreement with the agonist potencies determined in the functional assays using Na^+ containing buffer III or DMEM.

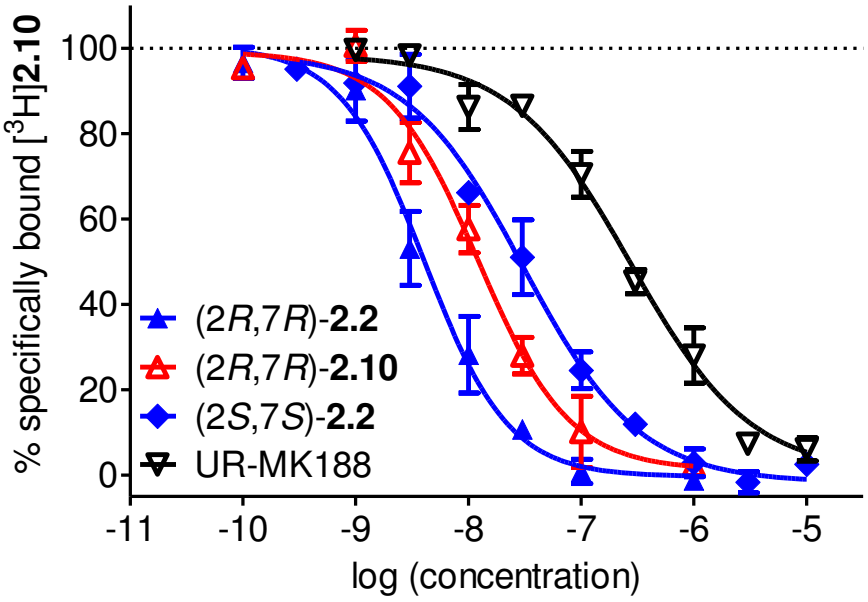


Figure 10. Radioligand displacement curves from competition binding experiments performed with [^{3}H]**2.10** at CHO-hY_4R-G_{qi5}-mtAEQ cells in Leibovitz' L15 medium. Displacement of [^{3}H]**2.10** (K_d = 9.8 nM, c = 10 nM). Data represent mean values ± SEM of at least 2 independent experiments, each performed in triplicate.

Interestingly, the affinity of the Y_4R antagonist UR-MK188 was not affected in the same way by an exchange of the binding buffer. The K_i value of UR-MK188 in competition binding experiments using the L-15 medium was even lower (K_i = 150 nM) than in experiments using buffer I. These results are compatible with recent insights obtained from GPCR crystal structures. Several high-resolution crystal structures described Na^+ ions as allosteric

stabilizers of inactive-state receptor conformations.[26,27] Therefore, Na^+ is expected to reduce the affinity of agonists binding to the active state.

As an alternative to [^{3}H]**2.10**, the radioligand [^{3}H]**2.18** is also suited for competition binding. The K_i values determined by displacement of [^{3}H]**2.18** were on average slightly lower (Table 3).

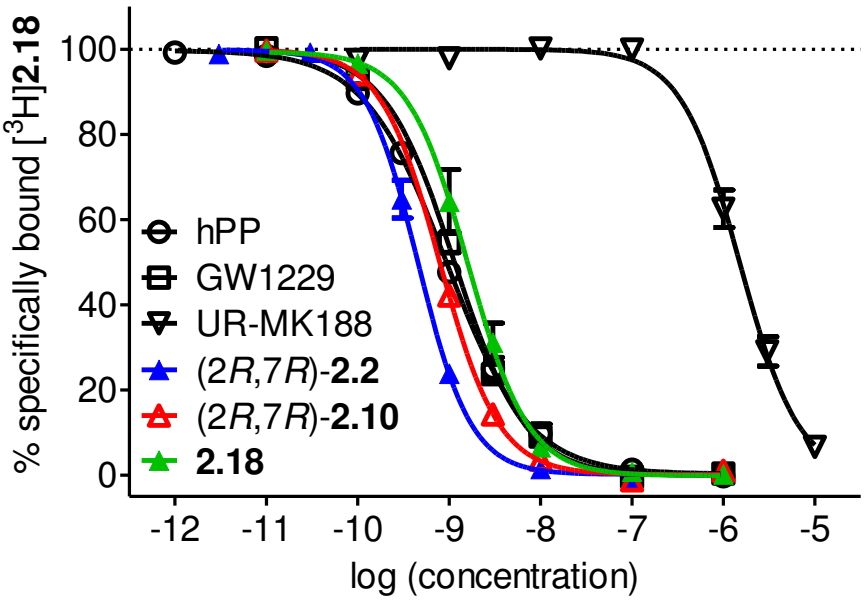


Figure 11. Radioligand displacement curves from competition binding experiments performed with [^{3}H]**2.18** at CHO-hY_4R-G_{qi5}-mtAEQ cells in buffer I. Displacement of [^{3}H]**2.18** (K_d = 0.67 nM, c = 1 nM). Data represent mean values ± SEM of at least 3 independent experiments, each performed in triplicate.

2.2.5.2 NPY Receptor Subtype Selectivity

The K_i values of (2*R*,7*R*)-**2.10** and **2.18**, the 'cold' analogues of the new Y_4R radioligands at the Y_1R, Y_2R and Y_5R were in the three-digit nanomolar range (Table 3). The 'heterodimeric' ligands **2.20** and **2.22** were comparable with (2*R*,7*R*)-**2.10** and **2.18** at the Y_1R and Y_5R, but only very weak binders at the Y_2R. K_i values were not determined for the 'homodimeric' ligand **2.21**, **2.27**, **2.28** and **2.30** at all NPY receptors due to low affinity. The same holds for compound **2.19** with exception of the Y_5R. Peptide dimers derived from the C-terminus of NPY such as GW1229 were among the first high affinity Y_1R antagonists described in literature.[13,28] Therefore, we selected (2*R*,7*R*)-**2.2** and **2.17** as examples for functional investigations at the Y_1R. In a standard Y_1R model, the Fura-2 calcium assay on HEL cells, both compounds revealed antagonism with K_b values of 9.5 nM and 25.3 nM, respectively (Table 3, footnote, and Figure 12).

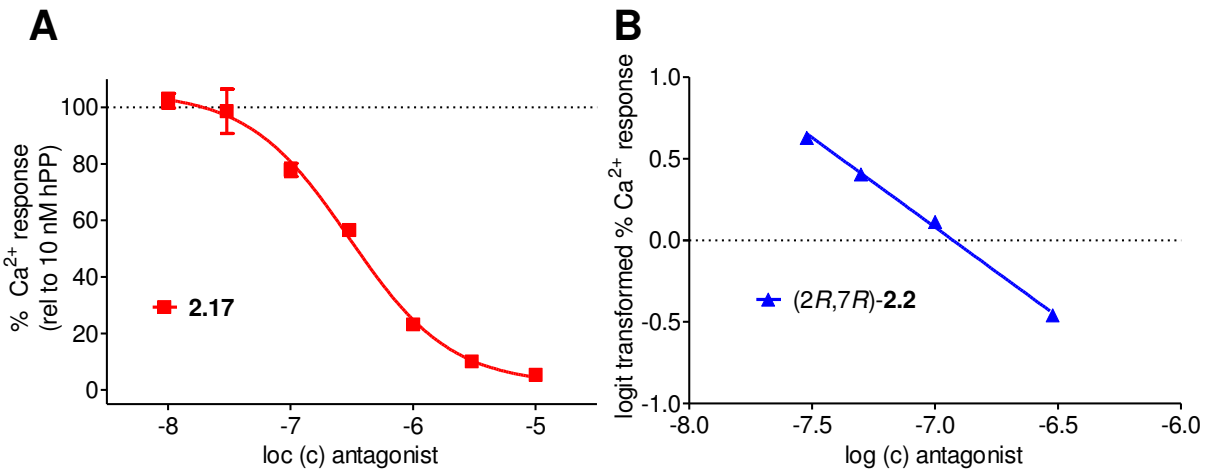


Figure 12: Inhibition of the pNPY (10 nM, EC_{50} = 0.87 nM) induced intracellular Ca^{2+} mobilization in HEL cells in a Fura-2 assay. (**A**) Inhibition of the pNPY induced Ca^{2+} signal by **2.17** (K_b = 25.3 ± 3.8 nM). Mean ± SEM from two independent experiments. (**B**) Logit transformed inhibition of the pNPY induced Ca^{2+} signal by (2*R*,7*R*)-**2.2** (K_b = 9.5 ± 0.12 nM). Mean ± SEM from two independent experiments.

In case of (2*R*,7*R*)-**2.2** the result disagrees with published data for the diastereomeric mixture **2.2**, which was reported to be an agonist in a cAMP assay on Y_1R expressing HEK 293 cells.[15] It may be speculated, whether the opposing qualities of action are related to the specific repertoire of G_q and G_i coupling[29] in HEL cells, endogenously expressing the Y_1R, compared to genetically engineered HEK293 cells.

Table 3. NPY receptor binding data.

	Y_1R	Y_2R		Y_4R, K_i [nM]		Y_5R
Compd.	K_i [nM][a]	K_i [nM][b]	[^{3}H]**2.10**[c]	[^{3}H]**2.10**[d]	[^{3}H]**2.18**[e]	K_i [nM][f]
PP	440[g]	>5000[g]	0.65 ± 0.13	-	0.37 ± 0.02	17[g]
GW1229	-	-	1.6 ± 0.34	-	1.3 ± 0.78	-
2.2	7.5[h]	1100[h]	1.06 ± 0.47 (0.05[h])	-	-	>1000[h]
(2*R*,7*R*)-**2.2**	440 ± 81[i]	830 ± 250	0.45 ± 0.14	2.0 ± 0.04	0.18 ± 0.02	1500 ± 300
(2*S*,7*S*)-**2.2**	930 ± 270	950 ± 520	2.3 ± 0.16	17 ± 4.3	-	2100 ± 980
UR-MK188	24[j]	920[j]	660 ± 140	150 ± 36	630 ± 80	>5000[j]
(2*R*,7*R*)-**2.10**	400 ± 46	420 ± 50	0.87 ± 0.16	6.3 ± 0.8	0.32 ± 0.02	710 ± 96
(2*S,*7*S*)-**2.10**	290 ± 110	750 ± 380	2.8 ± 0.44	-	-	400 ± 71
2.16	720 ± 100	1700 ± 70	3.5 ± 0.59[k]	-	-	280 ± 40
2.17	410 ± 150[l]	730 ± 150	1.2 ± 0.37[k]	-	-	140 ± 1.4
2.18	360 ± 46	1300 ± 760	1.2 ± 0.49[k]	-	0.66 ± 0.13	220 ± 40
2.19	>3000	>10000	>3000	-	-	550 ± 5.5
2.20	1400 ± 270	>3000	12 ± 1.6	-	-	220 ± 40
2.21	>10000	>10000	>3000	-	-	>10000

2.22	1600 ± 30	>3000	11 ± 1.4	-	-	460 ± 50
2.27	>5000	>5000	>5000	-	-	>5000
2.28	>5000	>5000	724 ± 168	-	-	2820 ± 217
2.29	3230 ± 690	1750 ± 394	12.2 ± 5.1	-	-	3440 ± 207
2.30	>5000	>5000	622 ± 150	-	-	>5000

[a]Radioligand competition binding assay with [^{3}H]UR-MK136[30] (K_d = 6.2 nM, c = 4 nM) using MCF-7-hY_1 cells in buffer II.[31] [b]Radioligand competition binding assay with [^{3}H]propionyl-pNPY[32] (K_d = 1.4 nM, c = 1 nM) using CHO-hY_2-G_{qi5}-mtAEQ cells in buffer I.[33] [c]Radioligand competition binding assay with [^{3}H]**2.10** (K_d = 0.67 nM, c = 0.6 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells in buffer I. [d]Radioligand competition binding assay with [^{3}H]**2.10** (K_d = 9.8 nM, c = 10 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells in L-15 medium. [e]Radioligand competition binding assay with [^{3}H]**2.18** (K_d = 0.67 nM, c = 1 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells in buffer I. [f]Radioligand competition binding assay with [^{3}H]propionyl-pNPY (K_d = 4.83 nM, c = 4 nM) using HEC-1b hY_5R cells in buffer II.[34] [g]K_i value reported by Berlicki et al.[25] [h]K_i value reported by Balasubramaniam et al.;[15] these authors used [^{125}I]PYY (hY_1R expressed in HEK 293 cells, hY_2R or hY_5R expressed in CHO cells) and [^{125}I]PP (hY_4R expressing CHO cells) for the determination of the binding data. [i]hY_1R antagonism at HEL cells (Fura-2 calcium assay): K_b = 9.5 ± 0.12 nM (Figure 12); in contrast to data reported in ref.[15] for the diastereomeric mixture **2.2** (cAMP assay, hY_1R expressing HEK 293 cells), no hY_1R agonist activity. [j]K_i value reported by Keller et al.[10] [k]Ref.[16]: K_i values determined by flow cytometry using fluorescence-labeled K^4-hPP in buffer I: **2.16**: 7.4 nM, **2.17**: 1.5 nM, **2.18**: 3.6 nM. [l]hY_1R antagonism at HEL cells (Fura-2 calcium assay): K_b = 25.3 ± 3.8 nM (Figure 12).

2.3 Conclusions

The synthesis of the enantiopure diaminosuberic acids (*R,R*)-**2.8** and (*S,S*)-**2.8**, accomplished in excellent yield via Grubbs' II catalyzed coupling, gave access to the individual diastereomers of compound **2.2** with (2*R,*7*R*)- or (2*S,*7*S*)-configuration in the linker. In the same way, octanedioic acid was used as an achiral linker to combine two identical or two different pentapeptide moieties, in which the Arg residues were either retained or replaced at one or two positions by Ala or by an amino-functionalized carbamoylated Arg. Interestingly, the latter turned out to compensate for the absence of the two amino groups in the connecting diacid with respect to affinity and potency (cf. compound **2.17**). Moreover, propionylation of one amino group in the linker of **2.2** (cf. compound **10**) or at the primary amino group in **2.17** gave access to the corresponding tritiated high affinity radioligands. [^{3}H]**2.10** ([^{3}H]UR-KK193) and [^{3}H]**2.18** ([^{3}H]UR-KK200) are useful new pharmacological tools for the Y_4R. (2*R,*7*R*)-**2.2** was superior to (2*S,*7*S*)-**2.2** and, in contrast to published data for the mixture **2.2**, affinities and agonist potencies were essentially in the same range. As demonstrated by saturation and competition binding experiments, the composition of the buffer may account for discrepancies between functional and binding data. It should be kept in mind that the affinity of peptidic Y_4R agonists may be considerably higher in the absence of sodium ions, conditions widely used for the investigation of NPY receptor ligands.

2.4 Experimental Section

2.4.1 General Experimental Conditions

Chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise indicated. MeCN for HPLC (gradient grade) was obtained from Merck (Darmstadt, Germany). DMF for peptide synthesis, NMP for peptide synthesis and HOBt hydrate were obtained from Acros Organics/Fisher Scientific (Nidderau, Germany). Fmoc-Sieber-PS resin (0.61 mmol/g), (*S*)-allylglycine, (*R*)-allylglycine, Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(*t*Bu)-OH and HBTU were purchased from Iris Biotech (Marktredwitz, Germany). Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Pro-OH, dicyclohexylcarbodiimide, dioxane, sodium hydroxide and methanol were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid, dichloromethane, diethyl ether, ethanol and Triton X-100 were obtained

from Sigma Aldrich (Deisenhofen, Germany), *N,N*-diisopropylethylamine (DIPEA) (99%) was from ABCR (Karlsruhe, Germany), di-*tert*-butyl dicarbonate (>97%) and 8-methoxy-8-oxooctanoic acid (98%) were from Alfa Aesar (Karlsruhe, Germany). Bovine serum albumin and bacitracin were purchased from Serva (Heidelberg, Germany), coelenterazin h was obtained from Biotrend (Cologne, Germany), human pancreatic polypeptide and porcine neuropeptide Y were synthesized by Prof. Dr. C. Cabrele (University Salzburg, Salzburg, Austria). GW1229 was a gift from Dr. A. J. Daniels (Glaxo Wellcome, NC, USA). The synthesis of UR-MK188,[10] **2.11**,[16] **2.12**,[22] and [^{3}H]propionyl-pNPY[32] was previously described. (4,5-DihydroIMES)(PCy_3)Cl_2Ru=CHPh (Grubbs' II catalyst) was synthesized according to literature procedure.[35] Millipore water was used throughout for the preparation of buffers and HPLC eluents. Polypropylene reaction vessels (1.5 or 2 mL) with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for the synthesis of the radioligands [^{3}H]**2.10** and [^{3}H]**2.18**, for small scale reactions (e.g. the preparation of **2.16**), the investigation of chemical stabilities and for the storage of stock solutions. TL chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates, silica gel 60 (40-63 μm, Merck) was used for column chromatography. Visualization was accomplished by UV light (λ = 254 nm or 366 nm) and ninhydrin/acetic acid solution. NMR-spectra were recorded on a Bruker Avance 300 (7.05 T, ^{1}H: 300.1 MHz, ^{13}C: 75.5 MHz) or a Bruker Avance 600 instrument with cryogenic probe (14.1 T, ^{1}H: 600.3 MHz, ^{13}C: 150.9 MHz) (Bruker, Karlsruhe, Germany). ATR-IR spectroscopy was performed on a Biorad Excalibur FTS 3000 spectrometer (Biorad, Munich, Germany), equipped with a Specac Golden Gate Diamond Single Reflection ATR-System. Mass spectra were recorded on a Finnigan MAT95 (EI-MS 70eV) or a Finnigan MAT SSQ 710 A (CI-MS (NH_3)). For HRMS an Agilent Q-TOF 6540 UHD (Agilent, Waldbronn, Germany) equipped with an ESI source was used. Optical rotations (given as specific rotation $[\alpha]^{20}$, [° cm^3 dm^{-1} g^{-1}], concentrations (c) given as g/100 mL) were determined in an MCP 500 (Anton Paar, Ostfildern-Scharnhausen, Germany) or a 241 polarimeter (Perkin Elmer, Rodgau, Germany) at 589 nm in a 1.0-dm cuvette. Preparative HPLC was performed on a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Kinetex-XB C_{18}, 5 μm, 250 mm × 21 mm, Phenomenex, Aschaffenburg, Germany) at a flow-rate of 15 mL/min using mixtures of acetonitrile and 0.1% aqueous TFA solution as mobile phase. A detection wavelength of 220 nm was used throughout. The collected fractions were lyophilised using an alpha 2-4 LD apparatus (Martin Christ, Osterode am Harz, Germany)

equipped with a RZ 6 rotary vane vacuum pump (vacuubrand, Wertheim, Germany). Analytical HPLC analysis was performed on a system from Merck-Hitachi (Hitachi, Düsseldorf, Germany) composed of a L-6200-A pump, a AS-2000A autosampler, a L-4000A UV detector, a D-6000 interface and a RP-column Kinetex-XB C_{18}, 5 μm, 250 mm × 4.6 mm (Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.8 mL/min. Mixtures of acetonitrile (A) and 0.1% aqueous TFA solution (B) were used as mobile phase. The following gradient was applied: A/B: 0-25 min: 10:90 - 60:40, 25-35 min: 60:40 - 95:5, 35-45 min: 95:5. Detection was performed at 220 nm, the oven temperature was 30 °C.

2.4.2 Chemistry: Experimental Protocols and Analytical data

General procedure for solid phase peptide synthesis. Peptides were synthesized by manual SPPS using the Fmoc strategy on an Fmoc-Sieber-PS resin. 5-ml or 20-ml Discardit II syringes (Becton Dickinson, Heidelberg, Germany) were equipped with 35 μm polyethylene frits (Roland Vetter Laborbedarf, Ammerbuch, Germany) and used as reaction vessels. DMF/NMP (8:2) was used as solvent. Protected standard *L*-amino acids (5 equiv) were pre-activated with HBTU (5 equiv)/HOBt (5 equiv)/DIPEA (10 equiv) for 5 min and added to the resin. 'Double' coupling at rt was performed for all standard amino acids for 45 min. After completed coupling of an Fmoc-aa, the resin was washed with DMF/NMP and treated with 20% piperidine in DMF/NMP (8:2) at rt (2×) for 10 min to remove the Fmoc group, followed by thorough washing of the resin.

General procedure for cleavage of protected peptides from the resin. After the last coupling step and Fmoc deprotection, the resin was washed with DCM. Side-chain protected peptides were cleaved from the resin with CH_2Cl_2/TFA (97:3) (rt, 10 × 6 min). The peptides were separated from the resin by filtration. The combined filtrates were collected in a round bottom flask containing water (10 times the volume of the combined filtrates). The organic solvent was removed on a rotary evaporator; the aqueous phase was lyophilised followed by purification by preparative HPLC.

(2*R*,7*R*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) hexakis(hydrotrifluoroacetate) ((2*R*,7*R*)-2.2). (*R,R*)-**2.8** (4.3 mg, 10.7 μmol), HBTU (8.5 mg, 2.1 equiv) and HOBt (3.5 mg, 2.1 equiv) were dissolved in anhydrous DMF (400 μL). DIPEA (10 μL, 5 equiv) was added and the mixture was stirred at room temperature for 5 min

followed by the addition of a solution of **2.9** (40 mg, 2.5 equiv). The resulting mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate was extracted with DCM (2 × 10 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 2 mL) was added and the mixture was stirred at room temperature for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 10:90–35:65, t_R = 15.8 min). Lyophilisation of the eluate afforded (2*R,*7*R*)-**2.2** as a white fluffy solid (7.5 mg, 29.3%). HRMS (*m/z*): $[M+4H]^{4+}$ calcd. for $C_{80}H_{128}N_{26}O_{16}$, 427.2495; found, 427.2510. RP-HPLC (220 nm): 96% (t_R = 18.93 min, *k* = 5.6). $C_{80}H_{124}N_{26}O_{16} \cdot C_{12}H_6F_{18}O_{12}$ (1706.04 + 684.14).

(2*S*,7*S*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) hexakis(hydrotrifluoroacetate) ((2*S*,7*S*)-2.2). (2*S,*7*S*)-**2.2** was prepared in analogy to (2*R,*7*R*)-**2.2** by using (*S,S*)-**2.8** (4.3 mg, 10.7 μmol). The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 10:90–35:65, t_R = 15.5 min). Lyophilisation of the eluate afforded (2*S,*7*S*)-**2.2** as a white fluffy solid (8.6 mg, 33.6%). HRMS (*m/z*): $[M+4H]^{4+}$ calcd. for $C_{80}H_{128}N_{26}O_{16}$, 427.2495; found, 427.2508. RP-HPLC (220 nm): 98% (t_R = 18.37 min, *k* = 5.4). $C_{80}H_{124}N_{26}O_{16} \cdot C_{12}H_6F_{18}O_{12}$ (1706.04 + 684.14).

(*R*)-2-[(*tert*-Butoxycarbonyl)amino]pent-4-enoic acid ((*R*)-2.4).[36]

(*R*)-2-Allylglycine, 1.0 g, 8.69 mmol) was dissolved in 1 M NaOH (20 mL) and dioxane (10 mL). Boc anhydride (2.32 g, 1.23 equiv.) was added under ice-cooling. The reaction mixture was allowed to warm up to ambient temperature, and stirring was continued for 18 h. After completion of the reaction, the mixture was concentrated under reduced pressure, and the aqueous phase was washed with diethyl ether to remove the excess of Boc anhydride. The pH was adjusted to 2.5 with 0.5 M hydrochloric acid, and the mixture was extracted with EtOAc (3 x 20 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The desired compound was obtained as viscous colorless oil (1.78 g, 95.2%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ 1.38 (s, 9H), 2.20 – 2.47 (m, 2H), 3.57 (s, 2H), 3.86 – 3.97 (m, 1H), 4.99 – 5.15 (m, 2H), 5.67 – 5.85 (m, 1H), 7.07 (d, 1H, *J* 8.24). LRMS (ESI): *m/z* = 140.04 $[M-H-C_4H_{10}O]^-$, 214.11 $[M-H]^-$.

(*S*)-2-[(*tert*-Butoxycarbonyl)amino]pent-4-enoic acid ((*S*)-2.4).[37]

Compound (*S*)-**2.4** was synthesized from (*S*)-2-allylglycine (1.0 g, 8.69 mmol) by analogy with the procedure for the preparation of (*R*)-**2.4.** The desired compound was obtained as viscous colorless oil (1.72 g, 92.0%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ 1.37 (s, 9H), 2.20 – 2.47 (m, 2H), 3.57 (s, 2H), 3.87 – 3.97 (m, 1H), 4.99 – 5.15 (m, 2H), 5.68 – 5.84 (m, 1H), 7.07 (d, 1H, *J* 8.09). LRMS (ESI): *m/z* = 140.04 [M-H-$C_4H_{10}O$]$^-$, 214.11 [M-H]$^-$.

Ethyl (*R*)-2-[(*tert*-butoxycarbonyl)amino]pent-4-enoate ((*R*)-2.5).[38]

(*R*)-**2.4** (350 mg, 1.63 mmol) and 4-(dimethylamino)pyridine (39.7 mg, 0.2 equiv.) were dissolved in DCM and EtOH (300 μL, 3 equiv.) was added. A solution of dicyclohexylcarbodiimide (352 mg, 1.05 equiv.) in DCM (5 mL) was added drop wise under ice-cooling. The mixture was allowed to warm up to ambient temperature and was stirred for 12 h. After completion of the reaction, the mixture was filtered and concentrated under reduced pressure. The residue was taken up in PE/EtOAc (10:1, 5 mL) and subjected to column chromatography (eluent: PE/EtOAc 10:1). The desired compound was obtained as viscous colorless oil (280 mg, 70.9%). TLC (PE/EtOAc 10:1): R_f = 0.32. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ 1.17 (t, 3H, J 7.08), 1.33 (s, 1H), 1.37 (s, 8H), 2.22 – 2.47 (m, 2H), 3.91 – 4.16 (m, 3H), 4.99 – 5.14 (m, 2H), 5.68 – 5.84 (m, 1H), 7.24 (d, 1H, *J* 7.69). LRMS (ESI): *m/z* = 144.10 [M-Boc+H]$^+$, 266.14 [M+NaH]$^+$.

Ethyl (*S*)-2-[(*tert*-butoxycarbonyl)amino]pent-4-enoate ((*S*)-2.5).

Compound (*S*)-**2.5** was synthesized from (*S*)-**2.4** (350 mg, 1.63 mmol) by analogy with the procedure for the preparation of (*R*)-**2.5**. The desired compound was obtained as viscous colorless oil (295 mg, 74.7%). TLC (PE/EtOAc 10:1): R_f = 0.32. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ 1.17 (t, 3H, J 7.13), 1.33 (s, 1H), 1.38 (s, 8H), 2.26 – 2.45 (m, 2H), 3.92 – 4.16 (m, 3H), 4.97 – 5.15 (m, 2H), 5.68 – 5.85 (m, 1H), 7.23 (d, 1H, *J* 7.78). LRMS (ESI): *m/z* = 144.10 [M-Boc+H]$^+$, 266.14 [M+Na]$^+$.

(2*R*,7*R*)-Diethyl 2,7-bis[(*tert*-butoxycarbonyl)amino]oct-4-enedioate ((*R,R*)-2.6). According to literature procedure[39] in a flame-dried Schlenk flask (*R*)-**2.5** (106 mg, 0.43 mmol, 2 equiv) was dissolved in anhydrous DCM (8 mL) under N_2-atmosphere. Grubbs' II catalyst second generation (18.5 mg, 0.022 mmol, 0.1 equiv) was added and the reaction mixture was stirred under reflux for 11 h until all starting material had been consumed. The solvent was removed under reduced pressure and the crude product was purified by column

chromatography (PE/EtOAc 5:1) to obtain (*R,R*)-**6** (89 mg, 0.19 mmol, 89%) as colorless oil. R_f = 0.23 (PE/EtOAc 5:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.21 (t, 6H, *J* 7.1 Hz), 1.38 (s, 18H), 2.31 - 2.49 (m, 4H), 4.13 (qd, 4H, *J* 1.8 Hz, 7.1 Hz), 4.2 – 4.31 (m, 2H), 5.01 – 5.11 (m, 2H), 5.31 – 5.44 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ (ppm) 14.2, 28.3, 35.5, 53.1, 61.3, 79.8, 128.5, 155.2, 171.9. IR (neat) 3369, 2978, 2937, 1703, 1498, 1367, 1330, 1248, 1159, 1095, 1051, 1021, 969, 857, 779 cm^{-1}. $[\alpha]_D^{20}$ -27.0 (c 1, chloroform). LRMS (ESI): *m/z* $[M+H]^+$ 459.3, $[M+Na]^+$ 481.3, $[2M+Na]^+$ 939.5. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{22}H_{39}N_2O_8]^+$ 459.2701, found: 459.2711. $C_{22}H_{38}N_2O_8$ (458.55).

(2*S*,7*S*)-Diethyl 2,7-bis[(*tert*-butoxycarbonyl)amino]oct-4-enedioate ((*S,S*)-2.6). Compound (*S,S*)-(+)-**2.6** was prepared according to (*R,R*)-**2.6** from (*S*)-**2.5** (105 mg, 0.43 mmol, 2 equiv) as starting material to obtain the product as a colorless oil (47.3 mg, 0.1 mmol, 49%). The spectroscopic data were identical to those for the enantiomer (*R,R*)-(-)-**2.6**. $[\alpha]_D^{20}$ +27.9 (c 1, chloroform).

(2*R*,7*R*)-Diethyl 2,7-bis[(*tert*-butoxycarbonyl)amino]octanedioate ((*R,R*)-2.7). In a flame dried Schlenk flask under nitrogen atmosphere (*R*)-**2.5** (300 mg, 1.23 mmol, 2 equiv) was dissolved in anhydrous DCM (18 mL). Grubbs' II catalyst (52 mg, 0.06 mmol, 0.1 equiv) was added, and the reaction mixture was stirred under reflux for 12 h. After all starting material was consumed, the solvent was removed under reduced pressure, and EtOH (10 mL) was added. The mixture was transferred into an autoclave and stirred under hydrogen at 20 bar at room temperature for 15 h. The reaction mixture was filtrated, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (PE/EtOAc 3:1) to yield (*R,R*)-**2.7** (268 mg, 0.58 mmol, 95% over two steps) as colorless oil. R_f = 0.3 (PE/EtOAc 3:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.21 (t, 6H, *J* 7.1 Hz), 1.24 – 1.33 (m, 4 H), 1.37 (s, 18H), 1.46 – 1.79 (m, 4 H), 4.05-4.24 (m, 4H + 2H), 4.94 – 5.04 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ (ppm) 14.2, 24.9, 28.3, 32.6, 53.3, 61.3, 79.8, 155.4, 172.8. IR (neat) 3354, 2978, 2937, 2870, 1699, 1505, 1457, 1367, 1248, 1159, 1021, 861, 779 cm^{-1}. $[\alpha]_D^{20}$ -14.8 (c 1, chloroform). LRMS (ESI): *m/z* $[M+H]^+$ 461.3, $[M+Na]^+$ 483.3, $[2M+Na]^+$ 943.5. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{22}H_{41}N_2O_8]^+$ 461.2857, found: 461.2865. $C_{22}H_{40}N_2O_8$ (460.56).

(2*S*,7*S*)-Diethyl 2,7-bis[(*tert*-butoxycarbonyl)amino]octanedioate ((*S,S*)-2.7). Compound (*S,S*)-(+)-**2.7** was prepared according to (*R,R*)-**2.7** by using (*S*)-**2.5** (303 mg, 1.2 mmol, 2 equiv) as starting material to obtain the product as a colorless oil (212 mg, 0.46 mmol, 74% over two steps). The spectroscopic data were identical to those for its enantiomer (*R,R*)-(-)-**2.7**. $[\alpha]_D^{20}$ +16.9 (c 1, chloroform).

(2*R*,7*R*)-2,7-bis[(*tert*-butoxycarbonyl)amino]octanedioic acid ((*R,R*)-2.8).[40] (*R,R*)-**2.7** (268 mg, 0.58 mmol, 1 equiv) was dissolved in EtOH (8 mL) and 1M NaOH solution (1.2 mL, 1.16 mmol, 2 equiv) was added dropwise. The reaction was stirred at room temperature for 21 h. When all the starting material had been consumed, the solution was adjusted to pH 2 by addition of 1M HCl solution. Water and EtOAc were added, and the phases were separated. The aqueous layer was extracted with EtOAc, and the combined organic phases were dried over $MgSO_4$, filtered and concentrated *in vacuo* to yield the product as a white solid (238 mg, 0.58 mmol, quant.). ^{1}H-NMR (300 MHz, CD_3OD): δ (ppm) 1.35 – 1.50 (m, 22H), 1.57 – 1.71 (m, 2H), 1.72 – 1.96 (m, 2H), 3.91 – 4.13 (m, 2H). ^{13}C-NMR (75 MHz, CD_3OD): δ (ppm) 26.5, 28.8, 32.7, 54.9, 80.5, 158.1, 176.5. IR (neat) 3295, 2978, 2933, 2363, 1789, 1707, 1680, 1509, 1449, 1394, 1370, 1312, 1244, 1159, 1077, 1047, 1017, 946, 846, 790, 758, 697 cm^{-1}. $[\alpha]_D^{20}$ +11.2 (c 1, DMF). LRMS (ESI): *m/z* = 403.2 $[M-H]^-$, 807.4 $[2M-H]^-$. HRMS (ESI): *m/z* $[M-H]^-$ calcd. for $[C_{18}H_{31}N_2O_8]^-$ 403.2086, found: 403.2096. $C_{18}H_{32}N_2O_8$ (404.46).

(2*S*,7*S*)-2,7-bis[(*tert*-butoxycarbonyl)amino]octanedioic acid ((*S,S*)-2.8).[41] Compound (*S,S*)-(+)-**2.8** was prepared according to (*R,R*)-**2.8** by using (*S,S*)-**2.7** (212 mg, 0.46 mmol, 2 equiv) as starting material to obtain the product as a white solid (190 mg, 0.46 mmol, quant.). The spectroscopic data were identical to those for the enantiomer (*R,R*)-**2.8**. $[\alpha]_D^{20}$ -9.8 (c 1, DMF, ref.[41] -15.2, 5% in DMF).

H-Tyr(*t*Bu)-Arg(Pbf)-Leu-Arg(Pbf)-Tyr(*t*Bu)-NH_2 hydrotrifluoroacetate (2.9).[16] The side chain protected pentapeptide **2.9** was prepared on a Sieber resin (loading: 0.61 mmol/g) according to the general procedure. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–16 min: MeCN/0.1% aq TFA 52:48–81:19, t_R = 11 min). Lyophilisation of the eluate afforded **2.9** as a white solid (200 mg, 56%). HRMS (ESI): *m/z* $[M+3H]^{3+}$ calcd. for $[C_{70}H_{107}N_{12}O_{13}S_2]^{3+}$ 462.5835, found 462.5838. $C_{70}H_{104}N_{12}O_{13}S_2 \cdot C_2HF_3O_2$ (1385.79 + 114.02).

(2*R*,7*R*)-2-Amino-7-(propionylamino)octanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) pentakis(hydrotrifluoroacetate) ((2*R*,7*R*)-2.10). (2*R,*7*R*)-**2.2** (4 mg, 1.67 μmol) was dissolved in 3% DIPEA in anhydrous DMF (400 μL). A solution of succinimidyl propionate in anhydrous DMF (10 mg/mL) was added in small portions (a total of 17 μL (0.98 μmol)). To prevent two-fold acylation, the reaction was monitored by HPLC and stopped when approximately 35% of the starting material was consumed. Water (5 mL) was added and the product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 10:90–35:65, t_R = 17.2 min). Lyophilisation of the eluate afforded (2*R,*7*R*)-**2.10** as a white fluffy solid (1.1 mg, 0.47 μmol). HRMS (*m/z*): $[M+4H]^{4+}$ calcd. for $C_{83}H_{132}N_{26}O_{17}$, 441.2560; found, 441.2575. RP-HPLC (220 nm): 97% (t_R = 20.00 min, k = 6.0). $C_{83}H_{124}N_{26}O_{17} \cdot C_{10}H_5F_{15}O_{10}$ (1762.10 + 570.12).

(2*S*,7*S*)-2-Amino-7-(propionylamino)octanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) pentakis(hydrotrifluoroacetate) ((2*S*,7*S*)-2.10). (2*S,*7*S*)-**2.10** was prepared by analogy with (2*R,*7*R*)-**2.10** using (2*S,*7*S*)-**2.2** (4 mg, 1.67 μmol) as starting material. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 10:90–35:65, t_R = 17.0 min). Lyophilisation of the eluate afforded (2*S,*7*S*)-**2.10** as a white fluffy solid (1.0 mg, 0.43 μmol). HRMS (*m/z*): $[M+4H]^{4+}$ calcd. for $C_{83}H_{132}N_{26}O_{17}$, 441.2560; found, 441.2577. RP-HPLC (220 nm): 97% (t_R = 19.56 min, k = 5.8). $C_{83}H_{124}N_{26}O_{17} \cdot C_{10}H_5F_{15}O_{10}$ (1762.10 + 570.12).

HOOC-$(CH_2)_6$-C(=O)-Tyr-Arg-Leu-Arg-Tyr-NH_2 (2.13).[16] Compound **2.13** was synthesized on a Sieber resin (loading: 0.61 mmol/g). Fmoc-amino acids were coupled according to the general procedure. The coupling of suberic acid monomethyl ester (196 mg, 1.04 mmol) was performed at 30 °C for 14 h (single coupling) using the solvent and coupling reagent as for Fmoc-amino acids. The peptide was cleaved from the resin according to the general procedure. The residue obtained after lyophilisation was dissolved in MeOH (5 mL), 0.5 M aq NaOH (1 mL) was added and the mixture was kept under reflux for 3 h. An aequous solution of citric acid (0.1 g/ml) (20 mL) was added causing the formation of a white precipitate. The suspension was stored in the refrigerator overnight, the solid was separated by filtration and purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–18 min: MeCN/0.1% aq TFA 52:48–90:10, t_R = 17 min). Lyophilisation of the eluate afforded **2.13** as a

white solid (120 mg, 30%). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{78}H_{118}N_{12}O_{16}S_2]^{2+}$ 771.411, found 771.4124. $C_{78}H_{116}N_{12}O_{16}S_2$ (1541.97).

Tyr(*t*Bu)-Ala-Leu-Arg(Pbf)-Tyr(*t*Bu)-amide hydrotrifluoroacetate (2.14). The side chain protected pentapeptide **2.14** was synthesized according to the general procedure (0.1 mmol Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–24 min: MeCN/0.1% aq TFA 38:62–90:10, t_R = 11 min). Lyophilisation of the eluate afforded **2.14** as a white solid (51 mg, 49%). HRMS (*m/z*): $[M+2H]^{2+}$ calcd. for $C_{54}H_{83}N_9O_{10}S$, 524.7986; found, 524.7999. $C_{54}H_{81}N_9O_{10}S \cdot C_2HF_3O_2$ (1048.36 + 114.02).

Tyr(*t*Bu)-Arg(Pbf)-Leu-Ala-Tyr(*t*Bu)-amide hydrotrifluoroacetate (2.15). The side chain protected pentapeptide **2.15** was synthesized according to the general procedure (0.1 mmol Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–24 min: MeCN/0.1% aq TFA 38:62–90:10, t_R = 10.5 min). Lyophilisation of the eluate afforded **2.15** as a white solid (58 mg, 55%). HRMS (*m/z*): $[M+2H]^{2+}$ calcd. for $C_{54}H_{83}N_9O_{10}S$, 524.7986; found, 524.7999. $C_{54}H_{81}N_9O_{10}S \cdot C_2HF_3O_2$ (1048.36 + 114.02).

Octanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) tetrakis(hydrotrifluoroacetate) (2.16).[16] Compound **2.9** (20 mg, 13.3 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Compound **2.12** (1.96 mg, 5.3 μmol) was added and the mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate extracted with CH_2Cl_2 (2 × 10 ml). The combined extracts were evaporated and the residue dried *in vacuo*. TFA/water (95:5 v/v) (2 mL) was added and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 14:86–52:48, t_R = 11 min). Lyophilisation of the eluate afforded **16** as a white fluffy solid (5.7 mg, 51%). ^{1}H-NMR (600 MHz, [D$_6$]DMSO): δ 0.83 (d, 6H, *J* 6.5 Hz), 0.87 (d, 6H, *J* 6.5 Hz), 1.03 (br s, 4H), 1.30 (br s, 4H), 1.37−1.56 (m, 16H), 1.56−1.73 (m, 6H), 1.95−2.05 (m, 4H), 2.57−2.65 (m, 2H), 2.68−2.75 (m, 2H), 2.81−2.89 (m, 4H), 3.02−3.13 (m, 8H), 4.17−4.24 (m, 2H), 4.24−4.36 (m, 6H), 4.39−4.46 (m, 2H), 6.59−6.65 (m, 8H), *ca* 6.9 (br s, 8H), 6.95−6.99 (m, 4H), 6.99−7.03 (m, 4H), 7.05 (s, 2H), *ca* 7.3 (br s, 8H), 7.36 (s, 2H), 7.49−7.57 (m, 4H), 7.74 (d, 2H, *J* 7.9 Hz), 7.85−7.94 (m, 4H), 8.03 (d, 2H, *J* 7.7 Hz), 8.17 (d, 2H, *J* 7.8 Hz), 9.16 (s, 2H), 9.17 (s, 2H). ^{13}C-

NMR (150 MHz, [D_6]DMSO): δ 21.4 (2 carb.), 23.1 (2 carb.), 24.1 (2 carb.), 24.9 (2 carb.), 25.0 (2 carb.), 25.1 (2 carb.), 28.4 (2 carb.), 28.9 (2 carb.), 29.0 (2 carb.), 35.2 (2 carb.), 36.6 (2 carb.), 36.8 (2 carb.), 40.4 (4 carb.), 40.7 (2 carb.), 50.9 (2 carb.), 52.1 (2 carb.), 52.2 (2 carb.), 53.9 (2 carb.), 54.1 (2 carb.), 114.8 (4 carb.), 114.9 (4 carb.), 127.5 (2 quat. carb.), 127.9 (2 quat. carb.), 129.9 (4 carb.), 130.0 (4 carb.), 155.7 (2 quat. carb.), 155.8 (2 quat. carb.), 155.6 (2 quat. carb.), 155.7 (2 quat. carb.), 170.8 (2 quat. carb.), 171.1 (2 quat. carb.), 171.8 (2 quat. carb.), 172.0 (2 quat. carb.), 172.3 (2 quat. carb.), 172.7 (2 quat. carb.). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $[C_{80}H_{126}N_{24}O_{16}]^{4+}$ 419.7440, found 419.7452. RP-HPLC (220 nm): 98% (t_R = 20.2 min, *k* = 6.0). $C_{80}H_{122}N_{24}O_{16} \cdot C_8H_4F_{12}O_8$ (1676.01 + 456.09).

At position Arg^2 *N*$^{\omega}$-[N-(4-aminobutyl)aminocarbonyl]-monosubstituted octanedioyl bis(Tyr-Arg-Leu-Arg-Tyr-amide) pentakis(hydrotrifluoroacetate) (2.17).[16] Compound **2.17** was synthesized on a Sieber resin (loading: 0.61 mmol/g). Fmoc-amino acids and arginine building block **2.11** were coupled according to the general procedure in the order Fmoc-Tyr(*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, **2.11** and Fmoc-Tyr(*t*Bu)-OH followed by the coupling of building block **2.13** (50 mg, 32 μmol) using HBTU/HOBt/DIPEA (1/1/2 equiv. to **2.13**) and anhydrous DMF (800 μl) as solvent (35 °C, 16 h). The peptide was cleaved from the resin according to the general procedure. The residue obtained after lyophilisation was dissolved in TFA/water (95:5 v/v) (2 mL), the mixture was stirred at rt for 2.5 h and poured into ice-cold diethyl ether (15 mL) causing the precipitation of the crude product, which was separated from the organic solvent by centrifugation and decantation. Purification by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 13:87–43:57, t_R = 16 min) afforded **2.17** as a white fluffy solid (22 mg, 41%). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 0.83 (d, 6H, *J* 6.5 Hz), 0.87 (d, 6H, *J* 6.5 Hz), 1.03 (br s, 4H), 1.30 (br s, 4H), 1.36−1.57 (m, 20H), 1.57−1.75 (m, 6H), 1.94−2.06 (m, 4H), 2.57−2.65 (m, 2H), 2.68−2.75 (m, 2H), 2.75−2.82 (m, 2H), 2.82−2.90 (m, 4H), 3.02−3.14 (m, 8H), 3.24 (br s, 2H), 4.17−4.23 (m, 2H), 4.24−4.36 (m, 6H), 4.39−4.46 (m, 2H), 6.59−6.65 (m, 8H), *ca* 6.95 (br s, 6H), 6.95−6.99 (m, 4H), 6.99−7.03 (m, 4H), 7.05 (s, 2H), *ca* 7.3 (br s, 6H), 7.33−7.39 (m, 2H), 7.52−7.63 (m, 4H), 7.67−7.79 (m, 4H), 7.84−7.95 (m, 4H), 7.99−8.08 (m, 2H), 8.17 (d, 1H, *J* 7.8 Hz), 8.20 (d, 1H, *J* 7.8 Hz), 8.45 (br s, 2H), 9.00 (s, 1H), 9.17 (br s, 4H), 10.38 (s, 1H). ^{13}C-NMR (150 MHz, [D_6]DMSO): δ 21.4 (2 carb.), 23.1, 23.1, 24.1 (2 carb.), 24.4, 24.9 (3 carb.), 25.0, 25.1 (2 carb.), 26.0, 28.4 (2 carb.), 28.9, 29.0 (3 carb.), 35.2 (2 carb.), 36.6 (2 carb.), 36.8 (2 carb.), 38.5, 38.6, 40.4 (3 carb.), 40.5, 40.7 (2 carb.), 51.0 (2 carb.), 50.1 (2 carb.), 52.26,

52.29, 53.89, 53.92, 54.1, 54.2, 114.8 (4 carb.), 114.9 (4 carb.), 115.6 (TFA), 117.6 (TFA), 127.5 (2 quat. carb.), 127.89, 127.91, 130.02 (4 carb.), 130.04 (4 carb.), 153.7, 153.8, 155.75 (2 quat. carb.), 155.82 (2 quat. carb.), 156.71 (2 quat. carb.), 156.73, 158.5 (q, *J* 33 Hz) (TFA), 170.81, 170.84, 171.08, 171.13, 171.8, 171.9, 172.0 (2 quat. carb.), 172.31, 172.34, 172.7 (2 quat. carb.). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $[C_{85}H_{136}N_{26}O_{17}]^{4+}$ 448.2639, found 448.2652. RP-HPLC (220 nm): 96%, (t_R = 19.3 min, *k* = 5.7). $C_{85}H_{132}N_{26}O_{17} \cdot C_{10}H_5F_{15}O_{10}$ (1790.16 + 570.11).

At position Arg^2 N^{ω}-[N-(4-propanoylaminobutyl)aminocarbonyl]-monosubstituted octanedioyl bis(Tyr-Arg-Leu-Arg-Tyr-amide) tetrakis(hydrotrifluoroacetate) (2.18).[16] Compound **2.17** (10 mg, 4.24 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Succinimidyl propionate (0.8 mg, 4.7 μmol) was added and the mixture was stirred at rt for 2 h. 0.2% aq TFA (9 mL) was added and the product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 12:88–43:57, t_R = 18 min). Lyophilisation of the eluate afforded **2.18** as a white fluffy solid (5.7 mg, 58%). ^{1}H-NMR (600 MHz, [D$_6$]DMSO): δ 0.83 (d, 6H, *J* 6.5 Hz), 0.87 (d, 6H, *J* 6.6 Hz), 0.98 (t, 3H, 7.7 Hz), 1.03 (br s, 4H), 1.30 (br s, 4H), 1.34−1.57 (m, 20H), 1.57−1.75 (m, 6H), 1.94−2.07 (m, 6H), 2.57−2.64 (m, 2H), 2.69−2.75 (m, 2H), 2.82−2.88 (m, 4H), 2.99−3.13 (m, 10H), 3.23 (br s, 2H), 4.17−4.24 (m, 2H), 4.24−4.36 (m, 6H), 4.40−4.46 (m, 2H), 6.59−6.65 (m, 8H), *ca* 6.85 (br s, 6H), 6.95−6.99 (m, 4H), 6.99−7.03 (m, 4H), 7.05 (s, 2H), *ca* 7.3 (br s, 6H), 7.37 (s, 2H), 7.46−7.53 (m, 4H), 7.69−7.77 (m, 3H), 7.84−7.93 (m, 4H), 8.03 (m, 2H), 8.18 (m, 2H), 8.40 (br s, 2H), 8.96 (s, 1H), 9.16 (s, 4H), 9.87 (s, 1H). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $[C_{88}H_{140}N_{26}O_{18}]^{4+}$ 462.2704, found 462.2717. RP-HPLC (220 nm): 96%, (t_R = 21.1 min, *k* = 6.3). $C_{88}H_{136}N_{26}O_{18} \cdot C_8H_4F_{12}O_8$ (1846.22 + 456.09).

Octanedioyl-bis(Tyr-Ala-Leu-Arg-Tyr-amide) bis(hydrotrifluoroacetate) (2.19). Compound **2.14** (16 mg, 13.8 μmol) was dissolved in anhydrous DMF/DIPEA (99:1, 600 μL). Compound **2.12** (2.5 mg, 6.78 μmol) was added and the mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate was extracted with DCM (2 × 10 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 10:90–62:38, t_R = 15.5

min). Lyophilisation of the eluate afforded **2.19** as a white fluffy solid (5.7 mg, 48.5%). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 0.82 (d, 6H, *J* 6.4 Hz), 0.86 (d, 6H, *J* 6.6 Hz), 1.03 (t, 4H, *J* 6.3 Hz), 1.15 (d, 6H, *J* 7.0 Hz), 1.26-1.34 (m, 4H), 1.38-1.56 (m, 10H), 1.56-1.63 (m, 2H), 1.63-1.71 (m, 2H), 1.94-2.03 (m, 4H), 2.56-2.63 (m, 2H), 2.67-2.73 (m, 2H), 2.81-2.88 (m, 4H), 3.04-3.12 (m, 4H), 4.14-4.21 (m, 2H), 4.24-4.32 (m, 6H), 4.38-4.45 (m, 2H), 6.57-6.64 (m, 8H), *ca* 6.8 (br s, 4H), 6.94-6.98 (m, 4H), 6.98-7.02 (m, 4H), 7.04 (s, 2H), *ca* 7.2 (br s, 4H), 7.31 (s, 2H), 7.43 (t, 2H, *J* 5.6 Hz), 7.65 (d, 2H, *J* 7.9 Hz), 7.88 (d, 2H, *J* 8.3 Hz), 7.91 (d, 2H, *J* 8.0 Hz), 8.02 (d, 2H, *J* 7.1 Hz), 8.11 (d, 2H, *J* 7.8 Hz), 9.13 (s, 4H). HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{74}H_{111}N_{18}O_{16}$, 502.6136; found, 502.6146. RP-HPLC (220 nm): 96% (t_R = 23.27 min, *k* = 7.1). $C_{74}H_{108}N_{18}O_{16} \cdot C_4H_2F_6O_4$ (1505.79 + 228.04).

Octanedioyl-1-(Tyr-Arg-Leu-Arg-Tyr-amide)-8-(Tyr-Ala-Leu-Arg-Tyr-amide) tris(hydrotrifluoroacetate) (2.20). Compound **2.13** (37.0 mg, 24.1 μmol), HOBt (3.69 mg, 1 equiv) and HBTU (9.14 mg, 1 equiv) were dissolved in 500 μL of DMF. DIPEA (14.5 μL, 3.5 equiv) was added, and the mixture was stirred for 5 min prior to the addition of a solution of **2.14** (30.6 mg, 1.1 equiv) in DMF (400 μL). The mixture was stirred at room temperature for 17 h, water (15 mL) was added, and the protected intermediate was extracted with DCM (2 ×20 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/H_2O (95:5, 3 mL) was added and the mixture was stirred at rt for 2.5 h. Water (150 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 10:90–62:38, t_R = 14.1 min). Lyophilisation of the eluate afforded **20** as a white fluffy solid (16.6 mg, 35.6%). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 0.83 (d, 6H, *J* 6.6 Hz), 0.86 (d, 6H, *J* 6.5 Hz), 1.00-1.07 (m, 4H), 1.19 (d, 3H, *J* 7.0 Hz), 1.25-1.34 (m, 4H), 1.36-1.55 (m, 13H), 1.56-1.72 (m, 5H), 1.95-2.03 (m, 4H), 2.55-2.63 (m, 2H), 2.67-2.74 (m, 2H), 2.81-2.89 (m, 4H), 3.01-3.12 (m, 6H), 4.16-4.23 (m, 2H), 4.23-4.35 (m, 6H), 4.37-4.44 (m, 2H), 6.57-6.65 (m, 8H), 6.94-7.02 (m, 8H), *ca.* 6.95 (br s, 6H), 7.04 (d, 2H, *J* 7.8 Hz), *ca.* 7.3 (br s, 6H), 7.34 (d, 2H, *J* 5.9 Hz), 7.53 (t, 1H, *J* 5.6 Hz), 7.56 (t, 2H, *J* 5.5 Hz), 7.74 (d, 1H, *J* 7.9 Hz), 7.77 (d, 1H, *J* 7.9 Hz), 7.85-7.95 (m, 5H), 8.01 (d, 1H, *J* 7.9 Hz), 8.11 (d, 1H, *J* 7.4 Hz), 8.15 (d, 1H, *J* 7.8 Hz), 9.16 (s, 4H). ^{13}C-NMR (150 MHz, [D_6]DMSO): δ 17.88, 21.37, 21.46, 23.07, 23.11, 24.12 (2 carb.), 24.79, 24.88, 25.02 (2 carb.), 25.06, 28.32 (2 carb.), 28.92, 28.96, 29.06, 35.14 (2 carb.), 36.53, 36.64, 36.75, 36.78, 40.41 (3 carb.), 40.53, 40.68, 48.06, 50.97, 51.07, 52.07, 52.09, 52.25, 53.92 (2 carb.), 54.03, 54.19, 114.75 (2 carb.), 114.77 (2 carb.), 114.85 (4 carb.), 127.48, 127.56, 127.90, 127.97,

129.97 (2 carb.), 130.01 (6 carb.), 155.69, 155.73, 155.79, 155.80, 156.68, 156.71 (2 quat. carb.), 170.76, 170.82, 171.13, 171.47, 171.86, 171.98, 171.99, 172.10, 172.25, 172.35, 172.68, 172.69. HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{77}H_{118}N_{21}O_{16}$, 530.9683; found, 530.9692. RP-HPLC (220 nm): 99% (t_R = 19.57 min, *k* = 5.8). $C_{77}H_{115}N_{21}O_{16} \cdot C_6H_3F_9O_6$ (1590.90 + 342.06).

Octanedioyl-bis(Tyr-Arg-Leu-Ala-Tyr-amide) bis(hydrotrifluoroacetate) (2.21). Compound **2.15** (12 mg, 10.4 μmol) was dissolved in anhydrous DMF/DIPEA (99:1, 400 μL). Compound **2.12** (1.9 mg, 5.1 μmol) was added and the mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate was extracted with DCM (2 × 10 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 10:90–62:38, t_R = 15.3 min). Lyophilisation of the eluate afforded **21** as a white fluffy solid (3.7 mg, 41.8%). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 0.82 (d, 6H, *J* 6.7 Hz), 0.86 (d, 6H, *J* 6.5 Hz), 1.03 (t, 4H, *J* 6.5 Hz), 1.19 (d, 6H, *J* 7.1 Hz), 1.26-1.34 (m, 4H), 1.35-1.53 (m, 10H), 1.54-1.68 (m, 4H), 1.98 (t, 4H, *J* 7.3 Hz), 2.56-2.62 (m, 2H), 2.67-2.73 (m, 2H), 2.81-2.88 (m, 4H), 3.02-3.08 (m, 4H), 4.19-4.29 (m, 6H), 4.30-4.35 (m, 2H), 4.37-4.43 (m, 2H), 6.57-6.64 (m, 8H), *ca* 6.7 (br s, 4H), 6.94-6.98 (m, 4H), 6.99-7.02 (m, 4H), 7.03 (s, 2H), *ca* 7.2 (br s, 4H), 7.34 (s, 2H), 7.41 (t, 2H, *J* 5.7 Hz), 7.76 (d, 2H, *J* 7.9 Hz), 7.85-7.93 (m, 6H), 8.09 (d, 2H, *J* 7.2 Hz), 9.13 (s, 4H). HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{74}H_{111}N_{18}O_{16}$, 502.6136; found, 502.6143. RP-HPLC (220 nm): 97% (t_R = 22.17 min, *k* = 6.7). $C_{74}H_{108}N_{18}O_{16} \cdot C_4H_2F_6O_4$ (1505.79 + 228.04).

Octanedioyl-1-(Tyr-Arg-Leu-Arg-Tyr-amide)-8-(Tyr-Arg-Leu-Ala-Tyr-amide) tris(hydrotrifluoroacetate) (2.22). Compound **2.13** (28.1 mg, 18.3 μmol), HOBt (2.8 mg, 1 equiv) and HBTU (6.94 mg, 1 equiv) were dissolved in 500 μL DMF. DIPEA (11 μL, 3.5 equiv) was added, and the mixture was stirred for 5 min followed by the addition of a solution of **2.15** (23.2 mg, 1.1 equiv) in DMF (400 μL). The mixture was stirred at room temperature for 18 h, water (15 mL) was added and the protected intermediate was extracted with DCM (2 x 20 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 3 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (150 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 10:90–62:38,

t_R = 14.2 min). Lyophilisation of the eluate afforded **2.22** as a white fluffy solid (12.5 mg, 35.3%). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 0.82 (d, 6H, *J* 6.5 Hz), 0.86 (d, 6H, *J* 6.6 Hz), 0.99-1.07 (m, 4H), 1.15 (d, 3H, *J* 7.1 Hz), 1.25-1.35 (m, 4H), 1.36-1.55 (m, 13H), 1.56-1.73 (m, 5H), 1.94-2.04 (m, 4H), 2.55-2.64 (m, 2H), 2.66-2.74 (m, 2H), 2.80-2.89 (m, 4H), 3.01-3.12 (m, 6H), 4.14-4.22 (m, 2H), 4.23-4.34 (m, 6H), 4.38-4.45 (m, 2H), 6.58-6.65(m, 8H), *ca.* 6.9 (br s, 6H), 6.93-7.02 (m, 8H), 7.04 (s, 2H), *ca.* 7.3 (br s, 6H), 7.31 (s, 1H), 7.35 (s, 1H), 7.52 (t, 1H, *J* 5.6 Hz), 7.53-7.57 (m, 2H), 7.66 (d, 1H, *J* 8.0 Hz), 7.74 (d, 1H, *J* 7.9 Hz), 7.85-7.95 (m, 4H), 7.99-8.05 (m, 2H), 8.11 (d, 1H, *J* 7.7 Hz), 8.15 (d, 1H, *J* 7.8 Hz), 9.16 (s, 4H). ^{13}C-NMR (150 MHz, [D_6]DMSO): δ 17.92, 21.36, 21.41, 23.09, 23.11, 24.10, 24.11, 24.87, 24.96, 25.02, 25.04 (2 carb.), 28.32 (2 carb.), 29.93, 28.97, 29.07, 35.15 (2 carb.), 36.56, 36.59, 36.71, 36.78, 40.42 (3 carb.), 40.59, 40.69, 48.38, 50.79, 50.95, 52.02, 52.08, 52.24, 53.91 (2 carb.), 54.07, 54.14, 114.75 (2 carb.), 114.76 (2 carb.), 114.82 (2 carb.), 114.85 (2 carb.), 127.47, 127.59, 127.89, 127.92, 129.98 (4 carb.), 130.01 (2 carb.), 130.05 (2 carb.), 155.70, 155.72, 155.76, 155.80, 156.67, 156.69, 156.70, 170.08, 171.06, 171.11, 171.64, 171.68 (2 quat. carb.), 171.98, 172.24, 172.33, 172.66, 172.67. HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{77}H_{118}N_{21}O_{16}$, 530.9683; found, 530.9689. RP-HPLC (220 nm): 99% (t_R = 19.97 min, *k* = 6.0) $C_{77}H_{115}N_{21}O_{16} \cdot C_6H_3F_9O_6$ (1590.90 + 342.06).

H-Tyr(*t*Bu)-Arg(Pbf)-Leu-Arg(Pbf)-Ala-NH_2 hydrotrifluoroacetate (2.23). The side chain protected pentapeptide **2.23** was prepared on a Sieber resin (loading: 0.61 mmol/g) according to the general procedure. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 43:57–81:19, t_R = 11.8 min). Lyophilisation of the eluate afforded **2.23** as a white solid (47 mg, 63%). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{60}H_{94}N_{12}O_{12}S_2]^{2+}$ 619.3272, found 619.3276. $C_{60}H_{92}N_{12}O_{12}S_2 \cdot C_2HF_3O_2$ (1239.60 + 114.02).

H-Tyr(*t*Bu)-Arg(Pbf)-Ala-Arg(Pbf)-Tyr(*t*Bu)-NH_2 hydrotrifluoroacetate (2.24). The side chain protected pentapeptide **2.24** was prepared on a Sieber resin (loading: 0.61 mmol/g) according to the general procedure. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–16 min: MeCN/0.1% aq TFA 43:57–81:19, t_R = 14.5 min). Lyophilisation of the eluate afforded **2.24** as a white solid (47 mg, 29%). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{67}H_{100}N_{12}O_{13}S_2]^{2+}$ 672.3492, found 462.5838. $C_{67}H_{98}N_{12}O_{13}S_2 \cdot C_2HF_3O_2$ (1385.79 + 114.02).

H-Ala-Arg(Pbf)-Leu-Arg(Pbf)-Tyr(*t*Bu)-NH_2 hydrotrifluoroacetate (2.25). The side chain protected pentapeptide **2.25** was prepared on a Sieber resin (loading: 0.61 mmol/g) according to the general procedure. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–18 min: MeCN/0.1% aq TFA 43:57–81:19, t_R = 12.4 min). Lyophilisation of the eluate afforded **2.25** as a white solid (36 mg, 48%). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{60}H_{94}N_{12}O_{12}S_2]^{2+}$ 619.3272, found 619.3278. $C_{60}H_{92}N_{12}O_{12}S_2$ · $C_2HF_3O_2$ (1239.60 + 114.02).

H-Thr(*t*Bu)-Arg(Pbf)-Pro-Arg(Pbf)-Tyr(*t*Bu)-NH_2 hydrotrifluoroacetate (2.26). The side chain protected pentapeptide **2.26** was prepared on a Sieber resin (loading: 0.61 mmol/g) according to the general procedure. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–18 min: MeCN/0.1% aq TFA 43:57–81:19, t_R = 12.3 min). Lyophilisation of the eluate afforded **2.26** as a white solid (76 mg, 87%). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{64}H_{100}N_{12}O_{13}S_2]^{2+}$ 654.3482, found 654.3489. $C_{64}H_{98}N_{12}O_{13}S_2$ · $C_2HF_3O_2$ (1307.68 + 114.02).

Octanedioyl-bis(Tyr-Arg-Leu-Arg-Ala-amide) tetrakis(hydrotrifluoroacetate) (2.27). Compound **2.23** (16.6 mg, 12.2 μmol) was dissolved in anhydrous DMF/DIPEA (99:1, 400 μL). Compound **2.12** (1.8 mg, 4.9 μmol) was added and the mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate was extracted with DCM (2 × 10 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 3:97–42:58, t_R = 16.6 min). Lyophilisation of the eluate afforded **2.27** as a white fluffy solid (4.1 mg, 43%). HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{68}H_{117}N_{24}O_{14}$, 497.9722; found, 497.9738. RP-HPLC (220 nm): 98% (t_R = 18.97 min, *k* = 5.6). $C_{68}H_{114}N_{24}O_{14}$ · $C_8H_4F_{12}O_8$ (1491.81 + 456.08).

Octanedioyl-bis(Tyr-Arg-Ala-Arg-Tyr-amide) tetrakis(hydrotrifluoroacetate) (2.28). Compound **2.24** (19.5 mg, 13 μmol) was dissolved in anhydrous DMF/DIPEA (99:1, 400 μL). Compound **2.12** (2.0 mg, 5.2 μmol) was added and the mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate was extracted with DCM (2 × 10 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100

mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 3:97–42:58, t_R = 15.2 min). Lyophilisation of the eluate afforded **2.28** as a white fluffy solid (5.2 mg, 49%). HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{74}H_{113}N_{24}O_{16}$, 531.2917; found, 531.2932. RP-HPLC (220 nm): 99% (t_R = 16.8 min, k = 4.9). $C_{74}H_{110}N_{24}O_{16} \cdot C_8H_4F_{12}O_8$ (1591.85 + 456.08).

Octanedioyl-bis(Ala-Arg-Leu-Arg-Tyr-amide) bis(hydrotrifluoroacetate) (2.29). Compound **2.25** (20.3 mg, 15 μmol) was dissolved in anhydrous DMF/DIPEA (99:1, 400 μL). Compound **2.12** (2.2 mg, 6.0 μmol) was added and the mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate was extracted with DCM (2 × 10 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 3:97–42:58, t_R = 16.4 min). Lyophilisation of the eluate afforded **2.29** as a white fluffy solid (6.0 mg, 51%). HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{68}H_{117}N_{24}O_{14}$, 497.9722; found, 497.9736. RP-HPLC (220 nm): 99% (t_R = 18.62 min, k = 5.5). $C_{68}H_{114}N_{24}O_{14} \cdot C_8H_4F_{12}O_8$ (1491.81 + 456.08).

Octanedioyl-bis(Thr-Arg-Pro-Ala-Tyr-amide) bis(hydrotrifluoroacetate) (2.30). Compound **2.26** (38.2 mg, 26.9 μmol) was dissolved in anhydrous DMF/DIPEA (99:1, 700 μL). Compound **2.12** (4.0 mg, 10.5 μmol) was added and the mixture was stirred at 35 °C for 16 h. Water (15 mL) was added and the protected intermediate was extracted with EtOAc (2 × 15 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 3:97–42:58, t_R = 13.0 min). Lyophilisation of the eluate afforded **2.30** as a white fluffy solid (11.4 mg, 56%). HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{68}H_{113}N_{24}O_{16}$, 507.2917; found, 507.2932. RP-HPLC (220 nm): 99% (t_R = 14.33 min, k = 4.0). $C_{68}H_{110}N_{24}O_{16} \cdot C_8H_4F_{12}O_8$ (1519.78 + 456.08).

2.4.3 Synthesis of the Radioligands [^{3}H]2.10 and [^{3}H]2.18

The radiolabeled peptides [^{3}H]**2.10** and [^{3}H]**2.18** were prepared by [^{3}H]propionylation of the precursor peptides (2*R*,7*R*)-**2.2** and **2.17**, respectively. A solution of succinimidyl [^{3}H]propionate (specific activity: 80 Ci/mmol, purchased from American Radiolabeled Chemicals, St. Louis, MO via Hartman Analytics, Braunschweig, Germany) (2.5 mCi, 5.5 μg, 32 nmol) in hexane/EtOAc (9:1) was transferred from the delivered ampoule into a 1.5 mL reaction vessel with screw cap, and the solvent was removed in a vacuum concentrator (30 °C, 30 min). A solution of the precursor peptide ((2*R*,7*R*)-**2.2**: 0.6 mg, 260 nmol, or **2.17**: 0.8 mg, 260 nmol) in anhydrous DMF containing 3% DIPEA (60 μL) was added, and the vessel was vigorously shaken at rt for 3 h. The mixture was acidified by addition of 2% aqueous TFA (80 μL) followed by addition of MeCN/H_2O (10:90) (400 μL). [^{3}H]**2.10** and [^{3}H]**2.18** were purified using a HPLC system from Waters (Eschborn, Germany) consisting of two 510 pumps, a pump control module, a 486 UV/VIS detector and a Flow-one beta series A-500 radiodetector (Packard, Meriden, USA). A Luna C_{18}(2) column (3 μm, 150 × 4.6 mm, Phenomenex, Aschaffenburg, Germany) was used as stationary phase at a flow rate of 0.8 mL/min. Mixtures of acetonitrile supplemented with 0.04% TFA (A) and 0.05% aq TFA (B) were used as mobile phase. The following linear gradient was applied: A/B: 0–20 min: 8:92 – 33:67, 20–22 min: 33:67 – 95:5, 22–32 min: 95:5. For the purification of each radiolabeled peptide four HPLC runs were performed. The radioligands (t_R = 17.3 min ([^{3}H]**2.10**) and t_R = 19.8 min ([^{3}H]**2.18**)) were collected in 2-mL reaction vessels with screw cap, and the volumes of the combined eluates were reduced by evaporation to approximately 600 μL. The same volume of ethanol (600 μL) was added, and the solution was diluted with 50% ethanol to a volume of 1500 μL for each radioligand. For the quantification of the radioligands, a four-point calibration was performed with (2*R*,7*R*)-**2.10** (0.15, 0.3, 0.45 and 0.6 μM; injection volume: 100 μL, UV-detection: 225 nm) or **2.18** (0.1, 0.2, 0.3 and 0.4 μM; injections volume: 100 μL, UV-detection: 225 nm) using the above mentioned HPLC system and gradient. Aliquots ([^{3}H]**2.10**: 2 μL, [^{3}H]**2.18**: 2 μL) of the respective radioligand solutions were added to acetonitrile/0.05% aqueous TFA (10:90) (128 μL). 100 μL of the resulting solutions were analyzed by HPLC and 5 × 3 μL were counted in 3 mL of liquid scintillator (Rotiszint eco plus, Carl Roth, Karlsruhe, Germany) with a LS 6500 liquid scintillation counter (Beckman Coulter, Krefeld, Germany). To determine the radiochemical purity and to prove the identity of the radioligands, a solution of the radiolabeled peptide (100 μL, 0.35 μM), spiked with 'cold'

(2*R*,7*R*)-**2.10** (5 μM) or **2.18** (5 μM), was analyzed using the same HPLC system as for quantification and simultaneous radiometric detection (Rotiszint eco plus/acetonitrile, 85:15 (v/v), flow rate: 4.0 mL/min). The radiochemical purities were > 99%. Analyses repeated after storage at -20 °C for 9 months revealed a radiochemical purities of 87% ([^{3}H]**2.10**) and 90% ([^{3}H]**2.18**), respectively. Calculated specific activity: 0.960 TBq/mmol (25.94 Ci/mmol, [^{3}H]**2.10**) or 0.976 TBq/mmol (26.37 Ci/mmol, [^{3}H]**2.18**). The activity concentration was adjusted to 15.00 MBq/ml by the addition of 50% ethanol amounting to final concentrations of 15.6 μM [^{3}H]**2.10**) and 15.4 μM ([^{3}H]**2.18**), respectively. Chemical yields: penta(hydrotrifluoroacetate) of [^{3}H]**2.10**: 75.6 μg, 32.4 nmol, and tetra(hydrotrifluoroacetate) of [^{3}H]**2.18**: 64.7 μg, 28.1 nmol. Radiochemical yields: [^{3}H]**2.10**: 0.842 mCi (31.14 MBq), 34%, [^{3}H]**2.18**: 0.741 mCi (27.39 MBq), 30%.

2.4.4 Investigation of the Chemical Stability

The chemical stability of 'cold' (2*R*,7*R*)-**2.10** and 'cold' **2.18** was investigated in buffer I at 22 ± 1 °C. The incubation was started by addition of 20 μL of a 1 mM aqueous solution of (2*R*,7*R*)-**2.10** or **2.18** to the buffer (780 μL or 580 μL) to give final concentrations of 25 μM ((2*R*,7*R*)-**2.10**) and 33.35 μM (**2.18**), respectively. After 0, 12 and 48 h, aliquots (100 μL) were taken, and 100 μL of 1% aqueous TFA were added. The resulting solutions were analyzed by RP-HPLC (cf. general experimental conditions).

2.4.5 Pharmacological Assays

Cells. The HEC-1B human endometrial cancer cell line and the MCF-7 (HTB 22) human breast cancer cells were from the American Type Culture Collection (Rockville, MD). A subclone of the MCF-7 cell line that shows higher Y_1R expression was established in our laboratory and used for binding experiments.[31] Human erythroleukemia (HEL) cells were kindly provided by Dr. M. C. Michel (Universitätsklinikum Essen, Germany). Human embryonal kidney cells (HEK-293 cells) and chinese hamster ovarian (CHO) cells were from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Genetically engineered cells, used for binding and functional assays, were generated and cultured as described (cf. brief description of protocols).

HEK293-hY$_4$-CRE Luc cells. HEK293 cells were stably co-transfected with the pGL4.29[luc2P/CRE/Hygro] plasmid (Promega, Mannheim, Germany) encoding hygromycin B resistance and the firefly luciferase, the transcription of which is controlled by the cAMP responsive element (HEK293-CRE Luc cells, ref.[24]) and pcDNA3.1 hY$_4$ vector (cDNA Resource Center; Bloomsberg, PA, USA) encoding the hY$_4$R (HEK293-hY$_4$-CRE Luc cells) and neomycin resistance. For transfection, the cells were seeded into a 24 well-plate (Becton Dickinson), so that they reached 60–70% confluency on the next day. The transfection mixture containing 2 μg of the DNA and 8 μL of FuGene HD transfection reagent (Roche Diagnostics, Mannheim, Germany) was prepared according to the manufacturer's protocol and added to the cells, followed by an incubation period of 48 h at 37 °C and 5% CO_2 in a water-saturated atmosphere.

Routinely performed examinations for mycoplasma contamination using the Venor GeM Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany) were negative for all cell types.

Cell Culture. Cells were cultured in 25- or 75-cm^2 flasks (Sarstedt, Nümbrecht, Germany) in a humidified atmosphere (95% air, 5% CO_2) at 37 °C. MCF-7-Y$_1$ cells,[30] HEL cells,[42] CHO-hY$_2$-G$_{qi5}$-mtAEQ cells[23] and CHO-hY$_4$-G$_{qi5}$-mtAEQ cells[23] were cultured as described previously. HEK293T-hY$_4$-CRE Luc cells were cultured in Dulbecco`s Modified Eagle Medium (DMEM) (Sigma-Aldrich, Deisenhofen, Germany) containing L-glutamine (Sigma-Aldrich), 4500 mg/L glucose, 3.7 g/L $NaHCO_3$ (Merck, Darmstadt, Germany), 110 mg/L sodium pyruvate (Serva, Heidelberg, Germany), 10 % fetal calf serum (FCS) (Biochrom, Berlin, Germany) and the selection antibiotics G418 (600 μg/mL) (Biochrom) and hygromycin B (250 μg/mL) (MoBiTec GmbH, Göttingen, Germany). HEC-1B cells expressing the human Y$_5$R were cultured as previously described.[34]

Buffers and Media Used in Binding and Functional Experiments. *Buffer I*. A sodium-free HEPES buffer (25 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) was used in binding experiments at the Y$_2$R and Y$_4$R. *Buffer II.* Binding experiments at the Y$_1$R and Y$_5$R were performed in a buffer containing 150 mM NaCl, 10 mM HEPES, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 5 mM KCl. *Buffer III*. For the aequorin assay on CHO cells stably expressing the Y$_4$R the buffer contained 120 mM NaCl, 25 mM HEPES, 1.5 mM $CaCl_2$, $MgCl_2$, 5 mM KCl, 10 mM D-glucose. *Leibovitz' L-15 medium* (containing 140 mM NaCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$,

amino acids, vitamins, pH 7.4) was used to perform additional binding experiments with selected compounds at the Y_4R. *DMEM* (without phenol red, containing 110 mM NaCl, 44 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.8 mM MgCl, 5.6 mM D-glucose, vitamins, amino acids, pH 7.4) was used to perform the luciferase assay.

Radioligand Binding Assays. Y_1R binding. Radioligand binding assays for all receptor subtypes were performed at 22 ± 1 °C. Competition binding experiments with the radioligand [^{3}H]UR-MK136 (K_d = 6.2 nM, c = 4 nM) were performed at intact MCF-7-Y_1 cells as previously described[30] with the following modifications: Experiments were carried out in 96-well plates with clear bottom (Corning Incorporated Life Sciences, Tewksbury, MA; Corning cat. no. 3610), and the volume per well was reduced to 100 μL. After incubation, the cells were washed with buffer II (200 μL) twice and covered with lysis solution (25 μL) consisting of urea, acetic acid and Triton-X-100 in water. The plates were shaken for 30 min prior to addition of liquid scintillator (Optiphase Supermix, PerkinElmer, Überlingen, Germany) (200 μL). The wells were sealed with a transparent film (permanent seal for microplates, PerkinElmer, prod. no. 1450–461). The plates were shaken to mix the scintillator and the aqueous phase and kept in the dark for at least 30 min. Radioactivity (dpm) was measured with a MicroBeta2 plate counter (PerkinElmer, Rodgau, Germany).

Y_2R binding. Competition binding experiments were performed at CHO-hY_2R-G_{qi5}-mtAEQ cells[33] with [^{3}H]propionyl-pNPY (K_d = 1.4 nM, c = 1 nM).[32] One day prior to the experiment, the cells were adjusted to a density of approximately 170 000 per mL in Ham's F12 (Sigma Aldrich) supplemented with 10 % FCS. 200 μL per well were seeded into 96-well plates with clear bottom (Corning Incorporated Life Sciences, Tewksbury, MA; cat. no. 3610). The cells were allowed to attach overnight at 37 °C, 5% CO_2 in a water-saturated atmosphere. On the day of the experiment, the confluency of the cells was >90%. The culture medium was removed by suction, the cells were washed with buffer I (200 μL) and covered with binding buffer (buffer I supplemented with 1% BSA and 0.1 mg/mL bacitracin) (80 μL). For radioligand displacement experiments, binding buffer containing the competitor 10-fold concentrated (10 μL) and binding buffer containing the radioligand 10-fold concentrated were added. The cells were incubated for 90 min, the buffer was removed by suction, and the cells were washed twice with ice-cold buffer I (200 μL), before 25 μL of lysis solution (25 mM tricine; glycerol 10 % (v/v); EGTA, 2 mM; 1% (v/v) Triton X-100; $MgSO_4 \cdot 7\ H_2O$, 5 mM;

DTT, 1 mM) were added. The plates were shaken for 30 min prior to addition of liquid scintillator (Optiphase Supermix, 200 μL). The sealed plates were processed as described above.

Y_4R binding. Binding assays with the radioligands [^{3}H]**2.10** or [^{3}H]**2.18** were performed at intact CHO-hY_4R-G_{qi5}-mtAEQ cells.[23] For saturation and competition binding experiments, cells were grown to 80% - 100% confluency, scraped off the culture flask and centrifuged for 5 min at 300 g. The culture medium was discarded, and the cells were resuspended at a density of 500,000 cells/mL in buffer I or Leibovitz' L-15 medium, both supplemented with 1% BSA and 0.1 mg/mL bacitracin. Both, saturation and competition experiments were performed in a final volume of 200 μL in Primaria 96-well plates (Corning Life Sciences, Oneonta, NY). Saturation binding experiments were performed in a concentration range of 0.14 - 11 nM, when using buffer I, or 0.5 - 30 nM, when using L-15 medium. Competition binding experiments were performed with increasing concentrations of unlabeled compounds and radioligand concentrations in the range of the respective K_d value (0.6 nM in case of [^{3}H]**2.10** in buffer I (sodium-free HEPES buffer), 10 nM of [^{3}H]**2.10** in L-15 medium, 1 nM of [^{3}H]**2.18** in buffer I). Nonspecific binding was determined in the presence of a 200-fold excess of (2*R,*7*R*)-**2.2** (when using [^{3}H]**2.10**) or **2.17** (when using [^{3}H]**2.18**), respectively. Incubation period was 90 min. Bound and free radioligand were separated by filtration through 0.3% polyethyleneimine pre-treated GF/C filters (Whatman, Maidstone, UK) using a Brandel Harvester (Brandel, Gaithersburg, MD, USA). Filter pieces for each well were punched out and transferred into 96-well plates 1450-401 (PerkinElmer) and scintillation cocktail (200 μL, Rotiscint eco plus) was added. After incubation in the dark for 60 min, radioactivity (dpm) was measured with a MicroBeta2 plate counter (PerkinElmer).

For kinetic experiments, the cells were adjusted to a density of approximately 170,000 per mL in Ham's F12 (Sigma Aldrich) supplemented with 10 % FCS. Cells were seeded in a volume of 200 µL per well into Primaria 96-well plates one day before the experiment. The cells were allowed to attach overnight at 37 °C, 5% CO_2 in a water-saturated atmosphere. On the day of the experiment, confluency of the cells was approximately 90%. The culture medium was removed by suction, the cells were washed with 200 μL of buffer I and covered with 160 μL of binding buffer (buffer I supplemented with 1% BSA and 0.1 mg/mL bacitracin) per well. Unspecific binding was determined in the presence of (2*R,*7*R*)-**2.2** in experiments with [^{3}H]**2.10** or **2.17** in experiments with [^{3}H]**2.18**. For association experiments, the radioligand

concentration was 1.5 nM. The incubation of the cells was stopped after different periods of time (2 – 180 min) by removing the radioligand-containing medium, and cells were washed twice with ice-cold buffer I (200 μL). In case of dissociation experiments, cells were pre-incubated with [^{3}H]**2.10** or [^{3}H]**2.18** at a concentration of 1.5 nM for 120 min. The solution was removed by suction and the cells were covered with binding buffer (200 μL) containing (2*R*,7*R*)-**2.2** (300 nM) or **2.17** (300 nM). After different periods of time (2 – 360 min) the cells were washed with ice-cold buffer I. After addition of lysis solution (25 μL), the plates were shaken for 30 min, and the content of the wells was transferred into 96-well plates 1450-401, followed by the addition of 200 μL of liquid scintillator (Optiphase Supermix). The plates were sealed and processed as described above.

Y_5R binding. Competition binding experiments were performed at HEC-1B-hY_5 cells[34] with [^{3}H]propionyl-pNPY (K_d = 4.8 nM, c = 4 nM).[32] One or two days prior to the experiment, the cells were seeded into 96-well plates with clear bottom (Corning cat. no. 3610). The cells were allowed to attach overnight at 37 °C, 5% CO_2 in a water-saturated atmosphere. On the day of the experiment, confluency of the cells was approximately 90%. The culture medium was removed by suction and the cells were washed with buffer II (200 μL) and covered with 80 μL of binding buffer (buffer II supplemented with 1% BSA and 0.1 mg/mL bacitracin). For displacement experiments, 10 μL of binding buffer containing the competitor (10-fold concentrated) and 10 μL of binding buffer containing the radioligand (10-fold concentrated) were added. The cells were incubated for 120 min. The solution was removed by suction and the cells were washed with ice-cold buffer II (2 × 200 μL) followed by the addition of lysis solution (25 μL). The plates were shaken for 30 min followed by the addition of 200 μL of liquid scintillator (Optiphase Supermix). The plates were sealed and processed as described above.

Functional Assays. Fura-2 Calcium Assay. The assay was performed for the functional characterization of selected ligands at the human Y_1 receptor using HEL cells and a LS-50B luminescence spectrometer (Perkin Elmer, Überlingen, Germany) as previously described.[42,43]

Aequorin Calcium Assay. The assay was performed on CHO-hY_4-G_{qi5}-mtAEQ cells in buffer III as previously described[23] using a GENios Pro plate reader (Tecan, Salzburg, Austria). Areas under the curve were calculated using SigmaPlot 12.5 software (Systat Software Inc.,

Chicago, IL). Luminescence data were normalized to the effect caused by 1 μM PP (measured in triplicate on every 96-well plate).

Luciferase Assay. The Luciferase assay was performed on HEK293-hY_4-CRE Luc cells. One day prior to the experiment, the cells were adjusted to a density of approximately 800,000 per mL in DMEM without phenol red supplemented with 5 % FCS. Cells were seeded in a volume of 160 μL per well into 96-well plates with clear bottom (Corning cat. no. 3610), and allowed to attach overnight at 37 °C, 5% CO_2 in a water-saturated atmosphere. A stock solution (10 mM) of forskolin (Sigma) in DMSO was used to prepare feed solutions in DMEM without phenol red (final DMSO concentration in the assay was 0.02%). After addition of 20 μL of forskolin solution (final concentration 2 μM), 20 μL of a 10-fold concentrated solution of the respective test compound were added. The cells were incubated at 37 °C in water saturated atmosphere containing 5 % CO_2 for 4.5 h. Afterwards, the medium was discarded, and 80 μL of lysis solution (pH adjusted to 7.8 with hydrochloric acid) were added to each well. The plates were shaken at 600 rpm for 30 min. Afterwards, 40 μL of the lysate were transferred into white 96-well plates (Greiner, Frickenhausen, Germany). Luminescence was measured with a GENios Pro microplate reader. Light emission was induced by injecting 80 μL of the luciferase assay buffer (25 mM Gly-Gly; $MgSO_4 \cdot 7\ H_2O$, 15 mM; KH_2PO_4, 15 mM; EGTA, 4 mM; ATP disodium salt, 2 mM; DTT, 2 mM; D-luciferin potassium salt (Synchem, Felsberg, Germany), 0.2 mg/mL; pH was adjusted to 7.8 with hydrochloric acid). Luminescence [RLU] was measured for 10 s.

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Chapter 3

Investigations with [^{3}H]UR-KK193 at Rat and Guinea Pig Y_4 Receptors

3.1 Introduction

Among the NPY receptors, the Y_4R shows the lowest degree of sequence identity across different species. For example, the rat Y_4R only shares a sequence identity of 75% with the human ortholog,[1] whereas the amino acid identity between human and rat Y_1Rs is 94%. The sequence of guinea pig Y_4R is much more similar to that of the human Y_4R (84% identity) than to the sequence of the rat Y_4R.[2] This correlates with the preferred ligand of the Y_4Rs, PP, for which rodents show the highest divergence of all known mammalian PP sequences.[3] Previously, the high-affinity agonist BVD-74D[4] (Figure 1) was described to have a regulatory effect on the food intake in fasted mice, suggesting BVD74-D as potential agent for the treatment of obesity.[5] However, due to the above-mentioned strong differences between human and rodent Y_4Rs, it is highly questionable whether these results can be transferred to humans. Binding studies with BVD-74D should be carried out at rodent Y_4Rs to be able to better assess the potential of BVD-74D to reduce the food intake in humans. Furthermore, by analogy with GPCR mutants, orthologous Y_4Rs might serve as a template to identify putative interaction partners between a peptide ligand and the human Y_4R.

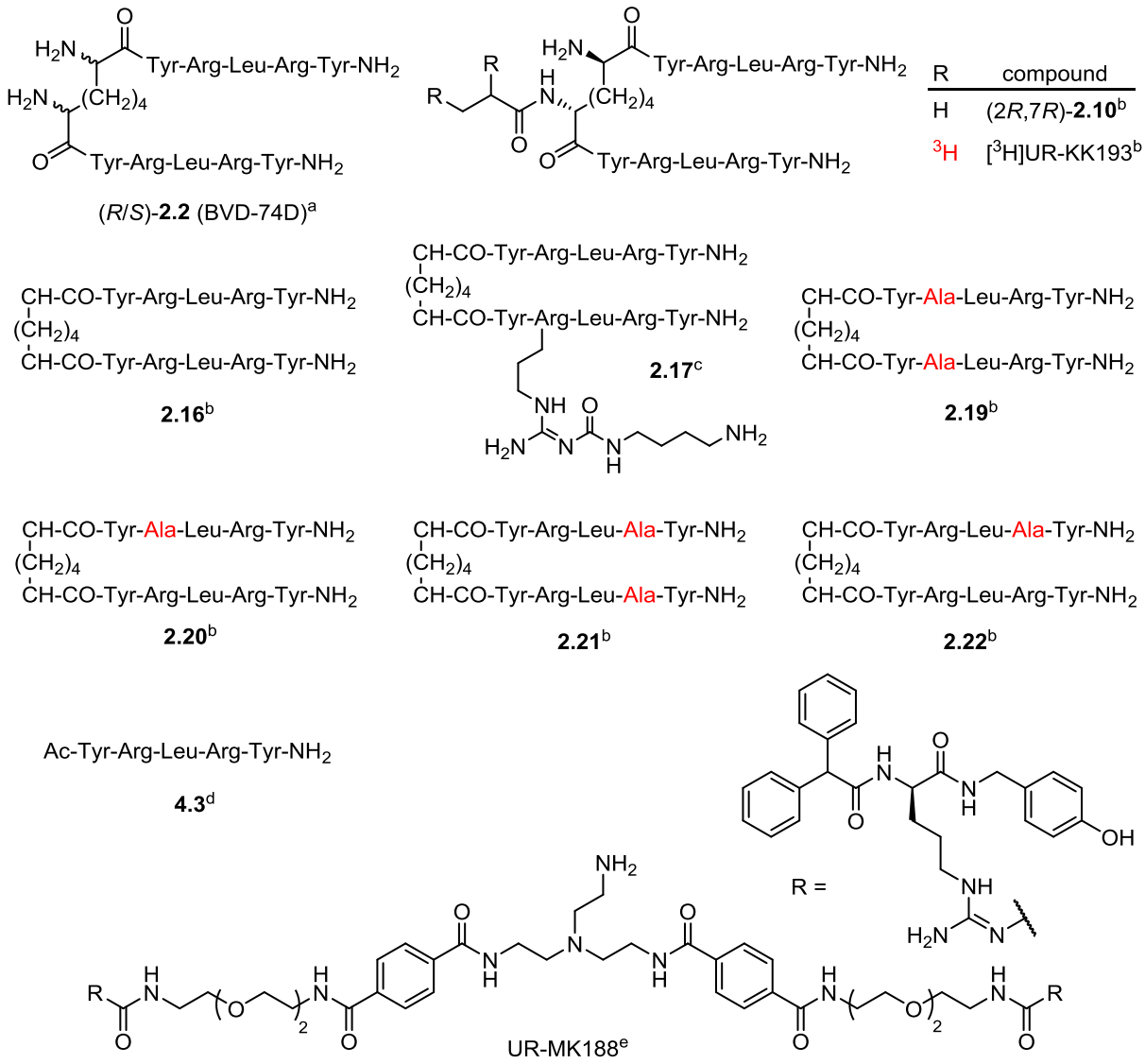

Figure 1: Structures of the Y$_4$R 'dimeric' pentapeptide agonists, the pentapeptide agonist **4.3** and the Y$_1$R/Y$_4$R antagonist UR-MK188. [a]Balasubramaniam et al[4]; [b]Kuhn et al[6]; [c]Keller et al[7]; [d]Berlicki et al[8]; Keller et al[9].

3.2 Results and Discussion

3.2.1 Saturation Binding of [^{3}H]UR-KK193 at Rat and Guinea Pig Y$_4$Rs

Saturation binding experiments with the tritiated radioligand [^{3}H]UR-KK193 were performed on homogenates of CHO cells stably expressing either the rY$_4$R or the gpY$_4$R in a sodium-free HEPES buffer. The affinity of [^{3}H]UR-KK193 at the rY$_4$R (K_d = 0.18 ± 0.01 nM) was higher than at the gpY$_4$R (K_d = 2.64 ± 0.5 nM) by a factor of 14. The number of specific binding sites per cell were 86500 ± 5700 (rY$_4$R) or 105500 ± 9200 (gpY$_4$R). At concentrations around the K_d values, nonspecific binding amounted to 10% (rY$_4$R) and 36% (gpY$_4$R), respectively, of the

total binding. Due to the high affinity of [^{3}H]UR-KK193 at the rY$_4$R and the low unspecific binding associated therewith, binding experiments at the rY$_4$R were especially robust.

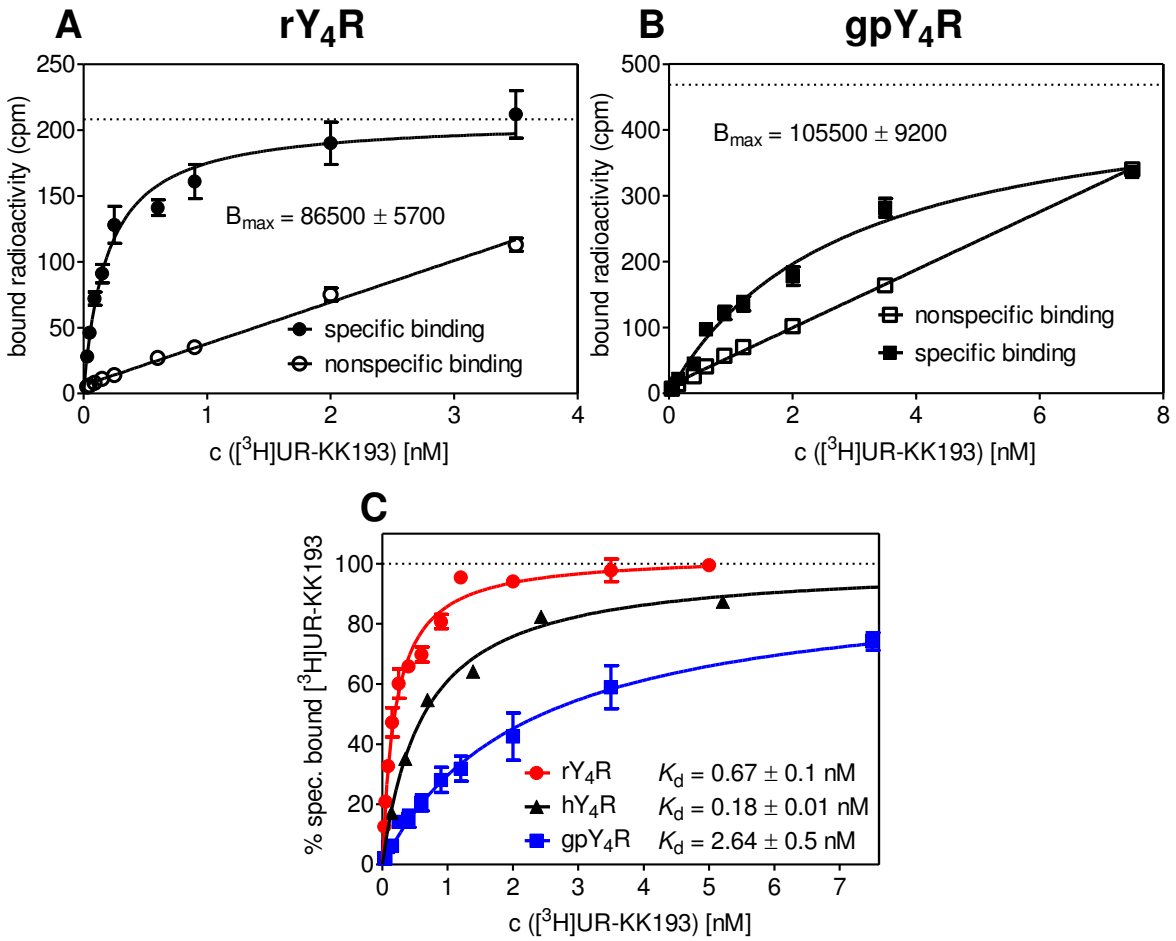

Figure 2: Representative saturation binding experiments with [^{3}H]UR-KK193 at CHO-rY$_4$R (A) and CHO-gpY$_4$R (B) cell homogenates. (C) Comparison of saturation binding experiments with [^{3}H]UR-KK193 at rY$_4$R (red), hY$_4$R (black) and gpY$_4$R (blue). Mean ± SEM of ≥ 3 independent experiments performed in triplicate.

3.2.2 Competition Binding Studies with [^{3}H]UR-KK193 at Rat and Guinea Pig Y$_4$Rs

The K_i values of several 'dimeric' pentapeptides and reference compounds were determined in competition binding experiments at the rY$_4$R and gpY$_4$R, respectively (Figure 3, for results see Table 1). The determination of K_i values at the hY$_4$R is described in chapter 2 (hPP, UR-MK188, (2*R*,7*R*)-**2.2**, (2*S*,7*S*)-**2.2**, (2*R*,7*R*)-**2.10**, **2.16**, **2.17**, **2.19**, **2.20**, **2.21**, **2.22**) and chapter 4 (**4.3**). The affinity of the endogenous ligand hPP to rY$_4$R and gpY$_4$R was nearly identical (K_i (rY$_4$R) = 0.15 nM, K_i (gpY$_4$R) = 0.17 nM). As observed for the radioligand [^{3}H]UR-KK193, the affinity of all investigated 'dimeric' pentapeptides to the rY$_4$R is higher than the affinity to

the gpY$_4$R by a factor of 10 to 20. For example, (2*R*,7*R*)-**2.2** showed K_i values in the picomolar range at the rY$_4$R (K_i = 0.044 nM) and substantially lower affinity to the gpY$_4$R (K_i = 1.08 nM). The same holds for the (2*S*,7*S*)-configured isomer (K_i (rY$_4$R) = 0.38 nM, K_i (gpY$_4$R) = 9.90 nM). The order of affinity within the investigated series of 'dimeric' pentapeptides was the same at all three Y$_4$R orthologs, that is, the affinity of each 'dimeric' pentapeptide to the hY$_4$R was in between those to the rY$_4$R and the gpY$_4$R. In contrast to the dimeric peptides, the difference in affinity to human, guinea pig and rat Y$_4$R was lower in case of peptide **4.3** and the dimeric antagonist UR-MK188. Compound **4.3** and UR-MK188 display similar affinities to the human and guinea pig Y$_4$Rs (**4.3**: K_i (hY$_4$R) = 337 nM, K_i (gpY$_4$R) = 267 nM; UR-MK188: K_i (hY$_4$R) = 660 nM, K_i (gpY$_4$R) = 643 nM). The affinity to the rY$_4$R was approximately 5 times higher for both compounds (**4.3**: K_i (rY$_4$R) = 55.6 nM, UR-MK188: K_i (rY$_4$R) = 124 nM).

Recently, a model of the hY$_4$R in complex with PP was constructed based on all available class A GPCR crystal structures. Several amino acids in the top of transmembrane helixes 2 (Tyr$^{2.64}$, Asp$^{2.68}$ and Trp$^{2.70}$), 6 (Asn$^{6.55}$ and Asp$^{6.59}$) and 7 (Asn$^{7.32}$ and Phe$^{7.35}$) were reported to form the core of the peptide binding pocket (Figure 4).[10] The reported amino acids and neighboring regions are highly conserved among human, guinea pig and rat Y$_4$Rs. Therefore it appears rather surprising that the affinity of the 'dimeric' peptides to rat and guinea pig Y$_4$Rs differs by a factor of 10 to 20, whereas the affinity of the endogenous ligand hPP is nearly identical. The different binding profiles could be explained by not or only partially overlapping binding sites. Furthermore, with respect to their size, the 'dimeric' peptides might occupy an additional allosteric interaction site that is less conserved among different Y$_4$ orthologous receptors. Glu$^{5.24}$ was reported to be crucial for the interaction of the hY$_2$R with the endogenous ligand NPY.[11] Like Asp$^{6.59}$, Glu$^{5.24}$ is highly conserved among mammalian Y$_4$Rs with exception of rat and mouse Y$_4$Rs (Figure 4). Due to the high affinity of all investigated compounds to the rY$_4$R, a contribution of Glu$^{5.24}$ to either the orthosteric binding pocket or an allosteric interaction site can be excluded in case of the Y$_4$R.

Taking into consideration the different affinity of (2*R*,7*R*)-**2.2** and (2*S*,7*S*)-**2.2** to hY$_4$R and rY$_4$R (Table 1), it remains a matter of speculation whether the regulatory effect of BVD-74D on food intake in mice (note: the rY$_4$R and mY$_4$R display a high sequence identity of 92%) can be extrapolated to humans. In addition, (2*R*,7*R*)-**2.2** is a weak antagonist at the hY$_1$R as well, whereas the affinity of BVD-74D to rodent Y$_1$, Y$_2$ and Y$_5$ receptors is unknown. Furthermore,

the y_6R is functionally expressed in mice.[12] As the y_6R is an inactive pseudogene in humans, it is often neglected in the interpretation of data from *in vivo* studies. In principal, the effect of BVD-74D on food intake in mice might be regulated via Y_1R, Y_2R, Y_5R or y_6R as well.

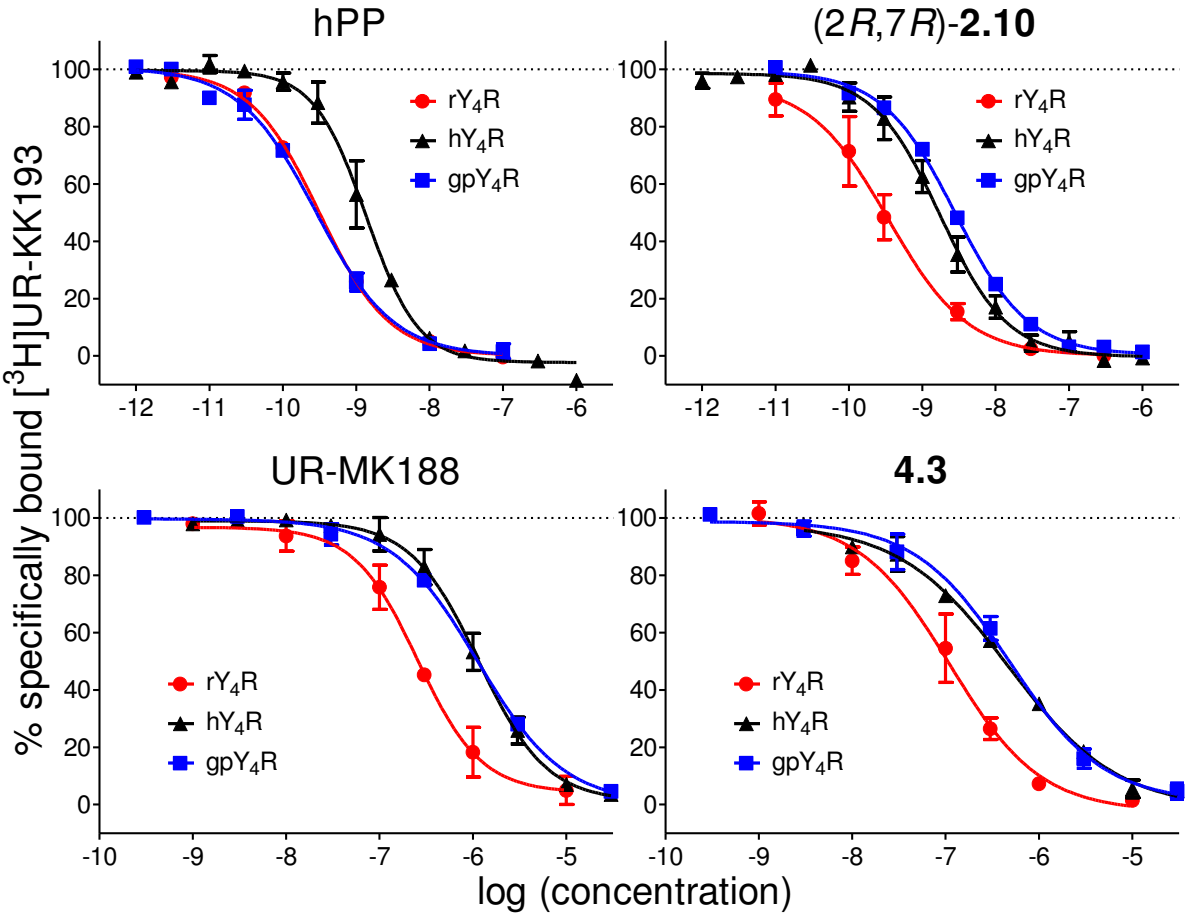

Figure 3: Radioligand displacement curves from competition binding experiments performed with [^{3}H]UR-KK193 at CHO-rY_4R (red, K_d = 0.18 nM, c = 0.2 nM) and CHO-gpY_4R (blue, K_d = 2.64 nM, c = 2 nM) cell homogenates or CHO-hY_4R-G_{qi5}-mtAEQ cells (K_d = 0.67 nM, c = 0.6 nM).

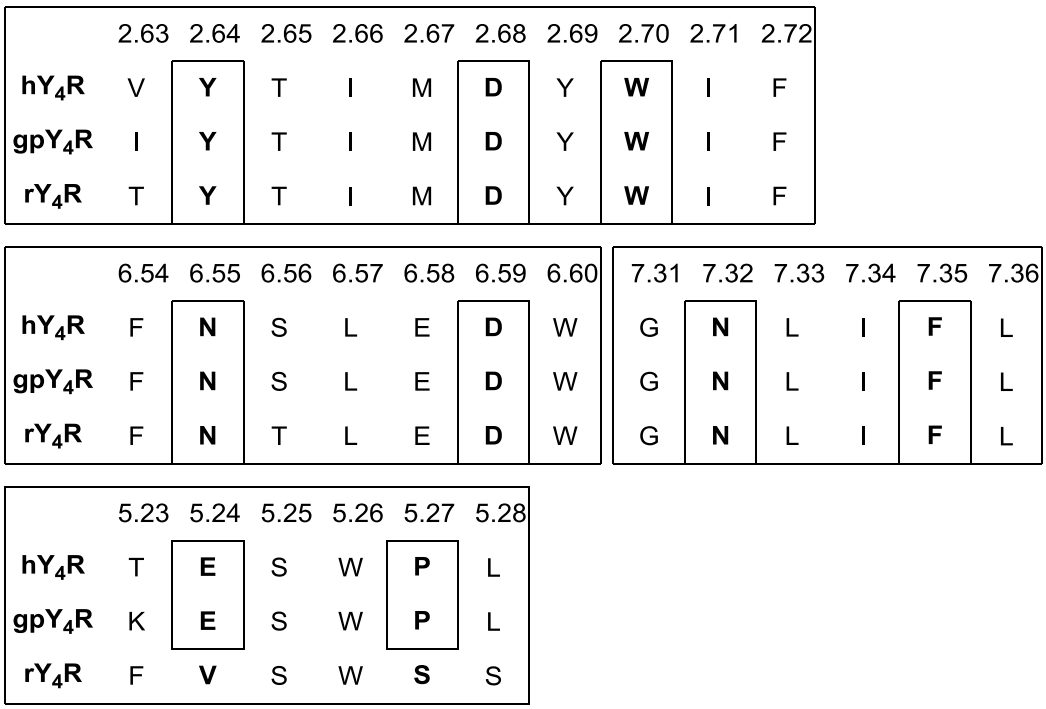

Figure 4: Alignment of Y$_4$R sequences that were reported to contribute to the Y$_4$R binding pocket.[10]

Table 1. NPY receptor binding data.

Compd.	hY$_4$R K_i [nM][a]	rY$_4$R K_i [nM][b]	gpY$_4$R K_i [nM][c]
[^{3}H]UR-KK193	0.67 ± 0.1 (K_d)	0.18 ± 0.01 (K_d)	2.64 ± 0.5 (K_d)
hPP	0.65 ± 0.13	0.15 ± 0.01	0.17 ± 0.02
UR-MK188	660 ± 140	124 ± 5.4	643 ± 5.7
4.3	337 ± 110	55.6 ± 20	267 ± 36
(2*R*,7*R*)-2.2	0.45 ± 0.14	0.044 ± 0.004	1.08 ± 0.13
(2*S*,7*S*)-2.2	2.3 ± 0.16	0.38 ± 0.16	9.90 ± 1.6
(2*R*,7*R*)-2.10	0.87 ± 0.16	0.16 ± 0.06	1.57 ± 0.06
2.16	3.5 ± 0.59	0.23 ± 0.02	19.8 ± 4.5
2.17	1.2 ± 0.37	0.21 ± 0.09	7.08 ± 1.1
2.19	>3000	>5000	>5000
2.20	12 ± 1.6	2.63 ± 0.27	47.6 ± 11
2.21	>3000	971 ± 180	>5000
2.22	11 ± 1.4	2.46 ± 0.64	110 ± 35

[a]Radioligand competition binding assay with [^{3}H]UR-KK193[6] (K_d = 0.18 nM, c = 0.2 nM) at CHO-rY$_4$R[13] cell homogenates. [b]Radioligand competition binding assay with [^{3}H]UR-KK193 (K_d = 0.67 nM, c = 0.6 nM) at CHO-hY$_4$R-G$_{qi5}$-mtAEQ cells.[14] [c]Radioligand competition binding assay with [^{3}H]UR-KK193 (K_d = 0.18 nM, c = 0.2 nM) at CHO-gpY$_4$R[2] cell homogenates.

3.3 Conclusions

The tritiated radioligand [^{3}H]UR-KK193 is a useful tool for binding studies at rat and guinea pig Y_4Rs. In contrast to the endogenous ligand hPP, the investigated 'dimeric' peptides bind with higher affinities to the rY_4R than to human and guinea pig Y_4Rs. Therefore, it is unclear whether the regulatory effect of BVD-74D on food intake in mice is reproducible in humans.

3.4 Experimental Section

3.4.1 Cell Culture

CHO-rY_4R[13] and CHO-gpY_4R[2] cells were cultured in 55- or 152-cm^2 dishes in a humidified atmosphere (95% air, 5% CO_2) at 37 °C in Dulbecco`s Modified Eagle Medium (DMEM) (Invitrogen) containing 10% FCS (Invitrogen), penicillin-streptomycin (100 IU/mL and 100 μg/mL) (Gibco) and amphotericin B (2.5 μg/mL) (Invitrogen).

3.4.2 Radioligand Binding Assays

Binding assays with the radioligand [^{3}H]UR-KK193 were performed at CHO-rY_4R or CHO-gpY_4R cell homogenates. Cells were grown to 90% - 100% confluency in 152 cm^2 dishes, scraped of the culture flask and centrifuged for 5 min at 300 g. The culture medium was discarded and the cells were resuspended in buffer (1 mL) (25 mM HEPES, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.4) containing 0.1 mg/mL bacitracin. The cells were homogenized using an Ultra-Turrax homogenizer under ice-cooling and subsequently diluted with buffer containing 0.1 mg/mL bacitracin and 1% BSA to a final volume of 22 mL. Both, saturation and competition experiments, were performed in a final volume of 200 μL in 96-well plates 1450-401 (PerkinElmer). Saturation binding experiments were performed in a concentration range from 0.03 to 7.5 nM. Competition binding experiments were performed with increasing concentrations of unlabeled compounds using the radioligand at a concentration in the range of the respective K_d value (0.2 nM in case of rY_4R, 2 nM in case of gpY_4R). Nonspecific binding was determined in the presence of a 200-fold excess of (2*R*,7*R*)-**2.2**. The incubation period was 90 min. Bound and free radioligand were separated by filtration through

polyethylenimine pre-treated GF/C filters (Whatman, Maidstone, UK) using a TOMTEC cell harvester (Orange, CT, USA), and the filters were dried at 60 °C. The dried filters were treated with MeltiLex A (Perkin Elmer) melt-on szintillator sheets and the remaining radioactivity was counted using a Wallac 1450 Betaplate counter (Perkin Elmer).

3.5 References

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Chapter 4

The Incorporation of Aza-Amino Acids or *D*-Amino Acids into NPY Y_4R Peptide Ligands Turns Agonists into Antagonists

Note: Prior to the submission of this thesis, parts of this chapter were submitted to publication in cooperation with partners:

The following experiments were performed by co-authors:

Timo Littmann: Characterization of final compounds in the β-arrestin 1 and β-arrestin 2 assay
Stefanie Dukorn: Characterization of (2*R*,7*R*)-**26** and **27** in the luciferase reporter gene assay

4.1 Introduction

Among the neuropeptide Y (NPY) receptors, designated Y_1R, Y_2R, Y_4R and Y_5R,[1] the Y_4R plays a special role, because it preferentially binds pancreatic polypeptide.[2] Compared to the Y_1R, Y_2R and Y_5R subtypes, by far less ligands (examples cf. Figure 1) are reported for the Y_4R, in particular, high-affinity Y_4R antagonists are still lacking.[3-6]

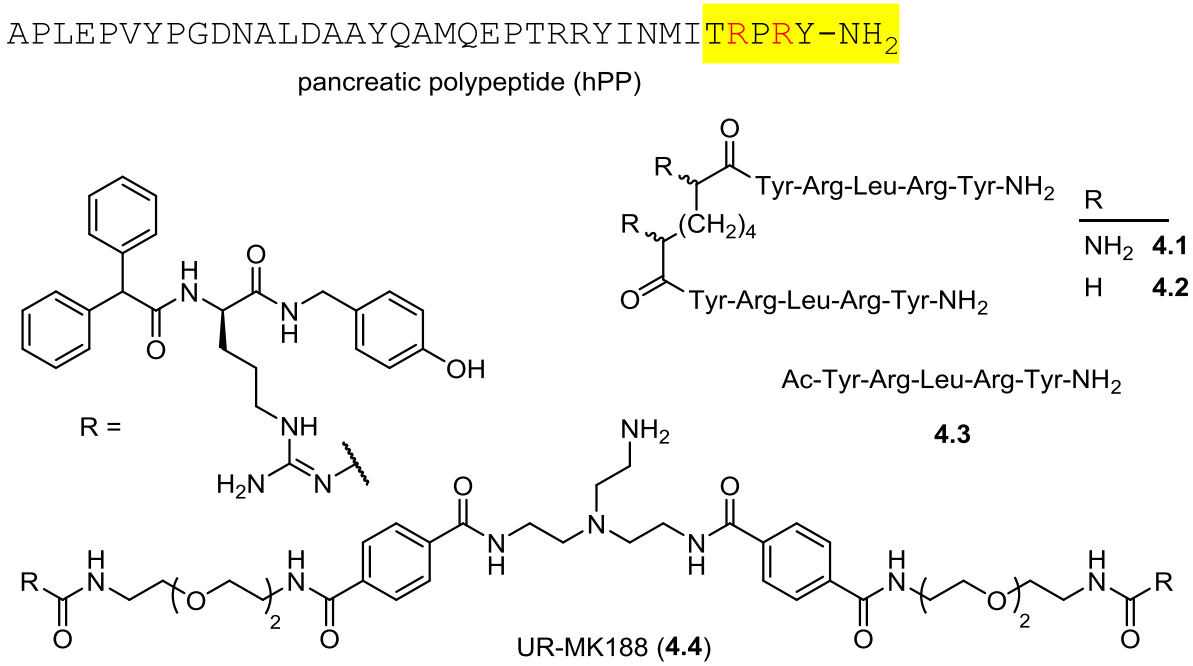


Figure 1: Amino acid sequence of human PP and structures of the described NPY Y_4R agonists **4.1**, **4.2** and **4.3** and the Y_1R/Y_4R antagonist UR-MK188.

Y_4R agonists are considered as potential anti-obesity agents.[7,8] The diastereomeric mixture of the cross-linked ("dimeric") pentapeptide **4.1** (BVD-74D), a mimic of the C-terminus of pancreatic polypeptide, was described as a high-affinity Y_4R agonist,[4] having an effect on the food intake in mice.[9] As reported previously, (2*R*,7*R*)-**4.1** (note: in the following, positions 2 and 7 refer to the stereo centers in the 2,7-diaminosuberic acid moiety) was by a factor of 3-10 more potent than (2*S*,7*S*)-**4.1**, depending on the type of assay.[10,11] Even though Y_4R agonists should come up with the higher clinical potential, antagonists are of interest as well, in particular, as pharmacological tools. The pentapeptide **4.3**,[6] a Y_4R agonist with an affinity in the two-digit nanomolar range (K_i = 50 nM), shares the same amino acid sequence with

the peptide moieties of **1**. The incorporation of cyclic β-amino acids such as (1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid resulted in partial agonism.[6]

To investigate the impact of backbone modifications in Y_4R agonists on functional activity in more detail, additional analogs of (2*R*,7*R*)-**4.1**, **4.2**[11] and **4.3** were prepared. Here we report on the synthesis of Y_4R ligands in which aza-amino acids or *D*-amino acids were introduced. The title compounds were characterized in binding and functional cellular assays with complementary readouts.

4.2 Results and Discussion

4.2.1 Chemistry

Aza-peptides contain at least one amino acid in which the α-carbon atom is replaced by nitrogen.[12] The semicarbazide substructure reduces conformational flexibility.[13,14] Several studies revealed that the incorporation of aza-amino acids into bioactive peptides may result in a longer duration of action or higher potencies compared to the parent peptide.[15-17] Aza-peptides can be prepared by the reaction of an *N*-protected *N*'-substituted hydrazine with an isocyanate.[12] However, the synthesis of aza-peptides on solid phase is compromised by an intramolecular side-reaction of resin-bound isocyanates resulting in the formation of hydantoins (Figure 2, **A**), lowering the yields of the target compounds and requiring time-consuming purification.[18] To circumvent hydantoin formation, *N*-Fmoc-aza[1]-dipeptides were prepared from *N*-protected *N*'-substituted hydrazines and benzyl ester protected amino acids, followed by hydrogenolytic debenzylation in solution (Figure 2, **B**).[19]

Two different Fmoc-protected hydrazines were synthesized to mimic the side chains of Orn or Leu, respectively (Scheme 1). 3-Aminopropan-1-ol was Boc-protected and subsequently oxidized under Parikh-Döring conditions using dimethyl sulfoxide as an oxidant to form the aldehyde **4.7**. Condensation of (9*H*-fluoren-9-yl)methyl hydrazinecarboxylate **4.8** and the aldehyde **4.7** or isobutyric aldehyde resulted in hydrazones **4.9** and **4.10**. Subsequent reduction using sodium cyanotrihydridoborate gave access to the substituted hydrazines **4.10** and **4.12**.

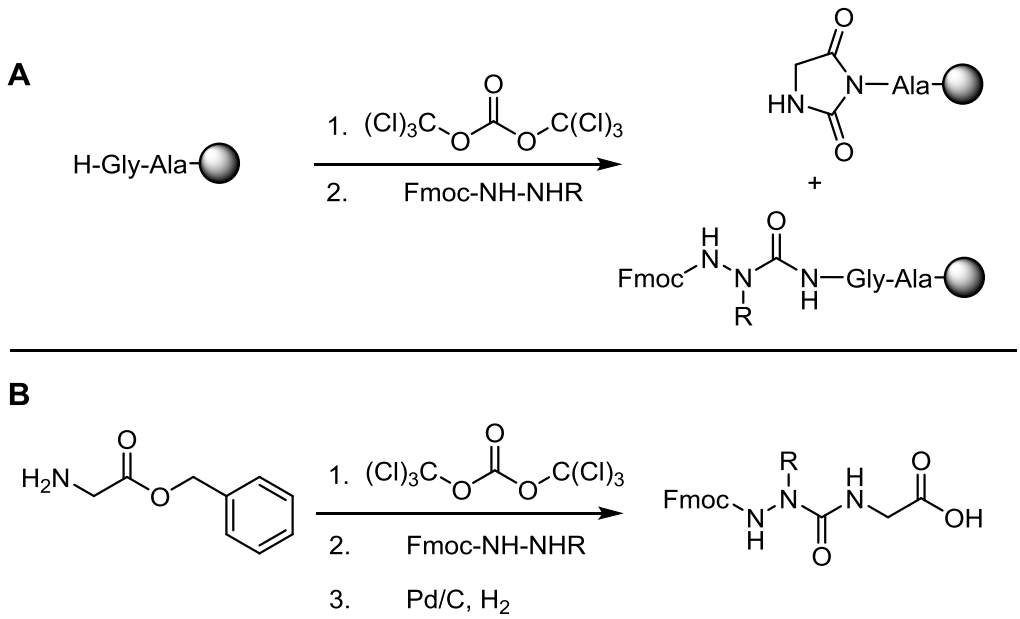
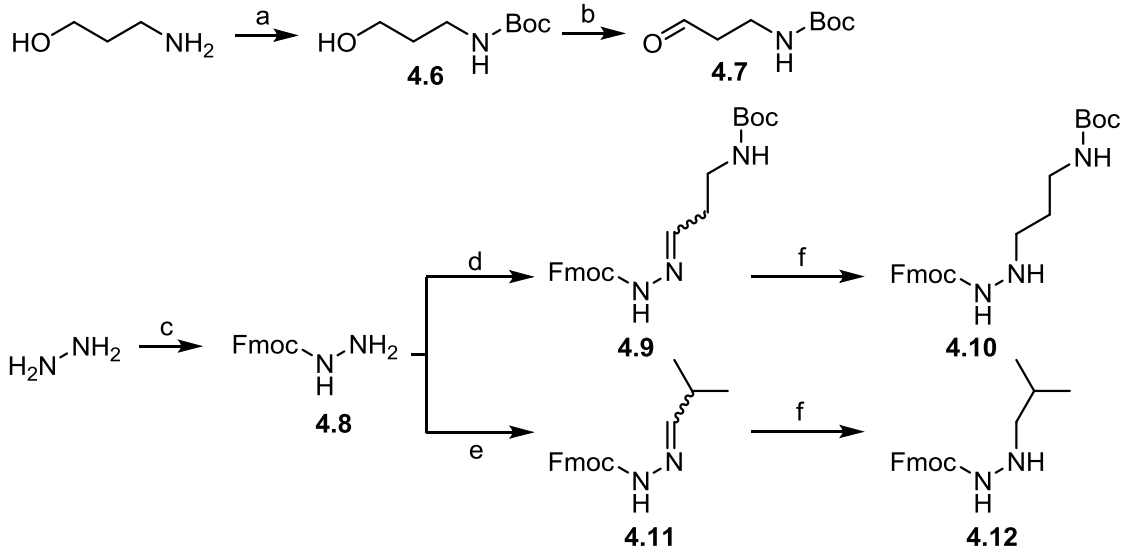


Figure 2: Synthesis of aza-peptides on a solid phase (**A**) or in solution (**B**).

Scheme 1. Synthesis of the hydrazines **4.10** and **4.12**[a]

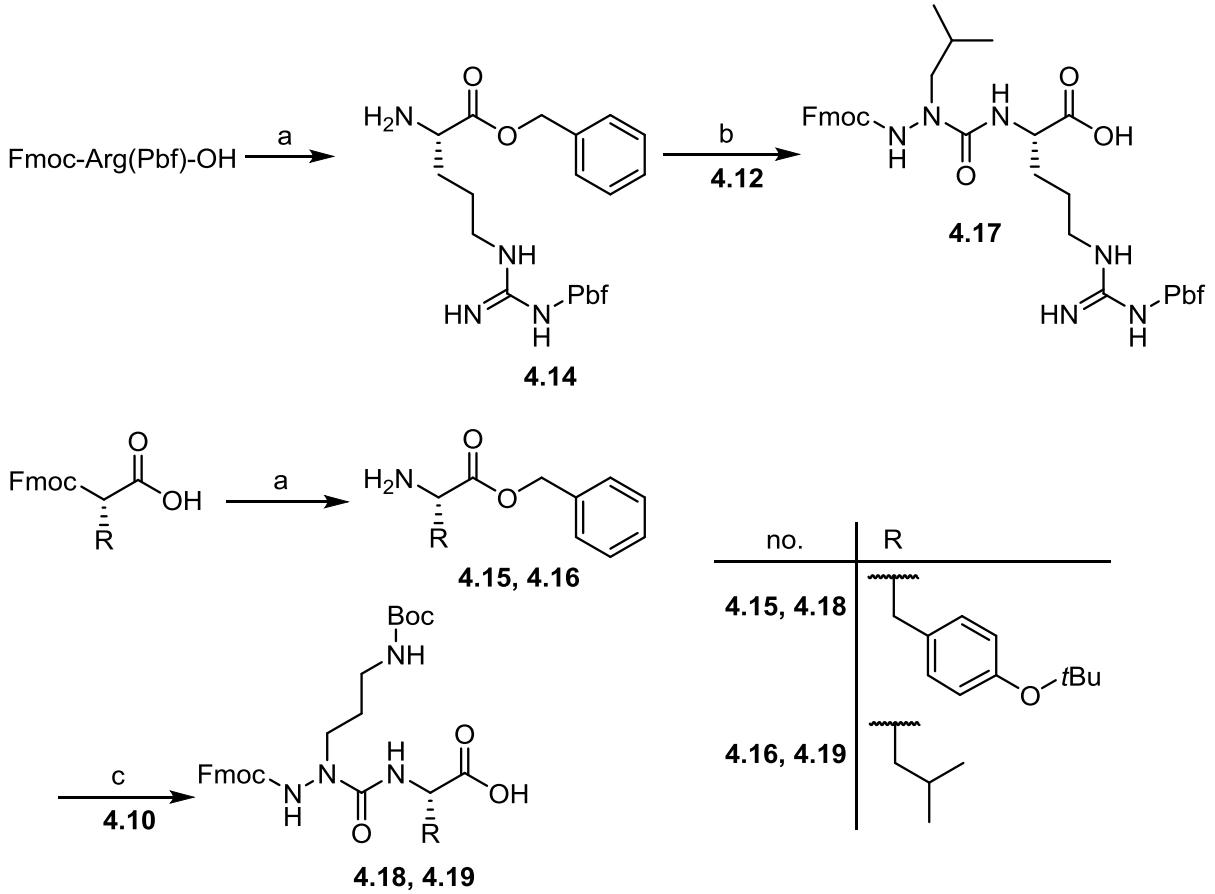


[a]Reagents and conditions: (a) Boc_2O, Na_2CO_3, THF, 0 °C, 10 min, followed by rt, 14 h; (b) Sulfur trioxide pyridine complex, DMSO, TEA, CH_2Cl_2, 0 °C, 1 h, followed by rt, 3 h; (c) Fmoc-Cl, MeCN/H_2O (1:1), 0 °C, 10 min, followed by rt, 12 h; (d) **4.7**, CH_2Cl_2, 14 h; (e) isobutyraldehyde, CH_2Cl_2, 50 h; (f) sodium cyanotrihydridoborate, CH_2Cl_2/MeOH (4:3), 2 M HCl (pH 2-3), rt, 2-6 h.

Benzyl ester protected amino acids **4.14**-**4.16** were treated with triphosgene to give the respective isocyanates *in situ* (Scheme 2). Without purification, the isocyanates were treated with hydrazines **4.10** and **4.12** affording the benzyl protected *N*-Fmoc-aza[1]-dipeptides in yields >70%. Subsequent ester cleavage by hydrogenation gave access to the *N*-Fmoc-aza[1]-dipeptides Fmoc-aza-Leu-Arg(Pbf)-OH (**4.17**), Fmoc-aza-Orn(Boc)-Tyr(*t*Bu)-OH (**4.18**) and Fmoc-aza-Orn(Boc)-Leu-OH (**4.19**). Dipeptides **4.17**-**4.19** were employed in solid phase

synthesis under the same conditions as for standard Fmoc-protected amino acids resulting in quantitative coupling efficiencies.

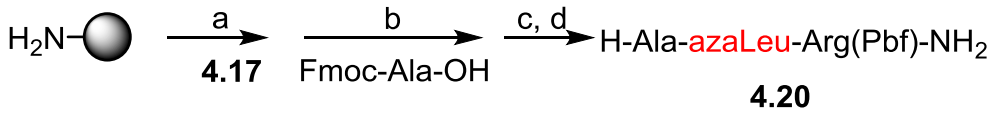
Scheme 2. Synthesis of *N*-Fmoc-aza[1]-dipeptides **4.17**-**4.19**[a]



[a]Reagents and conditions: (a) Benzyl alcohol, 4-DMAP, DCC, CH_2Cl_2, rt, 14 h, followed by DEA/CH_2Cl_2 (1:8), rt; (b) Triphosgene, DIPEA, CH_2Cl_2, rt, 90 min, followed by Pd/C, H_2, MeOH, rt, 1 h; (c) Triphosgene, DIPEA, CH_2Cl_2, rt, 90 min, followed by Pd/C, H_2, MeOH, rt, 1 h.

To optimize the coupling of a resin-bound aza[1]-peptide, the model peptide H-Ala-aza-Leu-Arg(Pbf)-NH_2 (**4.20**) was synthesized under various conditions (Scheme 3). When HBTU/HOBt was used as coupling reagent at 35 °C only 5 to 10% of the desired products were formed. The same holds for the application of the succinimidyl ester, Fmoc-Ala-OSu. The coupling efficiency was increased to approximately 50%, when the 7-azabenzotriazole derivative HATU was used as coupling reagent. The best results (> 50% coupling efficiency) were achieved by *in situ* activation of Fmoc-Ala-OH to the corresponding acyl chloride using triphosgene in THF. Surprisingly, when CH_2Cl_2 was used as a solvent instead of THF, only minor amounts of **4.20** were detected.

Scheme 3. Incorporation of aza-Leu into the model peptide **4.20** via SPPS.[a]

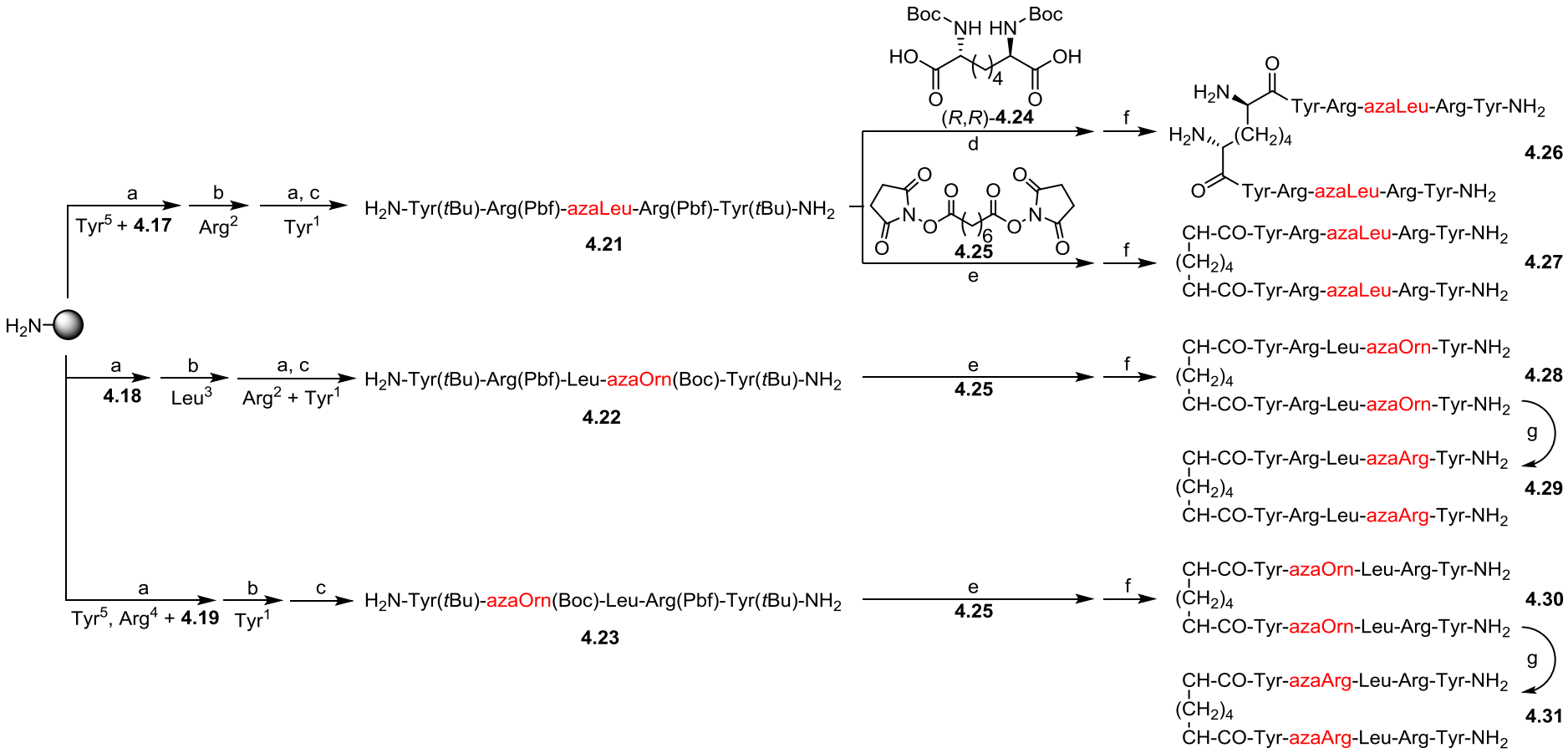


(b) conditions for the coupling of Fmoc-Ala-OH

coupling reagent	excess of amino acid	time	temp.	solvent	yield of **4.20** (%)
HBTU/HOBt	4 equiv.	10 h	35 °C	DMF/NMP	5-10
Fmoc-Ala-OSu	4 equiv.	10 h	35 °C	DMF/NMP	-
HATU	4 equiv.	10 h	30 °C	DMF/NMP	50-60
BTC	4 equiv.	12 h	30 °C	THF	50-60
BTC	4 equiv.	12 h	30 °C	DCM	0-5

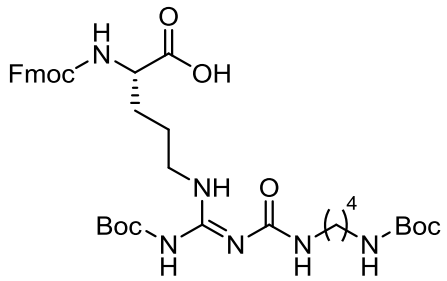
[a]Reagents and conditions: (a) SPPS (Fmoc strategy), **4.17**/HBTU/HOBt/DIPEA (3/3/3/6 equiv), solvent DMF, 35 °C, 14 h; Fmoc deprotection was carried out with 20% piperidine in DMF/NMP (8:2), rt, 2 × 10 min; (b) variation of the conditions according to the table; Fmoc deprotection as under (a); (c) CH_2Cl_2/TFA (97:3), 10 × 6 min.

Scheme 4. Synthesis of "homodimeric" aza-peptides **4.26-4.31**[a]



[a]Reagents and conditions: (a) SPPS (Fmoc strategy), Fmoc-aa/HBTU/HOBt/DIPEA (5/5/5/10 equiv), solvent DMF/NMP (8:2), 'double' coupling at rt, 60 min or dipeptide (**4.17**, **4.18** or **4.19**)/HBTU/HOBt/DIPEA (3/3/3/6), solvent: DMF, 35 °C, 14 h; Fmoc deprotection as under (a) in Scheme 3; (b) triphosgene, DIPEA, THF, 35 °C, 14 h; Fmoc deprotection as under (a) in Scheme 3; (c) CH_2Cl_2/TFA (97:3), 10 × 6 min; (d) HBTU, HOBt, DIPEA, anhydrous DMF, 35 °C, 16 h; (e) 1 equiv of **4.25** in 1% DIPEA in anhydrous DMF, followed by 2.5 equiv of **4.21**, **4.22** or **4.23**, 35 °C, 16 h; (f) TFA/H_2O (95:5), rt, 2.5 h; (g) *N*,*N*'-di-Boc-1*H*-pyrazole-1-carboxamidine, DMF, DIPEA, rt, 4 h; Boc-deprotection, TFA/CH_2Cl_2/H_2O (10/10/1), rt, 3 h.

The “dimeric” aza-peptide (2*R*,7*R*)-**4.26**, an aza-Leu analog of (2*R*,7*R*)-**4.1**, was prepared from the protected pentapeptide **4.21** and the diacid (*R*,*R*)-**4.24** (Scheme 4). For the preparation of the aza-peptides **4.27**-**4.31**, octanedioic acid disuccinimidyl ester (**4.25**) was used for cross-linking. Cross-linking of **4.21** with **4.25** yielded the “dimeric” aza-peptide **4.27**, an aza-Leu analog of **4.2**. Dimerization of the protected peptides **4.22** and **4.23** resulted in aza-peptides **4.28** and **4.30** containing ornithine instead of arginine. Subsequently, guanidinylation was performed with *N*,*N*'-di-Boc-1*H*-pyrazole-1-carboxamidine resulting in **4.29** and **4.31**, aza-Arg containing analogs of **4.2**.



4.32

Figure 3: Structure of the N^{ω}-carbamoylated arginine building block **4.32**.[20]

Very recently, we reported that the replacement of Arg^2 with the N^{ω}-carbamoylated arginine **4.32** (Figure 3) in one of the pentapeptide chains of **2** led to increased Y_4R affinity.[11] Similarly, **4.32** was used for the preparation of peptides **4.33**-**4.42** (Figure 4), which are analogs of the pentapeptide **4.3**, on an Fmoc-Sieber-PS resin by manual SPPS according to the Fmoc strategy.

compd no.	sequence
4.3	Ac-Tyr-Arg-Leu-Arg-Tyr-NH_2
4.33	Oct-Tyr-Arg-Leu-Arg-Tyr-NH_2
4.34	Ac-Tyr-Arg(carb)-Leu-Arg-Tyr-NH_2
4.35	Ac-Arg-Tyr-Arg(carb)-Leu-Arg-Tyr-NH_2
4.36	Ac-Arg-Tyr-Arg-Leu-Arg-Tyr-NH_2
4.37	Ac-Arg-Tyr-Arg-Leu-Arg(carb)-Tyr-NH_2
4.38	Ac-Arg-Tyr-Arg(carb)-Leu-Arg-*D*-Tyr-NH_2
4.39	Ac-Arg-Tyr-Arg(carb)-Leu-*D*-Arg-Tyr-NH_2
4.40	Ac-Arg-Tyr-Arg(carb)-*D*-Leu-Arg-Tyr-NH_2
4.41	Ac-Arg-*D*-Tyr-Arg(carb)-Leu-Arg-Tyr-NH_2
4.42	Ac-*D*-Arg-Tyr-Arg(carb)-Leu-Arg-Tyr-NH_2

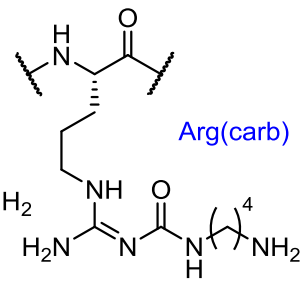
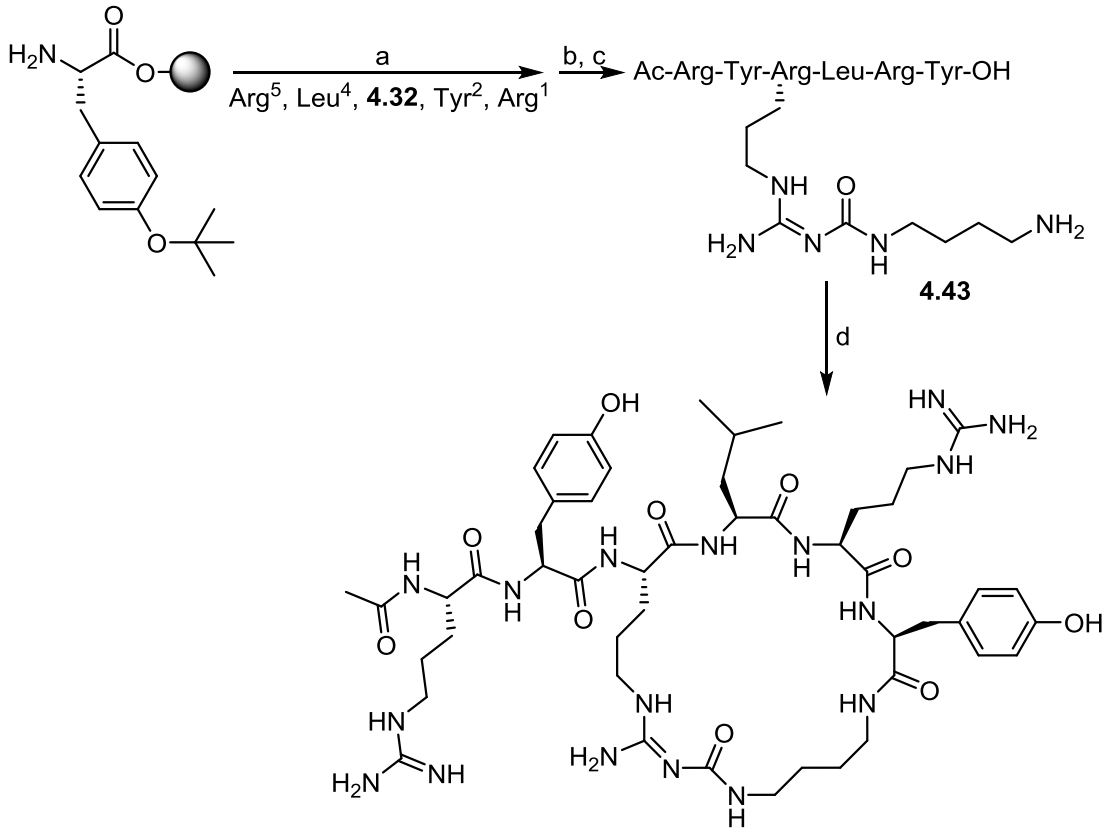


Figure 4: Primary structures of the synthesized peptides **4.33**-**4.42** (cf. "general procedure for solid phase peptide synthesis").

Finally, the applicability of **4.32** to the preparation of cyclic peptides via head-to-side-chain cyclization was investigated. The formation of head-to-tail cyclic peptides is often compromised by rather rigid backbones. However, the side-chain of **4.32** is very flexible, facilitating an approximation of the activated C-terminus and the terminal amino group. The hexapeptide **4.43** was prepared on an H-*L*-Tyr(*t*Bu)-2-CT resin followed by global side-chain deprotection (Scheme 5). Cyclization was achieved at a peptide concentration of 1 mM using PyBOP as coupling reagent. Under these conditions, the formation of peptide dimers was not observed.

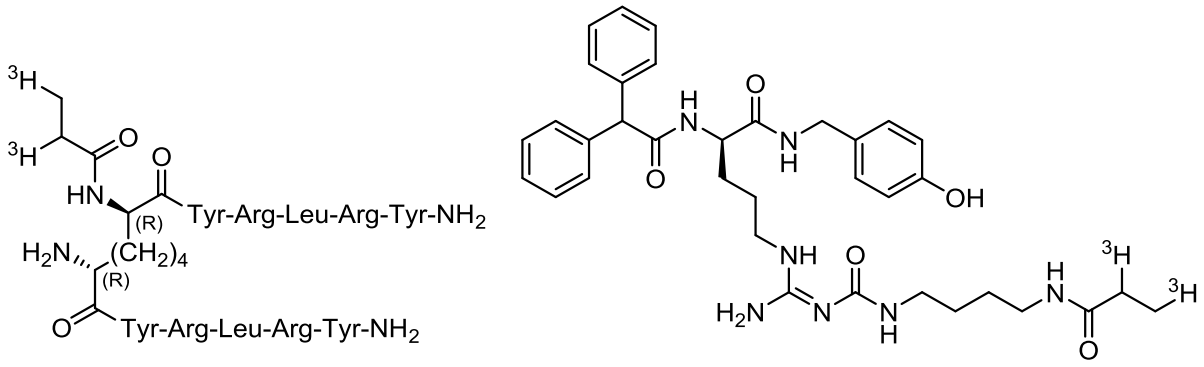
Scheme 5. Synthesis of the head-to-side-chain cyclic peptide **4.43**.[a]



[a]Reagents and conditions: (a) SPPS (Fmoc strategy), Fmoc-aa/HBTU/HOBt/DIPEA (5/5/5/10 equiv), solvent DMF/NMP (8:2), 'double' coupling at rt, 60 min or **4.32**/HBTU/HOBt/DIPEA (3/3/3/6), solvent: DMF, 35 °C, 14 h; Fmoc deprotection as under (a) in Scheme 3; (b) CH_2Cl_2/TFA (97:3), 10 × 6 min; (c) TFA/H_2O (95:5), rt, 2.5 h; (d) PyBOP, HOBt, DIPEA, DMF, rt, 24 h.

4.2.2 Competition Binding Studies at NPY Receptor Subtypes

4.2.2.1 Y_4R Binding



[^{3}H]UR-KK193 (**4.45**)
K_d (Y_4R) = 0.67 nM

[^{3}H]UR-MK136 (**4.46**)
K_d (Y_1R) = 6.2 nM

Figure 5: Structures of the Y_4R radioligand [^{3}H]UR-KK193 (**4.45**) and the Y_1R radioligand [3H]UR-MK136 (**4.46**).

K_i values of all target compounds were determined in competition binding studies on live cells expressing the Y_4R (CHO-hY_4R-G_{qi5}-mtAEQ cells) using the radioligand [^{3}H]UR-KK193 (**4.45**, Figure 5) (Table 1, Figure 6).[11] The affinities of the aza-peptides **4.27** (K_i = 134 nM), **4.29** (K_i = 52.7 nM) and **4.31** (K_i = 113 nM) were lower than the affinity of the respective reference compound **4.2** (K_i = 3.5 nM). (*R*,*R*)-2,7-diaminosuberic acid was superior to octanedioic acid as a linker in dimeric aza-peptides (cf. (2*R*,7*R*)-**4.26**: K_i = 30.8 nM) as in case of peptide **1** compared to **4.2**. The aza-Orn precursors of **4.29** and **4.31**, compounds **4.28** (K_i = 130 nM) and **4.30** (K_i = 385 nM) bind to the Y_4R as well, though with lower affinities compared to their aza-Arg counterparts.

Among the structural modifications of pentapeptide **4.3**, replacement of the *N*-terminal acetic acid with octanoic acid resulted in an increase in Y_4R affinity by a factor of almost six. The replacement of Arg^2 in **4.3** with the carbamoylated arginine **4.32** was even more favorable: The affinity of **4.34** (K_i = 19.5 nM) was 17-fold higher than the affinity of its parent compound **4.3**. Extension of the *N*-terminus by an additional arginine led to a further increase in affinity: the K_i value of compound **4.35** was in the single-digit nanomolar range (K_i = 2.87 nM). Up to now, comparable binding data were achieved only in case of cross-linked or significantly longer linear peptides. Furthermore, the introduction of an additional *N*-terminal arginine could compensate for the absence of the carbamoylated arginine **4.32**,

that is, **4.36** (K_i = 3.43 nM) showed nearly the same affinity as peptide **4.35**. Interestingly, the replacement of the arginine adjacent to the C-terminus with the modified arginine building block **4.32** was not tolerated (**4.37**: K_i = 501 nM).

Following the structural optimization of **4.3**, a *D*-amino acid scan was performed with the hexapeptide **4.35**. Whereas replacement of Arg^1 or Tyr^2 with *D*-Arg or *D*-Tyr, respectively, was tolerated (cf. **4.41**, **4.42**), replacement of Leu^4, Arg^5 or Tyr^6 by the corresponding *D*-amino acid led to a marked decrease in Y_4R affinity (cf. **4.38**-**4.40**).

Previous studies revealed that C-terminal amidation is crucial for Y_4R binding of the endogenous ligand hPP.[21] Similarly, the affinity of the carboxylic acid **4.43** (IC_{50} > 5000 nM) was by far lower than the affinity of the amide **4.35**. Cyclization of **4.43** resulted in an increase in Y_4R affinity. However, the cyclic peptide **4.44** (K_i = 1650 nM) was only a weak binder compared to the linear analog **4.35**.

4.2.3.2 NPY Receptor Subtype Selectivity

K_i values of all target compounds were also determined at Y_1, Y_2 and Y_5 receptors using the radioligands [^{3}H]propionyl-pNPY (Y_2R and Y_5R) or [^{3}H]UR-MK136 (**4.46**, Y_1R, Figure 5). Neither the linear peptides **4.34**-**4.43** nor the cyclic peptide **4.44** displayed remarkable affinity to Y_1, Y_2 or Y_5 receptors. Having a more than 1000-fold selectivity for the Y_4R over the other NPY receptors, peptide **4.35** proved to be superior to (2*R*,7*R*)-**4.1** and **4.2**. Only the pentapeptide **4.33** bound to the Y_1R with a K_i value in the submicromolar range (K_i (Y_1R) = 589 nM), that is, the lipophilic *N*-terminal octanoyl residue is disadvantageous with respect to Y_4R selectivity.

The cross-linked aza-peptides (2*R*,7*R*)-**4.26**, **4.27** and **4.28** displayed only low Y_1R, Y_2R and Y_5R affinities, whereas the affinity of **4.31** (K_i = 53 nM) at the Y_1R was markedly higher than that of the peptide analog **4.2**. In a Fura-2 Ca^{2+} assay on HEL cells, **4.31** revealed Y_1R antagonism (Figure 7) with a K_b value of 11.5 nM.

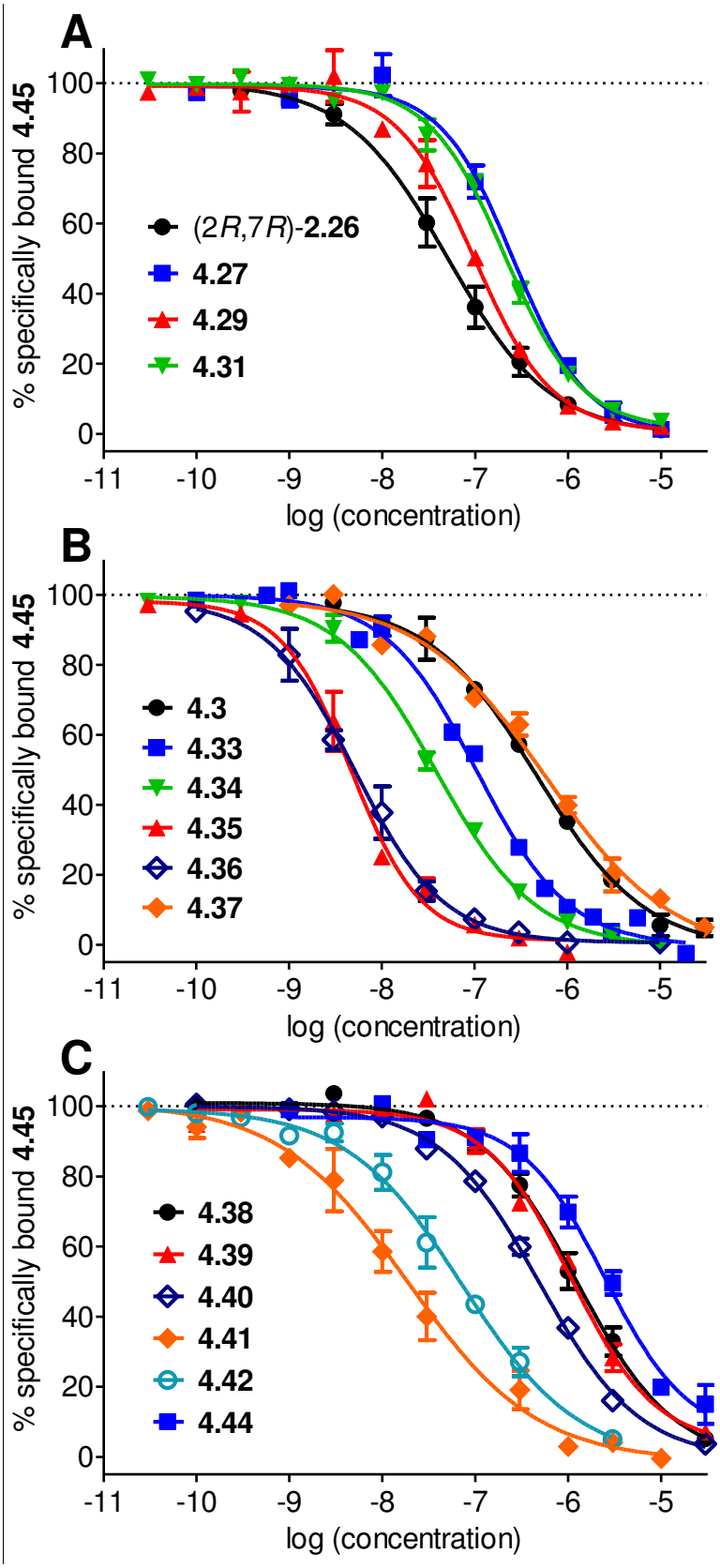


Figure 6. Competition binding performed with the radioligand **4.45** at CHO-hY_4R-G_{qi5}-mtAEQ cells. (**A**), (**B**) and (**C**) Displacement of **4.45** (K_d = 0.67 nM, c = 0.6 nM). Data represent mean values ± SEM of at least 3 independent experiments, each performed in triplicate.

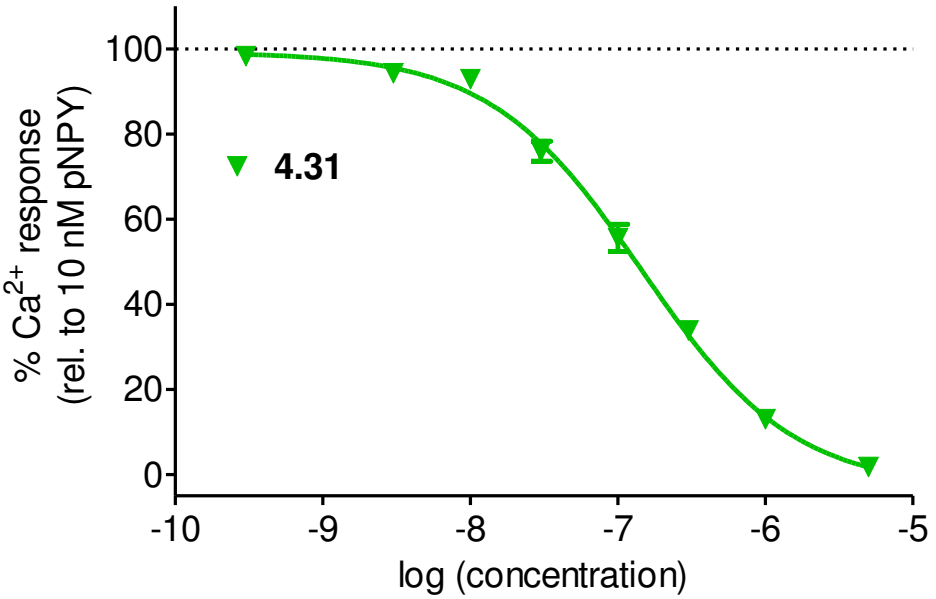


Figure 7. Y_1R antagonism of **4.31**. Inhibition of the pNPY (c = 10 nM, EC_{50} = 0.87 nM) induced intracellular Ca^{2+} mobilization in HEL cells by **4.31** (K_b = 11.5 ± 1.1 nM), determined in a Fura-2 assay. Mean ± SEM from three independent experiments.

Table 1. NPY receptor binding data.

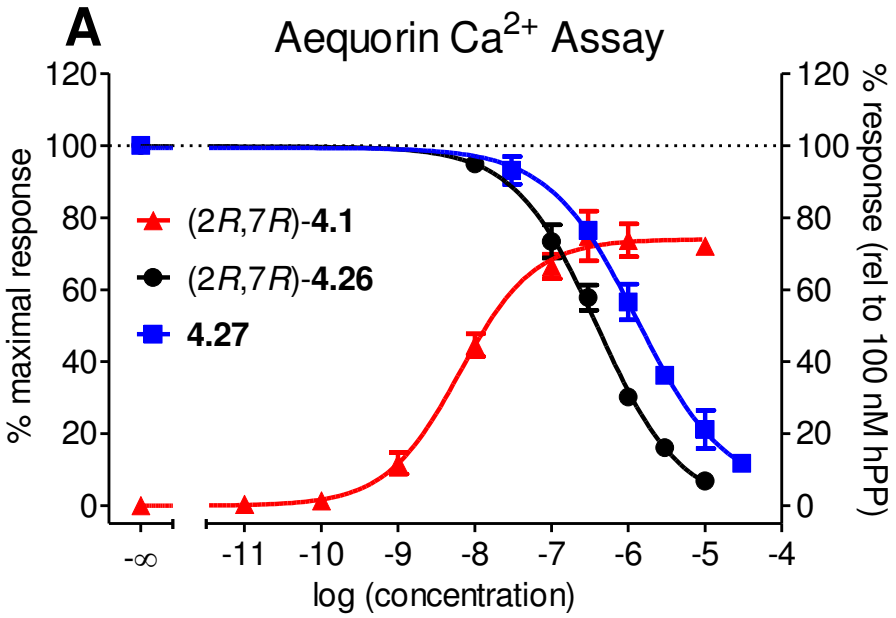
	Y_1R	**Y_2R**	**Y_4R**	**Y_5R**
Compd.	K_i [nM][a]	K_i [nM][b]	K_i [nM][c]	K_i [nM][d]
hPP	440[e]	>5000[e]	0.65[f]	17[e]
(2*R*,7*R*)-**4.1**	440[f]	830[f]	0.45[f]	1500[f]
4.2	720[f]	1700[f]	3.5[f]	280[f]
4.3	> 5000	> 5000	337 ± 110	> 5000
4.4	24[g]	920[g]	660[f]	> 5000[g]
(2*R*,7*R*)-**4.26**	1840 ± 380	> 5000	30.8 ± 7.5	> 5000
4.27	574 ± 49	> 5000	134 ± 20	2700 ± 490
4.28	n.d.	n.d.	130 ± 39	n.d.
4.29	3730 ± 350	> 5000	52.7 ± 7.1	3560 ± 440
4.30	n.d.	n.d.	385 ± 127	n.d.
4.31	53 ± 12	3270 ± 870	113 ± 14	2100 ± 240
4.33	589 ± 120	> 5000	56.9 ± 5.3	3440 ± 210
4.34	> 5000	> 5000	19.5 ± 2.6	> 5000
4.35	4830 ± 1400	> 5000	2.87 ± 0.78	> 5000

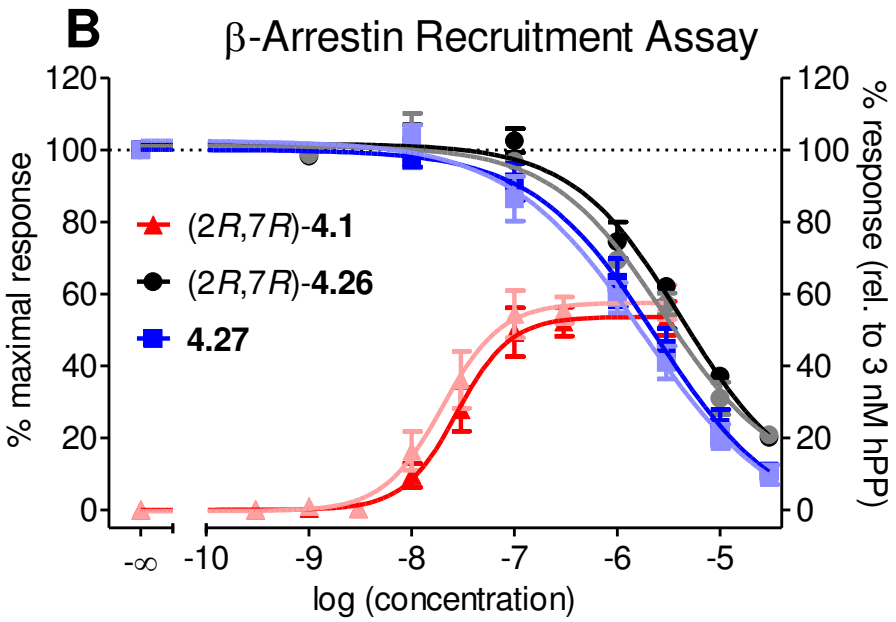
4.36	1180 ± 380	> 5000	3.43 ± 1.3	> 5000
4.37	1120 ± 270	> 5000	501 ± 200	> 5000
4.38	> 5000	> 5000	724 ± 140	> 5000
4.39	> 5000	> 5000	568 ± 76	> 5000
4.40	> 5000	> 5000	248 ± 23	> 5000
4.41	> 5000	> 5000	10.4 ± 3.3	> 5000
4.42	> 5000	> 5000	40.5± 15	> 5000
4.43	> 5000	> 5000	> 5000	> 5000
4.44	> 5000	> 5000	1650 ± 290	> 5000

[a]Radioligand competition binding assay with **4.46**[22] (K_d = 6.2 nM, c = 4 nM) using MCF-7-hY_1 cells.[23] [b]Radioligand competition binding assay with [^{3}H]propionyl-pNPY[24] (K_d = 1.4 nM, c = 1 nM) using CHO-hY_2-G_{qi5}-mtAEQ cells[25]. [c]Radioligand competition binding assay with **4.45**[11] (K_d = 0.67 nM, c = 0.6 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells.[26] [d]Radioligand competition binding assay with [^{3}H]propionyl-pNPY (K_d = 4.83 nM, c = 4 nM) using HEC-1b hY_5R cells[27]. [e]K_i value reported by Berlicki et al.[6] [f]K_i value reported by Kuhn et al.[11] [g]K_i value reported by Keller et al.[5]

4.2.3 Functional Studies at the Y_4R

The target compounds were investigated for Y_4R functional activity in an aequorin Ca^{2+} assay as well as β-arrestin 1 and β-arrestin 2 recruitment assays on genetically engineered CHO or HEK293T cells, respectively (data cf. Table 2, Figure 7 and Figure 8). (2*R*,7*R*)-**4.1** was a partial agonist in both, the Ca^{2+} assay and the β-arrestin recruitment assays (α = 0.62, (Ca^{2+} assay), α = 0.54 (β-arrestin 1) or 0.58 (β-arrestin 2)). The incorporation of aza-amino acids into the cross-linked peptides (2*R*,7*R*)-**4.1** and **4.2** had an impact on the quality of action in both assays. The "dimeric" aza-peptides (2*R*,7*R*)-**4.26**, **4.27**, **4.29** and **4.31** were Y_4R antagonists, able to suppress the response elicited by the endogenous ligand hPP (note: The aza-peptide **4.29** displayed very weak partial agonism with negligible efficacy (α = 0.07, EC_{50} = 542 nM (Ca^{2+} assay), α = 0.09, EC_{50} = 875 nM (β-arrestin 1), α = 0.12, EC_{50} = 473 (β-arrestin 2)).


A
Aequorin Ca2+ Assay
% maximal response
% response (rel to 100 nM hPP)
(2R,7R)-4.1
(2R,7R)-4.26
4.27
log (concentration)


B
β-Arrestin Recruitment Assay
% maximal response
% response (rel. to 3 nM hPP)
(2R,7R)-4.1
(2R,7R)-4.26
4.27
log (concentration)

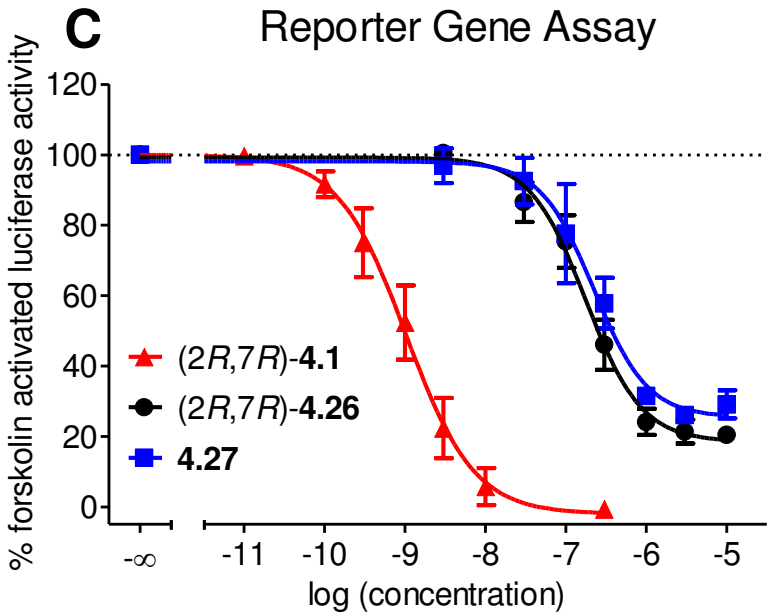

C
Reporter Gene Assay
% forskolin activated luciferase activity
(2R,7R)-4.1
(2R,7R)-4.26
4.27
log (concentration)

Figure 8. Y_4R functional activity of (2*R*,7*R*)-**4.1**, (2*R*,7*R*)-**4.26** and **4.27** determined in a calcium (aequorin) assay, a β-arrestin recruitment assay and a luciferase reporter gene assay. (**A**) Induced intracellular Ca^{2+} mobilization ((2*R*,7*R*)-**4.1**, plotted on left Y-axis) or inhibition of the hPP (EC_{50} = 9.7 nM, c = 100 nM) induced Ca^{2+} mobilization ((2*R*,7*R*)-**4.26**, **4.27**, plotted on right Y-axis) in CHO-hY_4R-mtAEQ-G_{qi5} cells. (**B**) Induced β-arrestin 1,2 recruitment ((2*R*,7*R*)-**4.1**, plotted on left Y-axis) or inhibition of the hPP (EC_{50} (Arr1) = 3.54 nM, EC_{50} (Arr2) = 2.74 nM, c = 3 nM) induced β-arrestin 1,2 recruitment ((2*R*,7*R*)-**4.26**, **4.27**, plotted on right Y-axis) in HEK293T-ARRB1-Y_4R cells (shown in dark colors) and HEK293T-ARRB2-Y_4R cells (shown in light colors). (**C**) Inhibition of the forskolin-stimulated (2 μM) luciferase activity (corresponding to 100%) in hY_4R expressing HEK293 cells with the maximum inhibitory effect of the endogenous ligand hPP which is set to 0% luciferase activity and corresponds to full agonism (α = 1.0). Data points shown for (**A**), (**B**), and (**C**) are the mean ± SEM of at least three independent experiments performed in triplicate.

Compounds (2*R*,7*R*)-**4.26** and **4.27** were also investigated for Y_4R agonism in a luciferase reporter gene assay. Surprisingly, both aza-peptides displayed partial agonism in this assay (α = 0.81, EC_{50} = 176 nM ((2*R*,7*R*)-**4.26**), α = 0.75, EC_{50} = 220 nM (**4.27**)). This can be explained by amplification of the signal in the luciferase assay, uncovering weak partial agonism which is elusive in assays with proximal readouts, a phenomenon demonstrated, for example, for histamine H_4 receptor ligands.[28] This becomes also obvious from the fact that the partial agonist (2*R*,7*R*)-**1** was a full agonist in this assay.[11] The pentapeptides **4.33**, **4.34** and the hexapeptides **4.35** and **4.36** were partial agonists achieving between 44 and 78% of the maximal response of hPP in both, the calcium and the arrestin assay. The introduction of *D*-amino acids into the C-terminal tripeptide of **4.35** led to Y_4R antagonism. Compound **4.40** in which Leu^4 was replaced by *D*-Leu displayed the highest antagonistic activity (K_b = 531 nM, Ca^{2+} assay). By contrast, Y_4R agonism was retained, though at a lower level, when *D*-amino acids were introduced into the N-terminal part of **4.35** (cf. **4.41**, **4.42**). Compound **4.37**, in which Arg^5 was replaced by the carbamoylated arginine **4.32**, and the cyclic hexapeptide **4.44** were identified as weak Y_4R antagonists.

In summary, the modification of the C-terminal part of the monomeric and crosslinked peptide partial agonists by the introduction of aza-amino acids or *D*-amino acids as well as the rigidization of the backbone by head-to-side-chain cyclization gave access to Y_4R antagonists. Unfortunately, these modifications went along with a decrease in Y_4R affinity. The antagonistic activities determined in the aequorin Ca^{2+} assay and the β-arrestin assays were in good agreement with the respective binding constants with exception of the K_b value of (2*R*,7*R*)-**4.26** in the β-arrestin assays. The EC_{50} values of the reported Y_4R partial

agonists were substantially higher than the K_i values. This effect was previously discussed and can be attributed to the presence or absence of sodium ions in the buffers.[11]

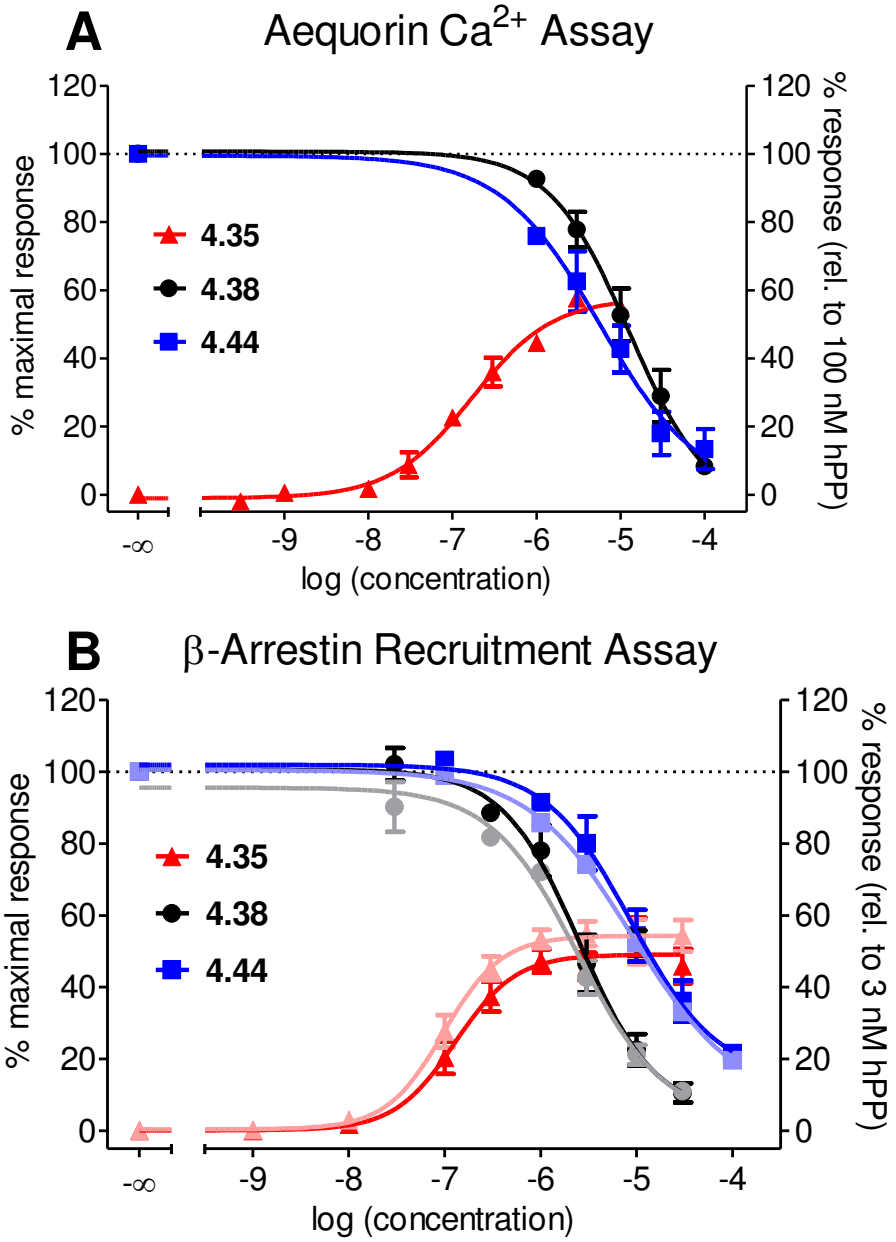


Figure 9. Y_4R functional activity of **4.35**, **4.38** and **4.44** determined in a calcium (aequorin) assay and a β-arrestin recruitment assay. (**A**) Induced intracellular Ca^{2+} mobilization (**4.35**, plotted on left Y-axis) or inhibition of the hPP (EC_{50} = 9.7 nM, c = 100 nM) induced intracellular Ca^{2+} mobilization (**4.38**, **4.44**, plotted on right Y-axis) in in CHO-hY_4R-mtAEQ-G_{qi5} cells. (**B**) Induced β-arrestin 1,2 recruitment (**4.35**, plotted on left Y-axis) or inhibition of the hPP (EC_{50} (Arr1) = 3.54 nM, EC_{50} (Arr2) = 2.74 nM, c = 3 nM) induced β-arrestin 1,2 recruitment (**4.38**, **4.44**, plotted on right Y-axis) in HEK293T-ARRB1-Y_4R cells (shown in dark colors) and HEK293T-ARRB2-Y_4R cells (shown in light colors). Data points shown for (**A**) and (**B**) are the mean ± SEM of at least three independent experiments performed in triplicate.

Table 2. NPY Y_4R agonist potencies (EC_{50}) and intrinsic activities (α) or antagonistic activities (K_b values).

	Aequorin Ca^{2+}[a]		**β-Arrestin 1**[b]		**β-Arrestin 2**[c]	
Compd.	EC_{50}/K_b [nM]	α	EC_{50}/K_b [nM]	α	EC_{50}/K_b [nM]	α
hPP	9.7[d]	1	3.54 ± 0.59	1	2.74 ± 0.23	1
(2*R*,7*R*)-**4.1**	6.9[d]	0.62	40.4 ± 11	0.54	31.8 ± 12	0.58
4.3	6390 ± 450	0.55	n.d.	n.d.	n.d.	n.d.
4	*20*[e]	n.a.	*102 ± 40*	n.a.	*96.3 ± 37*	n.a.
(2*R*,7*R*)-**4.26**[f]	*34.3 ± 4.5*	n.a.	*2940 ± 190*	n.a.	*632 ± 110*	n.a.
4.27[g]	*127 ±26*	n.a.	*591 ± 190*	n.a.	*340 ± 42*	n.a.
4.29	542 ± 110	0.07	875 ± 260	0.09	473 ±65	0.12
	50.6 ± 0.2	n.a.	n.d.	n.d.	n.d.	n.d.
4.31	*118.4 ± 47*	n.a.	n.d.	n.d.	n.d.	n.d.
4.33	2050 ± 84	0.44	2200 ± 130	0.56	1790 ± 1070	0.61
4.34	1460 ± 83	0.61	1070 ± 520	0.62	568 ± 120	0.60
4.35	187 ± 23	0.58	144 ± 28	0.48	124.1 ± 24	0.55
4.36	303 ± 68	0.78	187 ± 40	0.51	140 ± 45	0.54
4.37	*847 ± 180*	n.a.	*3590 ± 429*	n.a.	*3840 ± 55*	n.a.
4.38	*1660 ± 570*	n.a.	*708 ± 210*	n.a.	*533 ± 120*	n.a.
4.39	*737 ± 360*	n.a.	n.d.	n.d.	n.d.	n.d.
4.40	*531 ± 230*	n.a.	n.d.	n.d.	n.d.	n.d.
4.41	608 ± 140	0.61	2050 ± 780	0.46	710 ± 200	0.49
4.42	756 ± 230	0.63	2160 ± 600	0.45	624 ± 150	0.56
4.44	*1100 ± 520*	n.a.	*2920 ± 880*	n.a.	*1880 ± 320*	n.a.

[a]Aequorin calcium mobilization assay on CHO-hY_4-G_{qi5}-mtAEQ cells.[26] Antagonistic activities (K_b values given in italics) were determined in the presence of 100 nM hPP. [b,c]β-Arrestin 1,2 recruitment assay on HEK293T cells stably expressing the Y_4R and ARRB1 or ARRB2. Antagonistic activities (K_b values given in italics) were determined in the presence of 3 nM hPP. [d]EC_{50} value reported by Kuhn et al.[11] [e]K_b value reported by Keller et al.[5] [f]Y_4R agonism on HEK293-hY_4R-CRE Luc cells (luciferase reporter gene assay): EC_{50} = 176 ± 50 nM, α = 0.81. [g]Y_4R agonism on HEK293-hY_4R-CRE Luc cells (luciferase reporter gene assay): EC_{50} = 220 ± 73 nM, α = 0.75.

4.3 Conclusions

The affinity and potency of the Y_4R partial agonist **4.3** was considerably increased by the replacement of Arg^2 with the carbamoylated arginine **4.32** and the introduction of an additional arginine at the N-terminus. The latter turned out to compensate for the absence of the carbamoylated arginine. Several backbone modifications of the C-terminal tripeptide (e.g. the introduction of aza-amino acids or *D*-amino acids) as well as a rigidization *via* head-to-side-chain cyclization changed the quality of action of linear or "dimeric" Y_4R peptide ligands from partial agonism to antagonism. The resulting antagonists displayed lower Y_4R antagonistic activities than the reported Y_4R antagonist **4.4**. However, due to their extremely facile synthetic accessibility, linear peptides containing *D*-amino acids (e.g. **4.40** (K_i = 248 nM, K_b = 531 nM (aequorin Ca^{2+} assay)) are promising building blocks for further structural modifications and might pave the way for the development of peptide Y_4R antagonists with increased affinity and antagonistic activity.

4.4 Experimental Section

4.4.1 General Experimental Conditions

Chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise indicated. DMF for peptide synthesis, NMP for peptide synthesis, 4-DMAP, sodium cyanotrihydroborate and HOBt hydrate were from Acros Organics/Fisher Scientific (Nidderau, Germany). Fmoc-Sieber-PS resin (0.61 mmol/g), Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(*t*Bu)-OH, H-*L*-Tyr(*t*Bu)-2-CT resin (0.68 mmol/g), PyBOP and HBTU were from Iris Biotech (Marktredwitz, Germany). MeCN for HPLC (gradient grade), Fmoc-Leu-OH, DCC, sulfur trioxide pyridine complex, hydrazine hydroxide and methanol were from Merck (Darmstadt, Germany). Trifluoroacetic acid, triethylamin (TEA), triphosgene, Fmoc chloride, isobutyraldehyde, diethylamin (DEA), CH_2Cl_2, diethyl ether and Triton X-100 were from Sigma Aldrich (Deisenhofen, Germany), di-tert-butyl dicarbonate (>97%) and 3-aminopropan-1-ol were from Alfa Aesar (Karlsruhe, Germany). *N,N*-diisopropylethylamine (DIPEA) (99%) was from ABCR (Karlsruhe, Germany), and Fmoc-Ala-OSu was from Advanced Chemtech (Louisville, KY). Bovine serum albumin and bacitracin were from Serva (Heidelberg, Germany) and coelenterazin h was obtained from Biotrend (Cologne, Germany). Human pancreatic polypeptide and porcine neuropeptide Y were from Synpeptide (Shanghai, China). The synthesis of (2*R*,7*R*)-**4.1**,[11] **4.3**,[6] **4.4**,[5] **4.24**,[11] **4.25**,[29] **4.32**,[20] **4.45**,[11] **4.46**[22] and [^{3}H]propionyl-pNPY[24] was previously described. Millipore water was used throughout for the preparation of buffers and HPLC eluents. Polypropylene reaction vessels (1.5 or 2 mL) with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for small scale reactions (e.g. the preparation of **4.44**) and for the storage of stock solutions. TL chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates, silica gel 60 (40-63 µm, Merck) was used for column chromatography. Visualization was accomplished by UV light (λ = 254 nm or 366 nm) and with ninhydrin/acetic acid solution. NMR-spectra were recorded on a Bruker Avance 300 (7.05 T, ^{1}H: 300.1 MHz, ^{13}C: 75.5 MHz) or a Bruker Avance 600 instrument with cryogenic probe (14.1 T, ^{1}H: 600.3 MHz, ^{13}C: 150.9 MHz) (Bruker, Karlsruhe, Germany). Mass spectra were recorded on a Finnigan MAT95 (EI-MS 70eV) or a Finnigan MAT SSQ 710 A (CI-MS (NH_3)). For HRMS an Agilent Q-TOF 6540 UHD (Agilent, Waldbronn, Germany) equipped with an ESI source was used. Preparative HPLC was performed on a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-

2001 detector and a RP-column (Kinetex-XB C_{18}, 5 µm, 250 mm × 21 mm, Phenomenex, Aschaffenburg, Germany) at a flow-rate of 15 mL/min using mixtures of acetonitrile and 0.1% aqueous TFA solution as mobile phase. A detection wavelength of 220 nm was used throughout. The collected fractions were lyophilised using an alpha 2-4 LD apparatus (Martin Christ, Osterode am Harz, Germany) equipped with a RZ 6 rotary vane vacuum pump (vacuubrand, Wertheim, Germany). Analytical HPLC analysis was performed on a system from Merck-Hitachi (Hitachi, Düsseldorf, Germany) composed of a L-6200-A pump, a AS-2000A autosampler, a L-4000A UV detector, a D-6000 interface and a RP-column Kinetex-XB C_{18}, 5 µm, 250 mm × 4.6 mm (Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.8 mL/min. Mixtures of acetonitrile (A) and 0.1% aqueous TFA solution (B) were used as mobile phase. The following gradient was applied: A/B: 0-25 min: 10:90 - 60:40, 25-35 min: 60:40 - 95:5, 35-45 min: 95:5. Detection was performed at 220 nm, the oven temperature was 30 °C.

4.4.2 Chemistry: Experimental Protocols and Analytical Data

General procedure for solid phase peptide synthesis. Peptides were synthesized by manual SPPS using the Fmoc strategy on an Fmoc-Sieber-PS resin or an H-*L*-Tyr(*t*Bu)-2CT resin (**4.43**). 5-mL Discardit II syringes (Becton Dickinson, Heidelberg, Germany) were equipped with 35 μm polyethylene frits (Roland Vetter Laborbedarf, Ammerbuch, Germany) and used as reaction vessels. For the coupling of standard *D*- or *L*-amino acid to the *N*-terminus of an amino acid or the amino group of the Fmoc-Sieber-PS resin, DMF/NMP (8:2) was used as solvent. Fmoc amino acids (5-fold excess) were preactivated with HBTU/HOBt/DIPEA (5/5/10 equiv) for 5 min and added to the resin. 'Double' coupling at rt was performed for all standard amino acids for 45 min. The coupling of the arginine building block **4.32** and the dipeptides **4.17**, **4.18** and **4.19** (3-fold excess, preactivated with HBTU/HOBt/DIPEA (3/3/6 equiv) was performed at 35 °C for 14 h ('single coupling') using anhydrous DMF as solvent. For the coupling of Fmoc amino acids to a resin bound aza-amino acid, triphosgene was used as coupling reagent. Triphosgene (1.75 equiv) was dissolved in anhydrous THF and cooled to 0 °C. A solution of Fmoc amino acid (5 equiv) and DIPEA (15 equiv) in anhydrous THF was added dropwise causing the formation of a white precipitate. After addition, stirring was continued for 5 min. The suspension was centrifuged and the supernatant was added to the resin. Coupling was performed at 35 °C for 12 h.

After completed coupling, the resin was washed with DMF/NMP and treated with 20% piperidine in DMF/NMP (8:2) at rt (2 ×) for 10 min to remove the Fmoc group, followed by thorough washing of the resin.

General procedure for cleavage of protected peptides from the resin. After the last coupling step and Fmoc deprotection, the resin was washed with CH_2Cl_2. Side-chain protected peptides were cleaved from the resin with CH_2Cl_2/TFA (97:3) (rt, 10 × 6 min). The peptides were separated from the resin by filtration. The combined filtrates were collected in a round bottom flask containing water (10 times the volume of the combined filtrates). The organic solvent was removed on a rotary evaporator; the aqueous phase was lyophilised followed by purification by preparative HPLC.

***tert*-Butyl (3-hydroxypropyl)carbamate (4.6).**[30] 3-Aminopropan-1-ol (5 g, 66.5 mmol) was dissolved in THF (50 mL). An aqueous solution of Na_2CO_3 (8.42 g, 1.2 equiv, 10 mL) was added, and the mixture was cooled to 0 °C. Boc anhydride (14.82 g, 1.02 equiv) was added dropwise, and the mixture was stirred at room temperature for 14 h. The mixture was filtered over a pad of celite, and the volume of the filtrate was reduced to approximately 15 mL under reduced pressure. The residue was diluted with water (50 mL) and extracted with EtOAc (3 × 100 mL). The combined organic layers were dried over $MgSO_4$ followed by the removal of the solvent under reduced pressure. The desired compound was obtained as viscous oil (10.47 g, 80.8%). ^{1}H-NMR (400 MHz, [D_6]acetone): δ (ppm) 1.40 (s, 9H), 1.59 – 1.70 (m, 2H), 3.18 (dd, 2H, *J* 14.3 Hz, 6.6 Hz), 3.58 (dd, 2H, *J* 13.1 Hz, 5.8 Hz), 3.61 – 3.67 (m, 1H), 5.99 (br s, 1H). HRMS (ESI): *m/z* $[M+Na]^+$ calcd. for $[C_8H_{17}NNaO_3]^+$ 198.1101, found: 198.1097. $C_8H_{17}NO_3$ (175.23).

***tert*-Butyl (3-oxopropyl)carbamate (7).**[31] Compound **4.6** (10.3 g, 58.8 mmol), trimethylamine (28 mL) and DMSO (30 mL) were mixed with CH_2Cl_2 (100 mL). Sulfur trioxide pyridine complex (21 g, 132 mmol) was added under ice-cooling, and the mixture was stirred at 0 °C for 1 h and at room temperature for additional 3 h. Water (500 mL) was added, and the mixture was extracted with EtOAc (2 × 300 mL). The combined organic layers were dried over $MgSO_4$, and the solvent was removed on a rotary evaporator. The residue was taken up in hexane/EtOAc (3:1, 30 mL) and subjected to column chromatography (eluent: hexane/EtOAc: 3:1 → 1:1). The title compound was obtained as colorless oil (5.3 g, 58%). TLC

(light petroleum/EtOAc 2:1) R_f = 0.36. ^{1}H-NM R (300 MHz, [D_6]acetone): δ (ppm) 1.39 (s, 9H), 2.59 – 2.67 (m, 2H), 3.36 – 3.43 (m, 2H), 6.01 (br s, 1H), 9.75 (t, 1H, *J* 1.7 Hz). HRMS (ESI): *m/z* $[M+Na]^+$ calcd. for $[C_8H_{15}NNaO_3]^+$ 196.0944, found: 196.0945. $C_8H_{15}NO_3$ (173.21).

(9*H*-Fluoren-9-yl)methyl hydrazinecarboxylate (4.8).[32] Hydrazine hydroxide (0.97 g, 19.4 mmol) was dissolved in MeCN/H_2O (1:1, 10 mL). A solution of Fmoc-Cl (1 g, 3.9 mmol) in MeCN (20 mL) was added dropwise under ice-cooling causing the formation of a white precipitate. The mixture was stirred at room temperature for additional 12 h and filtered to yield the desired compound as a white solid (900 mg, 91.3%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 4.08 (br s, 2H), 4.16 – 4.34 (m, 3H), 7.27 – 7.48 (m, 4H), 7.69 (d, 2H, *J* 7.40 Hz), 7.89 (d, 2H, *J* 7.40 Hz), 8.36 (br s, 1H). HRMS (ESI): *m/z* $[2M+H]^+$ calcd. for $[C_{30}H_{29}N_4O_4]^+$ 509.2183, found: 509.2183. $C_{15}H_{14}N_2O_2$ (254.29).

(9*H*-Fluoren-9-yl)methyl 2-{3-[(*tert*-butoxycarbonyl)amino]propylidene}hydrazine-1-carboxylate (4.9).[33] Compound **6** (2.7 g, 10.5 mmol) was suspended in CH_2Cl_2 (120 mL). A solution of **4.8** (1.82 g, 10.5 mmol) in CH_2Cl_2 (20 mL) was added which in turn led to the dissolution of **4.6**. While stirring at room temperature for 14 h, a white, voluminous precipitate was formed. The volume of the mixture was reduced to approximately 50 mL and the precipitate was collected by filtration to yield **4.9** as white solid (3.6 g, 83.7%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.36 (s, 9H), 2.18 – 2.40 (m, 2H), 2.95 – 3.20 (m, 2H), 4.25 (t, 1H, *J* 6.3 Hz), 4.32 – 4.50 (m, 2H), 6.88 (t, 1H, *J* 5.6 Hz), 7.25 – 7.47 (m, 5H), 7.71 (d, 2H, *J* 7.3 Hz), 7.90 (d, 2H, *J* 7.3 Hz), 10.83 (s, 1H). HRMS (ESI): *m/z* $[M+Na]^+$ calcd. for $[C_{23}H_{27}N_3NaO_4]^+$ 432.1894, found: 432.1892. $C_{23}H_{27}N_3O_4$ (409.49).

(9*H*-Fluoren-9-yl)methyl 2-{3-[(*tert*-butoxycarbonyl)amino]propyl}hydrazine-1-carboxylate (4.10).[33] Compound **4.9** (2.75 g, 6.72 mmol) was dissolved in CH_2Cl_2/MeOH (3:2, 50 mL) and sodium cyanotrihydridoborate (550 mg, 8.74 mmol) was added. The pH was slowly adjusted to a value of 2-3 with 2 M aqueous HCl, and the mixture was stirred at room temperature for 6 h. After completion of the reaction (monitored by TLC (light petroleum/EtOAc 1:1): R_f = 0.44), the mixture was basified with Na_2CO_3. The precipitate was filtered off, and the volume of the filtrate was reduced to approximately 5 mL. The residue was taken up in hexane/EtOAc (1:2, 10 mL) and subjected to column chromatography (eluent: hexane/EtOAc: 4:3). After removal of the solvent on a rotary evaporator, the residue was triturated with EtOAc (3 mL), and the desired compound was obtained as white, crystalline

solid (1.05 g, 40%). ^{1}H-NMR (300 MHz, [D_6]acetone): δ (ppm) 1.40 (s, 9H), 1.55 – 1.72 (m, 2H), 2.80 – 2.94 (m, 2H), 3.10 – 3.23 (m, 2H), 4.24 (t, 1H, *J* 6.7 Hz), 4.28 – 4.44 (m, 3H), 5.99 (br s, 1H), 7.28 – 7.45 (m, 4H), 7.70 (d, 2H, *J* 7.3 Hz), 7.86 (d, 2H, *J* 7.5 Hz), 7.99 (br s, 1H). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{23}H_{30}N_3O_4]^+$ 412.2231, found: 412.2235. $C_{23}H_{29}N_3O_4$ (411.50).

(9*H*-Fluoren-9-yl)methyl 2-(2-methylpropylidene)hydrazine-1-carboxylate (4.11).[34] Compound **4.6** (890 mg, 3.45 mmol) was suspended in CH_2Cl_2 (20 mL). After addition of isobutyraldehyde (315 μL, 1 equiv) compound **4.6** dissolved, and the mixture was stirred for 50 h. The title compound was obtained as a white solid after removal of the volatiles under reduced pressure (1.06 g, quant). ^{1}H-NMR (300 MHz, [D_6]acetone): δ (ppm) 1.00 – 1.20 (m, 6H), 2.39 – 2.59 (m, 1H), 4.27 (t, 1H, *J* 7.0 Hz), 4.42 (d, 2H, *J* 7.0 Hz), 7.27 – 7.48 (m, 5H), 7.75 (br s, 2H), 7.87 (d, 2H, *J* 7.4 Hz), 9.72 (br s, 1H). HRMS (ESI): *m/z* $[M+Na]^+$ calcd. for $[C_{19}H_{20}N_2NaO_2]^+$ 331.1417, found: 331.1421. $C_{19}H_{20}N_2O_2$ (308.38).

(9*H*-Fluoren-9-yl)methyl 2-isobutylhydrazine-1-carboxylate (4.12).[32] Compound **4.11** (980 mg, 3.2 mmol) was dissolved in CH_2Cl_2/MeOH (4:3, 35 mL) and sodium cyanotrihydridoborate (260 mg, 4.1 mmol) was added. The pH was adjusted to a value of 2-3 with 2 M aqueous HCl and the mixture was stirred at room temperature for 2 h. After completion of the reaction, the mixture was basified with Na_2CO_3. The solids were removed by filtration, and the filtrate was extracted with EtOAc (150 mL). The organic layer was washed with water (2 ×) and brine. The title compound was obtained as a greyish solid (970 mg, 98%) after removal of the solvents. ^{1}H-NMR (300 MHz, [D_6]acetone): δ (ppm) 0.90 (d, 6H, *J* 6.6 Hz), 1.58 – 1.80 (m, 1H), 2.62 (br s, 2H), 4.13 – 4.28 (m, 2H), 4.35 (d, 2H, *J* 6.8 Hz), 7.26 – 7.45 (m, 4H), 7.69 (d, 2H, *J* 7.4 Hz), 7.85 (d, 2H, *J* 7.4 Hz), 7.96 (br s, 1H). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{19}H_{23}N_2O_2]^+$ 311.1754, found: 311.1757. $C_{19}H_{22}N_2O_2$ (310.40).

Benzyl N^{ω}-[(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl]-*L*-argininate (4.14).[35] Fmoc-Arg(Pbf)-OH (3 g, 4.62 mmol, 1 equiv), 4-dimethylaminopyridine (57 mg, 0.1 equiv) and benzyl alcohol (1 mL, 2 equiv) were dissolved in CH_2Cl_2 (25 mL), and a solution of DCC (1 g, 1.05 equiv) in CH_2Cl_2 (10 mL) was added dropwise under ice-cooling. The reaction mixture was stirred at room temperature for 14 h. After completion of the reaction (monitored by TLC (light petroleum/EtOAc 1:2): R_f = 0.40), the mixture was filtered, and the

filtrate was washed with 0.1 M HCl solution (30 mL) and brine (20 mL). The volume of the organic layer was adjusted to 160 mL with CH_2Cl_2, and diethylamine (20 mL) was added for removal of the Fmoc group. After all starting material had been consumed, the volatiles were removed on a rotary evaporator The residue was taken up in light petroleum/EtOAc (1:1, 10 mL) and subjected to column chromatography (eluent: light petroleum/EtOAc 1:1 → EtOAc/MeOH 4:1). The desired compound was obtained as a white, sticky solid (1.89 mg, 79.2%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.33–1.62 (m, 10H), 1.89 (br s, 2H), 1.99 (s, 3H, overlap with EtOAc), 2.42 (s, 3H), 2.47 (s, 3H), 2.93 (s, 2H), 2.97-3.10 (m, 2H), 3.26-3.34 (m, 1H), 5.06 (s, 2H), 6.43 (br s, 2H), 6.70 (br s, 1H), 7.27-7.42 (m, 5H). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{26}H_{37}N_4O_5S]^+$ 517.2479, found: 517.2543. $C_{26}H_{36}N_4O_5S$ (516.66).

Benzyl (*S*)-2-amino-3-[4-(tert-butoxy)phenyl]propanoate (4.15). Fmoc-Tyr(*t*Bu)-OH (2 g, 4.35 mmol, 1 equiv), 4-dimethylaminopyridine (53.2 mg, 0.1 equiv) and benzyl alcohol (1 mL, 2.2 equiv) were dissolved in CH_2Cl_2 (25 mL) and a solution of DCC (943 mg, 1.05 equiv) in CH_2Cl_2 (6 mL) was added dropwise under ice-cooling. The mixture was stirred at room temperature for 14 h. After completion of the reaction (monitored by TLC (light petroleum/EtOAc 2:1): R_f = 0.76), the mixture was filtered, and the filtrate was washed with 0.1 M HCl solution (30 mL) and brine (20 mL). The volume of the organic layer was adjusted to 70 mL with CH_2Cl_2, and diethylamine (12 mL) was added for removal of the Fmoc group. After all starting material had been consumed, the volatiles were removed on a rotary evaporator, and the residue was taken up in light petroleum/EtOAc (1:1, 10 mL) and subjected to column chromatography (eluent: light petroleum/EtOAc 1:1 → EtOAc/MeOH 4:1). The desired compound was obtained as a yellowish highly viscous oil (1.05 g, 73.7%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.26 (s, 9H), 1.80 (br s, 2H), 2.71 – 2.89 (m, 2H), 3.60 (t, 2H, *J* 6.9 Hz), 5.05 (s, 2H), 6.84 (d, 2H, *J* 8.5 Hz), 7.06 (d, 2H, *J* 8.4 Hz), 7.25 – 7.39 (m, 5H). ^{13}C-NMR (100.6 MHz, [D_6]DMSO): δ (ppm) 29.0 (3 carb), 56.4, 66.0, 78.0, 123.8 (2 carb), 128.40 (2 carb), 128.43, 128.8 (2 carb), 130.2 (2 carb), 132.9, 136.5, 153.9, 175.4. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{20}H_{26}NO_3]^+$ 328.1907, found: 328.1915. $C_{20}H_{25}NO_3$ (327.42).

Benzyl *L*-leucinate (4.16).[36] Fmoc-Leu-OH (1.54 g, 4.35 mmol, 1 equiv), 4-dimethylaminopyridine (53.2 mg, 0.1 equiv) and benzyl alcohol (1 mL, 2.2 equiv) were dissolved in CH_2Cl_2 (25 mL), and a solution of DCC (943 mg, 1.05 equiv) in CH_2Cl_2 (6 mL) was added dropwise under ice-cooling. The mixture was stirred at room temperature for 14 h.

After completion of the reaction (monitored by TLC (light petroleum/EtOAc 2:1): R_f = 0.80), the mixture was filtered and the filtrate was washed with 0.1 M HCl (30 mL) and brine (20 mL). The volume of the organic layer was adjusted to 70 mL with CH_2Cl_2, and diethylamine (12 mL) was added for removal of the Fmoc group. After all starting material had been consumed, the volatiles were removed on a rotary evaporator, the residue was taken up in light petroleum/EtOAc (1:1, 10 mL) and subjected to column chromatography (eluent: light petroleum/EtOAc 1:1 → EtOAc/MeOH 4:1). Compound **4.16** was obtained as a yellowish highly viscous oil (750 mg, 77.9%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 0.83 (d, 3H, *J* 6.6 Hz), 0.86 (d, 3H, *J* 6.6 Hz), 1.24–1.47 (m, 2H), 1.62–1.74 (m, 1H), 1.80 (br s, 2H), 3.28–3.42 (m, 1H), 5.10 (s, 2H), 7.28 – 7.43 (m, 5H). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{13}H_{20}NO_2]^+$ 222.1489, found: 222.1495. $C_{13}H_{19}NO_2$ (221.30).

***N*^{2}-(2-{[(9*H*-fluoren-9-yl)methoxy]carbonyl}-1-isobutylhydrazine-1-carbonyl)-*N*$^\omega$-[(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl]-*L*-arginine (4.17).** Triphosgene (152 mg, 0.4 equiv) was dissolved in CH_2Cl_2 (5 mL). A solution of **4.14** (145 mg, 0.442 mmol) and DIPEA (450 μL, 2 equiv) in CH_2Cl_2 (5 mL) was added dropwise under ice-cooling. The mixture was allowed to warm to ambient temperature under stirring for 30 min. A solution of **4.12** (200 mg, 1.1 equiv) in CH_2Cl_2 (2 mL) was added and stirring was continued for 90 min. The mixture was diluted with EtOAc (60 mL) and washed with water. The volatiles were removed under reduced pressure, the residue was taken up in EtOAc/hexane (2:1, 10 mL) and subjected to column chromatography (eluent: EtOAc/hexane (2:1)). The protected intermediate was dissolved in MeOH (10 mL), and Pd/C-catalyst (50 mg) was added. The mixture was stirred vigorously under hydrogen. After completion of the hydrogenation, the solids were removed by filtration through celite, the volume was reduced on a rotary evaporator. The desired compound was purified via column chromatography (eluent: CH_2Cl_2/MeOH (10:1) → CH_2Cl_2/MeOH (1:1) + 0.5% TFA). Removal of the volatiles under reduced pressure and by lyophilisation afforded **4.14** as white lyophilisate (370 mg, 88.5%). TLC (CH_2Cl_2/MeOH (10:0.6) + 1% AcOH): R_f = 0.28. ^{1}H-NMR (400 MHz, [D_4]methanol): δ (ppm) 0.85 (s, 6H), 1.41 (s, 6H), 1.47-1.69 (m, 3H), 1.69-1.79 (m, 1H), 1.79-1.92 (m, 1H), 2.06 (s, 3H), 2.49 (s, 3H), 2.56 (s, 3H), 2.95 (s, 2H), 3.15 (s, 2H), 4.12-4.30 (m, 2H), 4.52 (s, 2H), 7.28 (t, 2H, *J* 7.3 Hz), 7.37 (t, 2H, *J* 7.3 Hz), 7.54-7.70 (m, 2H), 7.77 (d, 2H, *J* 7.5 Hz). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{39}H_{51}N_6O_8S]^+$ 763.3484, found: 763.3494. $C_{39}H_{50}N_6O_8S$ (762.92).

(*S*)-5-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-2-[4-(*tert*-butoxy)benzyl]-12,12-dimethyl-4,10-dioxo-11-oxa-3,5,9-triazatridecanoic acid (4.18). Triphosgene (52.5 mg, 0.4 equiv) was dissolved in CH_2Cl_2 (2 mL). A solution of **4.15** (145 mg, 0.442 mmol) and DIPEA (150 μL, 2 equiv) in CH_2Cl_2 (2 mL) was added dropwise under ice-cooling. The mixture was allowed to warm to ambient temperature under stirring for 30 min. A solution of **4.10** (200 mg, 1.1 equiv) in CH_2Cl_2 (2 mL) was added, and stirring was continued for 90 min. The mixture was diluted with EtOAc (40 mL) and washed with water. The volatiles were removed under reduced pressure, the residue was taken up in EtOAc/hexane (1:1, 10 mL) and subjected to column chromatography (eluent: EtOAc/hexane (1:1), R_f = 0.57). The protected intermediate was dissolved in MeOH (10 mL), and Pd/C-catalyst (30 mg) was added. The mixture was stirred vigorously under hydrogen. After completion of the hydrogenation, the solids were removed by filtration through celite, the volume was reduced on a rotary evaporator. The desired compound was purified via column chromatography (eluent: CH_2Cl_2/MeOH (10:0.5) $\rightarrow$ CH_2Cl_2/MeOH (10:0.6) + 1% AcOH). Removal of the volatiles under reduced pressure and by lyophilisation afforded **4.18** as a white lyophilisate (221 mg, 74.1%). TLC (CH_2Cl_2/MeOH (10:0.6) + 0.5% AcOH): R_f = 0.35. ^{1}H-NMR (400 MHz, [D_4]methanol): δ (ppm) 1.20 (s, 9H), 1.42 (s, 9H), 1.51-1.66 (m, 2H), 2.81-3.20 (m, 5H), 3.69 (br s, 1H), 4.21 (s, 1H), 4.35-4.60 (m, 3H), 6.79 (s, 2H), 7.04 (s, 2H), 7.24-7.35 (m, 2H), 7.39 (t, 2H, *J* 7.2 Hz), 7.54-7.73 (m, 2H), 7.80 (d, 2H, *J* 7.5 Hz). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{37}H_{47}N_4O_8]^+$ 675.3388, found: 675.3403. $C_{37}H_{46}N_4O_8$ (674.80).

{[1-(9*H*-Fluoren-9-yl)-12,12-dimethyl-3,10-dioxo-2,11-dioxa-4,5,9-triazatridecan-5-yl]carbonyl}-*L*-leucine (4.19). Triphosgene (52.5 mg, 0.4 equiv) was dissolved in CH_2Cl_2 (2 mL). A solution of **4.16** (97.7 mg, 0.442 mmol) and DIPEA (150 μL, 2 equiv) in CH_2Cl_2 (2 mL) was added dropwise under ice-cooling. The mixture was allowed to warm to ambient temperature under stirring for 30 min. A solution of **4.10** (200 mg, 1.1 equiv) in CH_2Cl_2 (2 mL) was added and stirring was continued for 90 min. The mixture was diluted with EtOAc (40 mL) and washed with water. The volatiles were removed under reduced pressure and the residue was taken up in EtOAc/hexane (1:1, 10 mL) and subjected to column chromatography (eluent: EtOAc/hexane (1:1), R_f = 0.71). The protected intermediate was dissolved in MeOH (10 mL), and Pd/C-catalyst (30 mg) was added. The mixture was stirred vigorously under hydrogen. After completion of the hydrogenation, the solids were removed by filtration through celite, and the volume was reduced on a rotary evaporator. The desired

compound was purified via column chromatography (eluent: CH_2Cl_2/MeOH (10:0.4) → CH_2Cl_2/MeOH (10:0.6) + 0.1% TFA). Removal of the volatiles afforded **4.19** as a sticky solid (132 mg, 52.5%). TLC (CH_2Cl_2/MeOH (10:0.6) + 0.5% AcOH): R_f = 0.55. ^{1}H-NMR (400 MHz, [D_4]methanol): δ (ppm) 0.90 (d, 6H, *J* 4.4 Hz), 1.42 (s, 9H), 1.52-1.77 (m, 5H), 3.03 (s, 2H), 3.35-3.95 (m, 2H), 4.23 (t, 1H, *J* 6.3 Hz), 4.29 (t, 1H, *J* 7.0 Hz), 4.52 (s, 2H), 7.31 (t, 2H, *J* 7.3 Hz), 7.39 (t, 2H, *J* 7.4 Hz), 7.59-7.71 (m, 2H), 7.79 (d, 2H, *J* 7.5 Hz). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{30}H_{41}N_4O_7]^+$ 569.2970, found: 569.2969. $C_{30}H_{40}N_4O_7$ (568.67).

Tyr(*t*Bu)-Arg(Pbf)-azaLeu-Arg(Pbf)-Tyr(*t*Bu)-amide Hydrotrifluoroacetate (4.21). The side-chain protected pentapeptide **4.21** was synthesized according to the general procedure (100 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 42:58–78:22, t_R = 14.9 min). Lyophilisation of the eluate afforded **4.21** as a white solid (39 mg, 42.6%). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{69}H_{105}N_{13}O_{13}S_2]^{2+}$ 693.8693, found: 693.8708. $C_{69}H_{103}N_{13}O_{13}S_2 \cdot C_2HF_3O_2$ (1386.06 + 114.02).

Tyr(*t*Bu)-Arg(Pbf)-Leu-azaOrn(Boc)-Tyr(*t*Bu)-amide Hydrotrifluoroacetate (4.22). The side-chain protected pentapeptide **4.22** was synthesized according to the general procedure (100 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–20 min MeCN/0.1% aq TFA 42:58–78:22, t_R = 14.2 min). Lyophilisation of the eluate afforded **4.22** as a white solid (42 mg, 52.7%). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{60}H_{95}N_{11}O_{12}S]^{2+}$ 596.8436, found: 596.8445. $C_{60}H_{93}N_{11}O_{12}S \cdot C_2HF_3O_2$ (1192.53 + 114.02).

Tyr(*t*Bu)-azaOrn(Boc)-Leu-Arg(Pbf)-Tyr(*t*Bu)-amide Hydrotrifluoroacetate (4.23). The side-chain protected pentapeptide **4.23** was synthesized according to the general procedure (100 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–20 min MeCN/0.1% aq TFA 42:58–78:22, t_R = 14.6 min). Lyophilisation of the eluate afforded **4.23** as a white solid (36 mg, 45.2%). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{60}H_{95}N_{11}O_{12}S]^{2+}$ 596.8436, found: 596.8446. $C_{60}H_{93}N_{11}O_{12}S \cdot C_2HF_3O_2$ (1192.53 + 114.02).

(2*R*,7*R*)-Diaminooctanedioyl-bis(Tyr-Arg-azaLeu-Arg-Tyr-amide) Hexakis(hydrotrifluoroacetate) ((2*R*,7*R*)-4.26). Compound **4.24** (2.08 mg, 5.1 μmol), HBTU

(4.1 mg, 2.1 equiv) and HOBt (1.57 mg, 2 equiv) were dissolved in anhydrous DMF (400 μL). DIPEA (10 μL, 5 equiv) was added, and the mixture was stirred at room temperature for 5 min followed by the addition of a solution of **4.21** (19.3 mg, 12.8 μmol). The resulting mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. Subsequently, TFA/water (95:5, 2 mL) was added, and the mixture was stirred at room temperature for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 3:97–42:58, t_R = 15.6 min). Lyophilisation of the eluate afforded (2*R,*7*R*)-**4.26** as a white fluffy solid (3.45 mg, 28.3%). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $[C_{78}H_{126}N_{28}O_{16}]^{4+}$ 427.7471, found: 427.7485. RP-HPLC (220 nm): 99% (t_R = 17.72 min, *k* = 5.2). $C_{78}H_{122}N_{28}O_{16}{\cdot}C_{12}H_6F_{18}O_{12}$ (1708.01 + 684.12).

Octanedioyl-bis(Tyr-Arg-azaLeu-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (4.27). Compound **4.21** (28.8 mg, 19.2 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Compound **4.25** (2.83 mg, 7.7 μmol) was added, and the mixture was stirred at 35 °C for 16 h. Water (10 mL) was added, and the protected intermediate was extracted with CH_2Cl_2 (2 × 10 ml). The combined extracts were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5 v/v) (2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 3:97–42:58, t_R = 16.5 min). Lyophilisation of the eluate afforded **4.27** as a white fluffy solid (6.3 mg, 38.3%). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $C_{78}H_{124}N_{26}O_{16}$ 420.2417, found: 420.2433. RP-HPLC (220 nm): 95% (t_R = 19.13 min, *k* = 5.7). $C_{78}H_{120}N_{26}O_{16}{\cdot}C_8H_4F_{12}O_8$ (1677.98 + 456.08).

Octanedioyl-bis(Tyr-Arg-Leu-azaOrn-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (4.28). Compound **4.22** (16.7 mg, 12.8 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Compound **4.25** (1.9 mg, 5.1 μmol) was added, and the mixture was stirred at 35 °C for 16 h. After addition of water (10 mL), the protected intermediate was extracted with CH_2Cl_2 (2 × 10 ml). The combined extracts were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5 v/v) (2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative

HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 3:97–42:58, t_R = 16.1 min). Lyophilisation of the eluate afforded **4.28** as a white fluffy solid (4.2 mg, 40.2%). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $C_{76}H_{120}N_{22}O_{16}$ 399.2308, found: 399.2327. RP-HPLC (220 nm): 99% (t_R = 19.55 min, *k* = 5.8). $C_{76}H_{116}N_{22}O_{16}{\cdot}C_8H_4F_{12}O_8$ (1593.90 + 456.08).

Octanedioyl-bis(Tyr-Arg-Leu-azaArg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (4.29). Compound **4.28** (3.7 mg, 2.05 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (300 μL). A solution of *N*,*N*'-di-Boc-1*H*-pyrazole-1-carboxamidine (1 mg/10 μL, 16 μL, 2.5 equiv) was added, and the mixture was stirred at room temperature for 4 h. After addition of water (5 mL) the protected intermediate was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 3:97–52:48, t_R = 26.4 min). The eluates were subjected to lyophilisation, and the residue was taken up in TFA/ CH_2Cl_2/H_2O (5:5:0.5, 3 mL). The resulting mixture was stirred for 3 h. The volatiles were removed on a rotary evaporator and by lyophilisation. **4.29** was obtained as a white fluffy solid (1.62 mg, 37.0%). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $C_{78}H_{124}N_{26}O_{16}$ 420.2417, found: 420.2434. RP-HPLC (220 nm): > 99% (t_R = 20.15 min, *k* = 6.0). $C_{78}H_{120}N_{26}O_{16}{\cdot}C_8H_4F_{12}O_8$ (1677.98 + 456.08).

Octanedioyl-bis(Tyr-azaOrn-Leu-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (4.30). Compound **4.23** (16.7 mg, 12.8 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Compound **4.25** (1.88 mg, 5.1 μmol) was added, and the mixture was stirred at 35 °C for 16 h. After addition of water (10 mL), the protected intermediate was extracted with CH_2Cl_2 (2 × 10 ml). The combined extracts were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5 v/v) (2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 6:94–37:63, t_R = 17.4 min). Lyophilisation of the eluate afforded **4.30** as a white fluffy solid (3.9 mg, 37.3%). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $C_{76}H_{120}N_{22}O_{16}$ 399.2308, found: 399.2317. RP-HPLC (220 nm): 98% (t_R = 20.13 min, *k* = 6.0). $C_{76}H_{116}N_{22}O_{16}{\cdot}C_8H_4F_{12}O_8$ (1593.90 + 456.08).

Octanedioyl-bis(Tyr-azaArg-Leu-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (4.31). Compound **4.30** (3.0 mg, 1.66 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (300 μL). A solution of *N*,*N*'-di-Boc-1*H*-pyrazole-1-carboxamidine (1 mg/10 μL, 13 μL, 2.5 equiv) was added, and the mixture was stirred at room temperature for 4 h. After addition of water (5 mL), the protected intermediate was purified by preparative HPLC (column: Kinetex-XB

C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 3:97–52:48, t_R = 26.1 min). The solvents were removed by lyophilisation, the residue was taken up in TFA/ CH_2Cl_2/H_2O (5:5:0.5, 3 mL), and the resulting mixture was stirred for 3 h. The volatiles were removed on a rotary evaporator and by lyophilisation. The title compound was obtained as a white fluffy solid (1.50 mg, 42.3%). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $C_{78}H_{124}N_{26}O_{16}$ 420.2417, found: 420.2437. RP-HPLC (220 nm): 96% (t_R = 20.86 min, *k* = 6.3). $C_{78}H_{120}N_{26}O_{16}\cdot C_8H_4F_{12}O_8$ (1677.98 + 456.08).

Octanoyl-Tyr-Arg-Leu-Arg-Tyr-amide Bis(hydrotrifluoroacetate) (4.33). The pentapeptide **4.33** was synthesized according to the general procedure (82 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 22:78–51:49, t_R = 13.2 min). Lyophilisation of the eluate afforded **4.33** as a white solid (33.4 mg, 59.5%). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{44}H_{72}N_{12}O_8]^{2+}$ 448.2793, found: 448.2800. RP-HPLC (220 nm): 99% (t_R = 28.04 min, *k* = 8.8). $C_{44}H_{70}N_{12}O_8\cdot C_4H_2F_6O_4$ (895.13 + 228.04).

Ac-Tyr-*N*$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Leu-Arg-Tyr-amide Tris(hydrotrifluoroacetate) (4.34). The pentapeptide **4.34** was synthesized according to the general procedure (18.8 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 14.4 min). Lyophilisation of the eluate afforded **4.34** as a white solid (7.7 mg, 53.0%). HRMS (ESI): *m/z* $[M+3H]^{3+}$ calcd. for $[C_{43}H_{71}N_{14}O_9]^{3+}$ 309.1837, found: 309.1850. RP-HPLC (220 nm): 97% (t_R = 16.96 min, *k* = 4.9). $C_{43}H_{68}N_{14}O_9\cdot C_6H_3F_9O_6$ (925.12 + 342.06).

Ac-Arg-Tyr-*N*$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Leu-Arg-Tyr-amide Tetrakis(hydrotrifluoroacetate) (4.35). The hexapeptide **4.35** was synthesized according to the general procedure (18.8 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 13.6 min). Lyophilisation of the eluate afforded **4.35** as a white solid (9.3 mg, 52.7%). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $[C_{49}H_{84}N_{18}O_{10}]^{4+}$ 271.1649, found: 217.1657. RP-HPLC (220 nm): 97% (t_R = 15.22 min, *k* = 4.3). $C_{49}H_{80}N_{18}O_{10}\cdot C_8H_4F_{12}O_8$ (1081.31 + 456.08).

Ac-Arg-Tyr-Arg-Leu-Arg-Tyr-amide Tris(hydrotrifluoroacetate) (4.36). The hexapeptide **4.36** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 13.7 min). Lyophilisation of the eluate afforded **4.36** as a white solid (18.3 mg, 65.5%). HRMS (ESI): *m/z* $[M+3H]^{3+}$ calcd. for $[C_{44}H_{70}N_{16}O_9]^{3+}$ 323.1910, found: 323.1927. RP-HPLC (220 nm): 99% (t_R = 15.98 min, k = 4.6). $C_{44}H_{70}N_{16}O_9 \cdot C_6H_3F_9O_6$ (967.15 + 342.06).

Ac-Arg-Tyr-Arg-Leu-*N*$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Tyr-amide Tetrakis(hydrotrifluoroacetate) (4.37). The hexapeptide **4.37** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 13.6 min). Lyophilisation of the eluate afforded **4.37** as a white solid (7.2 mg, 21.9%). HRMS (ESI): *m/z* $[M+3H]^{3+}$ calcd. for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$ 361.2174, found: 361.2187. RP-HPLC (220 nm): 99% (t_R = 15.17 min, k = 4.3). $C_{49}H_{80}N_{18}O_{10} \cdot C_8H_4F_{12}O_8$ (1081.31 + 456.08).

Ac-Arg-Tyr-*N*$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Leu-Arg-*D*-Tyr-amide Tetrakis(hydrotrifluoroacetate) (4.38). The hexapeptide **4.38** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 13.6 min). Lyophilisation of the eluate afforded **4.38** as a white solid (5.7 mg, 17.4%). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $[C_{49}H_{84}N_{18}O_{10}]^{4+}$ 271.1649, found: 217.1666. RP-HPLC (220 nm): 98% (t_R = 15.25 min, k = 4.3). $C_{49}H_{80}N_{18}O_{10} \cdot C_8H_4F_{12}O_8$ (1081.31 + 456.08).

Ac-Arg-Tyr-*N*$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Leu-*D*-Arg-Tyr-amide Tetrakis(hydrotrifluoroacetate) (4.39). The hexapeptide **4.39** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 13.3 min). Lyophilisation of the eluate afforded **4.39** as a white solid (6.0 mg, 18.3%). HRMS (ESI): *m/z* $[M+3H]^{3+}$ calcd. for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$ 361.2174,

found: 361.2190. RP-HPLC (220 nm): 95% (t_R = 14.77 min, k = 4.1). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

Ac-Arg-Tyr-*N*$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-*D*-Leu-Arg-Tyr-amide

Tetrakis(hydrotrifluoroacetate) (4.40). The hexapeptide **4.40** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 13.4 min). Lyophilisation of the eluate afforded **4.40** as a white solid (6.6 mg, 20.1%). HRMS (ESI): *m/z* $[M+3H]^{3+}$ calcd. for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$ 361.2174, found: 361.2189. RP-HPLC (220 nm): 96% (t_R = 14.33 min, k = 4.0). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

Ac-Arg-*D*-Tyr-{*N*$^{\omega}$-[*N*-(4-aminobutyl)aminocarbonyl]}Arg-Leu-Arg-Tyr-amide

Tetrakis(hydrotrifluoroacetate) (4.41). The hexapeptide **4.41** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 13.5 min). Lyophilisation of the eluate afforded **4.41** as a white solid (6.7 mg, 20.5%). HRMS (ESI): *m/z* $[M+3H]^{3+}$ calcd. for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$ 361.2174, found: 361.2190. RP-HPLC (220 nm): 95% (t_R = 15.1 min, k = 4.3). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

Ac-*D*-Arg-Tyr-{*N*$^{\omega}$-[*N*-(4-aminobutyl)aminocarbonyl]}Arg-Leu-Arg-Tyr-amide

Tetrakis(hydrotrifluoroacetate) (4.42). The hexapeptide **4.42** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 13.5 min). Lyophilisation of the eluate afforded **4.42** as a white solid (7.0 mg, 21.4%). HRMS (ESI): *m/z* $[M+3H]^{3+}$ calcd. for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$ 361.2174, found: 361.2190. RP-HPLC (220 nm): 99% (t_R = 14.94 min, k = 4.2). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

Ac-Arg-Tyr-{*N*$^{\omega}$-[*N*-(4-aminobutyl)aminocarbonyl]}Arg-Leu-Arg-Tyr-OH

Tetrakis(hydrotrifluoroacetate) (4.43). The hexapeptide **4.43** was synthesized according to the general procedure (44.1 mg, H-*L*-Tyr(*t*Bu)-2 CT resin (loading: 0.68 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min

MeCN/0.1% aq TFA 13:87–42:58, t_R = 10.7 min). Lyophilisation of the eluate afforded **4.43** as a white solid (15.2 mg, 32.9 %). HRMS (ESI): *m/z* $[M+4H]^{4+}$ calcd. for $[C_{49}H_{83}N_{17}O_{11}]^{4+}$ 271.4109, found: 271.4124. RP-HPLC (220 nm): 95% (t_R = 16.28 min, *k* = 4.7). $C_{49}H_{79}N_{17}O_{11}\cdot C_8H_4F_{12}O_8$ (1082.28 + 456.08).

(*S*)-2-Acetamido-*N*-((*S*)-1-{[(12*S*,15*S*,18*S*,21*S*)-2-amino-15-(3-guanidinopropyl)-12-(4-hydroxybenzyl)-18-isobutyl-4,11,14,17,20-pentaoxo-1,3,5,10,13,16,19-heptaazacyclotetracos-2-en-21-yl]amino}-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)-5-guanidinopentanamide Tris(hydrotrifluoroacetate) (4.44). Compound **4.43** (9.0 mg, 5.85 μmol), HOBt (1.79 mg, 2 equiv) and DIPEA (0.9 μL, 8 equiv) were dissolved in anhydrous DMF (5850 μL) to give a 1 mM solution. A solution of PyBOP (3.65 mg, 1.2 equiv) was added dropwise. After stirring at room temperature for 24 h, 0.1% aq TFA (20 mL) was added and purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–18 min MeCN/0.1% aq TFA 3:97–42:58, t_R = 15.9 min). Lyophilisation of the eluate afforded **4.44** as a white solid (3.9 mg, 47.4 %). HRMS (ESI): *m/z* $[M+3H]^{3+}$ calcd. for $[C_{49}H_{80}N_{17}O_{10}]^{3+}$ 355.5419, found: 355.5434. RP-HPLC (220 nm): 95% (t_R = 18.4 min, *k* = 5.4). $C_{49}H_{77}N_{17}O_{10}\cdot C_6H_3F_9O_6$ (1064.27 + 342.06).

4.4.3 Pharmacological Assays

Cells. HEC-1B (HTB-113) human endometrial cancer cells, MCF-7 (HTB-22) human breast cancer cells were from the American Type Culture Collection (Rockville, MD). A subclone of the MCF-7 cell line that shows higher Y_1R expression was established in our laboratory and used for binding experiments.[23] Human erythroleukemia (HEL) cells were kindly provided by Dr. M. C. Michel (Universitätsklinikum Essen, Germany) and chinese hamster ovarian (CHO-K1, ACC-110) cells and human embryonal kidney cells (HEK-293T, 293T, ACC 635) cells were from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Genetically engineered cells, used for binding and functional assays, were generated and cultured as described (cf. brief description of protocols).

Routinely performed examinations for mycoplasma contamination using the Venor GeM Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany) were negative for all cell types.

HEK293T-ARRB1-hY$_4$R and HEK293T-ARRB2-hY$_4$R cells. The β-arrestin recruitment was quantified by a luciferase complementation assay based on the emerald luciferase (ELuc) from brazilian click-beetle *Pyrearinus termitilluminans*. The fusion construct of the human hY$_4$R (the hY$_4$R cDNA was from cDNA Resource Center, Bloomsburg, PA, USA) and the C-terminal luciferase fragment (hY$_4$R-ELucC) was generated using the previously described construct SSTR2-ELucC[37,38] by replacing the cDNA of SSTR2 by the cDNA of the hY$_4$R. HEK293T cells were stably transfected with the pcDNA3.1/myc-HIS (B) vector encoding the β-arrestin isoform 1 or 2, N-terminally fused with the N-terminus of the luciferase (ELucN-ARRB1 or ELucN-ARRB2, respectively)[37] and the pcDNA4/V5-HIS (B) vector encoding hY$_4$R-ELucC. Cells transfected with pcDNA3.1/myc-HIS (B) were cultivated in the presence of G418 (600 μg/mL) for up to 3 weeks until stable growth was observed. Afterwards, the cells were co-transfected with the pcDNA4/V5-HIS (B) vector encoding hY$_4$R-ELucC and selection pressure was applied using zeocin (40 μg/mL). To determine the hY$_4$R-ELucC expression, saturation binding studies were performed with both, HEK293T-ARRB1- hY$_4$R cells and HEK293T-ARRB2-hY$_4$R cells using the radioligand **45** (cf. Figure S6, S7, Supporting Information).

Cell Culture. Cells were cultured in 25- or 75-cm^2 flasks (Sarstedt, Nümbrecht, Germany) in a humidified atmosphere (95% air, 5% CO_2) at 37 °C. MCF-7-Y$_1$ cells,[22] HEL cells,[39] CHO-hY$_2$-G$_{qi5}$-mtAEQ cells,[25] CHO-hY$_4$-G$_{qi5}$-mtAEQ cells[26] and HEK293T-hY$_4$R-CRE Luc cells[11] were cultured as described previously. HEK293T-ARRB1-hY$_4$R and HEK293T-ARRB2-hY$_4$R cells were maintained in DMEM containing 10% FCS, 600 μg/mL G418 and 40 μg/mL zeocin.

Radioligand Binding Assays. Y$_1$R binding. The Radioligand binding assay was performed at intact MCF-7-Y$_1$ cells as previously described[22] with the following modifications: Experiments were carried out in 96-well plates with clear bottom (Corning Incorporated Life Sciences, Tewksbury, MA; Corning cat. no. 3610), and the volume per well was reduced to 100 μL. After incubation, the cells were washed with buffer (200 μL) twice and covered with lysis solution (25 μL) consisting of urea (8 M), acetic acid (3 M) and Triton-X-100 (1%) in water. The plates were shaken for 30 min prior to addition of liquid scintillator cocktail (Optiphase Supermix, PerkinElmer, Überlingen, Germany) (200 μL). The wells were sealed with a transparent film (permanent seal for microplates, PerkinElmer, prod. no. 1450–461). The plates were shaken and kept in the dark for at least 30 min. Radioactivity (dpm) was measured with a MicroBeta2 plate counter (PerkinElmer, Rodgau, Germany).

Y_2R binding. Competition binding experiments were performed at CHO-hY_2R-G_{qi5}-mtAEQ cells[25] with [^{3}H]propionyl-pNPY[24] (K_d = 1.4 nM, c = 1 nM) in a hypotonic HEPES buffer (25 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) as previously described.[11]

Y_4R binding. Competition binding experiments were performed at CHO-hY_4R-G_{qi5}-mtAEQ cells[26] with **4.45** (K_d = 0.67 nM, c = 0.6 nM) in a hypotonic HEPES buffer (25 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) as described in **Chapter 2.4.5**.

Y_5R binding. Competition binding experiments were performed at HEC-1B-hY_5 cells[27] with [^{3}H]propionyl-pNPY[24] (K_d = 4.8 nM, c = 4 nM) in an isotonic HEPES buffer (150 mM NaCl, 10 mM HEPES, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM KCl) as previously described.[11]

Fura-2 Calcium Assay. The assay was performed for the functional characterization of selected ligands at the human Y_1 receptor using human erythroleukemia (HEL) cells and a LS-50B luminescence spectrometer (Perkin Elmer, Überlingen, Germany) as previously described.[39,40]

Aequorin Calcium Assay. The assay was performed on CHO-hY_4-G_{qi5}-mtAEQ cells as previously described[26] using a GENios Pro plate reader (Tecan, Salzburg, Austria). Areas under the curve were calculated using SigmaPlot 12.5 software (Systat Software Inc., Chicago, IL).

Luciferase Assay. The Luciferase assay was performed on HEK293-hY_4-CRE Luc cells as previously described.[11]

β-Arrestin Recruitment Assay. The recruitment of β-arrestin was measured via split-luciferase complementation technique. The *N*-terminal part of a green light-emitting luciferase was fused to β-arrestin and the C-terminal part was fused to Y_4R. One day before the experiment, HEK293T-ARRB1-Y_4R and HEK293T-ARRB2-Y_4R cells were trypsinized (0.05% trypsin, 0.02% EDTA in PBS) and centrifuged (400 *g*, 5 min). The cells were re-suspended in DMEM without phenol red (Sigma, Steinheim, Germany) supplemented with 5% FCS, and 90 μL of the cell suspension were seeded in white, TC-treated, flat bottom 96-well microtiter plates (VWR, Ismaning, Germany) at a density of approximately 100,000 cells/well. The cells

were cultivated at 37 °C overnight in a water-saturated atmosphere containing 5% CO_2. Shortly before the experiment, the cells were removed from the incubator and allowed to equilibrate to room temperature, before 10 μL of agonist solution were added per well. The plates were shaken at 25 °C for 60 min. At the end of the incubation period, 50 μL of medium were replaced by 50 μL of Bright-Glo luciferase assay reagent (Promega, Mannheim, Germany). The plates were vigorously shaken (800 rpm) for 5 min. Bioluminescence was recorded for 1 s per well using the GENios Pro microplate reader (Tecan, Salzburg, Austria). For the determination of antagonistic activities, the cells were pre-incubated in the presence of the antagonist for 15 min. 10 μL of an hPP solution (30 nM, final concentration 3 nM) were added and incubation was continued at 25 °C for 60 min. The plates were further processed as in case of the agonist mode.

Data Analysis. All data are presented as mean ± SEM from at least 3 independent experiments performed in triplicate. Concentration response curves from the aequorin, β-arrestin or luciferase assay and concentration displacement curves from radioligand binding experiments were analyzed by four-parameter sigmoidal fits (GraphPad Prism 5.0, San Diego, CA). Agonist potencies are given as EC_{50} values, maximal responses (efficacies) are expressed as α value referred to the effect of 1 μM hPP (α = 1.0). K_i values were calculated from IC_{50} values using the Cheng-Prusoff equation.[41]

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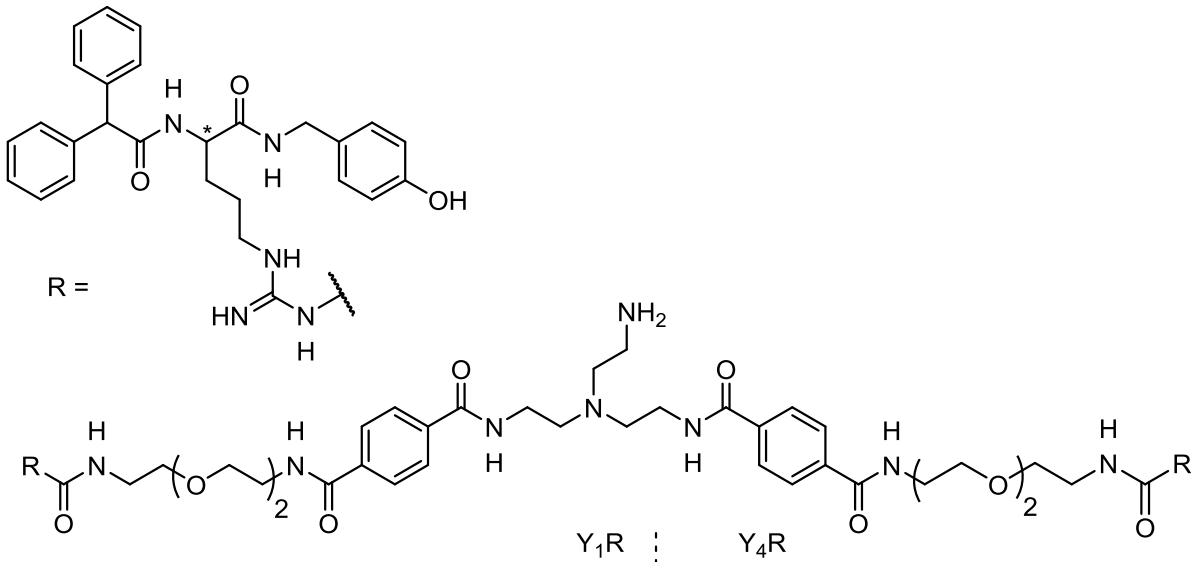
Chapter 5

Dimeric Argininamide-type Neuropeptide Y Receptor Ligands: The Influence of Truncated, Conformationally Constrained Linkers on Y_4R Affinity

5.1 Introduction

Whereas many non-peptide Y_1R and Y_5R antagonists and a few Y_2R antagonists with drug like properties have been reported (cf. general introduction (1.2)), highly potent and selective small molecule Y_4R antagonists are not known so far. Such compounds are required as pharmacological tools to further investigate the physiological and pathophysiological role of the Y_4R.

Surprisingly, within a series of bivalent N^G-acylated BIBP 3226 derivates which were initially designed as dimeric Y_1R antagonists, several compounds proved to possess Y_4R affinities and antagonistic activities in the two- to three-digit nanomolar range.[1] Modifications of the linkers regarding chemical nature, length, rigidity and attachment position resulted in the dimeric N^G-carbamoylated BIBP 3226 derivate UR-MK188.[2] Y_4R selectivity could be improved by changing the stereo configuration of UR-MK188. The *S*,*S*-configured enantiomer, UR-MEK388, binds to the Y_4R with an affinity comparable to that of the *R*,*R*-configured UR-MK188, but is inactive at the Y_1R.



		Y_1R	Y_4R	
compd.	*	K_i [nM]	K_i [nM]	K_b [nM]
UR-MK188	(*R*,*R*)	24	130	20
UR-MEK388	(*S*,*S*)	>5000	260	27
R-H (BIBP 3226)	(*R*)	1.3	>5000	-

Figure 1: Dimeric N^G-carbamoylated argininamides UR-MK188 and UR-MEK388 and the parent compound BIBP 3226.

Previous investigations on such dimeric argininamides, initially designed as bivalent Y_1R antagonists, suggest that the linker length should be reduced. Taking UR-MK188 as a lead structure, a series of bivalent Y_4R ligands with truncated, conformationally constrained linkers was prepared.

5.2 Retrosynthetic Analysis of Dimeric N^G-Carbamoylated Argininamides

Two series of bivalent BIBP 3226 derived ligands were planned, one containing an extended core structure, the other one containing a bent one. Each series comprised three ligands with linkers of different lengths. As an example, (*R*,*R*)-**5.1** is shown in Figure 2. (*R*,*R*)-**5.1** can be devided into four different substructures.

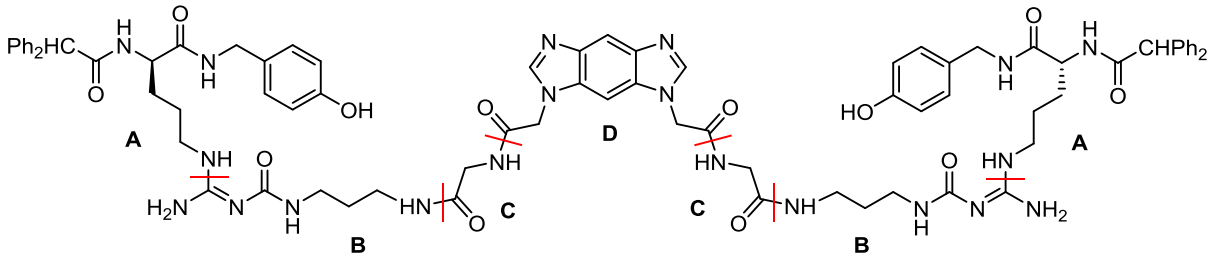


Figure 2: Retrosynthetic analysis of bivalent ligand (*R*,*R*)-**5.1**.

Building block **A** was prepared from Fmoc-Orn(Cbz)-OH in a four step synthesis. The rather flexible substructure **B** was attached to **A** by guanidinylation of the δ-amino group of the ornithinamide **A**. The N^G-carbamoylated argininamide (**A** + **B**) comprises the pharmacophoric moiety (BIBP 3226) and a rather flexible part to allow optimal spatial orientation of the pharmacophoric moieties when binding to the receptor. This part was kept identical throughout this series of bivalent ligands.

A connecting moiety **C**, consisting of one or three glycine residues was optionally used for the extension of the linkers. In the last step, the rigid core structure **D**, was coupled to the portion **A-C** containing the argininamide residues.

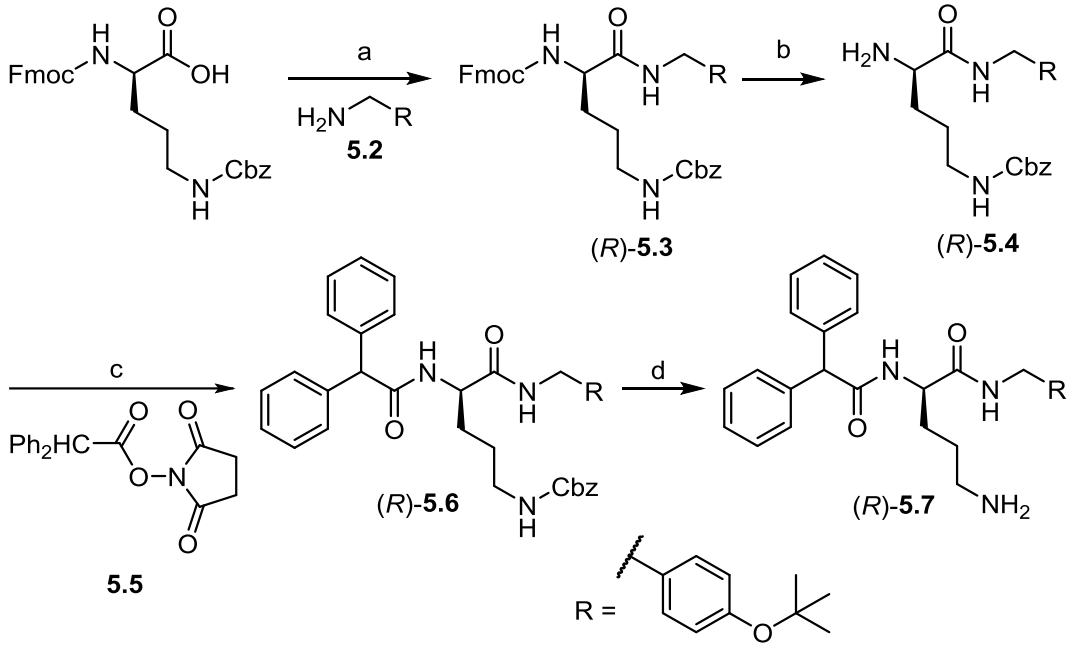
5.3 Results and Discussion

5.3.1 Chemistry

5.3.1.1 Synthesis of the N^G-(3-aminopropylcarbamoyl)argininamide (*R*)-5.13

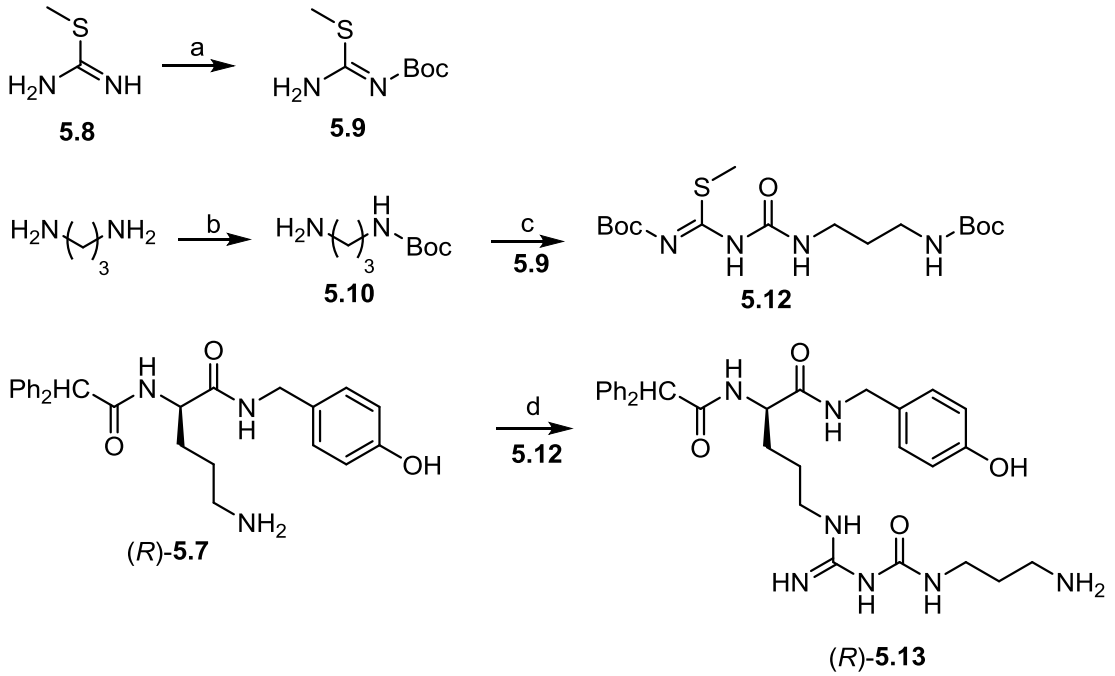
Carbamoylguanidines can be prepared from amines and *N*-protected, carbamoylated S-methylisothioureas. The amines (*R*)-**5.7** and (*S*)-**5.7**, which were used for guanidinylation, were prepared in a four step synthesis as previously described.[1,3] To allow for selective deprotection, three orthogonal protecting groups, Fmoc, Cbz and *tert*-butyl, were introduced. Amide coupling and deprotection were carried out by analogy with standard protocols (DEA for Fmoc cleavage, Pd/C, H_2 in MeOH for Cbz cleavage; CDI or succinimidyl esters for amide coupling).

Scheme 1: Preparation of the D-ornithinamide (*R*)-**5.7**.[a]



[a](*S*)-**5.7** was prepared according to the same protocol using (*S*)-**5.1** as starting material. Reagents and conditions: (a) CDI, DIPEA, THF, rt, 60 min, followed by **5.2**, rt, 18 h. (b) DEA, CH_2Cl_2, rt, 24 h. (c) **5.5**, DME/THF (85:15), 35 °C, 20 h. (d) H_2, Pd/C (10%), MeOH, rt, 20 h.

Scheme 2: Synthesis of guanidinylating reagent **5.12** and amine (*R*)-**5.13**[a]



[a](*S*)-**5.13** was prepared according to the same protocol using (*S*)-**5.7** as starting material. Reagents and conditions: (a) Boc_2O, TEA, chloroform, rt, 20 h. (b) Boc_2O, TEA, chloroform, rt, 40 h. (c) Triphosgene, CH_2Cl_2, DIPEA, rt, 45 min, followed by **5.1**, rt, 3 h. (d) $HgCl_2$, DIPEA, DMF, rt, 16 h, followed by TFA/ CH_2Cl_2/H_2O (10:10:1), rt, 2.5 h.

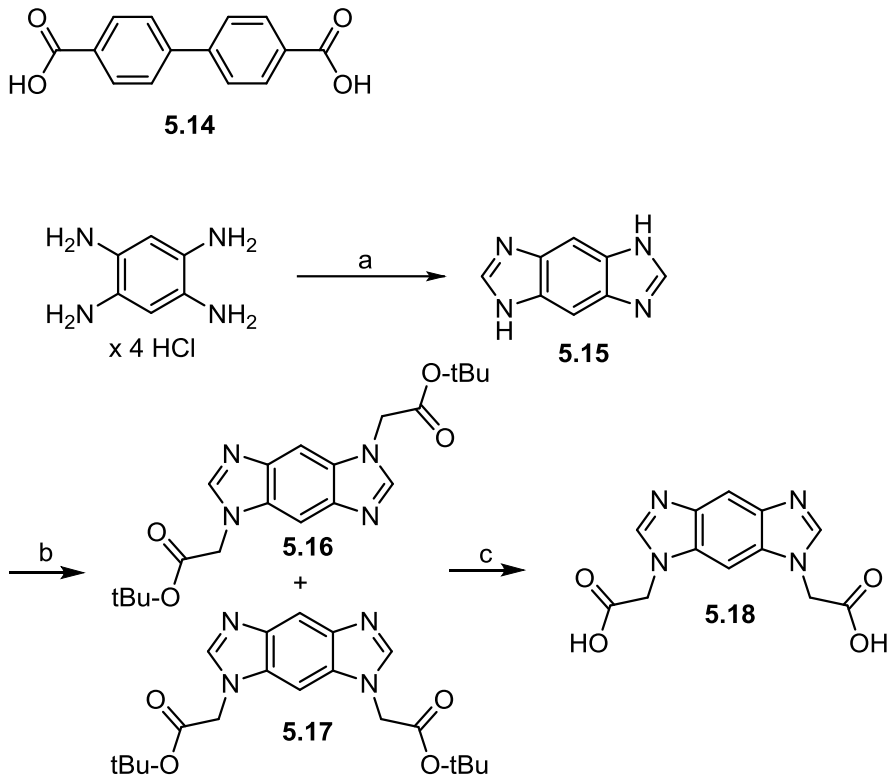
As guanidinylating reagent a carbamoylated S-methylisothiourea was used, which was prepared as outlined in Scheme 2. The mono-Boc-protected diamine **5.10** was in situ converted into the corresponding isocyanate with triphosgene and used for carbamoylation of the mono-Boc-protected isothiourea **5.9**. Subsequently, amines (*R*)-**5.7** and (*S*)-**5.7** were treated with the carbamoylated S-methyl isothiourea **5.12** in the presence of mercury(II) chloride to give the respective carbamoylated guanidines. Removal of the *tert*-butyl and Boc protecting groups under acidic conditions yielded the building blocks (*R*)-**5.13** and (*S*)-**5.13**.

5.3.1.2 Synthesis of the Bent Core Structure in Bivalent Ligands (*R*,*R*)/(*S*,*S*)-5.1, (*R*,*R*)/(*S*,*S*)-5.31 and (*R*,*R*)-5.32

Two different dicarboxylic acids with aromatic scaffolds were selected as rigid core structures of the linker. Biphenyl-4,4'-dicarboxylic acid (**5.14**) was commercially available. In search for a central core structure that sets the whole linker of the bivalent ligands into a bent conformation, 1,7-dihydroimidazo[4,5-*f*]benzimidazole (**5.15**) was chosen. The compound was easily accessible by heating benzene-1,2,4,5-tetraamine tetrahydrochloride

in formic acid at 100 °C. Subsequent alkylation of **5.15** with *tert*-butyl bromoacetate gave a 1:1 mixture of the desired 1,7-disubstituted product (**5.17**) and the 1,5-disubstituted isomer (**5.16**). The two isomers **5.16** and **5.17** could be easily separated by column chromatography using mixtures of CH_2Cl_2 and MeOH as mobile phases. Finally, the dicarboxylic acid **5.18** was obtained after cleavage of the *tert*-butyl protecting groups under acidic conditions.

Scheme 3: Structure of Biphenyl-4,4'-dicarboxylic acid (**5.14**) and the synthesis of dicarboxylic acid **5.18**[a]



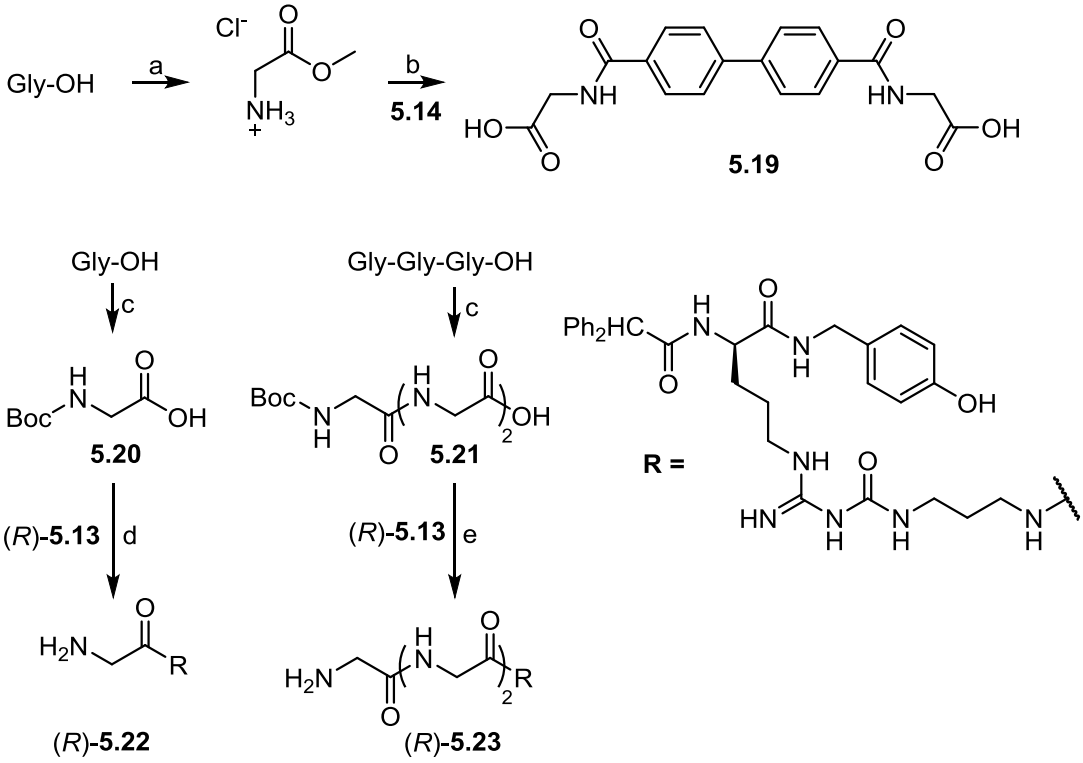
[a]Reagents and conditions: (a) Formic acid, 100 °C, 30 h. (b) NaH, anhydrous DMF, 80 °C, followed by *tert*-butyl bromoacetate, 30 min, 50 °C, 3 h. (c) TFA/ CH_2Cl_2/H_2O (10:10:1), rt, 4 h.

5.3.1.3 Synthesis of Intermediates for the Preparation of Bivalent Ligands with Extended Linkers

For the preparation of dimeric ligands with extended linkers, the intended glycine or triglycine moieties had to be attached either to the amines (*R*)-**5.13** and (*S*)-**5.13** or to the central rigid core structures **5.14** and **5.18**, respectively. In a first attempt glycine was protected as methyl ester and coupled to **5.14** using standard amide coupling protocols. The resulting dicarboxylic acid **5.19** could be isolated after cleavage of the methyl ester under basic conditions. However, due to poor solubility of both, starting material and product, in

various organic solvents, the purification of **5.19** was rather inconvenient. Therefore, the strategy for extension of the linker was changed. Instead of coupling ester-protected glycine or triglycine to the central part of the linker, Boc-protected glycine (**5.20**) and triglycine (**5.21**) were coupled to the amines (*R*)-**5.13** or (*S*)-**5.13**, respectively. In this way (*R*)-**5.22**, (*S*)-**5.22** and (*R*)-**5.23**, extended analogs of **5.13**, were obtained in acceptable yields.

Scheme 4: Synthesis of dicarboxylic acid **5.19** and amines (*R*)-**5.22** and (*R*)-**5.23**, extended analogs of **5.13**.[a]



[a](*S*)-**5.22** was prepared according to the same protocol using (*S*)-**5.13** as starting material. Reagents and conditions: (a) $SOCl_2$, MeOH, 0 °C, 10 min, reflux, 2 h. (b) Biphenyl-4,4'-dicarboxylic acid, CDI, DIPEA, anhydrous DMF, rt, 10 min, 70 °C, 3 h, followed by 10% NaOH, MeOH, 0 °C, 3 h. (c) Boc_2O, DIPEA, H_2O/dioxane (1:1), rt, 6 h. (d) CDI, DIPEA, DMF, (*R*)-**5.13**, 80 °C, 2 h, followed by CH_2Cl_2/TFA/H_2O (10:10:1), rt, 3 h. (e) TBTU, DIPEA, DMF, (*R*)-**5.13**, 70 °C (microwave conditions), 2 h, followed by TFA/ CH_2Cl_2/H_2O (10:10:1), rt, 3 h.

5.3.1.4 BIBP 3226 Derived Dimeric Ligands with Alternative Linker Attachment Positions

In addition to bivalent ligands like UR-MK188 in which the linker is attached to the guanidine nitrogen of BIBP 3226, bivalent ligands with different attachment positions should be considered. In this way, the size of the compounds can be reduced while maintaining the distance between the guanidine residues. Furthermore, the attachment of the linker to another substructure of BIBP 3226 increases the flexibility of the guanidine groups which might be favorable for the interaction with the receptor. Several different positions within BIBP 3226 are worth considering (cf. Figure 3).

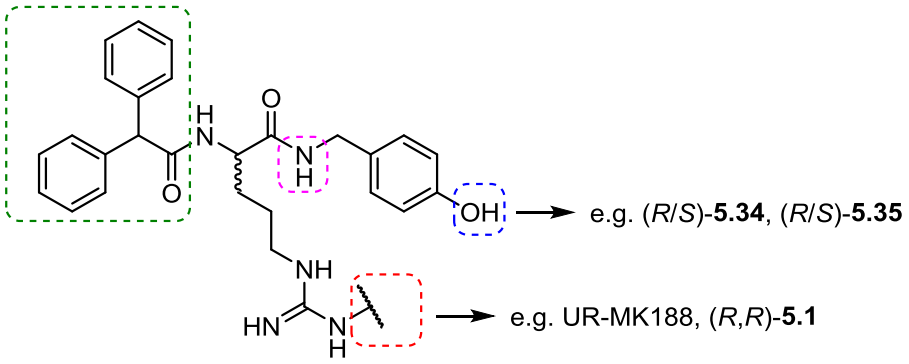
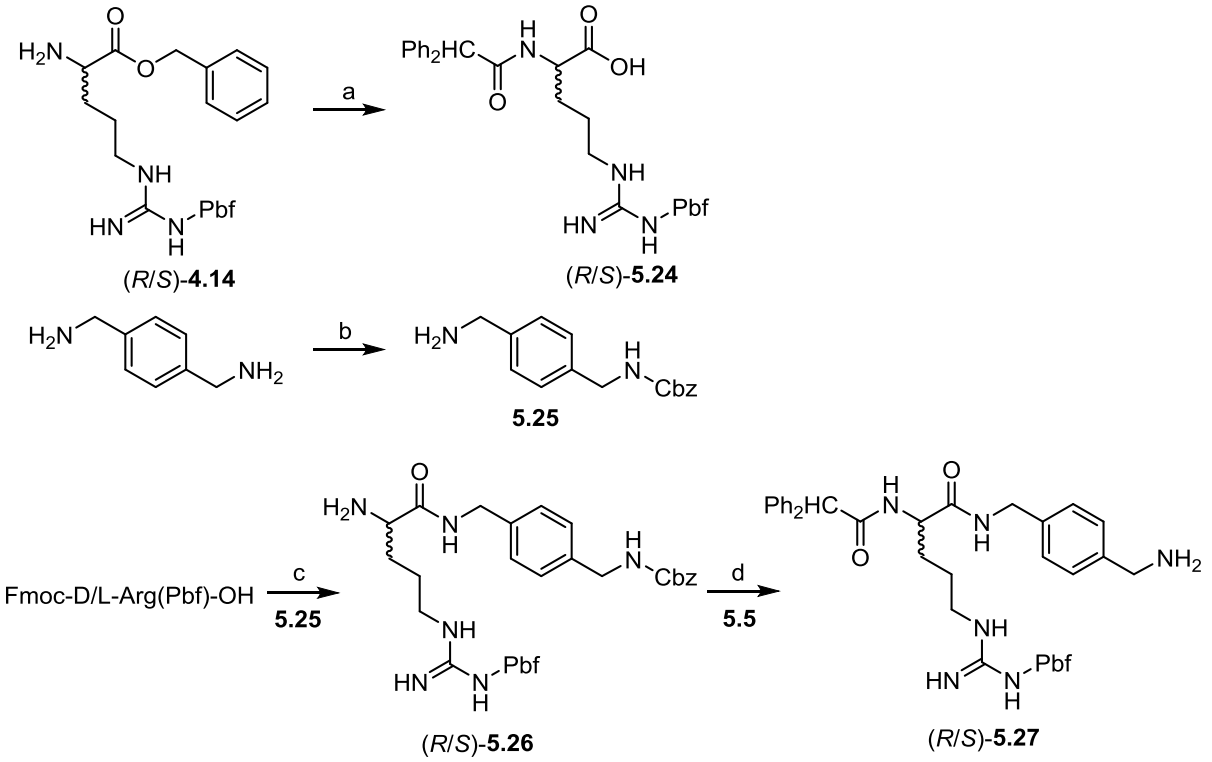


Figure 3: Possible positions of linker attachment for BIBP 3226 derived dimeric ligands.

Scheme 5 shows the synthesis of building blocks for the preparation of bivalent ligands in which the linker is attached to the carboxylic acid of the arginine in BIBP 3226. As the stereo discrimination of this set of bivalent ligands cannot be predicted, final compounds were prepared as racemic mixtures. Benzyl protected D/L-Arg(Pbf)-OH was acylated with diphenylacetic acid. Subsequent cleavage of the benzyl ester gave the carboxylic acid (*R*/*S*)-**5.24**. Mono-Cbz-protected 1,4-bis(aminomethyl)benzene (**5.25**) was coupled to Fmoc-D/L-Arg(Pbf)-OH. Subsequent Fmoc-deprotection with diethylamine resulted in the amine (*R*/*S*)-**5.26** which was acylated with *N*-succinimidyl diphenylacetate. The racemic mixture of amine **5.27** was obtained after Fmoc-deprotection.

Scheme 5: Preparation of building blocks for bivalent ligands with modified linker attachment sites[a]

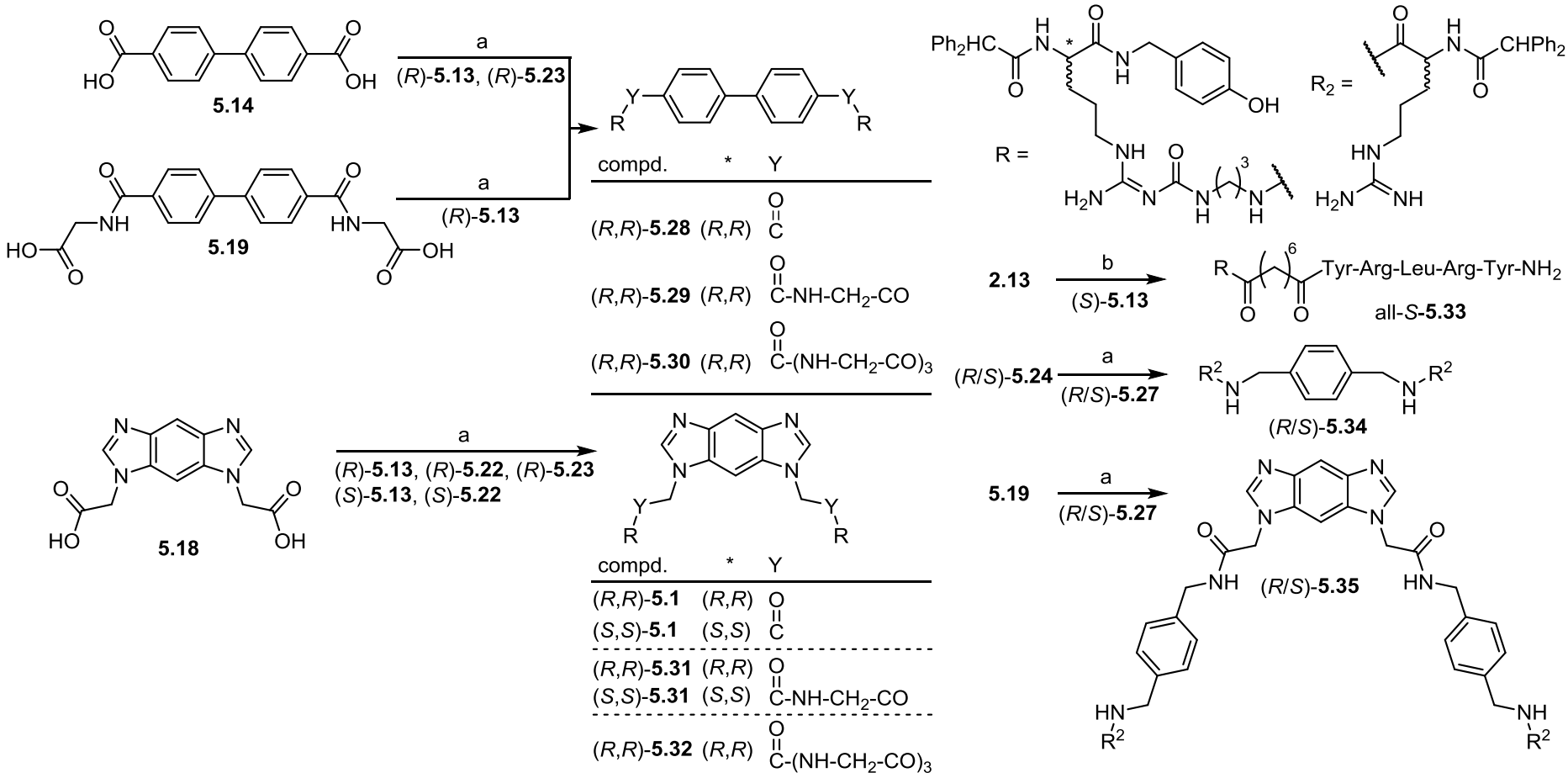


[a]Reagents and conditions: (a) Diphenylacetic acid, TBTU, DIPEA, CH_2Cl_2/DMF (5:3), rt, 14 h, followed by H_2, Pd/C, MeOH, rt. (b) Benzyl chloroformate, DIPEA, CH_2Cl_2, rt, 14 h. (c) **5.25**, *N*,*N*'-diisopropylcarbodiimide, DMF, 0 °C, 10 min, rt, 14 h, followed by CH_2Cl_2/DEA (8:1). (d) **5.5**, DIPEA, DME/THF (3:1), rt, 14 h, followed by, H_2, Pd/C, MeOH, rt.

5.3.1.5 Synthesis of Homo- and Heterodimeric Ligands

Finally, the dimeric argininamide-type potential Y_4R ligands (*R*,*R*)-**5.28**-**5.32**, (*R*,*R*)/(*S*,*S*)-**5.1** and (*S*,*S*)-**5.31** were prepared by amidation of the dicarboxylic acids **5.14**, **5.19** or **5.18** with the respective amine precursors (*R*)/(*S*)-**5.13**, (*R*)/(*S*)-**5.22** or (*R*)-**5.23** (Scheme 6). Satisfactory yields were achieved with TBTU as a coupling reagent under microwave irradiation at 70 °C. The bivalent ligands (*R*/*S*)-**5.34** and (*R*/*S*)-**5.35** were prepared from the acid (*R*/*S*)-**5.24** and the amine (*R*/*S*)-**5.27** or the diacid **5.19** and (*R*/*S*)-**5.27**, respectively, using the same coupling conditions as mentioned above (note: *R*/*S* denotes the mixture of *R*,*R*, *S*,*S*, and *R*,*S*-configured stereoisomers resulting from the coupling the the racemic argininamide derivative as building block). The hybrid compound all-*S*-**5.33** was prepared by coupling the amine (*S*)-**5.13** to the protected pentapeptide building block **2.13** and subsequent final deprotection under acidic conditions.

Scheme 6: Preparation of homodimeric ligands (*R*,*R*)-**5.28**-**5.32**, (*R*,*R*)/(*S*,*S*)-**5.1**, (*S*,*S*)-**5.31**, (*R*/*S*)-**5.34**, (*R*/*S*)-**5.35** and hybrid compound all-*S*-**5.33**[a]



[a]Reagents and conditions: (a) TBTU, DIPEA, DMF, rt 15 min, 70 °C, 30 min (microwave conditions). (b) HBTU, HOBt, DIPEA, DMF, rt, 10 min, 70 °C, 30 min, followed by TFA/H_2O (95:5), rt, 2.5 h.

5.3.2 Pharmacology: Binding and Functional Assays

K_i values of the dimeric ligands and all-*S*-**5.33** were determined in competition binding experiments on live cells expressing human Y_1, Y_2, Y_4 or Y_5 receptors to determine the NPY receptor affinity and subtype selectivity (data cf. Table 1).

The synthesized bivalent ligands (*R*,*R*)-**5.1** and (*R*,*R*)-**5.28**-**5.32** show binding to both Y_1R and Y_4R. The affinity to the Y_1R was considerable higher than to the Y_4R for all compounds mentioned above. The compounds with the highest Y_1R affinities, (*R*,*R*)-**5.31** and (*R*,*R*)-**5.32**,had K_i values of 41 nM and 80 nM, respectively, which is a 30- to 60-fold decrease in Y_1R affinity compared to the parent compound BIBP 3226 (K_i = 1.3 nM).

The monomeric parent compound BIBP 3226 is a high-affinity Y_1R antagonist, but inactive at the Y_4R. The bivalent ligand approach resulted in a tremendous increase in Y_4R binding. At the Y_4R, the compounds that share **5.18** as central rigid core were slightly superior to those comprising **5.14**. For example, compound (*R*,*R*)-**5.1** (Y_4R: K_i = 859 nM) had a higher affinity than (*R*,*R*)-**5.28** (Y_4R: K_i = 2020 nM). This trend was also observed for compounds (*R*,*R*)-**5.29** and (*R*,*R*)-**5.31** or (*R*,*R*)-**5.30** and (*R*,*R*)-**5.32**, respectively.

However, it is questionable whether the increase in affinity in case of compounds (*R*,*R*)-**5.1**, (*R*,*R*)-**5.31** and (*R*,*R*)-**5.32** is due to a bent conformation of the central core structure. For example, the biphenyl moiety in compounds (*R*,*R*)-**5.28**-**5.30** can rotate around the C-C bond between the benzene rings. This goes along with a higher loss of entropy upon binding, which could also be a reason for the lower affinity. Furthermore, the influence of the core structure on the spatial orientation of the whole linker should not be overestimated. The glycine chains are more rigid than previously used alkyl or glycol ether chains. However, when combined with the part adjacent to the pharmacophoric moieties, there is a high level of conformational flexibility.

In addition to rigidisation, variation of the linker length was performed to test the working hypothesis. Having a linker length of 41 atoms, UR-MK188 is among the dimeric argininamides with the longest linkers. Conformational analysis of the central part of the linker suggests a bent, V- or U-shaped conformation for this substructure. Considering the bent conformation of the central structure of UR-MK188, the argininamide moieties would bind to the receptor in much closer proximity than expected from a compound in an extended conformation. Furthermore, structures with shorter linkers (17 or 33 atoms)

revealed affinities in the nanomolar range as well. Recent observations suggest that linker lengths of 40 atoms and more are not necessary.[2] This is in line with the binding data of the bivalent ligands described herein. For instance, the three compounds from the series with bent linkers, compounds (*R*,*R*)-**5.1**, (*R*,*R*)-**5.31** and (*R*,*R*)-**5.32**, only show minor differences regarding Y_4R affinities, although they strongly differ in linker lengths (21 atoms, 27 atoms or even 39 atoms). The reduction of the linker length would be generally favorable for improving drug like properties of these molecules.

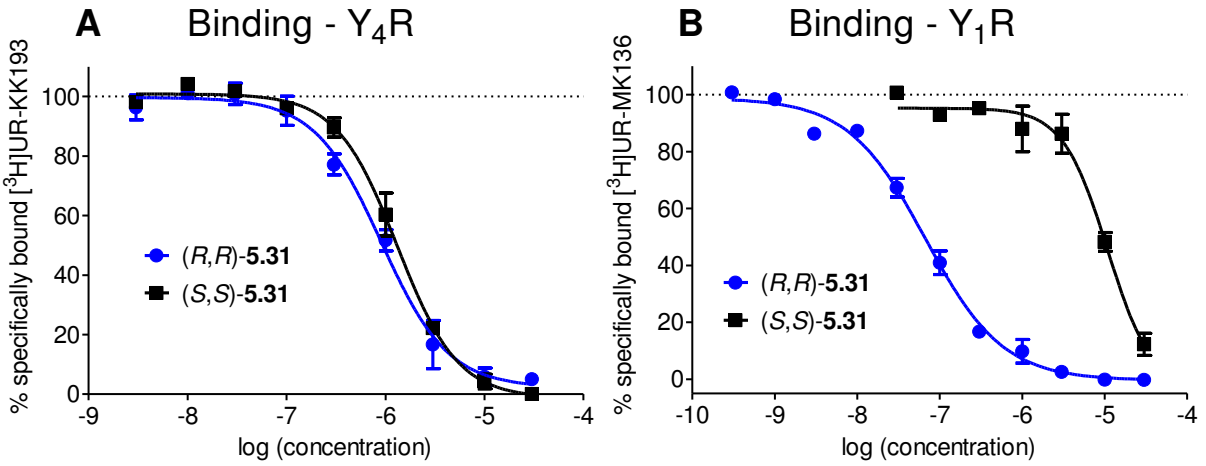


Figure 4: Radioligand displacement curves from competition binding experiments at Y_1R or Y_4R. (**A**) Displacement of ([^{3}H]UR-KK193[4] (K_d = 9.8 nM, c = 10 nM)) using CHO-hY_4R-G_{qi5}-mtAEQ cells[5] in L15 medium. Data represent mean values ± SEM of at least 3 independent experiments, each performed in triplicate. (**B**) Displacement of [^{3}H]UR-MK136[6] (K_d = 6.2 nM, c = 4 nM) using MCF-7-Y_1 cells. Data represent mean values ± SEM of at least 3 independent experiments, each performed in triplicate.

The *S*-configured enantiomer of BIBP 3226, BIBP 3435, was reported to bind to the Y_1R with a substantially lower affinity than the *R*-configured eutomer.[7] In order to exploit the stereodiscrimination of the Y_1R to gain Y_4R selectivity, an *S*-configured version of **5.1** and **5.31** was prepared. By analogy with the parent compound BIBP 3435, the Y_1R affinity of the *S*-configured dimeric ligands (K_i (Y_1R, (*S*,*S*)-**5.1**) = 3380 nM; K_i (Y_1R, (*S*,*S*)-**5.31**) = 5320 nM) was significantly lower than the affinity of the *R*-configured counterparts. In contrast, Y_2R, Y_4R and Y_5R affinity remained essentially unaffected (*S*,*S*)-**5.1** (K_i (Y_4R) = 614 nM) and (*S*,*S*)-**5.31** (K_i (Y_4R) = 725 nM) proved to be equipotent with the *R*-configured enantiomers (*R*,*R*)-**5.1** and (*R*,*R*)-**5.31**, respectively.

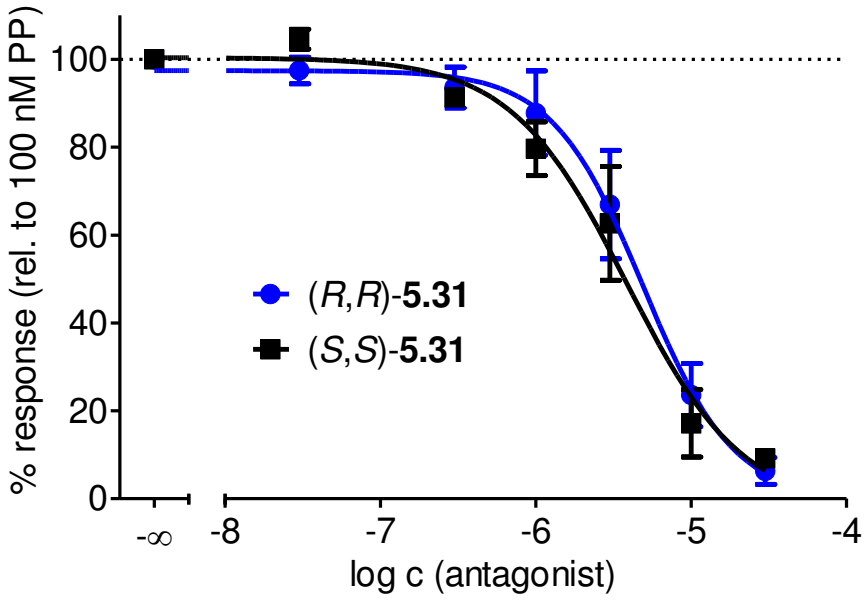
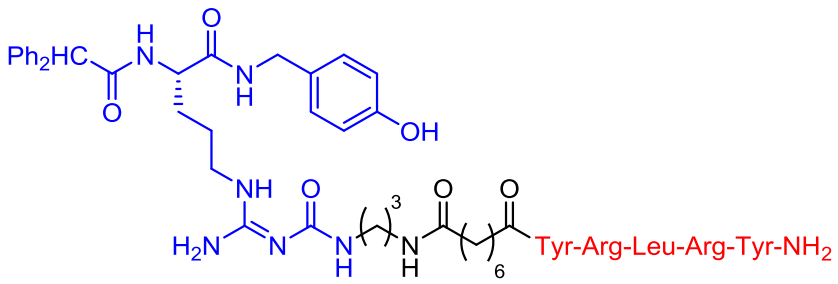


Figure 5: Inhibition of the PP (100 nM) stimulated Ca^{2+} response on CHO-hY_4R-mtAEQ-G_{qi5} cells. Mean values ± SEM were from at least 3 independent experiments (performed in triplicate).

Additionally, (*S*,*S*)-**5.31** and (*R*,*R*)-**5.31** were investigated for Y_4R antagonism in an aequorin Ca^{2+} assay. Like UR-MK188, both compounds antagonized the effect of PP at the Y_4R. The K_b values were 453 nM ((*R*,*R*)-**5.31**) or 368 nM ((*S*,*S*)-**5.31**), respectively. In summary, the replacement of the *R*-configured argininamide moieties by the *S*-configured enantiomers is a convenient way to shift the selectivity of this type of ligands from Y_1R to Y_4R.

The K_i values determined for the bivalent ligands (*R*/*S*)-**5.34** and (*R*/*S*)-**5.35** at the Y_4R were in the one-digit micromolar range. Moreover, (*R*/*S*)-**5.34** was not Y_4R selective, as the affinity to the Y_2R (K_i = 864 nM) was increased. The K_i value of (*R*/*S*)-**5.35** (K_i (Y_4R) = 1460 nM) was in the same range as those of the UR-MK188 derived bivalent ligands ((*R*,*R*)/(*S*,*S*)-**5.1**, (*S*,*S*)-**5.31**, (*R*,*R*)-**5.28**-**5.32**). Apparently, carbamoylation of the guanidine residues as well as the 4-(aminomethyl)phenol moieties are not crucial for interaction with the receptor. Y_4R affinity was not increased by the exchange of the linker attachment position. However, the synthetic accessibility was improved, simplifying structural variations at the diphenylacetic acid residues.

The heterodimeric ligand all-*S*-**5.33** (Figure 6) takes a special role among the investigated compounds. All-*S*-**5.33** comprises two different pharmacophores: a part that is derived from the antagonist UR-MK188 and a peptide that was described as Y_4R agonist.[8]



all-*S*-**5.33**

Figure 6: Heterodimeric Y_4R ligand all-*S*-**5.33**

The affinity of all-*S*-**5.33** (K_i (Y_4R) = 332 nM) was slightly lower than that of UR-MK188. Functional experiments revealed that all-*S*-**5.33** is an agonist that elicits a higher maximal response (E_{Max} = 2.15 (aequorin assay), E_{Max} = 1.95 (DMR assay)) than the endogenous ligand PP (aequorin assay) or the modified endogenous ligand K_4hPP (dynamic mass redistribution assay). In both cases, EC_{50} values (EC_{50} = 488 nM (Aequorin assay), EC_{50} = 483 nM (DMR assay)) were in good agreement with the K_i value.

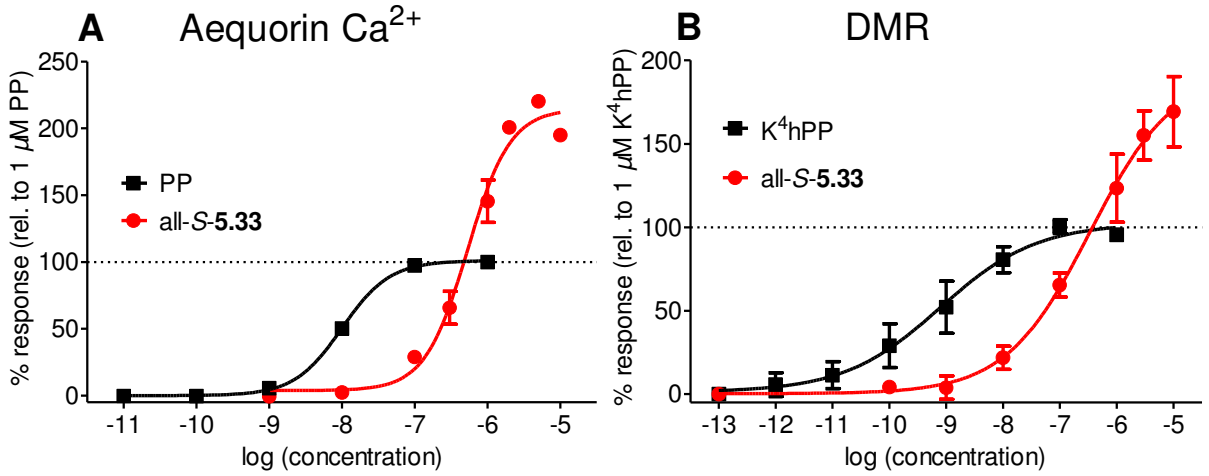


Figure 7: Y_4R agonism of all-*S*-**5.33** and PP or K^4hPP determined in an aequorin Ca^{2+} assay and a dynamic mass redistribution (DMR) assay. (**A**) Concentration response curves from an aequorin assay on CHO-hY_4R-mtAEQ-G_{qi5} cells. Mean values ± SEM were from at least 3 independent experiments (performed in triplicate). (**B**) Concentration response curves from a dynamic mass redistribution assay on CHO-hY_4R-mtAEQ-G_{qi5} cells. Mean values ± SEM were from at least 3 independent experiments (performed in triplicate).

Compounds eliciting a higher maximal functional response than the endogenous agonists are referred to as superagonists. Superagonism is a phenomenon that has been rarely described for GPCR ligands[9-11]. Overall, GPCRs should have substantial conformational freedom to achieve degrees of activation that are higher than the natural state that is formed by the complex of receptor and endogenous ligand. However, it remains unclear whether the occurance of superagonism in cell based functional assays has any physiological relevance,

since the activation of only a fraction of receptors might be sufficient to elicit the full response *in vivo*.

Recently we demonstrated that the affinity of Y_4R ligands is affected by the concentration of sodium ions in the binding buffer.[4] The affinity of Y_4R agonists was increased in the absence of sodium ions, whereas the affinity of the antagonist UR-MK188 was reduced. The same trend was observed for all UR-MK188 derived bivalent ligands and the homodimeric compounds (*R*/*S*)-**5.34** and (*R*/*S*)-**5.35** described in this chapter. For example, the K_i values of (*R*,*R*)-**5.1** (K_i = 859 nM (L-15 medium), K_i = 3270 nM (sodium free buffer)) or (*R*/*S*)-**5.35** (K_i = 1460 nM (L-15 medium), K_i = 3470 nM (sodium free buffer)) were lower in the sodium containing L-15 medium than in the sodium free buffer. In contrast, the superagonist all-*S*-**5.33** had a three-fold higher affinity in sodium free buffer compared to L-15 medium.

Table 1. NPY receptor binding and functional data[a]

Compd.	Y_1R K_i [nM][a]	Y_2R K_i [nM][b]	Y_4R K_i [nM][c]	K_i [nM][d]	K_b [nM][e]	EC_{50} [nM][f]	Y_5R K_i [nM][g]	Linker length[h]
UR-MK188	24[i]	1100[i]	150[k]	660[k]	20[i]	-	>5000[i]	41
(*R*,*R*)-**5.28**	225 ± 80	>10000	2020 ± 240	2980 ± 1800	-	-	>10000	22
(*R*,*R*)-**5.29**	500 ± 188	>10000	1300 ± 260	3750 ± 500	-	-	>10000	28
(*R*,*R*)-**5.1**	82 ± 38	4950 ± 1100	859 ± 99	3270 ± 1300	-	-	>10000	21
(*S*,*S*)-**5.1**	3880 ± 810	2690 ± 1000	614 ± 58	1890 ± 220	-	-	>10000	21
(*R*,*R*)-**5.30**	99 ± 3	>10000	2960 ± 340	>10000	-	-	>10000	40
(*R*,*R*)-**5.31**	41 ± 6	5570 ± 1500	577 ± 100	3750 ± 540	453 ± 140	-	>10000	27
(*S*,*S*)-**5.31**	5320 ± 950	5540 ± 2200	725 ± 100	2960 ± 380	368 ± 110	-	>10000	27
(*R*,*R*)-**5.32**	80 ± 14	>10000	1090 ± 130	6100 ± 3600	-	-	>10000	39
(*R*/*S*)-**5.34**	1510 ± 340	864 ± 210	4030 ± 1300	>10000	-	-	>10000	20
(*R*/*S*)-**5.35**	683 ± 180	9420 ± 4000	1460 ± 370	3470 ± 1800	-	-	>10000	37
All-*S*-**5.33**	2060 ± 490	4950 ± 870	332 ± 100	116 ± 33	488 ± 49 (EC_{50})	483 ± 167	1290 ± 150	-

[a]Radioligand competition binding assay with [^{3}H]UR-MK136[6] (K_d = 6.2 nM, c = 4 nM) using MCF-7-hY_1 cells. [1212] [b]Radioligand competition binding assay with [^{3}H]propionyl-pNPY[13] (K_d = 1.4 nM, c = 1 nM) using CHO-hY_2-G_{qi5}-mtAEQ cells.[14] [c]Radioligand competition binding assay with [^{3}H]**2.10** [4] (K_d = 9.8 nM, c = 10 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells[5] in L-15 medium. [d]Radioligand competition binding assay with [^{3}H]**2.10** (K_d = 0.67 nM, c = 0.6 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells in buffer I. [e]Aequorin calcium mobilization assay on CHO-hY_4-G_{qi5}-mtAEQ cells.[5] [f]Dynamic mass redistribution assay on CHO-hY_4-G_{qi5}-mtAEQ cells. [g]Radioligand competition binding assay with [^{3}H]propionyl-pNPY (K_d = 4.83 nM, c = 4 nM) using HEC-1b hY_5R cells[15]. [h]Number of atoms separating the guanidine moieties of the bivalent ligands. [i]K_i value reported by Berlicki et al.[8] [k]K_i value reported by Kuhn et al.[4]

5.4 Conclusions

The most potent Y_4R antagonist UR-MK188 known so far was used as a lead structure to explore the potential of dimeric ligands with truncated, conformationally constrained linkers. Aromatic substructures in the core of the linker were tolerated, and a truncation of the linker length from 39 to 21 atoms did not affect Y_4R affinity. An exchange of the linker attachment position from the guanidine group to the carboxylic acid of the arginine was tolerated with respect to Y_4R affinity. All-*S*-**5.33** is the first Y_4R agonist capable of activating the receptor with a higher efficacy than the endogenous ligand PP. However, additional experiments, e.g. cytotoxicity assays, functional assays with different readouts in the presence or absence of an antagonist as well as functional assays at wild type cells should be performed to confirm superagonism and to exclude off-target effects.

5.5 Experimental Section

5.5.1 General Experimental Conditions

Chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise indicated. MeCN for HPLC (gradient grade), mercury(II) chloride, glycine, thionyl chloride, formic acid and 1,4-benzenedimethanamine were purchased form Merck (Darmstadt, Germany), HOBt (98%, contains 11-16% H_2O) and diphenylacetic acid were from Acros Organics/Fisher Scientific (Nidderau, Germany). Trifluoroacetic acid, palladium on activated charcoal (loading 10%), trimethylamine, triphosgene, carbonyldiimidazole, *tert*-butyl bromoacetate and diisopropyl carbodiimide were obtained from Sigma Aldrich (Deisenhofen, Germany), *N*,*N*-diisopropylethylamine (DIPEA) (99%) was from ABCR (Karlsruhe, Germany). Triglycine (99%) and boc anhydride were from Alfa Aesar (Karlsruhe, Germany). Fmoc-Arg(Pbf)-OH, Fmoc-D-Arg(Pbf)-OH, HBTU and TBTU were from Iris Biotech (Marktredwitz, Germany). Bovine serum albumin and bacitracin were from Serva (Heidelberg, Germany), human pancreatic polypeptide and porcine neuropeptide Y were from Synpeptide (Shanghai, China). Compounds **5.2**[3] and **5.5**[3] were synthesized and provided by Dr. Max Keller (University of Regensburg, Germany). The synthesis of [^{3}H]**2.10**[4] and [^{3}H]propionyl-pNPY[13] was previously described. Millipore water was used throughout for the preparation of buffers and HPLC eluents. Polypropylene

reaction vessels (1.5 or 2 mL) with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for the storage of stock solutions. TL chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates, silica gel 60 (40-63 μm, Merck) was used for column chromatography. Visualization was accomplished by UV light (λ = 254 nm or 366 nm). NMR-spectra were recorded on a Bruker Avance 300 (7.05 T, ^{1}H: 300.1 MHz, ^{13}C: 75.5 MHz), a Bruker Avance III HD 400 (^{1}H: 400 MHz, ^{13}C: 101 MHz) or a Bruker Avance 600 instrument with cryogenic probe (14.1 T, ^{1}H: 600.3 MHz, ^{13}C: 150.9 MHz) (Bruker, Karlsruhe, Germany). Mass spectra were recorded on a Finnigan MAT95 (EI-MS 70eV) or a Finnigan MAT SSQ 710 A (CI-MS (NH_3)). For HRMS an Agilent Q-TOF 6540 UHD (Agilent, Waldbronn, Germany) equipped with an ESI source was used. Preparative HPLC was performed on a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Kinetex-XB C_{18}, 5 μm, 250 mm × 21 mm, Phenomenex, Aschaffenburg, Germany or Nucleodur-100 C_{18}, 250 mm × 21 mm, Macherey-Nagel, Düren) at a flow-rate of 15 mL/min using mixtures of acetonitrile and 0.1% aqueous TFA solution as mobile phase. A detection wavelength of 220 nm was used throughout. The collected fractions were lyophilised using an alpha 2-4 LD apparatus (Martin Christ, Osterode am Harz, Germany) equipped with a RZ 6 rotary vane vacuum pump (vacuubrand, Wertheim, Germany). Analytical HPLC analysis was performed on a system from Merck-Hitachi (Hitachi, Düsseldorf, Germany) composed of a L-6200-A pump, a AS-2000A autosampler, a L-4000A UV detector, a D-6000 interface and a RP-column (Kinetex-XB C_{18}, 5 μm, 250 mm × 4.6 mm, Phenomenex, Aschaffenburg, Germany or Eurospher-100 C_{18} column, 250 mm × 4 mm, 5 μm, Knauer, Berlin) at a flow rate of 0.8 mL/min. Mixtures of acetonitrile (A) and 0.1% aqueous TFA solution (B) were used as mobile phase. The following gradient was applied: A/B: 0-25 min: 20:80 - 95:5, 25-35 min: 95:5 - 95:5. Detection was performed at 220 nm, the oven temperature was 30 °C.

5.5.2 Chemistry: Experimental Protocols and Analytical Data

(2*R*)-2-(2,2-Diphenylacetamido)-5-{[({[3-(2-{7-[({3-[({*N*-[(4*R*)-4-(2,2-diphenylacetamido)-4-{[(4-hydroxyphenyl)methyl]carbamoyl}butyl]carbamimidoyl}carbamoyl)amino]propyl}-carbamoyl)methyl]-1*H*,7*H*-imidazo[4,5-*f*]benzimidazol-1-yl}acetamido)propyl]carbamoyl}-amino)methanimidoyl]amino}-*N*-[(4-hydroxyphenyl)methyl]pentanamide ((*R*,*R*)-5.1). **5.18** (9.7 mg, 25 μmol) was suspended in anhydrous DMF (0.6 mL). TBTU (18 mg, 2.2 equiv) and DIPEA (9 μL, 2 equiv) were added, and the mixture was stirred for 15 min leading to the dissolution of the suspended starting material. A solution of (*R*)-**5.13** (50 mg, 2.5 equiv) and DIPEA (23 μL, 5 equiv) in anhydrous DMF (0.4 mL) was added and the mixture was subjected to microwave irradiation (70 °C, 30 min). The desired compound was purified by preparative HPLC (column: Nucleodur-100 C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 14:86–62:38, t_R = 14.7 min). Lyophilisation of the eluate afforded (*R*,*R*)-**5.13** as a white fluffy solid (6 mg, 15%). ^{1}H-NMR (600 MHz, COSY, [D$_4$]methanol): δ (ppm) 1.45 – 1.60 (m, 4H), 1.66 – 1.76 (m, 6H), 1.78 – 1.86 (m, 2H), 3.13 – 3.24 (m, 8H), 3.27 – 3.34 (m, overlap with solvent, 4H), 4.22 (q, 4H, *J* 14.6 Hz, 10.8 Hz), 4.41 (q, 2H, *J* 5.8 Hz, 2.7 Hz), 5.07 (s, 2H), 5.20 (s, 4H), 6.67 (d, 4H, *J* 8.6 Hz), 7.02 (d, 4H, *J* 8.6 Hz), 7.19 – 7.39 (m, 20H), 7.98 (s, 1H), 8.10 (s, 1H), 8.88 (s, 2H). ^{13}C-NMR (150.9 MHz, HSQC, HMBC, [D$_4$]methanol): δ (ppm) 25.7 (2 carb.), 30.1 (2 carb.), 30.2 (2 carb.), 37.8 (2 carb.), 37.9 (2 carb.), 41.7 (2 carb.), 43.7 (2 carb.), 49.6 (2 carb.), 54.4 (2 carb.), 58.7 (2 carb.), 94.6, 105.3, 116.3 (4 carb.), 128.1 (4 carb.), 128.2 (4 carb.), 129.5 (4 carb.), 129.6 (4 carb.), 129.8 (4 carb.), 129.9 (4 carb.), 130.3 (2 carb.), 133.2 (2 carb.), 136.9 (2 carb.), 140.8 (2 carb.), 140.9 (2 carb.), 155.6 (4 carb.), 157.8 (2 carb.), 168.0 (2 carb.), 173.4 (2 carb.), 174.88 (2 carb.). RP-HPLC (220 nm): 99% (t_R = 13.4 min, *k* = 3.7). LRMS (ESI): *m/z* (%) 693.34 (15) $[M+2H]^{2+}$, 462.56 (100) $[M+3H]^{3+}$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{74}H_{85}N_{18}O_{10}^+$: 1385.6691, found: 1385.6695. $C_{74}H_{84}N_{18}O_{10} \cdot C_4H_2F_6O_4$ (1385.60 + 228.04).

(2*S*)-2-(2,2-Diphenylacetamido)-5-{[({[3-(2-{7-[({3-[({*N*-[(4*S*)-4-(2,2-diphenylacetamido)-4-{[(4-hydroxyphenyl)methyl]carbamoyl}butyl]carbamimidoyl}carbamoyl)amino]propyl}-carbamoyl)methyl]-1*H*,7*H*-imidazo[4,5-*f*]benzimidazol-1-yl}acetamido)propyl]carbamoyl}-amino)methanimidoyl]amino}-*N*-[(4-hydroxyphenyl)methyl]pentanamide ((*S*,*S*)-5.1). Compound (*S*,*S*)-**5.1** was synthesized from **5.18** and (*S*)-**5.13** (50 mg, 62 μmol) by analogy with the procedure for the preparation of (*R*,*R*)-**5.1**. The desired compound was obtained as

a white lyophilisate (9.2 mg, 23%). The spectroscopic data were identical to those for (*R*,*R*)-**5.1**. RP-HPLC (220 nm): >99% (t_R = 14.0 min, *k* = 3.9).

(2*R*)-5-Amino-*N*-{[4-(*tert*-butoxy)phenyl]methyl}-2-(2,2-diphenylacetamido)pentanamide ((*R*)-5.7).[3] Pd/C-catalyst (0.35 g) was added a suspension of (*R*)-**5.6** (3.50 g, 5.63 mmol) in MeOH (30 mL). The mixture was stirred vigorously under hydrogen (reaction vessel was connected to a hydrogen balloon). The progress of the reaction could be observed, since the product dissolved in MeOH, whereas the starting material was poorly soluble. After completion of the hydrogenation (monitored by TLC (CH_2Cl_2/MeOH (10:1)), the solids were removed by filtration through celite, the volume was reduced on a rotary evaporator and the residue was dried in vacuum to yield (*R*)-**5.7** (2.49 g, 91%) as a pale grey foam. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.19 – 1.39 (m, 12H), 1.44 – 1.75 (m, 3H), 4.12 – 4.24 (m, 2H), 4.29 (q, 1H, *J* 7.9 Hz, 6.0 Hz), 5.11 (s, 1H), 6.81 – 6.91 (m, 2H), 7.05 – 7.13 (m, 2H), 7.16 – 7.37 (m, 10H), 8.39 – 8.55 (m, 2H). LRMS (ESI): *m/z* (%) 488.29 (100) $[M+H]^+$. $C_{30}H_{37}N_3O_3$ (487.64).

(2*S*)-5-Amino-*N*-{[4-(tert-butoxy)phenyl]methyl}-2-(2,2-diphenylacetamido)pentanamide ((*S*)-5.7).[1] Compound (*S*)-**5.7**was synthesized from (*S*)-**5.6** (1.89 g, 3.04 mmol) by analogy with the procedure for the preparation of (*R*)-**5.7**. The desired compound was obtained as white foam (1.06 g, 72%). The spectroscopic data were identical to those for (*R*)-**5.7**.

***tert*-Butyl *N*-[(methylsulfanyl)methanimidoyl]carbamate (5.9).**[16] S-Methylisothiourea hydroiodide (2.3 g, 10.55 mmol) and TEA (1 equiv) were dissolved in chloroform (25 mL). A solution of Boc anhydride (2.3 g, 1 equiv) in chloroform (15 mL) was added over a period of 30 min, and the mixture was stirred at room temperature overnight. After completion of the reaction, the mixture was washed with water (30 mL) and brine (20 mL). The solvent was removed under reduced pressure, and the residue was taken up in EtOAc/PE (1:2, 5 mL). The desired compound was purified by column chromatography (EtOAc/PE (1:5 → 1:1)). The Boc protected intermediate was obtained as a white solid (1.1 g, 55%). TLC (PE/EtOAc 1:1): R_f = 0.52. T_M 77 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.50 (s, 9H), 2.45 (s, 3H). LRMS (ESI): *m/z* (%) 191.08 (10) $[M+H]^+$, 135.02 (100) $[M-C_4H_8]^+$. $C_7H_{14}N_2O_2S$ (190.26).

***tert*-Butyl *N*-(3-aminopropyl)carbamate (5.10).**[17] 1,3-Diaminopropane (7.4 g, 100 mmol) was dissolved in chloroform (20 mL). After addition of a catalytic amount of TEA, a solution of Boc anhydride (2.18 g, 10 mmol) in chloroform (15 mL) was added dropwise over 30 min, and the mixture was stirred at room temperature for 40 h. The solvent was removed under

reduced pressure, and the residue was taken up in EtOAc (100 mL). The solution was washed with saturated aqueous $NaHCO_3$ solution (50 mL), H_2O/brine (2:1, 50 mL) and brine (30 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. Drying of the residue *in vacuo* afforded the desired Boc protected diamine as a yellowish oil (1.1 g, 63%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.36 (s, 9H), 1.48 – 1.62 (m, 2H), 2.70 (t, 2H, *J* 6.6 Hz), 3.05 – 3.18 (m, 2H). GCMS (CI (NH_3)) : *m/z* (%) 175.1 (100) $[M+H]^+$, 118.0 (40) $[M-C_4H_8]^+$. $C_8H_{18}N_2O_2$ (174.24).

***N*-*tert*-Butoxycarbonyl-*N*'-[*N*-(3-*tert*-butoxycarbonylaminopropyl)aminocarbonyl]-*S*-methylisothiourea (5.12).**[18] All used glass materials were dried in an oven at 120 °C, starting materials had been stored in a desiccator. Triphosgene (0.86 g, 2.9 mmol) was dissolved in anhydrous CH_2Cl_2 (15 mL) at room temperature. A solution of **5.10** in anhydrous CH_2Cl_2 (25 mL) was added dropwise over 30 min. Subsequently, a solution of **5.9** in anhydrous CH_2Cl_2 (15 mL) was added in one portion, and the mixture was stirred for 3 h. After completion, the mixture was concentrated to a volume of approximately 5 mL and subjected to column chromatography (CH_2Cl_2/EtOAc, 50:1 → 1:1). Fractions containing product and starting material were collected and subjected to column chromatography using the same gradient again. Evaporation of the solvent yielded the desired compound as a yellow, highly viscous oil (1.15 g, 55.6%). TLC (PE/EtOAc 1:1): R_f = 0.44. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.37 (s, 9H), 1.44 (s, 9H), 1.53 (dt, 2H, *J* 16.3, 8.1 Hz, 2H), 2.22-2.33 (m, 3H), 2.90 (dt, 2H, *J* 13.6, 6.9 Hz), 3.03 (q, 2H, *J* 6.7 Hz), 6.76 (dt, 2H, *J* 11.6, 5.6), 7.75 (t, 1H, *J* 5.9 Hz). ^{13}C-NMR (75.5 MHz, [D_6]DMSO): δ (ppm) 13.5, 27.5, 28.1, 29.5, 37.0, 37.5, 77.3, 82.0, 150.1, 155.5, 161.1, 164.7. LRMS (ESI): *m/z* (%) 391.20 (100) $[M+H]^+$, 291.15 (10) $[M-Boc]^+$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{16}H_{31}N_4O_5S^+$: 391.2009, found: 391.2010. $C_{16}H_{30}N_4O_5S$ (390.50).

(*R*)-5-{3-[(3-aminopropyl)carbamoyl]guanidino}-2-(2,2-diphenylacetamido)-*N*-(4-hydroxybenzyl)pentanamide ((*R*)-5.13). (*R*)-**5.7** (0.24 g, 0.49 mmol) and **5.12** (0.21 g, 0.54 mmol) were dissolved in anhydrous DMF (12 mL). DIPEA (1.2 equiv), and $HgCl_2$ (0.2 g, 1.5 equiv) was added which caused the formation of a white precipitate. The mixture was stirred at room temperature for 16 h. The solids were separated by centrifugation, and the supernatant was diluted with water (200 mL) and extracted with EtOAc (3 x 150 mL). The combined organic layers were dried over $MgSO_4$, concentrated to a volume of about 5 mL on a rotary evaporator and subjected to column chromatography (CH_2Cl_2/EtOAc (2:1)). After

evaporation of the solvent, the protected intermediate was obtained as a white, glue-like solid that was dissolved in TFA/CH_2Cl_2/H_2O (10:10:1, 10 mL) and stirred at room temperature for 2.5 h. After completion, the mixture was diluted with CH_2Cl_2 (100 mL), and the solvents were removed under reduced pressure. This procedure was repeated three times with CH_2Cl_2. The residue was taken up in water (150 mL) and subjected to lyophilization. The title compound was obtained as a white, hygroscopic lyophilisate (0.22 mg, 57%). ^{1}H-NMR (75.5 MHz, [D_4]methanol): δ (ppm) 1.32 – 1.60 (m, 4H), 1.65 – 1.77 (m, 2H), 2.73 – 2.86 (m, 2H), 3.10 – 3.15 (m, 4H), 4.06 – 4.23 (m, 2H), 4.27 – 4.38 (m, 1H), 5.13 (s, 1H), 6.61 – 7.04 (m, 4H), 7.17 – 7.33 (m, 10H). ^{13}C-NMR (300 MHz, [D_4]methanol): δ (ppm) 25.7, 28.7, 30.3, 37.6, 38.3, 41.7, 43.7, 54.3, 58.7, 116.3, 128.1, 128.2, 129.5, 129.6, 129.8, 129.9, 130.0, 130.3, 140.8, 140.9, 155.7, 156.0, 157.8, 173.4, 174.9. LRMS (ESI): *m/z* (%) 574.31 (20) $[M+H]^+$, 287.66 (100) $[M+2H]^{2+}$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{31}H_{40}N_7O_4^+$: 574.3136, found: 574.3131. $C_{31}H_{39}N_7O_4 \cdot C_4H_2F_6O_4$ (573.70 + 228.04).

(*S*)-5-{3-[(3-aminopropyl)carbamoyl]guanidine}-2-(2,2-diphenylacetamido)-*N*-(4-hydroxybenzyl)pentanamide ((*S*)-5.13). Compound (*S*)-**5.13** was synthesized from (*S*)-**5.7** (0.3 g, 0.61 mmol) by analogy with the procedure for the preparation of (*R*)-**5.13**. The desired compound was obtained as a white lyophilisate (0.25 g, 51%). The spectroscopic data were identical to those for (*R*)-**5.13**.

1,5-Dihydrobenzo[1,2-*d*:4,5-*d'*]diimidazole (5.15).[19] Benzene-1,2,4,5-tetraamine tetrahydrochloride (710 mg, 2.52 mmol) was dissolved in formic acid (35 mL) and stirred at 100 °C for 30 h. The reaction mixture was allowed to cool down to ambient temperature, and the volume was concentrated to about 1 mL on a rotary evaporator. Addition of aqueous $NaHCO_3$ solution caused the precipitation of the product, which was collected by filtration, washed with cold water and dried *in vacuo*. The title compound was obtained as a pale brown solid (0.44 g, 110%). T_M > 300°C. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 7.68 (s, 2H), 8.20 (s, 2H), 12.23 (s, 2H). LRMS (ESI): *m/z* (%) 159.07 (100) $[M+H]^+$, 80.04 (20) $[M+2H]^{2+}$. $C_8H_6N_4$ (158.16).

2,2'-(Benzo[1,2-*d*:4,5-*d'*]diimidazole-1,7-diyl)diacetic acid (5.18). 5.15 (85 mg, 0.54 mmol) was dissolved in anhydrous DMF (4 mL). NaH (60% suspension, 52 mg, 2.4 equiv) was added, and the mixture was stirred at 80 °C for 20 min. After cooling down to 60 °C, *tert*-butyl bromoacetate (214.5 mg, 2.04 equiv) was added dropwise over 10 min, and stirring was

continued for 2 h. Water (80 mL) was added, and the mixture was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with water (50 mL) and water/brine (1:3, 25 mL), and the volatiles were removed under reduced pressure. The residue was taken up in CH_2Cl_2/MeOH (10:1) and subjected to column chromatography (eluent: CH_2Cl_2/MeOH (20:1). Two fractions were isolated containing the desired product (**5.17**) and the corresponding 1,5-disubstituted isomer (**5.16**). Removal of the solvent from the second fraction under reduced pressure and drying *in vacuo* gave the desired 1,7-disubstituted *tert*-butyl protected intermediate as a pale yellow solid that was dissolved in TFA/CH_2Cl_2/H_2O (10:10:1, 4.2 mL) and stirred at room temperature for 4 h. After completion of deprotection, the mixture was diluted with CH_2Cl_2 (30 mL), and the solvents were removed under reduced pressure. This procedure was repeated three times with CH_2Cl_2. The residue was suspended in MeOH (10 mL) and sonicated for 5 min. Diethyl ether (10 mL) was added ,and the mixture was stored in a freezer overnight. The solids were collected by filtration, washed with ice-cold diethyl ether and dried *in vacuo* to give the desired diacid **5.18** as a pale yellow solid (33 mg, 22%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 5.15 (s, 4H), 7.82 (s, 1H), 7.90 (s, 1H), 8,32 (s, 2H), 13.26 (s, 2H). ^{13}C-NMR (75.5 MHz, [D_6]DMSO): δ (ppm) 45.6 (2 carb.), 90.3, 107.4, 132.2 (2 carb.), 139.4 (2 carb.), 145.3 (2 carb.), 169.7 (2 carb.). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{12}H_{11}N_4O_4^+$: 275.0775, found: 275.0777. $C_{12}H_{10}N_4O_4$ (274.24).

Glycine methyl ester hydrochloride.[20] To a suspension of glycine (1.96 g, 26.1 mmol) in MeOH (10 mL) was added $SOCl_2$ (3 equiv) dropwise under ice cooling. Subsequently, the mixture was stirred under reflux for 2 h. The volatiles were evaporated *in vacuo* to give glycine methyl ester hydrochloride as a white solid (3.15 g, 96.6%). T_M = 176 °C. ^{1}H-NMR (300 MHz, [D_4]methanol): δ (ppm) 3.83 (s, 3H), 3.85 (s, 2H). $C_3H_8ClNO_2$ (125.55).

2,2'-[(Biphenyl-4,4'-dicarbonyl)bis(azanediyl)]diacetic acid (5.19). Biphenyl-4,4'-dicarboxylic acid (29 mg, 0.12 mmol) was suspended in anhydrous DMF (1 mL). The dicarboxylic acid dissolved upon addition of CDI (42.8 mg, 2.2 equiv) and DIPEA (42 μL, 2 equiv). The mixture was stirred at room temperature for 10 min. After addition of glycine methyl ester hydrochloride (60 mg, 4 equiv) and DIPEA (64 μL, 3 equiv), the temperature was raised to 70 °C and stirring was continued for 3 h. After completion of the reaction (monitored by TLC (CH_2Cl_2/MeOH 10:1)), the mixture was quenched with water (15 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with water (10 mL) and

water/brine (2:8, 10 mL). The volatiles were removed under reduced pressure, and the residue was dried *in vacuo* overnight. The methyl-protected intermediate was suspended in a mixture of MeOH (2 mL) and 10% aqueous NaOH solution (0.4 mL) and stirred under ice cooling for 3 h. During the reaction, the suspended starting material dissolved slowly. After completion of the reaction (monitored by TLC (CH_2Cl_2/MeOH (5:1)), the mixture was filtered through celite. When the pH of the filtrate was adjusted to a value of 3 with 1 N HCl solution a white solid precipitated. After storage in a refrigerator for 2 d, the solids were collected by filtration, suspended in 0.1% aqueous TFA solution (10 mL) and subjected to lyophilization. Compound **5.19** was obtained as a white lyophilisate (16 mg, 37%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 3.95 (d, 4H *J* 5.9 Hz), 7.88 (d, 4H, *J* 8.5 Hz), 8.00 (d, 4H, *J* 8.5 Hz), 8.95 (t, 2H, *J* 5.9 Hz), 12.62 (s, 2H). ^{13}C-NMR (75.5 MHz, [D_6]DMSO): δ (ppm) 41.1 (2 carb.), 126.7 (2 carb.), 127.9 (2 carb.), 133.0 (2 carb.), 141.7 (2 carb.), 165.9 (2 carb.), 171.2 (2 carb.). LRMS (ESI): *m/z* (%) 713.21 (5) $[2M+H]^+$, 357.11 (100) $[M+H]^+$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{18}H_{17}N_2O_7^+$: 3571081, found: 357.1083. $C_{18}H_{16}N_2O_6$ (356.33).

***N-tert*-Butoxycarbonyl-glycine (5.20).**[21] Glycine (80 mg, 1.06 mmol) and DIPEA (275 μL, 1.5 equiv) were dissolved in H_2O/dioxane (1:1, 10 mL). A solution of Boc anhydride (232 mg, 1 equiv) in dioxane (1 mL) was added, and the mixture was stirred at room temperature for 6 h. The volatiles were removed on a rotary evaporator, and the residue was taken up in H_2O (20 mL). The aqueous phase was washed with ether (20 mL), acidified with 1 N HCl solution to a pH value of 2 and extracted with EtOAc (2 × 20 mL). The volatiles were removed under reduced pressure, and the title compound was obtained as white solid (140 mg, 75%). ^{1}H-NMR (300 MHz, MeOD): δ (ppm) 1.44 (s, 9H), 3.75 (s, 2H). LRMS (ESI): *m/z* (%) 349.15 (55) $[2M-H]^-$, 174.07 (100) $[M-H]^-$. $C_7H_{13}NO_4$ (175.18).

***N-tert*-Butoxycarbonyl-glycylglycylglycine (5.21).**[22] Triglycine (100 mg, 0.53 mmol) and DIPEA (138 μL, 1.5 equiv) were dissolved in H_2O/dioxane (1:1, 5 mL). A solution of Boc anhydride (116 mg, 1 equiv) in dioxane (1 mL) was added, and the mixture was stirred at room temperature for 6 h. The volatiles were removed on a rotary evaporator, and the residue was taken up in H_2O (20 mL). The aqueous phase was washed with ether (20 mL), acidified with 1 N HCl solution to a pH value of 2 and extracted with EtOAc (2 × 20 mL). The volatiles were removed under reduced pressure, and the title compound was obtained as white glue-like solid (63 mg, 41%). ^{1}H-NMR (400 MHz, [D_6]DMSO): δ (ppm) 1.38 (s, 9H), 3.58

(d, 2H, *J* 6.0 Hz), 3.71 – 3.78 (m, 4H), 6.99 (t, 1H, *J* 5.8 Hz), 8.05 (t, 1H, *J* 5.7 Hz), 8.13 (t, 1H, *J* 5.8 Hz). ^{13}C-NMR (400 MHz, [D$_6$]DMSO): δ (ppm) 28.6 (3 carb.), 41.0, 42.2, 43.8, 78.6, 156.3, 169.6, 170.1, 171.5. LRMS (ESI): *m/z* (%) 579.26 (40) $[2M+H]^+$, 290.13 (30) $[M+H]^+$, 234.07 (100) $[M-C_8H_8]^+$, 190.08 (50) $[M-Boc]^+$. $C_{11}H_{19}N_3O_6$ (289.29).

(2*R*)-5-{[({[3-(2-Aminoacetamido)propyl]carbamoyl}amino)methanimidoyl]amino}-2-(2,2-diphenylacetamido)-*N*-[(4-hydroxyphenyl)methyl]pentanamide di(hydrotrifluoroacetate) ((*R*)-5.22). Compound **5.20** (32.8 mg, 0.188 mmol) and DIPEA (1 equiv) were dissolved in anhydrous DMF (1 mL). CDI (33 mg, 1.1 equiv) was added, and the mixture was stirred at room temperature under argon atmosphere for 15 min. A solution of (*R*)-**5.13** (100 mg, 0.125 mmol) and DIPEA (1 equiv) in anhydrous DMF (0.5 mL) was added, and stirring was continued at 80 °C for 2 h. The protected intermediate was purified by preparative HPLC (column: Nucleodur-100 C18 250 × 21 mm; gradient: 0–18 min: MeCN/0.1% aq TFA 24:76–52:48, t_R = 16.2 min). After removal of the solvents on a rotary evaporator followed by lyophilisation, the intermediate was taken up in CH_2Cl_2/TFA/H_2O (10:10:1, 5 mL) and stirred at room temperature for 3 h. After completion of deprotection, the mixture was diluted with CH_2Cl_2 (30 mL), and the solvents were removed under reduced pressure. This procedure was repeated three times with CH_2Cl_2. The residue was taken up in water (50 mL). Lyophilisation afforded (*R*)-**5.22** as a white fluffy solid (45 mg, 42%). ^{1}H-NMR (600 MHz, COSY, [D$_4$]methanol): δ (ppm) 1.50 – 1.63 (m, 2H), 1.67 – 1.78 (m, 3H), 1.81 – 1.88 (m, 1H), 3.18 – 3.34 (m, overlap with solvent, 6H), 3.66 (s, 2H), 4.25 (q, 2H, *J* 14.6, 5.8), 4.43 (q, 1H, *J* 6.1, 2.6), 5.07 (s, 1H), 6.71 (d, 2H, *J* 8.6 Hz), 7.06 (d, 2H, *J* 8.6 Hz), 7.22 – 7.32 (m, 10H). ^{13}C-NMR (150.9 MHz, HSQC, HMBC, [D$_4$]methanol): δ (ppm) 25.7, 30.3 (2 carb.), 37.9, 38.2, 41.4, 41.7, 43.7, 54.2, 58.7, 116.3 (2 carb.), 128.1 (2 carb.), 128.2 (2 carb.), 129.5 (2 carb.), 129.6 (2 carb.), 129.8 (2 carb.), 129.9 (2 carb.), 130.0 (2 carb.), 130.3, 140.8, 140.9, 155.7 (2 carb.), 157.9, 167.3, 173.3, 174.9. LRMS (ESI): *m/z* (%) 631.34 $[M+H]^+$ (25), 316.17 $[M+2H]^{2+}$ (100). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{33}H_{43}N_8O_5^+$: 631.3351, found: 631.3354. $C_{33}H_{42}N_8O_5 \cdot C_4H_2F_6O_4$ (630.75 + 228.04).

(2*S*)-5-{[({[3-(2-Aminoacetamido)propyl]carbamoyl}amino)methanimidoyl]amino}-2-(2,2-diphenylacetamido)-*N*-[(4-hydroxyphenyl)methyl]pentanamide di(hydrotrifluoroacetate) ((*S*)-5.22). Compound (*S*)-**5.22** was synthesized from **5.20** and (*S*)-**5.13** (100 mg, 0.125 mmol) by analogy with the procedure for the preparation of (*R*)-**5.22**. The desired compound was

obtained as a white lyophilisate (51 mg, 48%). The spectroscopic data were identical to those for (*R*)-**5.22**.

(2*R*)-5-[({[(3-{2-[2-(2-Aminoacetamido)acetamido]acetamido}propyl)carbamoyl]amino}-methanimidoyl)amino]-2-(2,2-diphenylacetamido)-*N*-[(4-hydroxyphenyl)methyl]pentanamide ((*R*)-5.23). Compound **5.21** (31.1 mg, 0.1 mmol), TBTU (35.3 mg, 1.1 equiv) and DIPEA (10 μL, 1 equiv) were dissolved in anhydrous DMF (0.6 mL) and the mixture was stirred in a microwave vial under argon atmosphere at room temperature for 15 min. After addition of a solution of (*R*)-**5.13** (80 mg, 1 equiv) and DIPEA (10 μL, 1 equiv) in anhydrous DMF (0.4 mL), the mixture was subjected to microwave irradiation (70 °C, 2 h). The Boc-protected intermediate was purified by preparative HPLC (column: Nucleodur-100 C18 250 × 21 mm; gradient: 0–18 min: MeCN/0.1% aq TFA 24:76–52:48, t_R = 14.2 min). After removal of the solvents on a rotary evaporator and by lyophilisation, the intermediate was taken up in CH_2Cl_2/TFA/H_2O (10:10:1, 5 mL) and stirred at room temperature for 3 h. After completion, the mixture was diluted with CH_2Cl_2 (30 mL), and the solvents were removed under reduced pressure. This procedure was repeated three times with CH_2Cl_2. The residue was taken up in water (50 mL). Lyophilisation afforded (*R*)-**5.23** as a white fluffy solid (35 mg, 25.3%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.35 – 1.48 (m, 2H), 1.50 – 1.71 (m, 4H), 3.03 – 3.13 (m, 4H), 3.14 – 3.22 (m, 2H), 3.60 (q, 2H *J* 5.8, 5.5 Hz), 3.68 (d, 2H, *J* 5.8 Hz), 3.84 (d, 2H, *J* 5.5 Hz), 4.05 – 4.21 (m, 2H), 4.32 (q, 1H, *J* 7.7 Hz), 5.11 (s, 1H), 6.66 (d, 2H, *J* 8.5 Hz), 7.00 (d, 2H, *J* 8.5 Hz), 7.17 – 7.32 (m, 10H), 7.49 (t, 1H, *J* 5.3 Hz), 7.90 (t, 1H, *J* 5.5 Hz), 7.94 – 8.06 (m, 3H), 8.27 (t, 1H, *J* 5.8 Hz), 8.35 – 8.43 (m, 2H), 8.50 (d, 1H, *J* 8.2 Hz), 8.61 (t, 1H, *J* 5.7 Hz), 8.89 (s, 1H), 9.30 (s, 1H), 9.98 (s, 1H). LRMS (ESI): *m/z* (%) 745.37 (10) $[M+H]^+$, 373.19 (100) $[M+2H]^{2+}$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{37}H_{49}N_{10}O_7^+$: 745.3780, found: 745,3778. $C_{37}H_{48}N_{10}O_7 \cdot C_4H_2F_6O_4$ (744.85 + 228.04).

N^2-(2,2-Diphenylacetyl)-N^ω-[(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)-sulfonyl]arginine ((*R*/*S*)-5.24). Diphenylacetic acid (90.4 mg, 0.43 mmol), TBTU (137 mg, 1 equiv) and DIPEA (111 μL, 1.5 equiv) were dissolved in CH_2Cl_2/DMF (5:3, 8 mL), and the mixture was stirred at room temperature for 15 min. (*R*/*S*)-**4.14** (200 mg, 0.9 equiv) and DIPEA (74 μL, 1 equiv) were added, and stirring was continued for 14 h. EtOAc (40 mL) was added, and the mixture was washed with water (2 × 40 mL). The volume of the organic phase was reduced and the benzyl-protected intermediate was purified by column

chromatography (eluent: EtOAc/PE (2:1)). The intermediate was dissolved in methanol (20 mL) followed by the addition of Pd/C (30 mg). The mixture was stirred vigorously under hydrogen (reaction vessel was connected to a hydrogen balloon). After completion of the reaction, the solids were removed by filtration through celite, and the solvent was removed under reduced pressure. (*R*/*S*)-**5.24** was obtained as white, hardened foam (138 mg, 58%). ^{1}H-NMR (400 MHz, [D_4]methanol): δ (ppm) 1.42 (d, 6H, *J* 1.3 Hz), 1.44 – 1.53 (m, 2H), 1.58 – 1.73 (m, 1H), 1.77 – 1.91 (m, 1H), 2.06 (s, 3H), 2.50 (s, 3H), 2.56 (s, 3H), 2.96 (s, 2H), 3.10 (t, 2H, *J* 6.5 Hz), 4.33 – 4.43 (m, 1H), 5.06 (s, 1H), 7.14 – 7.34 (m, 10H). ^{13}C-NMR (400 MHz, [D_4]methanol): δ (ppm) 11.2, 17.0, 18.3, 25.6, 27.3 (2 carb.), 28.4, 40.1, 42.6, 52.1, 57.3, 86.3, 117.2, 124.8, 126.6, 126.8, 128.0 (2 carb.), 128.1 (2 carb.), 128.5 (2 carb.), 128.6 (2 carb.), 132.2, 132.7, 138.1, 139.5, 139.6, 156.5, 158.6, 173.4, 173.6.HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{33}H_{41}N_4O_6S^+$: 621.2741, found: 621.2749. $C_{33}H_{40}N_4O_6S$ (620.76).

Benzyl [4-(aminomethyl)benzyl]carbamate (5.25).[23] Benzene-1,4-dimethanamine (2 g, 1.47 mmol) and DIPEA (500 μL, 0.2 equiv) were dissolved in CH_2Cl_2 (10 mL). A solution of benzyl chloroformate (833 mg, 0.33 equiv) in CH_2Cl_2 (10 mL) was added dropwise. The mixture was stirred at room temperature for 14 h. The solvent was removed on a rotary evaporator, and the residue was taken up in EtOAc (50 mL) and washed with 1 M aqueous NaOH solution (2 × 50 mL). The volume of the organic phase was reduced and the desired compound was purified by column chromatography (eluent: MeOH/CH_2Cl_2 (1:2)). **5.25** was obtained as a yellow viscous oil (760 mg, 57%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 2.22 (br s, 2H), 3.68 (s, 2H), 4.17 (d, 2H, *J* 6.2 Hz), 5.03 (s, 2H), 7.12 (d, 2H, *J* 8.1 Hz), 7.26 (d, 2H, *J* 8.2 Hz), 7.29-7.42 (m, 5H), 7.81 (t, 1H, *J* 6.2 Hz). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{16}H_{19}N_2O_2^+$: 271.1441, found: 271.1443. $C_{16}H_{18}N_2O_2$ (270.33).

Benzyl {4-[(2-amino-5-{3-[(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl]guanidino}pentanamido)methyl]benzyl}carbamate ((*R*/*S*)-5.26). Fmoc-D/L-Arg(Pbf)-OH (1.46 g, 2.25 mmol), **5.25** (620 mg, 1.02 equiv) and HOBt (541 mg, 1.8 equiv) were dissolved in DMF (30 mL). A solution of *N,N'*-diisopropylcarbodiimide (290 mg, 1.02 equiv) in DMF (2 mL) was added slowly under ice cooling, and the resulting mixture was stirred at room temperature for 14 h. Water (350 mL) was added, and the mixture was extracted with EtOAc (2 × 300 mL). The combined organic phases were dried over $MgSO_4$, and the volume was reduced on a rotary evaporator. The protected intermediate was

purified by column chromatography (eluent: EtOAc/PE (1:1)), and the volatiles were removed on a rotary evaporator. The residue was dissolved in CH_2Cl_2/DEA (8:1, 90 mL) and stirred for 16 h. The volatiles were removed under reduced pressure, and the desired compound was purified by column chromatography (eluent: EtOAc/PE (1:1) → CH_2Cl_2/7N NH_3 in MeOH (20:1)) affording the title compound as a pale yellow solid (960 mg, 63%). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.30-1.72 (m, 10 H), 2.00 (s, 3H), 2.43 (s, 3H), 2.49 (s, 3H, overlap with solvent), 2.95 (s, 2H), 2.99-3.10 (m, 2H), 3.17 (s, 2H), 3.41 (t, 1H, *J* 5.5 Hz), 4.17 (d, 2H, *J* 6.1 Hz), 4.22-4.31 (m, 2H), 5.03 (s, 2H), 6.50 (br s, 1H), 6.98 (br s, 1H), 7.14-7.24 (m, 4H), 7.27-7.40 (m, 5H), 7.93 (t, 1H, *J* 6.2 Hz), 8.63 (t, 1H, *J* 5.7 Hz). ^{13}C-NMR (75.5 MHz, [D_6]DMSO): δ (ppm) 12.2, 17.5, 18.9, 25.1, 28.2 (carb.), 30.6, 41.7, 42.3, 43.4, 48.5, 53.3, 65.2, 86.2, 116.2, 124.2, 126.9 (2 carb.), 127.2 (2 carb.), 127.6 (2 carb.), 127.7, 128.2 (2 carb.), 131.1, 137.0, 137.2, 137.5, 138.3, 156.0, 156.2, 157.3, 172.0. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{35}H_{47}N_6O_6S^+$: 679.3276, found: 679.3272. $C_{35}H_{46}N_6O_6S$ (678.85).

***N*-[4-(Aminomethyl)benzyl]-2-(2,2-diphenylacetamido)-5-({3-[(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl]guanidino}pentanamide ((*R*/*S*)-5.27).** (*R*/*S*)-**5.26** (960 mg, 1.42 mmol) and DIPEA (250 μL, 1 equiv) were dissolved in DME/THF (3:1, 20 mL). A solution of **5.5** (570 mg, 1.3 equiv) in DME (10 mL) was added, and the mixture was stirred at room temperature for 14 h. The reaction mixture was concentrated under reduced pressure, and the Cbz-protected intermediate was purified by column chromatography (eluent: CH_2Cl_2/MeOH (30:1)). The intermediate compound was dissolved in MeOH (15 mL) and Pd/C (90 mg) was added. The mixture was stirred vigorously under hydrogen (reaction vessel was connected to a hydrogen balloon). After completion of the reaction (monitored by TLC: CH_2Cl_2/7N NH_3 in MeOH (10:0.8)), the solids were removed by filtration through celite, and the solvent was removed on a rotary evaporator. Purification by column chromatography (eluent: CH_2Cl_2/MeOH (20:1) → CH_2Cl_2/7N NH_3 in MeOH (3:1)) gave the title compound as a white hardened foam (400 mg, 38%). TLC (CH_2Cl_2/7N NH_3 in MeOH 12:1) R_f = 0.29. ^{1}H-NMR (400 MHz, [D_4]methanol): δ (ppm) 1.32 – 1.50 (m, 8H), 1.58 – 1.70 (m, 1H), 1.71 – 1.84 (m, 1H), 2.05 (s, 3H), 2.50 (s, 3H), 2.56 (s, 3H), 2.94 (s, 2H), 3.00 – 3.16 (m, 2H), 3.97 (s, 2H), 4.25 – 4.46 (m, 3H), 5.09 (s, 1H), 7.10 – 7.42 (m, 14H). ^{13}C-NMR (400 MHz, [D_4]methanol): δ (ppm) 11.2, 17.1, 18.3, 25.7, 27.4 (2 carb.), 28.9, 39.8, 42.2, 42.6, 43.3, 53.4, 57.2, 86.3, 117.1, 124.7, 126.6, 126.8, 127.6 (2 carb.), 128.1 (2 carb.), 128.2 (2 carb.), 128.4 (2 carb.), 128.5 (2 carb.), 128.6 (2 carb.), 129.3, 132.1, 133.0, 134.2, 138.0, 139.0, 139.6, 156.7, 158.5, 172.7,

173.3. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{41}H_{51}N_6O_5S^+$: 739.3636, found: 739.3644. $C_{41}H_{50}N_6O_5S$ (738.95).

***N*^{4},*N*$^{4'}$-Bis[(*R*)-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-9-imino-3,11-dioxo-2,8,10,12-tetraazapentadecan-15-yl]-biphenyl-4,4'-dicarboxamide ((*R*,*R*)-5.28).** Biphenyl-4,4'-dicarboxylic acid (7.7 mg, 0.032 mmol) was suspended in anhydrous DMF (0.6 mL). TBTU (22.6 mg, 2.2 equiv) and DIPEA (11 μL, 2 equiv) were added and the mixture was stirred for 15 min which led to the dissolution of the suspended starting material. A solution of (*R*)-**5.13** (64.1 mg, 2.5 equiv) and DIPEA (28 μL, 5 equiv) in anhydrous DMF (0.4 mL) was added, and the mixture was subjected to microwave irradiation (70 °C, 30 min). The desired compound was purified by preparative HPLC (column: Nucleodur-100 C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 15:85–79:21, t_R = 16.3 min). Lyophilisation of the eluate afforded (*R*,*R*)-**5.28** as a white fluffy solid (20.7 mg, 41%). ^{1}H-NMR (600 MHz, COSY, [D$_4$]methanol): δ (ppm) 1.48 – 1.63 (m, 4H), 1.65 – 1.73 (m, 2H), 1.79 – 1.89 (m, 6H), 3.15 – 3.26 (m, 4H), 3.29 – 3.34 (m, 4H), 3.47 (t, 4H, *J* 6.6 Hz), 4.23 (q, 4H, *J* 14.6 Hz, 8.6 Hz), 4.42 (t, 2H, *J* 6.9 Hz), 5.07 (s, 2H), 6.7 (d, 4H, *J* 8.6), 7.05 (d, 4H, *J* 8.6 Hz), 7.18 – 7.32 (m, 20H), 7.72 – 7.95 (m, 8H). ^{13}C-NMR (150.9 MHz, HSQC, HMBC, [D$_4$]methanol): δ (ppm) 25.7 (2 carb.), 30.3 (2 carb.), 30.4 (2 carb.), 38.2 (2 carb.) 38.3 (2 carb.), 41.7 (2 carb.), 43.7 (2 carb.), 54.3 (2 carb.), 58.7 (2 carb.), 116.3 (4 carb.), 128.1 (4 carb.), 128.2 (4 carb.), 129.0 (4 carb.), 129.5 (4 carb.), 129.6 (4 carb.), 129.8 (4 carb.), 129.9 (4 carb.), 130.0 (4 carb.), 130.3 (2 carb.), 135.0 (2 carb.), 140.8 (2 carb.), 140.9 (2 carb.), 144.3 (2 carb.), 155.6 (2 carb.), 155.7 (2 carb.), 157.8 (2 carb.) 169.9 (2 carb.), 173.3 (2 carb.), 174.9 (2 carb.). RP-HPLC (220 nm): 98% (t_R = 15.8 min, *k* = 4.5). LRMS (ESI): *m/z* (%) 1353.63 (1) $[M+H]^+$, 677.33 (100) $[M+2H]^{2+}$. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{76}H_{85}N_{14}O_{10}^+$: 1353.6568, found: 1353.6559. $C_{76}H_{84}N_{14}O_{10} \cdot C_4H_2F_6O_4$ (1353.60 + 228.04).

***N*^{4},*N*$^{4'}$-Bis[(*R*)-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-9-imino-3,11,17-trioxo-2,8,10,12,16-pentaazaoctadecan-18-yl]-biphenyl-4,4'-dicarboxamide ((*R*,*R*)-5.29).** Compound **5.19** (10 mg, 0.028 mmol) was dissolved in anhydrous DMF (0.6 mL), TBTU (19.8 mg, 2.2 equiv) and DIPEA (10 μL, 2 equiv) were added, and the mixture was stirred for 15 min. A solution of (*R*)-**5.13** (56.1 mg, 2.5 equiv) and DIPEA (25 μL, 5 equiv) in anhydrous DMF (0.4 mL) was added, and the mixture was subjected to microwave irradiation (70 °C, 30 min). The desired compound was purified by preparative HPLC (column: Nucleodur-100 C18 250 ×

21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 15:85–79:21, t_R = 13.2 min). Lyophilisation of the eluate afforded (*R*,*R*)-**5.29** as a white fluffy solid (15.8 mg, 33%). ^{1}H-NMR (600 MHz, COSY, [D$_6$]DMSO,): δ (ppm) 1.35 – 1.48 (m, 4H), 1.49 – 1.55 (m, 2H), 1.56 – 1.62 (m, 4H), 1.63 – 1.70 (m, 2H), 3.08 – 3.14 (m, 8H), 3.15 – 3.21 (m, 4H), 3.86 (d, 4H, *J* 5.6 Hz), 4.08 – 4.19 (m, 4H), 4.32 (q, 2H, *J* 8.0 Hz, 6.3 Hz), 5.11 (s, 2H), 6.66 (d, 4H, *J* 8.6 Hz), 6.99 (d, 4H, *J* 8.6 Hz), 7.17 – 7.24 (m, 4H), 7.24 – 7.30 (m, 16H), 7.49 (t, 2H, *J* 5.3 Hz), 7.85 (d, 4H, *J* 8.4 Hz, 4H), 7.97 – 8.02 (m, 6H), 8.35 (t, 2H, *J* 5.8 Hz), 8.38 (s, 2H), 8.47 (d, 2H, *J* 8.1 Hz), 8.83 (t, 2H, *J* 5.7 Hz), 8.90 (s, 2H), 9.29 (s, 2H), 10.02 (s, 2H). ^{13}C-NMR (150.9 MHz, HSQC, HMBC, [D$_6$]DMSO): δ (ppm) 24.6 (2 carb.), 29.2 (2 carb.), 29.4 (2 carb.), 36.0 (2 carb.), 36.9 (2 carb.), 40.3 (2 carb.), 41.6 (2 carb.), 42.7 (4 carb.), 52.2 (2 carb.), 55.9 (2 carb.), 115.0 (4 carb.), 126.5 (4 carb.), 126.6 (4 carb.), 128.1 (4 carb.), 128.1 (4 carb.), 128.2 (4 carb.), 128.4 (4 carb.), 128.5 (4 carb.), 128.5 (4 carb.), 129.1 (2 carb.), 133.3 (2 carb.), 140.2 (2 carb.), 140.4 (2 carb.), 141.7 (2 carb.), 153.5 (2 carb.), 153.7 (2 carb.), 156.2 (2 carb.), 166.0 (2 carb.), 169.1 (2 carb.), 170.9 (2 carb.), 171.0 (2 carb.). RP-HPLC (220 nm): 98% (t_R = 14.7 min, *k* = 4.1). LRMS (ESI): *m/z* (%) 734.35 (100) $[M+2H]^{2+}$. HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $C_{80}H_{92}N_{16}O_{12}^{2+}$: 734.3534, found: 734.3537. $C_{80}H_{90}N_{16}O_{12} \cdot C_4H_2F_6O_4$ (1476.7 + 228.04).

N^4,$N^{4'}$-Bis[(*R*)-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-9-imino-3,11,17,20,23-pentaoxo-2,8,10,12,16,19,22-heptaazatetracosan-24-yl]-biphenyl-4,4'-dicarboxamide ((*R*,*R*)-5.30). Biphenyl-4,4'-dicarboxylic acid (5.5 mg, 22.6 μmol) was suspended in anhydrous DMF (0.6 mL). TBTU (16.0 mg, 2.2 equiv), DIPEA (8 μL, 2 equiv) was added, and the mixture was stirred for 15 min, leading to the dissolution of the suspended starting material. A solution of (*R*)-**5.23** (55 mg, 2.5 equiv) and DIPEA (20 μL, 5 equiv) in anhydrous DMF (0.4 mL) was added, and the mixture was subjected to microwave irradiation (70 °C, 30 min). The desired compound was purified by preparative HPLC (column: Nucleodur-100 C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 14:86–62:38, t_R = 15.1 min). Lyophilisation of the eluate afforded (*R*,*R*)-**5.30** as a white fluffy solid (10 mg, 32%). ^{1}H-NMR (600 MHz, COSY, [D$_6$]DMSO): δ (ppm) 1.35 – 1.46 (m, 2H), 1.49 – 1.58 (m, 6H), 1.62 – 1.69 (m, 2H), 3.04 – 3.11 (m, 8H), 3.14 – 3.21 (m, 4H), 3.68 (d, 4H, *J* 5.7 Hz), 3.76 (d, 4H, *J* 5.6 Hz), 3.95 (d, 4H, *J* 5.6 Hz), 4.08 – 4.19 (m, 4H), 4.32 (q, 2H, *J* 7.7 Hz, 6.5 Hz), 5.11 (s, 2H), 6.66 (d, 4H, *J* 8.4 Hz), 6.99 (d, 4H, *J* 8.4 Hz), 7.18 – 7.23 (m, 4H), 7.25 – 7.29 (m, 16H), 7.42 – 7.46 (m, 2H), 7.78 (t, 2H, *J* 5.6 Hz), 7.85 (d, 4H, *J* 8.4 Hz), 7.99 (d, 4H, *J* 8.4), 8.16 (t, 2H, *J* 5.8 Hz), 8.30 (t, 2H, *J* 5.6 Hz, 2H), 8.33 – 8.40 (m, 4H), 8.47 (t, 2H, *J* 8.1 Hz), 8.87 (s, 2H), 8.90 (t, 2H, *J* 5.6 Hz), 9.28 (s, 2H), 9.89

(s, 2H). RP-HPLC (220 nm): 98% (t_R = 14.1 min, k = 3.9). LRMS (ESI): *m/z* (%) 848.40 (100) $[M+2H]^2$. HRMS (ESI): *m/z* $[M+H]^{2+}$ calcd. for $C_{88}H_{104}N_{20}O_{16}^{2+}$: 848.3964, found: 848.3967. $C_{88}H_{102}N_{20}O_{16} \cdot C_4H_2F_6O_4$ (1695.91 + 228.04).

(2*R*)-2-(2,2-Diphenylacetamido)-5-{[({[3-(2-{2-[7-({[({3-[({*N*-[(4*R*)-4-(2,2-diphenylacetamido)-4-{[(4-hydroxyphenyl)methyl]carbamoyl}butyl]carbamimidoyl}-carbamoyl)amino]propyl}carbamoyl)methyl]carbamoyl}methyl)-1*H*,7*H*-imidazo[4,5-*f*]benzimidazol-1-yl]acetamido}acetamido)propyl]carbamoyl}amino)methanimidoyl]-amino}-*N*-[(4-hydroxyphenyl)methyl]pentanamide ((*R*,*R*)-5.31). Compound **5.18** (6 mg, 21.9 μmol) was suspended in anhydrous DMF (0.6 mL). TBTU (15.5 mg, 2.2 equiv), DIPEA (8 μL, 2 equiv) was added, and the mixture was stirred for 15 min leading to the dissolution of the suspended starting material. A solution of (*R*)-**5.22** (47 mg, 2.5 equiv) and DIPEA (20 μL, 5 equiv) in anhydrous DMF (0.4 mL) was added, and the mixture was subjected to microwave irradiation (70 °C, 30 min). The desired compound was purified by preparative HPLC (column: Nucleodur-100 C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 14:86–62:38, t_R = 14.6 min). Lyophilisation of the eluate afforded (*R*,*R*)-**5.31** as a white fluffy solid (11 mg, 29%). ^{1}H-NMR (600 MHz, COSY, [D$_4$]methanol): δ (ppm) 1.46 – 1.61 (m, 4H), 1.63 – 1.73 (m, 6H), 1.78 – 1.86 (m, 2H), 3.11 – 3.20 (m, 8H), 3.22 – 3.27 (m, 4H), 3.93 (s, 4H), 4.22 (q, 4H, *J* 14.7 Hz, 9.3 Hz), 4.42 (q, 2H, *J* 6.1 Hz, 2.1 Hz), 5.06 (s, 2H), 5.29 (s, 4H), 6.66 (d, 4H, *J* 8.2 Hz), 7.02 (d, 4H, *J* 8.2 Hz), 7.17 – 7.30 (m, 20H), 8.06 (s, 1H), 8.16 (s, 1H), 8.88 (s, 2H). RP-HPLC (220 nm): 97% (t_R = 13.0 min, k = 3.5). LRMS (ESI): *m/z* (%) 750.36 (20) $[M+2H]^{2+}$, 500.58 (100) $[M+3H]^{3+}$. HRMS (ESI): *m/z* $[M+H]^{2+}$ calcd. for $C_{78}H_{92}N_{20}O_{12}^{2+}$: 750.3596, found: 750.3601. $C_{78}H_{90}N_{20}O_{12} \cdot C_4H_2F_6O_4$ (1499.71 + 228.04).

(2*S*)-2-(2,2-Diphenylacetamido)-5-{[({[3-(2-{2-[7-({[({3-[({*N*-[(4*S*)-4-(2,2-diphenylacetamido)-4-{[(4-hydroxyphenyl)methyl]carbamoyl}butyl]-carbamimidoyl}carbamoyl)amino]propyl}carbamoyl)methyl]carbamoyl}methyl)-1*H*,7*H*-imidazo[4,5-*f*]benzimidazol-1-yl]acetamido}acetamido)propyl]carbamoyl}amino)-methanimidoyl]amino}-*N*-[(4-hydroxyphenyl)methyl]pentanamide ((*S*,*S*)-5.31). Compound (*S*,*S*)-**5.31** was synthesized from **5.18** (6.4 mg, 23.4 μmol) and (*S*)-**5.22** (50 mg, 58.3 μmol) by analogy with the procedure for the preparation of (*R*,*R*)-**5.31**. The desired compound was obtained as a white lyophilisate (12.3 mg, 31%). The spectroscopic data were identical to those for (*R*,*R*)-**5.31**. RP-HPLC (220 nm): 99% (t_R = 13.1 min, k = 3.6).

(*R*)-17-(2,2-Diphenylacetamido)-N^1-[3-(2-{7-[(*R*)-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-9-imino-3,11,17,20,23,26-hexaoxo-2,8,10,12,16,18,21,24-octaazaheptacosan-27-yl]benzo[1,2-*d*:4,5-*d'*]diimidazol-1(7*H*)-yl}acetamido)-2-oxopropyl]-N^{18}-(4-hydroxybenzyl)-12-imino-4,10-dioxo-3,5,9,11,13-pentaazaoctadecanediamide ((*R*,*R*)-5.32). Compound **5.18** (3.39 mg, 12.4 μmol) was suspended in anhydrous DMF (0.6 mL). TBTU (8.8 mg, 2.2 equiv), DIPEA (5 μL, 2 equiv) was added, and the mixture was stirred for 15 min leading to the dissolution of the suspended starting material. A solution of (*R*)-**5.23** (30 mg, 2.5 equiv) and DIPEA (12 μL, 5 equiv) in anhydrous DMF (0.4 mL) was added, and the mixture was subjected to microwave irradiation (70 °C, 30 min). The desired compound was purified by preparative HPLC (column: Nucleodur-100 C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 14:86–62:38, t_R = 14.6 min). Lyophilisation of the eluate afforded (*R*,*R*)-**5.32** as a white fluffy solid (10 mg, 41%). ^{1}H-NMR (600 MHz, COSY, [D$_6$]DMSO): δ (ppm) 1.34 – 1.48 (m, 4H), 1.49 – 1.59 (m, 6H), 1.62 – 1.70 (m, 2H), 3.07 (m, 8H), 3.18 (q, 4H, *J* 6.1 Hz, 5.9 Hz), 3.64 (d, 4H, *J* 5.6 Hz), 3.78 (d, 4H, *J* 5.5 Hz), 3.84 (d, 4H, *J* 5.4 Hz), 4.08 – 4.19 (m, 4H), 4.32 (q, 2H, *J* 7.9 Hz, 6.3 Hz), 5.11 (s, 2H), 5.20 (s, 4H), 6.66 (d, 4H, *J* 8.5 Hz), 6.99 (d, 4H, *J* 8.5 Hz), 7.18 – 7.24 (m, 4H), 7.25 – 7.30 (m, 16H), 7.47 (t, 2H, *J* 5.1 Hz), 7.83 (t, 2H, *J* 5.6 Hz), 8.12 – 8.15 (m, 3H), 8.31 (t, 2H, *J* 5.7 Hz), 8.33 – 8.43 (m, 5H), 8.47 (d, 2H, *J* 8.1 Hz), 8.73 (t, 2H, *J* 5.1 Hz), 8.89 (s, 2H), 9.29 (s, 2H), 10.05 (s, 2H). RP-HPLC (220 nm): 98% (t_R = 12.6 min, *k* = 3.4). LRMS (ESI): *m/z* (%) 864.40 (10) $[M+2H]^{2+}$, 576.60 (100) $[M+3H]^{3+}$. HRMS (ESI): *m/z* $[M+H]^{2+}$ calcd. for $C_{86}H_{104}N_{24}O_{16}^{2+}$: 864.4026, found: 864.9037. $C_{86}H_{102}N_{24}O_{16} \cdot C_4H_2F_6O_4$ (1727.91 + 228.04).

N^1-((6*S*,9*S*,12*S*,15*S*)-1-Amino-6-{[(*S*)-1-amino-3-(4-hydroxyphenyl)-1-oxopropan-2-yl]carbamoyl}-12-(3-guanidinopropyl)-16-(4-hydroxyphenyl)-1-imino-9-isobutyl-8,11,14-trioxo-2,7,10,13-tetraazahexadecan-15-yl)-N^8-[(*S*)-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-9-imino-3,11-dioxo-2,8,10,12-tetraazapentadecan-15-yl]octanediamide tri(hydrotrifluoroacetate) (all-*S*-5.33). Compound **2.13** (30 mg, 19.5 μmol), HOBt (2.76 mg, 1.05 equiv), HBTU (7.8 mg, 1.05 equiv) and DIPEA (8.4 μl, 2.5 equiv) were dissolved in anhydrous DMF (500 μL) and incubated for 10 min at rt. A solution of (*S*)-**5.13** (23.4 mg, 1.5 equiv) and DIPEA (5 μL, 1.5 equiv) in DMF (300 μL) was added, and the mixture was stirred at 70 °C under microwave irradiation for 30 min. After addition of water (8 mL), a white solid precipitated. The solids were collected by filtration, dried in vacuo and stirred for 2.5 h in TFA/H_2O (95:5, 2 mL). The mixture was poured into ice-cold diethyl ether (15 mL) to afford a

white precipitate. After centrifugation, the supernatant was discarded, and the residue was dissolved in H_2O (8 mL). The desired compound was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 24:76–62:38, t_R = 17.8 min). Lyophilisation of the eluate afforded all-*S*-**5.33** as a white fluffy solid (20 mg, 49%). ^{1}H-NMR (400 MHz, [D_6]DMSO): δ (ppm) 0.77 – 0.94 (m, 6H), 1.03 – 1.22 (m, 4H), 1.27 – 1.78 (m, 21H), 1.94 – 2.11 (m, 4H), 2.78 – 2.92 (m, 2H), 2.97 – 3.15 (m, 8H), 3.16 – 3.25 (m, 2H), 4.05 – 4.48 (m, 8H), 5.13 (s, 1H), 6.57 – 6.73 (m, 6H), 6.92 – 7.16 (m, 10H), 7.18 – 7.25 (m, 3H), 7.26 – 7.33 (m, 9H), 7.36 (s, 2H), 7.51 (t, *J* 5.0 Hz, 1H), 7.54 – 7.64 (m, 2H), 7.75 (d, *J* 7.8 Hz, 1H), 7.82 (t, *J* 5.5 Hz, 1H), 7.89 (d, *J* 8.0 Hz, 1H), 7.94 (d, *J* 8.0 Hz, 1H), 8.03 (d, *J* 7.6 Hz, 1H), 8.16 (d, *J* 7.9 Hz, 1H), 8.36 (t, *J* 5.9 Hz, 1H), 8.41 (br s, 2H), 8.49 (d, *J* 8.0 Hz, 1H), 8.94 (br s, 1H), 9.21 (br s, 3H), 10.17 (s, 1H). RP-HPLC (220 nm): 95% (t_R = 28.58 min, *k* = 9.0). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{75}H_{108}N_{19}O_{13}^{3+}$: 494.2786, found: 494.2795. $C_{75}H_{105}N_{19}O_{13} \cdot C_6H_3F_9O_6$ (1480.79 + 342.06).

***N*,*N*'-[1,4-Phenylenebis(methylene)]bis[2-(2,2-diphenylacetamido)-5-guanidinopentanamide] di(hydrotrifluoroacetate) ((*R*/*S*)-5.34).** (*R*/*S*)-**5.24** (16.1 mg, 25.9 μmol), HOBt (4 mg, 1 equiv), TBTU (8.3 mg, 1 equiv) and DIPEA (7 μl, 1.5 equiv) were dissolved in anhydrous DMF (1 mL) and incubated for 15 min at room temperature. A solution of (*R*/*S*)-**5.27** (18.2 mg, 0.95 equiv) and DIPEA (7 μL, 1.5 equiv) in DMF (500 μL) was added, and the mixture was stirred at 70 °C under microwave irradiation for 30 min. Water (20 mL) was added, and the mixture was extracted with EtOAc (2 × 15 mL). The organic phases were combined, and the solvent was removed under reduced pressure. The residue was dissolved in TFA/H_2O (95:5, 3 mL) and stirred at room temperature for 3 h. The mixture was poured into ice-cold diethyl ether (15 mL) affording a white precipitate. After centrifugation, the supernatant was discarded, and the precipitate was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–18 min: MeCN/0.1% aq TFA 24:76–62:38, t_R = 12.3 min). Lyophilisation of the eluate afforded (*R*/*S*)-**5.34** as a white fluffy solid (14.2 mg, 54%). ^{1}H-NMR (600 MHz, COSY, [D_6]DMSO): δ (ppm) 1.30-1.48 (m, 4H), 1.48-1.59 (m, 2H), 1.62-1.74 (m, 2H), 3.00-3.10 (m, 4H), 4.16-4.29 (m, 4H), 4.30-4.37 (m, 2H), 5.12 (s, 2H), *ca.* 6.95 (br s, 4H), 7.11 (s, 4H), 7.17-7.23 (m, 4H), 7.24-7.37 (m, 16H), 7.61 (t, 2H, *J* 5.7 Hz), 8.47 (t, 2H, *J* 6.0 Hz), 8.50 (d, 2H, *J* 8.0 Hz). ^{13}C-NMR (150.9 MHz, HSQC, HMBC, [D_6]DMSO): δ (ppm) 25.1 (2 carb.), 29.4 (2 carb.), 40.3 (2 carb.), 41.8 (2 carb.), 52.4 (2 carb.), 55.9 (2 carb.), 126.5 (2 carb.), 126.6 (2 carb.), 127.0 (4 carb.), 128.1 (4 carb.), 128.2 (4 carb.),

128.4 (4 carb.), 128.5 (4 carb.), 137.6 (2 carb.), 140.3 (2 carb.), 140.5 (2 carb.), 156.7 (2 carb.), 171.0 (2 carb.), 171.2 (2 carb.). RP-HPLC (220 nm): 99% (t_R = 14.2 min, k = 3.9). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $C_{48}H_{58}N_{10}O_4^{2+}$: 419.2316, found: 419.2324. $C_{48}H_{56}N_{10}O_4 \cdot C_4H_2F_6O_4$ (837.04 + 228.04).

***N*,*N*'-{[({[2,2'-(benzo[1,2-*d*:4,5-*d'*]diimidazole-1,7-diyl)bis(acetyl)]bis(azanediyl)}bis(methylene))bis(4,1-phenylene)]bis(methylene)}bis(2-(2,2-diphenylacetamido)-5-guanidinopentanamide) di(hydrotrifluoroacetate) ((*R*/*S*)-5.35).** Compound **5.18** (3.8 mg, 13.9 μmol) was suspended in anhydrous DMF (0.6 mL), TBTU (9.4 mg, 2.1 equiv), HOBt (4.5 mg, 2.1 equiv) and DIPEA (6 μL, 2.5 equiv) were added, and the mixture was stirred for 10 min resulting in the dissolution of the suspended starting material. A solution of (*R*/*S*)-**5.27** (25.7 mg, 2.5 equiv) and DIPEA (6 μL, 2.5 equiv) in anhydrous DMF (0.5 mL) was added, and the mixture was subjected to microwave irradiation (70 °C, 30 min). Water (20 mL) was added, and the mixture was extracted with EtOAc (2 × 20 mL). The volatiles were removed under reduced pressure, the residue was dissolved in TFA/H_2O (95:5, 3 mL), and the solution was stirred at room temperature for 3 h. Water (100 mL) was added followed by lyophilisation. The desired compound was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–18 min: MeCN/0.1% aq TFA 14:86–62:38, t_R = 14.6 min). Lyophilisation of the eluate afforded (*R*/*S*)-**5.35** as a white fluffy solid (9.4 mg, 47%). ^{1}H-NMR (400 MHz, [D_4]methanol): δ (ppm) 1.40 – 1.61 (m, 4H), 1.62 – 1.74 (m, 2H), 1.75 – 1.90 (m, 2H), 3.00 – 3.20 (m, 4H), 4.30 (s, 4H), 4.34 – 4.45 (m, 6H), 5.07 (s, 2H), 5.18 (s, 4H), 7.05 – 7.35 (m, 28H), 7.81 (br s, 1H), 8.12 (br s, 1H), 8.93 (br s, 2H). RP-HPLC (220 nm): >99% (t_R = 12.2 min, k = 3.3). HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $C_{68}H_{76}N_{16}O_6$: 606.3061, found: 606.3069. $C_{68}H_{74}N_{16}O_6 \cdot C_4H_2F_6O_4$ (1211.44 + 228.04).

5.5.3 Pharmacology

Buffers and media used in binding assays. Cf. chapter 2.4.5

Radioligand binding assays. Cf. chapter 2.4.5

Aequorin assay. The assay was performed on CHO-hY_4-G_{qi5}-mtAEQ cells as previously described[5] using a GENios Pro plate reader (Tecan, Salzburg, Austria). Areas under the curve were calculated using SigmaPlot 12.5 software (Systat Software Inc., Chicago, IL).

Dynamic mass redistribution (DMR) assay. One day prior to the experiment, CHO-hY_4R-G_{qi5}-mtAEQ cells were trypsinized and centrifuged (400 *g*, 5 min). The cells were re-suspended in HAM's F12 nutrient mixture supplemented with 10% FCS at a density of 500,000 cells/ mL, and 40 μL of the cell suspension were seeded in uncoated 384-well EnSpire microplates. The cells were allowed to attach overnight at 37 °C, 5% CO_2 in a water-saturated atmosphere. The cells were washed four times with serum-free L-15 medium supplemented with 1% BSA and 0.1 mg/mL bacitracin and allowed to equilibrate in a volume of 30 μL of the same medium per well in the multimode reader at 37 °C for about 60 min. After recording baseline date, 10 μL of the test substances dissolved in L-15 medium (4-fold concentrated) were added with a multichannel electronic finnpipette (Thermo Fisher Scientific, Waltham, MA, USA) and the cellular response was recorded continuously for 40 min. Data processing was performed as previously described.[24]

5.6 References

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Chapter 6

Synthesis and Pharmacological Characterization of CYM 9484 Derivatives as NPY Y_2R Ligands

6.1 Introduction

The thiourea CYM 9484 (**6.4**, Figure 1) is a high affinity (IC_{50} = 19 nM) Y_2R antagonist.[1] Structure activity relationships from other series of Y_2R ligands suggest that the introduction of a basic moiety into **6.4** might further increase the Y_2R affinity. Moreover, the water solubility of **6.4** is rather low. The introduction of a basic moiety as well as the replacement of the lipophilic benhydrylpiperidine portion with a more polar substructure, e.g. the "western part" of the Y_2R antagonist JNJ 31020028[2] (Figure 1) should increase the solubility and improve the applicability of **6.4** as a pharmacological tool, e.g. as a stabilizing ligand for co-crystallization with the Y_2R.

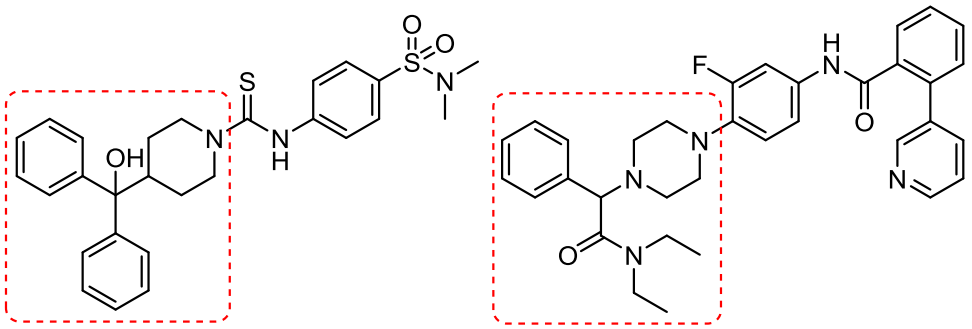


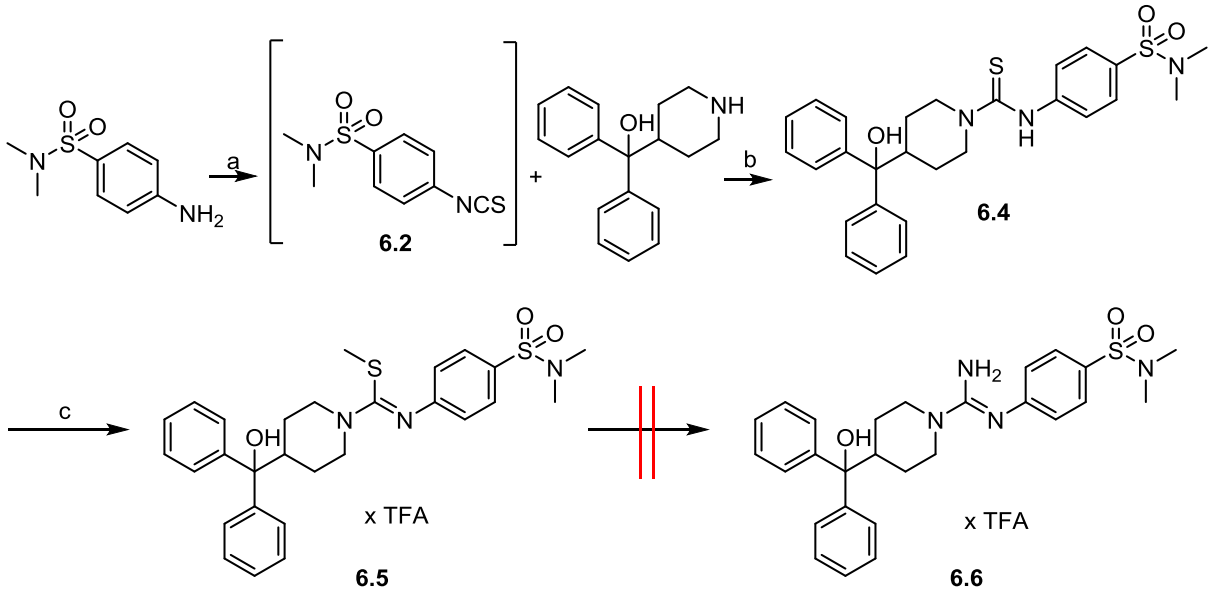
Figure 1: Y_2R antagonists CYM 9484 and JNJ 31020028.

6.2 Results and Discussion

6.2.1 Chemistry

The thiourea CYM 9484 (**6.4**) (Scheme 1) was prepared in a one-pot reaction by analogy with procedures from literature.[1] Methylation of the thiourea with iodomethane gave the isothiourea **6.5**, which was expected to be a suitable guanidinylating reagent. However, in contrast to *S*-methylisothioureas derived from primary amines, **6.5** was not reactive in a mercury(II) chloride mediated guanidinylation reaction. Presumably, the disubstitution at the nitrogen atom in the piperidine ring of **6.5** prevents the elimination of methanethiol and thus the formation of an activated carbodiimide intermediate.

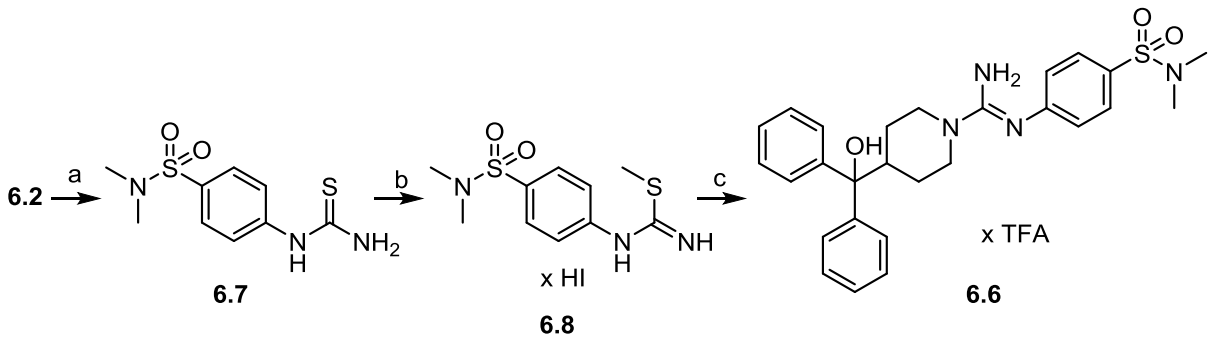
Scheme 1: Synthesis of the thiourea CYM9484 (**6.4**) and the isothiourea analog (**6.5**)[a]



[a]Reagents and conditions: (a) Di(2-pyridyl) thionocarbonate, CH_2Cl_2, rt, 6h. (b) CH_2Cl_2, rt, 15 min. (c) iodomethane, DMF, rt, 21 h.

To circumvent this problem, the thiourea **6.7** (Scheme 2) was prepared by treating the intermediate isothiocyanate **6.2** with an aqueous ammonia solution. Compound **6.7** was *S*-methylated and subsequently treated with *α*,*α*-diphenyl-4-piperidinemethanol to give the desired guanidine **6.6**. The application of mercury(II) chloride was not necessary.

Scheme 2: Synthesis of the guanidine analog (**6.6**) of **6.4**[a]

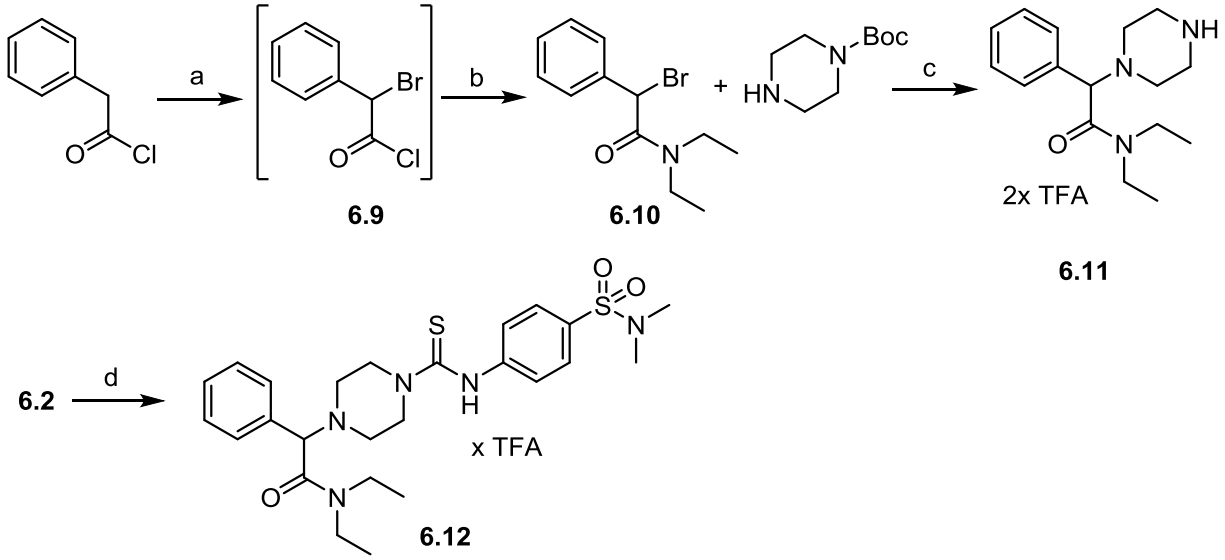


[a]Reagents and conditions: (a) ammonia solution (32%), CH_2Cl_2, rt, 40 min. (b) iodomethane, MeOH, rt, 36 h. (c) *α*,*α*-diphenyl-4-piperidinemethanol, *t*-BuOH/DMF, reflux, 2 h.

The thiourea **6.12** (Scheme 3) is a hybrid compound comprising structural moieties of **6.4** and JNJ 31020028 (Figure 1).[3] Apart from the slightly higher Y_2R affinity, the water solubility of JNJ 31020028 is substantially better. Similarly, the introduction of the “eastern part” of JNJ 31020028 into **6.4** was considered a promising approach to improve both, Y_2R affinity

and water solubility. Phenylacetyl chloride was brominated under Wohl-Ziegler conditions to form the intermediate **6.9** that was treated with diethylamine without prior purification resulting in the amide **6.10**. Alkylation of mono-Boc-protected piperazine with compound **6.10** under microwave irradiation and subsequent Boc deprotection gave compound **6.11**. Finally, the thiourea **6.12** was prepared from **6.11** and 4-Amino-*N*,*N*-dimethylbenzenesulfonamide by analogy with the synthesis of **6.4**.

Scheme 3: Synthesis of the thiourea **6.12**.[a]



[a]Reagents and conditions: (a) NBS, AIBN, chloroform, reflux, 5 h. (b) diethylamine, DIPEA, chloroform, rt, 3 h. (c) 1-Boc-piperazine, MeCN, microwave conditions, 110 °C, 30 min. (d) **6.11**, DIPEA, CH_2Cl_2, rt, 1 h.

6.2.2 Competition Binding Studies at NPY Receptor Subtypes

The target compounds **6.4**, **6.5**, **6.6** and **6.12** were investigated for Y_2R affinity and subtype selectivity in radioligand binding assays (Y_1R, Y_2R, Y_4R and Y_5R) and a fluorescent ligand binding assay (Y_2R) (for results see Table 1). The K_i value of **6.4** at the Y_2R determined by displacement of the fluorescent ligand Cy5-pNPY was in good agreement with published data (K_i = 23.9 nM (determined with Cy5-pNPY), IC_{50} = 19 nM[1]). Methylation of the thiourea did not affect Y_2R affinity, that is, **6.5** (K_i = 18.5 nM) had the same affinity as **6.4**. Suprisingly, the guanidine analog of CYM 9484, compound **6.6**, showed a considerably decreased Y_2R affinity (K_i > 10000 nM). This result does not support the hypothesis that the introduction of a basic moiety into CYM 9484 might improve the interaction with the Y_2R. The hybrid compound **6.12** (K_i = 407 nM) still binds to the Y_2R in the nanomolar range, but has a

considerably lower affinity than **6.4**. The binding constants determined in the radioligand binding assay and the fluorescent ligand binding assay were essentially in the same range (**6.4**: K_i = 69.7 nM (radioligand binding), K_i = 23.9 nM (fluorescent ligand binding)).

The K_i values of **6.4** and **6.12** at Y_1R, Y_4R and Y_5R were not determined due to low affinity. The same holds for compound **6.6** with exception of the Y_5R (K_i = 5330 nM). The isothiourea **6.5** was inactive at Y_1R and Y_5R, but a weak binder at the Y_4R (K_i = 5200 nM). Since we could previously show that the affinity of Y_4R ligands is affected by the concentration of sodium ions in the binding buffer, Y_4R affinity of **6.5** was additionally investigated in the sodium containing L-15 medium. However, the affinity of **6.5** in the L-15 medium was lower (IC_{50} > 30000 nM) than in the sodium-free buffer. Therefore, **6.5** was not considered a promising lead compound for further structural optimizations regarding Y_4R affinity.

Table 1. NPY receptor binding data.

Compd.	Y_1R K_i [nM][a]	Y_2R K_i [nM][b]	K_i [nM][c]	Y_4R K_i [nM][d]	Y_5R K_i [nM][e]
6.4	>10000	69.7 ± 20.1	23.9 ± 2.6	>10000	>10000
6.5	>10000	87.5 ± 6.1	18.5 ± 5.1	5200 ± 90[f]	>10000
6.6	>10000	>10000	>10000	>10000	5330 ± 390
6.12	>10000	935 ± 322	407 ± 129	>10000	>10000

[a]Radioligand competition binding assay with [^{3}H]UR-MK136[4] (K_d = 6.2 nM, c = 4 nM) using MCF-7-hY_1 cells in buffer II.[5] [b]Radioligand competition binding assay with [^{3}H]propionyl-pNPY[6] (K_d = 1.4 nM, c = 1 nM) using CHO-hY_2-G_{qi5}-mtAEQ cells[7] in buffer I. [c]Flow cytometric binding assay with Cy5-pNPY (K_d = 5.2 nM, c = 5 nM) using CHO-hY_2-G_{qi5}-mtAEQ cells in buffer I.[7] [c]Radioligand competition binding assay with [^{3}H]**2.10**[8] (K_d = 0.67 nM, c = 0.6 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells[9] in buffer I. [e]Radioligand competition binding assay with [^{3}H]propionyl-pNPY (K_d = 4.83 nM, c = 4 nM) using HEC-1b hY_5R cells[10] in buffer II. [f]Y_4R binding with [^{3}H]**2.10**[8] (K_d = 9.8 nM, c = 10 nM) at CHO-hY_4R-G_{qi5}-mtAEQ cells in Leibovitz' L15-medium: IC_{50} > 30000 nM.

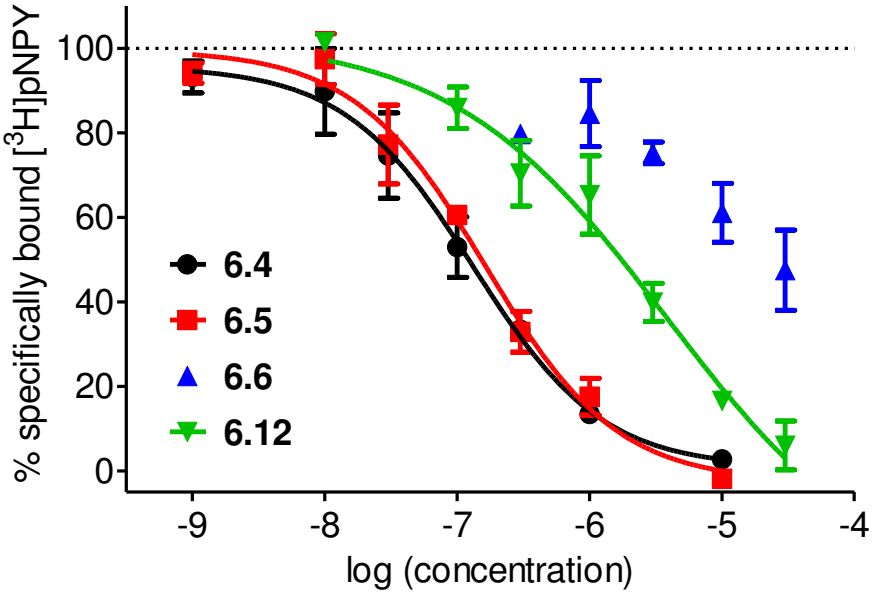


Figure 2: Competition binding performed with the fluorescent ligand Cy5-pNPY at CHO-hY_2R-G_{qi5}-mtAEQ cells. Displacement of Cy5-pNPY (K_d = 5.2 nM, c = 5 nM). Data represent mean values ± SEM of at least 2 independent experiments, each performed in triplicate.

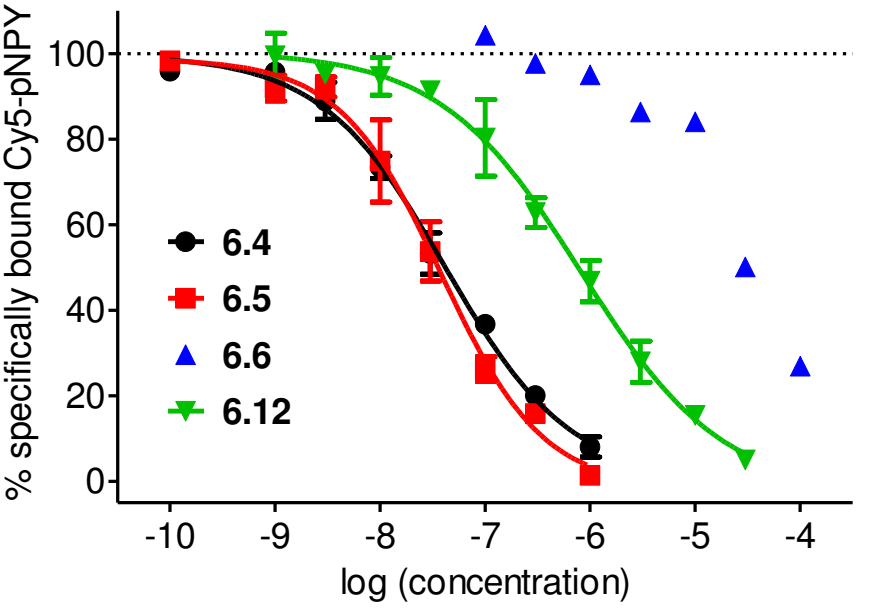


Figure 3: Competition binding performed with the radioligand [^{3}H]pNPY at CHO-hY_2R-G_{qi5}-mtAEQ cells. Displacement of [^{3}H]pNPY (K_d = 1.4 nM, c = 1 nM). Data represent mean values ± SEM of at least 3 independent experiments, each performed in triplicate.

6.3 Conclusions

Whereas guanidinylation of the isothiourea **6.5** was not successful, the synthesis of the guanidine analog of the thiourea **6.4**, compound **6.6**, was accomplished *via* the guanidinylation of *α*,*α*-diphenyl-4-piperidinemethanol with the isothiourea **6.8**. However, the working hypothesis was not confirmed: the replacement of the thiourea moiety in CYM 9484 with a basic guanidine substructure did not result in increased Y_2R affinity.

6.4 Experimental Section

6.4.1 General Experimental Conditions

Chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise indicated. MeCN for HPLC (gradient grade) and ammonia solution (32%) were purchased form Merck (Darmstadt, Germany) and 2,2'-Azobis(2-methylpropionitrile) was from Acros Organics/Fisher Scientific (Nidderau, Germany). Trifluoroacetic acid, dichloromethane, diethyl ether, dimethylformamide, iodomethane (99%), Di-2-pyridyl thionocarbonate (98%), *N*-bromosuccinimide (99%), *α*,*α*-diphenyl-4-piperidinemethanol and phenylacetyl chloride (98%) were obtained from Sigma Aldrich (Deisenhofen, Germany), *N*,*N*-diisopropylethylamine (DIPEA) (99%) was from ABCR (Karlsruhe, Germany). 4-Amino-*N*,*N*-dimethylbenzenesulfonamide (97%) was from Alfa Aesar (Karlsruhe, Germany) and *tert*-butanol was from Carl Roth (Karlsruhe, Germany). Bovine serum albumin and bacitracin were purchased from Serva (Heidelberg, Germany), human pancreatic polypeptide and porcine neuropeptide Y were from Synpeptide (Shanghai, China). 1-Boc-piperidine was synthesized by Dr. Paul Baumeister. The synthesis of [^{3}H]**2.10**[8] and [^{3}H]propionyl-pNPY[6] was previously described. Millipore water was used throughout for the preparation of buffers and HPLC eluents. Polypropylene reaction vessels (1.5 or 2 mL) with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for the storage of stock solutions. TL chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates, silica gel 60 (40-63 μm, Merck) was used for column chromatography. Visualization was accomplished by UV light (λ = 254 nm or 366 nm). NMR-spectra were recorded on a Bruker Avance 300 (7.05 T, ^{1}H: 300.1 MHz, ^{13}C: 75.5 MHz), a Bruker Avance III HD 400 (^{1}H:

400 MHz, ^{13}C: 101 MHz) or a Bruker Avance 600 instrument with cryogenic probe (14.1 T, ^{1}H: 600.3 MHz, ^{13}C: 150.9 MHz) (Bruker, Karlsruhe, Germany). Mass spectra were recorded on a Finnigan MAT95 (EI-MS 70eV) or a Finnigan MAT SSQ 710 A (CI-MS (NH_3)). For HRMS an Agilent Q-TOF 6540 UHD (Agilent, Waldbronn, Germany) equipped with an ESI source was used. Preparative HPLC was performed on a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Kinetex-XB C_{18}, 5 μm, 250 mm × 21 mm, Phenomenex, Aschaffenburg, Germany) at a flow-rate of 16 mL/min using mixtures of acetonitrile and 0.1% aqueous TFA solution as mobile phase. A detection wavelength of 220 nm was used throughout. The collected fractions were lyophilised using an alpha 2-4 LD apparatus (Martin Christ, Osterode am Harz, Germany) equipped with a RZ 6 rotary vane vacuum pump (vacuubrand, Wertheim, Germany). Analytical HPLC analysis was performed on a system from Merck-Hitachi (Hitachi, Düsseldorf, Germany) composed of a L-6200-A pump, a AS-2000A autosampler, a L-4000A UV detector, a D-6000 interface and a RP-column Kinetex-XB C_{18}, 5 μm, 250 mm × 4.6 mm (Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.8 mL/min. Mixtures of acetonitrile (A) and 0.1% aqueous TFA solution (B) were used as mobile phase. The following gradient was applied: A/B: 0-25 min: 20:80 - 95:5, 25-35 min: 95:5 - 95:5. Detection was performed at 220 nm, the oven temperature was 30 °C.

6.4.2 Chemistry: Experimental Protocols and Analytical Data

***N*-[4-(*N*,*N*-Dimethylsulfamoyl)phenyl]-4-(hydroxydiphenylmethyl)piperidine-1-carbothioamide (6.4).**[1] Compound **6.1** (50 mg, 0.25 mmol) was dissolved in CH_2Cl_2 (4 mL), di(2-pyridyl) thionocarbonate (58 mg, 1 equiv) was added, and the mixture was stirred for 6 h to give the isocyanate **6.2**. After addition of α,α-diphenyl-4-piperidinemethanol (94 mg, 1.4 equiv) stirring was continued for 15 min. The mixture was diluted with CH_2Cl_2 (15 mL) and washed with water (2 × 15 mL). The solvent was removed on a rotary evaporator and the residue was taken up in EtOAc/Hexane (6:1, 3.5 mL). Purification via column chromatography (eluent: Hexane/EtOAc (2:1)) afforded the desired compound as a grey solid (110 mg, 87%). TLC (PE/EtOAc 1:1): R_f = 0.56. ^{1}H-NMR (300 MHz, [D_6]DMSO): 1.28-1.62 (m, 4H), 2.58 (s, 6H), 2.94 (t, 1H, *J* 11.5 Hz), 3.11 (t, 2H, *J* 11.7 Hz), 4.72 (d, 2H, *J* 12.3 Hz), 5.44 (s, 1H), 7.15 (t, 2H, *J* 7.2 Hz), 7.29 (t, 4H, *J* 7.6 Hz), 7.50-7.60 (m, 6H), 7.60-7.67 (m, 2H), 9.52

(s, 1H).HRMS (m/z): $[M+H]^+$ calcd. for $C_{27}H_{32}N_3O_3S_2^+$, 510.1874; found, 510.1884. RP-HPLC (220 nm): 99% (t_R = 22.09 min, k = 6.7). $C_{27}H_{31}N_3O_3S_2$ (509.68).

Methyl *N*-[4-(*N*,*N*-dimethylsulfamoyl)phenyl]-4-(hydroxydiphenylmethyl)piperidine-1-carbimidothioate (6.5). 6.4 (50 mg, 0.098 mmol) was dissolved in DMF (1 mL), and a solution of iodomethane (13.9 mg, 1 equiv) in DMF (300 μL) was slowly added at room temperature. The resulting mixture was stirred at room temperature for 21 h. The mixture was taken up in H_2O (20 mL), DIPEA (20 μL) was added, and the mixture was extracted with EtOAc (3 × 20 mL). The organic layers were combined, and the volume was reduced to approximately 4 mL. Purification via column chromatography (eluent: Hexane/EtOAc (1:1)) afforded the title compound as a white solid (40 mg, 78%). TLC (PE/EtOAc 2:1): R_f = 0.34. ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 1.33 (d, 2H, *J* 12.5 Hz), 1.43-1.54 (m, 2H), 2.02 (s, 3H), 2.54 (s, 6H), 2.80-2.88 (m, 1H), 2.91-2.99 (m, 2H), 4.24 (d, 2H, *J* 12.9 Hz), 5.36 (s, 1H), 6.92-6.97 (m, 2H), 7.13 (t, 2H, *J* 7.4 Hz), 7.27 (t, 4H, *J* 7.6 Hz), 7.52-7.57 (m, 6H). ^{13}C-NMR (150.9 MHz, [D_6]DMSO): δ 15.6, 26.1 (2 carb.), 37.7 (2 carb.), 43.4, 47.9 (2 carb.), 78.5, 121.6 (2 carb.), 125.7 (4 carb.), 125.9 (2 carb.), 126.0, 127.8 (4 carb.), 128.6 (2 carb.), 147.0 (2 carb.), 154.4, 156.5. HRMS (m/z): $[M+H]^+$ calcd. for $C_{27}H_{34}N_3O_3S_2^+$, 524.2036; found, 524.2039. RP-HPLC (220 nm): >99% (t_R = 16.91 min, k = 4.9). $C_{28}H_{33}N_3O_3S_2$ (523.71).

***N*'-[4-(*N*,*N*-Dimethylsulfamoyl)phenyl]-4-(hydroxydiphenylmethyl)piperidine-1-carboximidamide hydrotrifluoroacetate (6.6).** Compound **6.8** (30 mg, 0.075 mmol) was dissolved in *t*-BuOH/DMF (1:1, 6 mL), *α*,*α*-diphenyl-4-piperidinemethanol (24 mg, 1.2 equiv) was added, and the mixture was stirred under reflux for 2 h. The desired compound was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 14:86–62:38, t_R = 16.8 min). Lyophilisation of the eluate afforded **6.6** as a white fluffy solid (17.7 mg, 39%). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 1.39 (d, 2H, *J* 12.5 Hz), 1.49-1.53 (m, 2H), 2.60 (s, 6H), 2.89-2.96 (m, 1H), 3.16 (t, 2H, *J* 12.5 Hz), 3.92 (d, 2H, *J* 13.2 Hz), 5.51 (br s, 1H), 7.15 (t, 2H, *J* 7.3 Hz), 7.28 (t, 4H, *J* 8.1 Hz), 7.36 (d, 2H, *J* 8.8 Hz), 7.53 (d, 4H, *J* 7.4 Hz), 7.34 (d, 2H, *J* 8.8 Hz), 8.27 (s, 2H), 9.93 (s, 1H). ^{13}C-NMR (150.9 MHz, [D_6]DMSO): δ 25.9 (2 carb.), 37.6 (2 carb.), 42.1, 46.9 (2 carb.), 78.5, 121.6 (2 carb.), 125.7 (4 carb.), 126.1 (2 carb.), 127.9 (2 carb.), 129.2 (2 carb.), 129.9, 142.3, 146.8 (2 carb.), 153.5. HRMS (m/z): $[M+H]^+$ calcd. for $C_{27}H_{33}N_4O_3S^+$, 493.2268; found, 493.2283. RP-HPLC (220 nm): >99% (t_R = 15.83 min, k = 4.5). $C_{27}H_{32}N_4O_3S \cdot C_2H_1F_3O_2$ (492.64 + 114.02).

***N*,*N*-Dimethyl-4-thioureidobenzenesulfonamide (6.7).** Compound **6.1** (100 mg, 0.5 mmol) was dissolved in CH_2Cl_2 (8 mL), di(2-pyridyl) thionocarbonate (116 mg, 1 equiv) was added, and the mixture was stirred for 10 h to form the isocyanate **6.2**. Ammonia solution (32%) was added and vigorous stirring was continued for 40 min. After addition of EtOAc (30 mL), the mixture was washed with water (2 × 20 mL), the volume of the organic phase was reduced on a rotary evaporator, and the desired compound was purified by column chromatography (eluent: EtOAc/MeOH (20:1)). Compound **6.7** was obtained as a yellow solid (120 mg, 93%). TLC (EtOAc): R_f = 0.67. ^{1}H-NMR (400 MHz, [D_6]DMSO): δ 2.60 (s, 6H), 7.50 (br s, 1H), 7.66 (d, 2H, *J* 8.8 Hz), 7.81 (d, 2H, *J* 8.8 Hz), 8.02 (br s, 1H), 10.1 (s, 1H). ^{13}C-NMR (100.6 MHz, [D_6]DMSO): δ 38.1 (2 carb.), 121.9 (2 carb.), 128.8 (2 carb.), 129.2, 144.2 181.7. HRMS (m/z): $[M+H]^+$ calcd. for $C_9H_{14}N_3O_2S_2^+$, 260.0522; found, 260.0517. $C_9H_{13}N_3O_2S_2$ (259.35).

Methyl [4-(*N*,*N*-dimethylsulfamoyl)phenyl]carbamimidothioate hydroiodide (6.8). Compound **6.7** (110 mg, 0.42 mmol) was dissolved in MeOH (8 mL). A solution of iodomethane (60.3 mg, 1 equiv) in MeOH (2 mL) was slowly added, and the mixture was stirred at room temperature for 36 h. After completion of the reaction (monitored by TLC (CH_2Cl_2/MeOH (10:0.8))), the volume of the mixture was reduced to approximately 2 mL, and ice-cold diethyl ether (10 mL) was added. After storage in a freezer for 14 h, the precipitated compound **6.8** was isolated by filtration as a white solid (150 mg, 93%). ^{1}H-NMR (400 MHz, [D_4]methanol): δ 2.74 (s, 6H), 2.78 (s, 3H), 7.65 (d, 2H, *J* 8.8 Hz), 7.94 (s, 2H, *J* 8.8 Hz). ^{13}C-NMR (100.6 MHz, [D_4]methanol): δ 13.2, 36.9 (2 carb.), 125.9 (2 carb.), 129.6 (2 carb.), 135.5, 138.9, 171.5. HRMS (m/z): $[M+H]^+$ calcd. for $C_{10}H_{16}N_3O_2S_2^+$, 274.0678; found, 274.0676. $C_{10}H_{15}N_3O_2S_2 \cdot HI$ (273.37 + 127.91).

2-Bromo-*N*,*N*-diethyl-2-phenylacetamide (6.10).[11] Phenylacetyl chloride (200 mg, 1.3 mmol), NBS (232 mg, 1 equiv) and AIBN (15 mg, 0.06 equiv) were suspended in chloroform (3 mL), and the mixture was stirred under reflux conditions. After 5 h, PE (10 mL) was added, and the solids were filtered off. The volume of the filtrate was reduced on a rotary evaporator, and the residue was taken up in chloroform (2 mL), followed by the addition of DIPEA (221 μL, 1 equiv) and diethylamine (136 μL, 1 equiv). After stirring at room temperature for additional 3 h, the mixture was washed with water (2 × 10 mL). Purification by column chromatography (eluent: Hexane/EtOAc (1:1)) afforded the title compound as a

white glue-like solid (202 mg, 58%). TLC (PE/EtOAc 2:1): R_f = 0.68. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.09-1.19 (m, 6H), 3.27-3.48 (m, 4H), 5.67 (s, 1H), 7.30-7.60 (m, 5H). GCMS (CI): *m/z* = 192.1 $[M-Br+2H]^+$ (100), 270.1 $[M+H]^+$ (85), 272.0 $[M+H]^+$ (85). $C_{12}H_{16}BrNO$ (270.17).

***N*,*N*-Diethyl-2-phenyl-2-(piperazin-1-yl)acetamide di(hydrotrifluoroacetate) (6.11).**[12] Compound **6.10** (100 mg, 0.37 mmol), 1-Boc-piperazine (90 mg, 1.3 equiv) and DIPEA (84 μL, 1.3 equiv) were dissolved in MeCN (4 mL). The mixture was stirred under microwave conditions (110 °C, 30 min). Purification by column chromatography (eluent: EtOAc/Hexane (1:1)) afforded the Boc-protected intermediate that was dissolved in CH_2Cl_2/TFA/H_2O (10:10:1, 10 mL). The mixture was stirred at room temperature for 3 h. Removal of the volatiles under reduced pressure afforded compound **6.11** as a hygroscopic white solid (134 mg, 72%). ^{1}H-NMR (300 MHz, $[D_6]$DMSO): δ 0.83 (t, 3H, *J* 7.00 Hz), 1.00 (t, 3H, *J* 7.00 Hz), 2.96 (br s, 4H), 3.10-3.37 (m, 8H, overlap with H_2O), 5.14 (s, 1H), 7.35-7.56 (m, 5H), 8.89 (br s, 1H), 9.17 (br s, 1H). LRMS (ESI): *m/z* = 275.9 $[M+H]^+$ (100). $C_{16}H_{25}N_3O \cdot C_4H_2F_6O_4$ (275.39 + 228.04).

2-(4-{[4-(*N*,*N*-dimethylsulfamoyl)phenyl]carbamothioyl}piperazin-1-yl)-*N*,*N*-diethyl-2-phenylacetamide di(hydrotrifluoroacetate) (6.12). Compound **6.1** (25 mg, 0.125 mmol) was dissolved in CH_2Cl_2 (3 mL) and di(2-pyridyl) thionocarbonate (29 mg, 1 equiv) was added. The mixture was stirred for 6 h to form the isocyanate **6.2**. Compound **6.11** (63 mg, 1 equiv) and DIPEA (56 μL, 2.5 equiv) were added and stirring was continued for 1 h. The mixture was diluted with CH_2Cl_2 (15 mL) and washed with water (2 × 15 mL). The solvent was removed on a rotary evaporator and the desired compound was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 14:86–62:38, t_R = 14 min). Lyophilisation of the eluate afforded **6.12** as a white fluffy solid (34.6 mg, 43%). ^{1}H-NMR (600 MHz, $[D_6]$DMSO): δ 0.85 (t, 3H, *J* 7.0 Hz), 1.01 (t, 3H, *J* 7.1 Hz), 2.59 (s, 6H), 3.16-3.25 (m, 3H), 3.26-3.34 (m, 2H), 3.35-3.43 (m, 2H), 3.58 (br s, 3H), 4.68 (br s, 1H), 4.89 (br s, 1H), 5.60 (s, 1H), 7.50-7.59 (m, 5H), 7.61 (d, 2H, *J* 8.8 Hz), 7.67 (d, 2H, *J* 8.8 Hz), 9.98 (s, 1H), 10.65 (br s, 1H). ^{13}C-NMR (150.9 MHz, $[D_6]$DMSO): δ 12.4, 13.7, 37.6 (2 carb.), 40.0 (2 carb.), 40.3 (2 carb.), 41.3 (2 carb.), 68.1, 123,3 (2 carb.), 127.9 (2 carb.), 129.1, 129.7 (4 carb.), 130.6, 145.1, 164.8, 181.3. HRMS (m/z): $[M+H]^+$ calcd. for $C_{25}H_{36}N_5O_3S_2^+$, 518.2254; found, 518.2264. RP-HPLC (220 nm): 99% (t_R = 13.79 min, *k* = 3.8). $C_{25}H_{35}N_5O_3S_2 \cdot C_4H_2F_6O_4$ (517.71 + 228.04).

6.4.3 Pharmacology

Buffers and media used in binding assays. Cf. chapter 2.4.5

Radioligand binding assays. Cf. chapter 2.4.5

Fluorescent ligand binding assays. The flow cytometric binding assay with Cy5-pNPY (K_d = 5.2 nM, c = 5 nM) at CHO-hY_2-G_{qi5}-mtAEQ cells was performed with a FACSCalibur^{TM} flow cytometer (Becton D ickinson, Heidelberg, Germany), equipped with an argon laser (488 nm) and a red diode laser (635 nm) as previously described.[13]

6.5 References

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Chapter 7

Summary

The neuropeptide Y family comprises three 36-amino acid peptides, namely neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP). In humans, the three peptides activate four functionally expressed G-protein coupled receptors (Y_1R, Y_2R, Y_4R and Y_5R) and contribute to the regulation of numerous (patho)physiological processes such as feeding behavior, pain sensitivity or the formation of mental disorders. Among the NPY receptors, the Y_4R is unique, because it preferentially binds PP over NPY or PYY. Recently, Y_4R agonists were proposed as anti-obesity agents. However, due to the lack of appropriate pharmacological tools, the physiological role of the Y_4R is still far from being understood. Therefore, this work aimed at the development of selective high affinity Y_4R ligands, agonists and antagonists, and labeled Y_4R ligands.

Bivalent, N^G-carbamoylated argininamides such as UR-MK188 represent the only class of Y_4R antagonists with affinities in the nanomolar range known so far. UR-MK188 (K_i = 130 nM, K_b = 20 nM) was used as a lead structure for the development of truncated, centrally constrained dimeric ligands. Aromatic substructures in the core of the linker were tolerated. Bivalent ligands with a central bent 1,7-disubstituted imidazo[4,5-f]benzimidazol moiety had slightly higher affinities than those with a 4,4'-disubstituted biphenyl moiety. A truncation of the linker length from 39 to 21 atoms did not compromise the Y_4R affinity of the investigated ligands. An exchange of the linker attachment position from the guanidine group to the carboxylic acid of the argininamides improved the synthetic availability, but resulted in Y_4R bivalent ligands with slightly reduced affinities.

The diastereomeric mixture of *D*/*L*-2,7-diaminooctanedioyl-bis(YRLRY-NH_2) (BVD-74D) was described as a high affinity Y_4R agonist. The pure diastereomers (2*R*,7*R*)-BVD-74D and (2*S*,7*S*)-BVD-74D and a series of homo- and heterodimeric analogs in which octanedioic acid was used as an achiral linker were prepared. To investigate the role of the amino acid residues, selected amino acids were replaced by Ala. (2*R*,7*R*)-BVD-74D was superior to (2*S*,7*S*)-BVD-74D in binding and functional cellular assays, and the presence of at least one unaltered tetrapeptide sequence Arg-Leu-Arg-Tyr-NH_2 was indispensable with regard to Y_4R affinity. [^{3}H]Propionylation of one amino group in the linker of (2*R*,7*R*)-BVD-74D resulted in the high affinity Y_4R radioligand [^{3}H]UR-KK193 (K_d (Y_4R) = 0.67 nM), a useful new tool for the Y_4R. Binding experiments with [^{3}H]UR-KK193 revealed that the affinity of peptide Y_4R

agonists may be considerably higher in the absence of sodium ions, conditions widely used for the investigation of NPY receptor ligands.

Recently, analogs of the C-terminus of PP containing artificial cyclic β-amino acids such as [cpen^{34}]pNPY(32-36), were described as partial Y_4R agonists. With respect to the development of Y_4R antagonists, aza-amino acids and *D*-amino acids where introduced into peptide agonist precursors. The incorporation of aza-amino acids, either azaArg or azaLeu, into the above-mentioned 'dimeric' peptides changed the quality of action from agonism to antagonism in functional assays with complementary readouts. The amino acid sequence of the Y_4R agonist Ac-YRLRY-NH_2 was optimized with respect to Y_4R affinity by the replacement of Arg^2 with an N^G-carbamoylated arginine building block and the extension of the *N*-terminus by an additional arginine. A *D*-amino acid scan was performed with this hexapeptide. Hexapeptides, in which either one of the three C-terminal *L*-amino acids had been replaced with the respective *D*-amino acid, were Y_4R antagonists. Due to their extremely facile synthetic availability, linear peptides containing *D*-amino acids are promising building blocks for further structural modifications and might pave the way for the development of peptide Y_4R antagonists with increased affinity and antagonistic activity.

In summary, the high-affinity ligands and radiolabeled molecular tools described in this thesis should contribute to gain deeper insight into the physiological role and the signaling mechanisms of the neuropeptide Y Y_4 receptor.

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Einige der experimentellen Arbeiten wurden in Zusammenarbeit mit anderen Personen durchgeführt. Entsprechende Vermerke finden sich in den entsprechenden Kapiteln (Chapter 2, Chapter 4). Eine detaillierte Auflistung aller Kooperationen enthält zudem der Abschnitt „Acknowledgements and declaration of collaborations“.

Weitere Personen waren an der inhaltlich-materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir, weder unmittelbar noch mittelbar, geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

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