Molecular Tools for the NPY Y_1 Receptor: Dimerization Probes, Vesicles, Catalytically Active Derivatives and Irreversible Ligands

Dissertation

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

vorgelegt von

Stefan Weiß

aus Straubing

2011

The experimental part of this work was carried out between November 2007 and December 2010 under the supervision of Prof. Dr. Burkhard König at the Institute of Organic Chemistry, University of Regensburg.

The PhD thesis was submitted on:	27.01.2011
The colloquium took place on:	28.02.2011

Board of Examiners:	Prof. Dr. A. Göpferich	(Chairman)
	Prof. Dr. B. König	(1st Referee)
	Prof. Dr. A. Buschauer	(2nd Referee)
	Prof. Dr. H. Brunner	(Examiner)

Acknowledgements

Especially I want to thank my supervisor Prof. Dr. Burkhard König for giving me the opportunity to perform these interesting investigations and for the useful discussions and his hints to improve my scientific work.

I also want to thank Prof. Dr. Armin Buschauer and Prof. Dr. Günther Bernhardt for the helpful discussions and the very good cooperation in the projects.

Many thanks go to the people in the departement of pharmacy:

Dr. Max Keller for the excellent collaboration, the interesting discussions and the support especially in the beginning of the thesis

Miroslaw Lopuch for introducing me in cell culture, microscopy experiments and for his cooperativeness

Melanie Kaske and Nathalie Pop for helping me with FACS techniques

Brigitte Wenzl and Elviria Schreiber for performing important cell assays for my thesis

And all other people from the department of pharmacy for their collaboration

My gratitude goes to all coworkers in the department of organic chemistry:

Dr. Andreas Späth and Peter Raster for the good collaboration, the interesting discussions about chemistry and the culinary specialties served on silver plates

Florian Schmidt, Florian Kinzl, Tobias “Fliesn” Lang and Andreas Müller for the collaboration in the lab

Karin Lehner for the casual goodies between the work

Dr. Rudi Vasold and Simone Strauß for HPLC investigations and measurements

And all other people from the department of organic chemistry for their collaboration

I want to thank all coworkers of the central analytical department, especially Dr. Thomas Burgemeister for the assistance by the interpretation of 2D NMR spectra, Wolfgang Söllner and Joseph Kiermaier for recording mass spectra.

Thanks to Dr. Anja Lechner for assistance by FACS measurements in the department of immunology at the Universitätsklinikum Regensburg

I want to thank the DFG for financial support within the GRK 760.

And finally I want to thank my familiy for their support.

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

Regensburg, _____

Stefan Weiß

„The game of life“, S. Weiss, 2006

„Eines Tages wird man offiziell zugeben müssen, dass das, was wir Wirklichkeit getauft haben, eine noch größere Illusion ist als die Welt des Traumes.“

Salvador Dali

Für meine Familie

und Jeannine

Table of Contents

3 Towards Target Specific Fluorescent Liposomes for the Neuropeptide Y_1 Receptor

4 Towards the Catalytic Staining of NPY Y_1 Receptors on Living MCF-7 Cells with DMAP Modified BIBP 3226 Derivatives

5 Ligand Dependent Irreversible Blockade of the Neuropeptide Y_1 Receptor

A Appendix

CHAPTER 1

N^G-Acyl-argininamides as NPY Y_1 Receptor Antagonists: Influence of Stucturally Diverse Acyl Substituents on Stability and Affinity

1.1 Introduction

G Protein-coupled receptors (GPCRs) represent a major class of biological targets in drug discovery. Focusing on neuropeptide Y (NPY) and histamine receptors as models of aminergic and peptidergic GPCRs, respectively, we are particularly interested in bioisosteric approaches to develop special receptor subtype-selective tools, e. g., bivalent ligands, prodrugs, radiolabeled and fluorescent compounds.[1-6] Strongly basic groups such as guanidines (including Arg residues) are essential structural features of numerous GPCR ligands, but are unfavourable with respect to bioavailability and brain penetration. This is also true for many high affinity NPY and histamine receptor agonists and antagonists.[7, 8] Therefore, special effort was put into the search for bioisosteric replacements of strongly basic functional groups. Recently, we reported on the exploitation of guanidine - acylguanidine bioisosterism with respect to histamine H_2, H_3 and H_4 receptor ligands[5, 9-12] and arginine-type NPY Y_1 receptor antagonists.[1, 3, 4, 6, 7, 13, 14]

NPY is a highly conserved peptide which plays an important role as a neurotransmitter in the central and peripheral nervous system.[15] In humans, four receptor subtypes, referred to as NPY Y_1, Y_2, Y_4 and Y_5 receptors, mediate the biological effects of NPY. For instance, in the periphery NPY Y_1 receptor (Y_1R) stimulation causes an increase in blood pressure. In the central nervous system (CNS) Y_1R activation elicits anxiolytic and sedative effects and is involved in the stimulation of food intake.[15]

Arginine derivatives such as the Y_1R antagonist BIBP3226[16] (**1a**, Figure 1) have been proven as valuable pharmacological tools regardless of properties being far from drug-like. Interestingly, the reduction of the basicity of the Arg-derived Y_1R antagonist **1a** by introducing electron-withdrawing N^G-substituents such as acyl groups turned out to be a promising general route to guanidine derivatives with increased potency, to fluorescent ligands and radioactive tracers.[3-7, 14] Even bulky fluorophores attached to the guanidine via spacers of different size were tolerated; a moderate decrease in affinity did not compromise suitability of the compounds as pharmacological tools. However, depending on the chemical

nature of the linker cleavage of the acylguanidine group may occur as demonstrated for a model compound.[17]

In continuation of our studies on the structure-activity relationships of argininamide-type Y_1R antagonists we synthesized a series of N^G-acylated analogues of **1a** in order to explore the impact of structurally diverse substituents on stability and pharmacological activity in vitro (Y_1R binding and antagonism). As the small library of Y_1R antagonists was synthesized to cover a wide range of distribution coefficients (logD) the BIBP 3226 skeleton was substituted with hydrophobic alkyl chains or sugars and amines to alter the polarity of the parent compound (Figure 1).

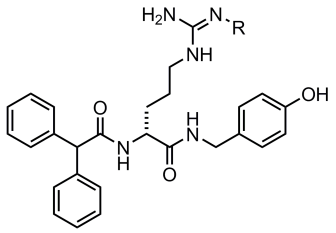
1.2 Results and Discussion

1.2.1 Synthesis of the N^G-acylated argininamides

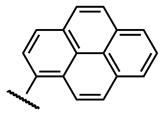
The N^G-acylated argininamides were efficiently prepared according to the general synthetic route shown in Scheme 1. *N*-Boc-*S*-methylisothiourea (**3**) was acylated with the respective carboxylic acids yielding the isothiourea derived guanidinylation reagents **4b**-**4g**, **4i**, **4k**-**4q**, **4s-4u**. Guanidinylation of D-ornithinamide **5** and subsequent removal of protecting groups gave the envisaged N^G-acylated argininamides. Amine **5** is available from D-ornithine in six steps in 32% overall yield.[4]

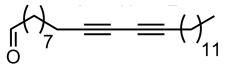
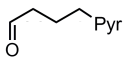
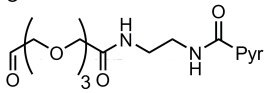
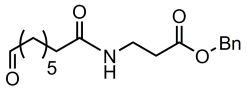
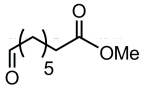
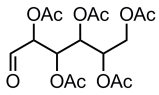
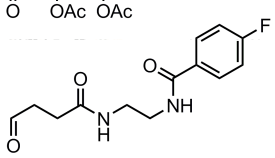
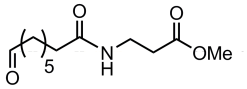
Synthetic strategies for the preparation of carboxylic acids **7**, **8**, **11**, **14**, **17**, **20**, **22**, **27**, **28**, **30**, and **36** are depicted in Schemes 2 – 4. Acids **7** and **8**, containing a triazole entity, were prepared through a Cu(I)-catalysed "click-reaction" between 4-azidobutanoic acid and ^{t}Bu-protected propargyl alcohol or Boc-protected propargyl amine (Scheme 2). Carboxylic acid **14** was prepared via guanidinylation of 6-aminohexanoic acid methyl ester with *N*,*N*'-diBoc-*S*-methylisothiourea followed by ester hydrolysis (Scheme 2). For the synthesis of succinic acid derivative **17** ethylenediamine was one-fold guanidinylated with *N*,*N*'-diBoc-*S*-methyl-isothiourea yielding amine **16**, which was treated with succinic anhydride (Scheme 2). Acid precursors **20** and **22** were obtained through the treatment of amines **19** and **21** with succinic anhydride (Scheme 2). The suberic acid derivatives **27** and **28** were prepared through amidation of octandioic acid mono-benzyl ester with amines **24** and **21**, respectively, followed by benzyl ester cleavage (Scheme 3). Octandioic acid derivative **30** was obtained via amidation of non-protected suberic acid with 3-aminopropanoic acid benzyl ester (Scheme 3). The synthesis of carboxylic acid **36** started from pyrene-1-carboxylic acid (**31**), which was amidated with mono-Boc-protected ethylenediamine (**21**) in moderate yield (Scheme 4). The

Boc group was removed with hydrochloric acid yielding amine **33**, which was coupled with 3,6,9-trioxaundecandioic acid mono-benzyl ester (**34**) to give compound **35**. Hydrogenolysis of **35** resulted in acid **36** (Scheme 4).



Pyr =



no.	R	logD
1a	H (BIBP 3226)	0.7 (1.4)
1b		12.5
1c		9.4
1d		7.6 (5.3)
1e		7.2
1f		6.0 (3.1)
1g		4.6 (3.2)
1h		3.9
1i		3.5 (2.9)
1j		3.5
1k		2.9

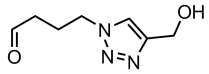
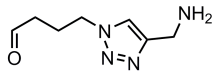
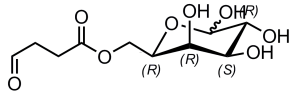
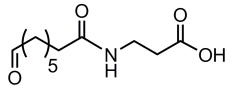
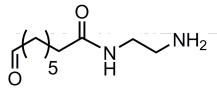
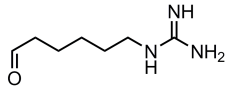
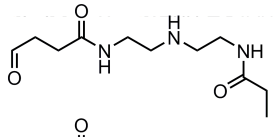
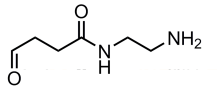
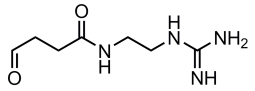
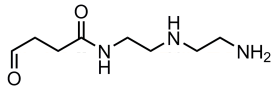
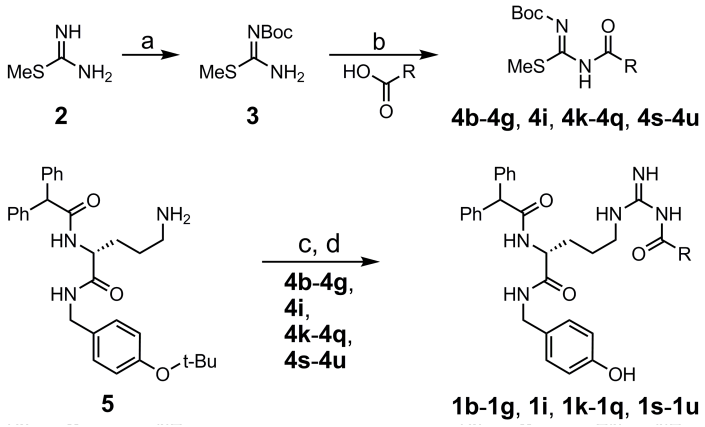
no.	R	logD
1l		1.8 (1.4)
1m		1.7
1n		0.9 (1.0)
1o		0.7
1p		-0.3 (1.3)
1q		-0.3 (1.4)
1r		-0.6
1s		-1.3
1t		-1.4
1u		-1.8 (1.3)

Figure 1. Structures of N^G-acylated derivatives of the Y_1R antagonist BIBP 3226 (**1a**) ranked according to calculated logD values (ACD-labs software version 12, pH = 7.4); experimental logD values from HPLC measurements[18] in parentheses.

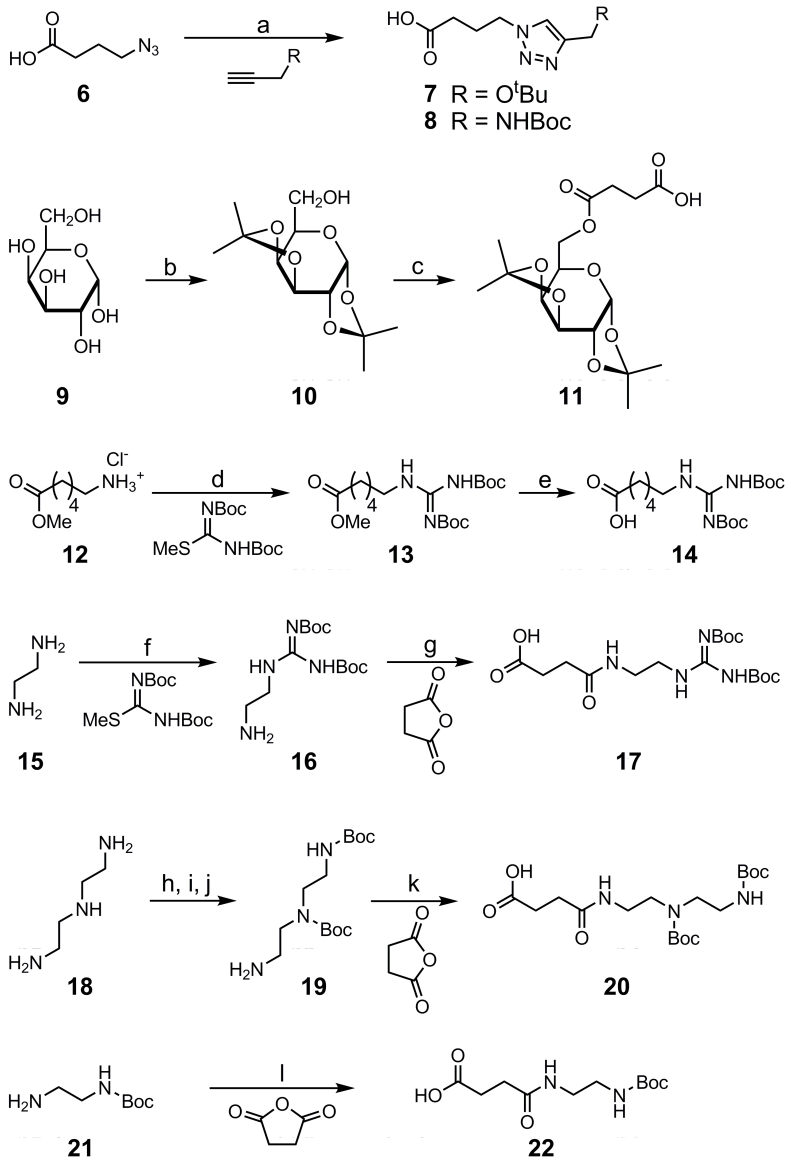
D-Gluconic acid derivative **1i** was synthesized from penta-acetylated D-gluconic acid. Unfortunately, deprotection of the hydroxyl groups failed. The base-labile acylguanidine moiety prevents basic selective cleavage of the acetyl protecting groups. Enzymatic deprotection using a lipase preparation (Novozym 435) was also unsuccessful. To circumvent

such problems, sugar derivative **1n**, containing a galactose entity, was prepared. The synthesis of the respective carboxylic acid **11** is shown in Scheme 2. Two acetal protecting groups were introduced in the first step and the remaining primary hydroxyl function was esterified with succinic anhydride. The acetal groups are compatible with the general synthetic strategy outlined in Scheme 1 and can be easily removed in the final deprotection step with TFA/DCM.

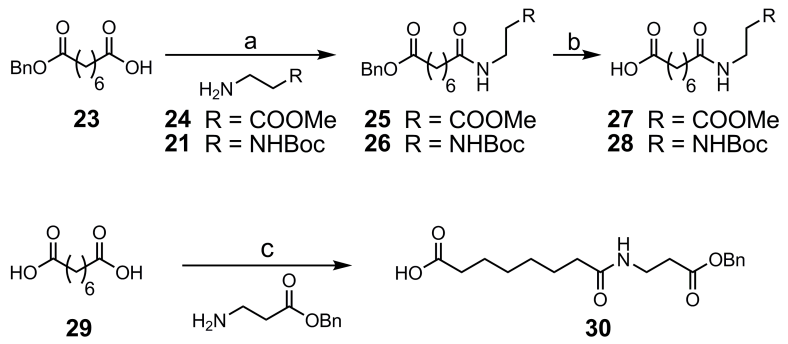
BIBP 3226 derivatives **1j** and **1r** were prepared through 4-fluorobenzoylation and propionylation of amines **1s** and **1u**, respectively (Scheme 5).



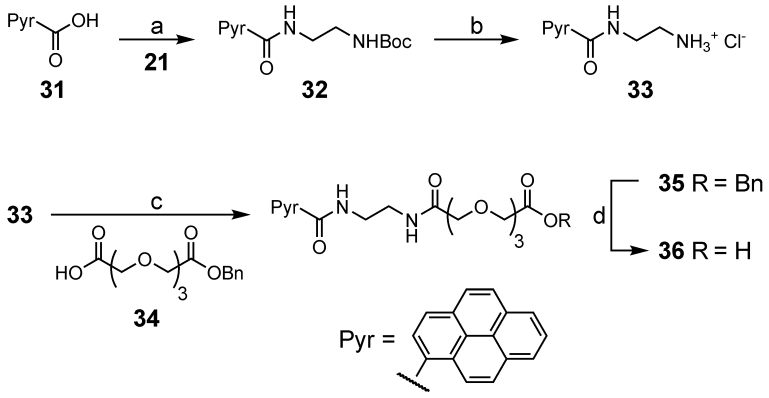
Scheme 1. General route for the synthesis of N^G-acylated BIBP 3226 derivatives **1b**-**1g**, **1i**, **1k**-**1q** and **1s**-**1u**. a) Boc_2O, NaOH, ^{t}BuOH, 90%; b) DIPEA, EDC, HOBT, DCM, (**1s**: TBTU, DIPEA, DMF), 31 - 95%; c) $HgCl_2$, NEt_3, DMF, 39 - 78%; d) TFA/DCM 1:1, quantitative yield.



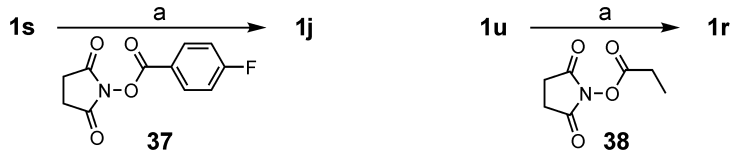
Scheme 2. Synthesis of carboxylic acids **7**, **8**, **11**, **14**, **17**, **20** and **22**. a) $CuSO_4$ (5 mol%), Na-ascorbate (10 mol%), MeOH, H_2O, 67%-86%; b) acetone, iodine, 71%; c) succinic anhydride, DMAP, NEt_3, 73%; d) $HgCl_2$, NEt_3, DMF, 84%; e) THF, NaOH, 71%; f) $CHCl_3$, 16 h, 97%; g) THF, NEt_3, 49%; h) CF_3COOEt, DCM; i) Boc_2O, DCM; j) K_2CO_3, MeOH, H_2O, 47% overall; k) succinic anhydride, THF, NEt_3, 77%; l) DCM, 99%.



Scheme 3. Synthesis of suberic acid derivatives **27**, **28** and **30**. a) DCM, EDC, HOBt, DIPEA, 62%; b) Pd-C, H_2, 90% - 94%; c) DCM, EDC, HOBt, DIPEA, 44%.



Scheme 4. Synthesis of the pyrene-1-carboxamide derivative **36**. a) DCM, EDC, HOBt, DIPEA, 52%; b) MeOH, HCl, 83%; c) DMF, EDC, HOBt, DIPEA, 59%; d) Pd-C, MeOH, quantitative.



Scheme 5. Synthesis of the N^G-acylated BIBP 3226 derivatives **1j** and **1r**. a) NEt_3, DMF, 65% (**1j**), 21% (**1r**).

1.2.2 Stability and Y_1R antagonistic activity

As the acylguanidine moiety of N^G-acylated argininamide-type Y_1R antagonists may be considered as the most probable cleavage site the stability of the presented BIBP 3226 (**1a**) derivatives was investigated with respect to the formation of **1a** at physiological pH of 7.4. A

release of **1a** under the conditions of the pharmacological assays has to be taken into account as **1a** is a highly potent Y_1R antagonist (K_i = 1.3 nM) and could pretend a higher potency of the investigated compounds. Therefore the stability of the N^G-acylated argininamides was investigated at pH 7.4 on the time scale of the assays. Formation of **1a** was not observed for the strongly hydrophobic compounds **1c**, **1d** and **1e**, which are devoid of hetero atoms in their N^G-acyl substituents. The hydrophobic ligands **1f** and **1g**, both containing an amide group in the acyl substituent, showed a very minor decomposition over 90 min (< 0.5%). Instability was more pronounced (decomposition up to 2.6% over 90 min) for the more polar compounds (**1h**, **1i**, **1k** – **1q**) bearing amide, ester, triazole, sugar, amine, carboxylic or guanidine functions in the acyl residue.

Exceptionally high instabilities were found for argininamides with succinyl attached to the guanidine (**1j**, **1r** – **1u**). Compounds **1r** – **1u** quantitatively decomposed to **1a** over 90 min (Table 1). This process was exemplarily explored by RP-HPLC-MS using the hydrophobic compound **1j**, which allows the detection of the acyl substituent upon cleavage. As becomes obvious from Figure 2 compound **1j** is cleaved via an intramolecular attack of the succinic amide nitrogen at the carbonyl group attached to the guanidine resulting in the *N*-alkyl succinimide derivative **39**. The lability of the acylguanidines bearing nucleophiles in position 5 or 6 of the acyl side chain is in accordance with the recently reported reactivity of 5-aminopentanoyl substituted guanidine.[17]

All compounds proved to be stable at acidic pH (0.1 % aqueous trifluoroacetic acid, pH 2-3). Under these conditions the acylguanidine group is almost quantitatively protonated due to pK_a values in the range of 7 – 8. Obviously, this changes the susceptibility against hydrolysis and prevents intramolecular attacks of nucleophiles at the carbonyl group, respectively.

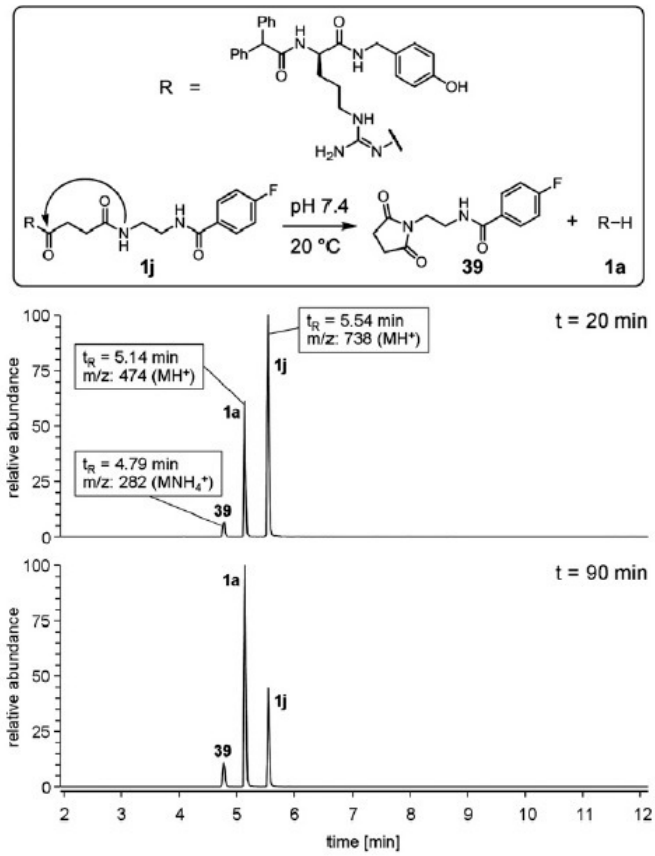


Figure 2. Exploration of the decomposition mechanism of BIBP 3226 derivative **1j** incubated in an aqueous buffer (pH 7.4) at 20 °C. LC-MS analysis was performed after an incubation period of 20 and 90 min. **1j** is decomposed to BIBP 3226 (**1a**) and the succinimide derivative **39** through an intramolecular attack of the succinic amide nitrogen at the guanidine linked carbonyl group. Chromatograms were acquired by single ion monitoring analysis.

Except for the highly unstable compounds **1j** and **1r** – **1u** the N^G-acylated argininamides were characterised in terms of Y_1R antagonism (K_b values) and Y_1R affinity (K_i values) using a Fura-2 assay on human erythroleukemia (HEL) cells[19] and a radioligand binding assay on SK-N-MC human neuroblastoma cells,[4] respectively. K_b and K_i values are summarized in Table 1. Except for **1h** (K_i = 0.9 nM) all N^G-acylated argininamides showed a reduced Y_1R affinity compared to the parent compound **1a** (K_i = 1.3 nM, Table 1). The decrease in affinity was most pronounced (up to three orders of magnitude) for the most hydrophobic compounds (**1b** – **1e**, Table 1), which have limited solubility and might interact with the cell membrane.

Direct attachment of pyrene-1-carboxylic acid to the guanidine-N (compound **1e**) resulted in complete loss of Y_1R affinity. By contrast, high Y_1R affinity can be retained, if the pyrene

moiety is attached to the guanidine group through a linker (compounds **1d** and **1f**, Figure 1, Table 1).

The Y_1R affinity of the polar sugar and guanidine substituted compounds **1n** and **1q** is about 35 times lower compared to the affinity of **1a**. The argininamide **1a** may be considered a mimic of the C-terminal dipeptide in NPY (...-Arg^{35}-Tyr^{36}-NH_2). It may be speculated that the introduction of a second guanidine moiety (as provided by compound **1q**) could enhance binding affinity through mimicking the second arginine residue (Arg^{33}).[20] However, the K_i value of compound **1q** was only 51 nM – perhaps due to an inappropriate distance between the two guanidine groups.

The hydroxy substituted Y_1R antagonist **1l** (K_i = 3.0 nM), proved to be as potent as **1a**. It is conceivable that the hydroxy group mimics Thr^{32} or Tyr^{27} of the natural ligand NPY. Both amino acids are very important for the binding of NPY to the Y_1R, as identified by alanine scan.[21] The amine analogue of **1l**, compound **1m**, binds with similar affinity to the Y_1R (K_i = 6.7 nM).

As partial decomposition of compounds **1i**, **1k** and **1o** - **1q** occurs (up to 2.6% over 90 min), the determined K_i values of these five compounds have to be considered with reservation, as the affinity of the cleavage product **1a** (K_i = 1.3 nM) and the assay periods (from preparation of solutions to read out: 60 – 90 min) have to be taken into account.

With respect to the prediction of the affinity of new compounds, three issues have to be taken into account: substitution with bulky groups directly at the BIBP 3226 backbone results in a complete loss of affinity, that can be compensated with a spacer of adequate length. Derivatives with a small aromatic or heteroaromatic substituent (**1g, 1l, 1m**) show slightly higher binding affinity compared to the more flexible derivatives (**1g vs. 1k, 1o, 1p; 1m vs. 1q**). Relatively small hydrophobic substituents, as in **1h** (K_i = 0.9 nM) and the radioligand [^{3}H]-UR-MK114 (K_i = 1.3 nM),[4] result in similar and even higer affinity compared to BIBP 3226. Interactions of the acyl substituent with the Y_1R are difficult to predict, because these residues are presumably oriented toward flexible extracellular loop regions[7] and, depending on chemical nature and size of the acyl residue, an interaction with membrane lipids cannot be ruled out.

Table 1. LogD values, stability, Y_1R antagonistic activity (K_b) and Y_1R binding affinity (K_i) of BIBP 3226 derivatives **1b** – **1u**.

no.	logD[a]	% decomposition (pH 7.4, 20 °C) after 20 / 90 min	Y_1R antagonism K_b[b] [nM]	Y_1R affinity K_i[c] [nM]
1a[d]	0.7 (1.4)	--	1.5 ± 0.2	1.3 ± 0.2
1b	12.5	n.d.	140 ± 10	1500 ± 120
1c	9.4	0 / 0	170 ± 8	460 ± 19
1d	7.6 (5.3)	0 / 0	n.d.[e]	270 ± 70
1e	7.2	0 / 0	n.d.[e]	inactive
1f	6.0 (3.1)	0 / < 0.5	n.d.[e]	59 ± 16
1g	4.6 (3.2)	0 / < 0.5	1.10 ± 0.04	40 ± 9
1h	3.9	0 / 0.8	0.06 ± 0.01[f]	0.9 ± 0.1[f]
1i	3.5 (2.9)	0.9 /2.6	83 ± 8	110 ± 38
1j	3.5	30 /69	n.d.[g]	n.d.[g]
1k	2.9	0 /0.7	170 ± 18	260 ± 82
1l	1.8 (1.4)	0.9 /2.1	0.40 ± 0.03	3.0 ± 0.5
1m	1.7	0.6 / 1.7	14 ± 4	6.7 ± 0.1
1n	0.9 (1.0)	< 0.5 /1.3	510 ± 190	41 ± 5
1o	0.7	< 0.5 /1.4	450 ± 52	73 ± 11
1p	-0.3 (1.3)	< 0.5 /1.4	230 ± 64	130 ± 10
1q	-0.3 (1.4)	< 0.5 /1.6	18 ± 8	51 ± 18
1r	-0.6	64 /100	n.d.[g]	n.d.[g]
1s	-1.3	51 /100	n.d.[g]	n.d.[g]
1t	-1.4	61 /100	n.d.[g]	n.d.[g]
1u	-1.8 (1.3)	40 /100	n.d.[g]	n.d.[g]

[a] Calculated with ACD-labs software version 12, pH = 7.4; in brackets: experimental logD determined with HPLC measurements. [b] K_b values for inhibition of NPY (10 nM) induced calcium mobilization in HEL cells (Fura-2 assay); all mean values ± SEM from two or three (1k, 1n, 1o, 1p, 1u) independent experiments. [c] K_i values determined from the displacement of 1.5 nM [^{3}H]-UR-MK114[4] on SK-N-MC cells; all mean values ± SEM from two or three (1c, 1f, 1g, 1k, 1l, 1p) independent experiments. [d] BIBP 3226. [e] Due to their fluorescent properties pyrene ligands are not compatible with the Fura-2 assay. [f] Keller *et al.*[1] [g] not determined due to the high instability.

1.3 Conclusion

The stability of the N^G-acylated argininamides strongly depends on the nature of the acyl residue. It becomes obvious from the high instability of the succinyl derivatives (**1j**, **1r** – **1u**) that cleavage is favoured when the residues harbour nucleophilic functional groups capable of an intramolecular attack on the acyl carbonyl. This is also conceivable for argininamides decomposing to a minor extent and bearing N^G-acyl residues containing amide, ester, triazole, sugar, amine, carboxylic or guanidine functions (**1h**, **1i**, **1k** – **1q**). This has to be taken into account in pharmacological investigations, in particular, when cleavage products are highly bioactive as in the case of **1a** (K_i = 1.3 nM). However, despite these limitations broad

structural variation of N^G-acyl substituents was tolerated (affinities in the nM range). Most of the investigated compounds proved to be sufficiently stable at pH 7.4 to determine reliable in vitro pharmacological data. In conclusion, the N^G-acylation of the stongly basic guanidine group in argininamides is a successful bioisosteric approach to more drug-like properties provided that the outlined stability considerations are taken care of. This is not restricted to the NPY field, as guanidine groups are crucial structural features of many different biologically active compounds including GPCR ligands

1.4 Experimental

1.4.1 General experimental conditions

Unless otherwise noted, solvents (analytical grade) were purchased from commercial suppliers and used without further purification. Ethyl acetate (EA), petrol ether (PE, 60 – 70 °C), methanol and dichloromethane were obtained in technical grade and distilled before application. Acetonitrile (MeCN) for HPLC was obtained from Merck (Darmstadt, Germany). Pentacosa-10,12-diynoic acid (Sigma-Aldrich Chemie GmbH, Munich, Germany), 4-(pyren-1-yl)butanoic acid (Sigma-Aldrich Chemie GmbH, Munich, Germany), hexadecanoic acid (Riedel-de Haen, Seelze, Germany), suberic acid (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany) and D-(+)-galactose (Merck, Darmstadt, Germany) were purchased. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18 (250 × 21 mm, 5 µm; Macherey-Nagel, Germany) and a Eurospher-100 C18 (250 × 32 mm, 5 µm; Knauer, Germany) served as RP-columns at flow rates of 20 and 38 mL/min, respectively. Mixtures of MeCN and diluted aqueous TFA (0.1 %) were used as mobile phase. ^{1}H-NMR spectra were recorded at 300 MHz on a Bruker Avance 300 spectrometer or at 600 MHz on a Bruker Avance III 600 with cryogenic probehead (Bruker, Karlsruhe, Germany). ^{13}C-NMR spectra were recorded at 75 MHz on a Bruker Avance 300 spectrometer. All chemical shifts values are reported in ppm. UV/VIS spectra were recorded with a Varian Cary BIO 50 UV/VIS/NIR spectrophotometer (Varian Inc., CA, USA). Mass spectra: Finnigan SSQ 710A (EI), Finnigan MAT 95 (CI), Finnigan MAT TSQ 7000 (Thermo FINNIGAN, USA) (ES/LC-MS). LC-system for LC-MS: Agilent 1100 (Palo Alto, USA). LC-MS method I (LC-MS-I): Column: Phenomex Luna C18, 3.0 µm, 100 x 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.30 mL/min; solvent A (water + 0.1% TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 1 min [A/B 95/5], 11 min [A/B 2/98], 18 min [A/B 2/98], 19 min [A/B 95/5], 24 min

[A/B 95/5]. LC-MS method II: Column: Phenomex Luna C18, 2.5 µm, 50 x 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.40 mL/min; solvent A (water + 0.1% TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 8 min [A/B 2/98], 11 min [A/B 2/98], 12 min [A/B 95/5], 15 min [A/B 95/5]. Analytical HPLC (HPLC): Compounds **1c**-**1i**, **1k**-**1q**, **1t**, **1u**: Phenomex Luna C18, 3.0 µm, 150 x 2 mm (Phenomenex, Aschaffenburg, Germany); flow: 0.30 mL/min; solvent A (H_2O + 0.0059%TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 30 min [A/B 2/98]; **1j**, **1r**, **1s**: Eurospher-100 C18, 5 µm, 250 x 4.0 mm (Knauer, Berlin, Germany); flow: 0.80 mL/min; solvent A (water + 0.05% TFA), solvent B (MeCN); gradient: 0 min [A/B 85/15], 28 min [A/B 45/55], 33 min [A/B 5/95], 40 min [A/B 5/95]. Melting points were determined with a Lambda Photometrics Optimelt MPA100 apparatus (Lambda photometrics, Harpenden, UK), they are not corrected. Thin layer chromatography (TLC) was performed on alumina plates coated with silica gel (Merck silica gel 60 F_{245}, thickness 0.2 mm). Column chromatography (CC) was performed with Merck Geduran SI 60 silica gel as the stationary phase.

The synthesis of **38**, **1a** and **1h** was described elsewhere.[1, 4] Compounds **3**,[2] **5**,[4] **6**,[22] penta-acetyl gluconic acid,[23] ^{t}Bu-protected propargyl alcohol,[24] ^{t}Bu-protected propargyl amine,[25] **10**,[26] **11**,[27] **14**,[28] **16**,[29] **19**,[30] **21**,[31] **23**,[32] **31**,[33] **37**[4] were prepared according to literature procedures.

1.4.2 Synthetic protocols and analytical data of 7, 8, 17, 20, 22, 25-28, 30 and 32-36

4-[4-(*tert*-Butoxymethyl)-1H-1,2,3-triazol-1-yl]butanoic acid (7)

4-Azidobutanoic acid (419 mg, 3.25 mmol) was mixed with Boc-protected propargyl alcohol (364 mg, 3.25 mmol) in 5 mL MeOH. Then ascorbic acid (65 mg, 0.33 mmol) dissolved in 1 mL H_2O and copper sulphate pentahydrate (8 mg, 0.03 mmol) dissolved in 1 mL H_2O were added and the reaction mixture was heated to reflux overnight. Next day MeOH was evaporated completely and dichloromethane (30 mL) and sat. aqueous $NaHSO_4$ solution (20 mL) were added. The organic layer was collected and then diluted NaOH (40 mL, 1 mol/L) was added. The aqueous layer was collected and acidified with $NaHSO_4$ solution until pH < 2. Dichloromethane (40 mL) was added and the acid was extracted. The organic phase was dried over $MgSO_4$, the solvent was evaporated and a white solid was obtained (674 mg, 86%), m.p. 81 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.26 (s, 9H), 2.15 – 2.25 (m, 2H), 2.39 (t, J = 6.88, 2H), 4.42 (t, J = 6.94, 2H), 4.58 (s, 2H), 7.55 (s, 1H), 10.31 (bs, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 25.3, 27.5, 30.5, 49.3, 56.2, 74.1, 122.5, 146.8, 176.4. **$C_{11}H_{19}N_3O_3$: MS** (CI, NH_3): *m/z*(%) 242(100, MH+).

4-{4-[(*tert*-Butoxycarbonylamino)methyl]-1H-1,2,3-triazol-1-yl}butanoic acid (8)

4-Azidobutanoic acid (0.89 g, 6.90 mmol) was mixed with Boc-protected propargylamine (1.07 g, 6.90 mmol) in MeOH (10 mL). NaOH (1 mol/L) was added until the pH value was between 6 and 8. Then ascorbic acid (65 mg, 0.33 mmol) dissolved in 1 mL H_2O and copper sulphate pentahydrate (8 mg, 0.03 mmol) dissolved in 1 mL H_2O were added and the reaction mixture was heated to reflux overnight. Next day MeOH was evaporated under reduced pressure and the residue was diluted with 80 mL EA and 80 mL of aqueous $NaHSO_4$ solution (5%w). The organic layer was separated, dried over $MgSO_4$ and the solvent was evaporated. The crude material was recrystallised from EA yielding a white crystalline solid (1.31 g, 67%), m.p. 117 °C. **^{1}H NMR** (300 MHz, CD_3OD): 1.43 (s, 9H), 2.10 – 2.20 (m, 2H), 2.25 – 2.35 (m, 2H), 4.29 (s, 2H), 4.44 (t, J = 6.89, 2H), 7.82 (s, 1H). **^{13}C NMR** (75 MHz, CD_3OD): 26.7, 29.0, 31.5, 36.9, 50.6, 80.5, 124.2, 147.3, 158.3, 176.1. **$C_{12}H_{20}N_4O_4$: MS** (LC-MS-II): *m/z*(%) [t_R = 5.1 min]: 285(35, MH+), 569(100).

6-(*tert*-Butoxycarbonylamino)-2,2-dimethyl-4,11-dioxo-3-oxa-5,7,10-triaza-tetradec-5-en-14-oic acid (17)

Compound **16** (300 mg, 0.99 mmol) and succinic anhydride (119 mg, 1.19 mmol) were dissolved in a mixture of THF (10 mL) and NEt_3 (150 mg, 1.49 mmol) and stirred overnight. Next day water was added (10 mL) and the mixture was stirred for one hour. THF was evaporated completely and the residue was diluted with water (20 mL). Aqueous $NaHSO_4$ solution (5%w, 10 mL) was added and the mixture was extracted with dichloromethane (2 x 50 mL). The organic layer was dried over $MgSO_4$ and the solvent was evaporated yielding a white solid (197 mg, 49%), m.p. 77-80 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.47 (s, 9H), 1.49 (s, 9H), 2.50 – 2.60 (m, 2H), 2.60 – 2.70 (m, 2H), 3.35 – 3.45 (m, 2H), 3.45 – 3.60 (m, 2H), 7.00 – 8.40 (bs, 1H), 8.50 (bs, 1H), 8.67 (bs, 1H), 11.38 (bs, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 28.0, 28.2, 30.3, 30.5, 40.3, 41.9, 79.9, 83.9, 153.0, 157.6, 162.5, 172.9, 175.1. **$C_{17}H_{30}N_4O_7$: MS** (LC-MS-I): *m/z*(%) [t_R = 10.3 min]: 403(100, MH+), 805(10).

4-{2-[(2-*tert*-Butoxycarbonylaminoethyl)(*tert*-butoxycarbonyl)amino]ethylamino}-4-oxobutanoic acid (20)

Compound **19** (1.00 g, 3.30 mmol) was dissolved in 5 mL THF and a solution of succinic anhydride (0.33 g, 3.30 mmol) in 5 mL THF was added. Then NEt_3 (0.50 g, 4.95 mmol) was added to the mixture. The mixture was stirred overnight at ambient temperature. Next day THF was removed completely and the crude product was dissolved in 20 mL

dichloromethane. It was washed with aqueous $NaHSO_4$ sol. (5%w, 30 mL), dried over $MgSO_4$ and the solvent was evaporated giving a white solid (1.03 g, 77%), m.p. 90 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.40 (s, 9H), 1.43 (s, 9H), 2.48 (bs, 2H), 2.64 (bs, 2H), 3.10 - 3.40 (m, 8H), 4.95 – 5.25 (m, 1H), 7.00 – 7.30 (m, 1H), 9.31 (bs, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 20.4, 29.7, 30.6, 39.3, 39.6, 47.0, 47.8, 79.6, 80.7, 156.6, 156.7, 172.8, 175.7. **$C_{18}H_{33}N_3O_7$: MS** (ES): *m/z*(%) 204(5), 245(45), 304(50), 348(25), 404(100, MH+), 426(5), 808(7), 825(15).

3-(2-*tert*-Butoxycarbonylaminoethyl)aminocarbonylpropanoic acid (22)

Succinic anhydride (0.41 g, 4.06 mmol) and NEt_3 (56 µL, 0.41 mmol) were added to a solution of amine **21** (0.65 g, 4.06 mmol) in dichloromethane (3 mL). The mixture was heated to 60 °C for 25 min (microwave synthesizer (Biotage Initiator 8)). After removal of the solvent under reduced pressure the product (insoluble in dichloromethane) was afforded as a white solid (1.05 g, 99%). **^{1}H NMR** (300 MHz, DMSO-d6): 1.37 (s, 9H), 2.25 – 2.32 (m, 2H), 2.37 – 2.44 (m, 2H), 2.91 – 2.99 (m, 2H), 3.00 – 3.08 (m, 2H), 6.78 (t, J = 5.45, 1H), 7.85 (t, J = 5.29, 1H), 12.08 (bs, 1H). **^{13}C NMR** (75 MHz, DMSO-d6): 28.1, 29.0, 29.9, 38.6, 39.5, 77.5, 155.5, 171.0, 173.8. **$C_{11}H_{20}N_2O_5$:** (LC-MS-II): *m/z*(%) [t_R = 4.63 min]: 261(40, MH+), 278(6, MNH_4+), 521(35, 2MH+), 538(100, $2MNH_4$+).

Benzyl 8-(3-methoxy-3-oxopropylamino)-8-oxooctanoate (25)

Acid **23** (250 mg, 0.95 mmol), DIPEA (368 mg, 2.85 mmol), and HOBT · H_2O (142 mg, 1.05 mmol) were dissolved in ice-cold dichloromethane (20 mL) and EDC (147 mg, 0.95 mmol) was added under nitrogen atmosphere. After 15 minutes amine **24** (98 mg, 0.95 mmol) was added. The reaction mixture was stirred overnight. Next day the reaction mixture was washed with aqueous $NaHSO_4$ sol. (5%w, 20 mL) and sat. aqueous $NaHCO_3$ solution (20 mL). The solvent was dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography (PE/EA 3:1 -> EA R_f = 0.4 [EA]) yielding the product as a white solid (253 mg, 76%), m.p. 48 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.31 (m, 4H), 1.60 (m, 4H), 2.10 (m, 2H), 2.30 (t, J = 7.48, 2H), 2.50 (m, 2H), 3.50 (dd, J1 = 6.07, J2 = 11.99, 2H), 3.68 (s, 3H), 5.10 (s, 2H), 6.00 – 6.10 (bs, 1H), 7.30 – 7.38 (m, 5H). **^{13}C NMR** (75 MHz, $CDCl_3$): 24.0, 25.4, 28.75, 28.80, 33.9, 34.2, 34.7, 36.6, 51.8, 66.1, 128.2, 128.6, 136.1, 173.0, 173.2, 173.6. **$C_{19}H_{27}NO_5$: MS** (CI, NH_3): *m/z*(%) 350(100, MH+).

Benzyl 8-[2-(*tert*-butoxycarbonylamino)ethylamino]-8-oxooctanoate (26)

Compound **23** (264 mg, 1.00 mmol) was dissolved in 10 mL of dichloromethane. HOBT · H_2O (149 mg, 1.10 mmol) and DIPEA (387 mg, 3.00 mmol) were added under nitrogen atmosphere. The mixture was stirred and cooled in an ice bath. EDC (171 mg, 1.10 mmol) was added and after 15 min amine **21** (160 mg, 1.00 mmol) was added. The mixture was stirred overnight. Next day DCM was added (10 mL), the organic phase was washed once with aqueous $NaHSO_4$ (5%w, 20 mL) and sat. aqueous $NaHCO_3$ sol. (20 mL), was dried over $MgSO_4$ and the organic layer was evaporated. The crude product was purified by column chromatography (PE/EA 1:1 -> EA R_f = 0.3 [EA]). A white, wax-like solid (251 mg, 62%), m.p. 60 °C was obtained. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.25 – 1.25 (m, 4H), 1.43 (s, 9H), 1.55 – 1.70 (m, 4H), 2.10 – 2.17 (m, 2H), 2.30 – 2.37 (m, 2H), 3.20 – 3.30 (m, 2H), 3.30 – 3.40 (m, 2H), 4.97 (bs, 1H), 5.10 (s, 2H), 6.20 (bs, 1H), 7.30 – 7.40 (m, 5H). **^{13}C NMR** (75 MHz, $CDCl_3$): 24.7, 25.4, 28.4, 28.7, 28.8, 34.1, 36.5, 40.3, 40.7, 66.1, 79.6, 128.2, 128.6, 136.1, 157.0, 173.6, 173.8. **$C_{22}H_{34}N_2O_5$: MS** (ES): *m/z*(%) 407(100, MH+).

8-(3-Methoxy-3-oxopropylamino)-8-oxooctanoic acid (27)

Compound **25** (210 mg, 0.60 mmol) was dissolved in MeOH, placed into an autoclave and Pd/C (25 mg) was added after flushing the autoclave with N_2. The reaction mixture was stirred overnight at room temperature and 12 bar hydrogen pressure. Pd/C was removed by filtration over celite, MeOH was evaporated and a white solid was obtained (146 mg, 94%), m.p. 73 °C. **^{1}H NMR** (300 MHz, CD_3OD): 1.34 (m, 4H), 1.59 (m, 4H), 2.16 (t, J = 7.45, 2H,), 2.27 (t, J = 7.39, 2H), 2.52 (t, J = 6.64, 2H), 3.41 (t, J = 6.64, 2H), 3.67 (s, 3H). **^{13}C NMR** (75 MHz, CD_3OD): 26.0, 26.9, 29.90, 29.92, 34.8, 34.9, 36.4, 36.9, 52.2, 173.9, 176.4, 177.7. **$C_{12}H_{21}NO_5$: MS** (CI, NH_3): *m/z*(%) 260(100, MH+), 277(41).

8-[2-(*tert*-Butoxycarbonylamino)ethylamino]-8-oxooctanoic acid (28)

Compound **26** (172 mg, 0.42 mmol) was dissolved in 5 mL MeOH. Pd-C (17 mg) was added and the mixture was stirred at 15 bar hydrogen pressure in an autoclave overnight. Pd/C was filtered off using celite and MeOH was evaporated. The product was obtained as a white solid (120 mg, 90%), m.p. 95 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.15 – 1.25 (m, 4H), 1.31 (s, 9H), 1.40 – 1.55 (m, 4H), 2.00 – 2.10 (m, 2H), 2.10 – 2.20 (m, 2H), 3.00 – 3.10 (m, 2H), 3.10 – 3.17 (m, 2H), 4.05 (bs, 3H). **^{13}C NMR** (75 MHz, $CDCl_3$): 24.5, 25.3, 28.1, 28.5, 28.6, 33.9, 36.1, 39.6, 39.7, 79.6, 157.1, 174.9, 176.5. **$C_{15}H_{28}N_2O_5$: MS** (ES): *m/z*(%) 217(100), 261(45), 317(85, MH+), 339(15), 633(10), 650(25).

8-[3-(Benzyloxy)-3-oxopropylamino]-8-oxooctanoic acid (30)

Octanedioic acid (500 mg, 2.87 mmol), DIPEA (1.11 g, 8.61 mmol) and HOBT · H_2O (427 mg, 3.16 mmol) were dissolved in ice-cold dichloromethane (20 mL) and EDC (490 mg, 3.16 mmol) was added under nitrogen atmosphere. After 15 min 3-aminopropanoic acid benzyl ester (617 mg, 2.87 mmol) was added and the ice bath was removed. The mixture was stirred overnight. Next day an aqueous $NaHSO_4$ solution (5%w, 20 mL) was added and the crude product was extracted with dichloromethane (2 x 20 mL), the organic phase was dried over $MgSO_4$, filtered and evaporated. The crude material was purified by column chromatography, (EA -> EA/MeOH 9:1). The obtained material is a 2:1 mixture of the desired product (420 mg, 44%) and the appropriate octanedioic acid diamide. The mixture was used in the next synthesis step without further purification.

***tert*-Butyl 2-(pyrene-1-carbonylamino)ethylcarbamate (32)**

Pyrene-1-carboxylic acid (246 mg, 1.00 mmol), DIPEA (387 mg, 3.00 mmol) and HOBT · H_2O (149 mg, 1.10 mmol) were dissolved in ice-cold dichloromethane (20 mL). EDC (171 mg, 1.10 mmol) was added under nitrogen atmosphere. After 15 min Boc-protected ethylenediamine (160 mg, 1.0 mmol) was added and the ice bath was removed. The mixture was stirred overnight. Next day the reaction mixture was diluted with dichloromethane (40 mL), washed with aqueous $NaHSO_4$ solution (5%w, 40 mL) and sat. aquoeus $NaHCO_3$ solution (1 x 40 mL). The organic layer was dried over $MgSO_4$ and evaporated. The crude compound was recrystallised from EA yielding the product as a yellow solid (213 mg, 55%), m.p. > 190 °C. **^{1}H NMR** (300 MHz, DMSO-d6): 1.41 (s, 9H), 3.20 – 3.30 (m, 2H), 3.40 – 3.50 (m, 2H), 7.00 (t, J = 5.57, 1H), 8.05 – 8.30 (m, 5H), 8.30 – 8.40 (m, 3H), 8.50 – 8.54 (m, 1H), 8.71 (t, J = 5.37, 1H). **^{13}C NMR** (75 MHz, DMSO-d6): 28.2, 39.58, 39.61, 77.6, 123.5, 123.7, 124.2, 124.7, 125.3, 125.5, 125.7, 126.5, 127.1, 127.9, 128.2, 130.1, 130.6, 131.5, 131.8, 155.7, 168.9. **$C_{24}H_{24}N_2O_3$: MS** (EI): m/z(%) 201(58), 229(100), 245(57), 258(15), 389(22, MH+).

2-(Pyren-1-carbonylamino)ethanaminium chloride (33)

Compound **32** (549 mg, 1.41 mmol) was suspended in a mixture of 20 mL MeOH and 5 mL HCl (37%). It was stirred 30 min at 70 °C. The solvent was evaporated completely and a yellow solid was obtained (445 mg, 97%), m.p. > 190 °C. **^{1}H NMR** (300 MHz, DMSO-d6): 3.05 – 3.20 (m, 2H), 3.65 – 3.75 (m, 2H), 8.05 – 8.15 (m, 1H), 8.15 – 8.30 (m, 3H), 8.30 – 8.35 (m, 4H), 8.35 – 8.50 (bs, 3H), 8.62 (m, 1H), 9.02 (t, J = 5.33, 1H). **^{13}C NMR** (75 MHz,

DMSO-d6): 37.3, 38.4, 123.5, 123.7, 124.2, 124.7, 125.6, 125.7, 125.8, 126.5, 127.1, 128.0, 128.3, 130.0, 130.6, 130.8, 131.7, 169.2. **$C_{19}H_{16}N_2O$: MS** (CI, NH_3): m/z(%) 289(100, MH+).

3-Oxo-1-phenyl-2,5,8,11-tetraoxatridecan-13-oic acid (34)

3,6,9-Trioxaundecanedioic acid (5.55 g, 25 mmol), benzyl alcohol (2.70 mg, 25 mmol) and toluene sulfonic acid (43 mg, 0.25 mmol) were combined in a 250 mL flask and toluene (70 mL) was added. The flask was connected to a Dean-Stark apparatus. The mixture was heated to reflux and the reaction was completed after 1 h. Sat. aqueous $NaHCO_3$ (50 mL) solution was added and the phases were separated. The aqueous layer was collected and extracted with EA (3 x 100 mL). The organic layer was dried over $MgSO_4$ and evaporated. The product is a viscous oil (1.87 g, 24%). **^{1}H NMR** (300 MHz, CD_3OD): 3.60 – 3.75 (m, 8H), 4.10 (s, 2H), 4.20 (s, 2H), 5.17 (s, 2H), 7.30 – 7.40 (m, 5H). **^{13}C NMR** (75 MHz, CD_3OD): 66.7, 68.5, 68.6, 70.3, 70.5, 70.7, 70.9, 128.44, 128.47, 128.52, 128.63, 128.65, 135.3, 170.5, 173.2. **$C_{15}H_{20}O_7$: MS** (CI, NH_3): m/z(%) 242(6), 286(28), 313(2, MH+), 330(100).

Benzyl 1,6-dioxo-1-(pyren-1-yl)-8,11,14-trioxa-2,5-diazahexadecan-16-oate (35)

Compound **34** (462 mg, 1.48 mmol), DIPEA (476 mg, 3.69 mmol) and HOBT · H_2O (200 mg, 1.48 mmol) were dissolved in DMF (10 mL) and EDC (229 mg, 1.48 mmol) was added (ice bath). After 15 min compound **33** (400 mg, 1.23 mmol) was added and the mixture was stirred overnight. Next day DMF was removed completely, the residue was dissolved in dichloromethane (20 mL) and washed with aqueous $NaHSO_4$ solution (5%w, 20 mL), sat. aqueous $NaHCO_3$ solution (1 x 20 mL) and brine (1 x 20 mL). The crude product was purified with column chromatography (PE/EA 1:1 -> EA/MeOH 4:1 R_f = 0.3 [EA/MeOH 9:1]). The product is a yellow solid (420 mg, 59%), m.p. > 190 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 3.45 – 3.80 (m, 12H), 3.97 (s, 2H), 4.02 (s, 2H), 5.07 (s, 2H), 7.20 – 7.40 (m, 6H), 7.64 (t, J = 5.57, 1H), 7.95 – 8.10 (m, 6H), 8.14 – 8.19 (m, 2H), 8.55 – 8.60 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 38.8, 40.9, 66.6, 68.4, 70.1, 70.27, 70.32, 70.7, 70.9, 124.3, 124.4, 124.6, 124.69, 124.7, 125.65, 125.72, 126.3, 127.1, 128.39, 128.47, 128.51, 128.55, 128.62, 130.7, 130.95, 131.12, 132.4, 135.2, 170.1, 170.4, 171.4. **$C_{34}H_{34}N_2O_7$: MS** (ES): m/z(%) 583(100, MH+), 605(60), 621(10). **UV** (MeOH): λ(ε) 233($65\cdot10^3$), 242($89\cdot10^3$), 265($34\cdot10^3$), 275($51\cdot10^3$), 326($31\cdot10^3$), 340($41\cdot10^3$). **Fluorescence** (MeOH): λ(type) 383 (monomer), 401 (monomer)

1,6-Dioxo-1-(pyren-1-yl)-8,11,14-trioxa-2,5-diazahexadecan-16-oic acid (36)

Compound **35** (203 mg, 0.35 mmol) was dissolved in 5 mL of MeOH. Pd/C (10 mg) was added and the mixture was stirred in an autoclave at 1 bar hydrogen pressure for 4 h. After that time Pd/C was filtered off with celite and the solvent was removed. The product is a yellow solid (172 mg, 100%), m.p. > 190 °C. **^{1}H NMR** (300 MHz, CD_3OD): 3.50 – 3.58 (m, 4H), 3.58 – 3.65 (m, 4H), 3.65 – 3.75 (m, 4H), 4.01 (s, 2H), 4.03 (s, 2H), 8.00 – 8.30 (m, 8H), 8.45 – 8.55 (m, 1H). **^{13}C NMR** (75 MHz, DMSO-d6): 38.1, 39.2, 67.5, 69.4, 69.6, 69.7, 69.9, 70.2, 123.5, 123.7, 124.3, 124.7, 125.2, 125.5, 125.7, 126.5, 127.1, 127.7, 128.0, 128.2, 130.1, 130.6, 131.5, 131.8, 169.0, 169.6, 171.6. **$C_{27}H_{28}N_2O_7$: MS** (ES): m/z(%) 493(100, MH+), 510(10), 515(50).

General procedure for the preparation of compounds 4b-4g, 4i, 4k-4q, 4t, 4u

The appropriate carboxylic acid (1 mmol), HOBT · H_2O (1.2 mmol), EDC (1.2 mmol) and DIPEA (2 mmol) were combined in a flask under nitrogen atmosphere with 10 mL of cold DMF. After 15 min compound **3** (1 mmol) was added and the mixture was stirred overnight at room temperature. Next day the solvent was removed completely and the crude material was purified with column chromatography.

***tert*-Butyl (pentacosa-10,12-diynamido)methylthiomethylenecarbamate (4b)**

Column chromatography: (PE/EA 9:1, R_f = 0.3). The product is a white solid (489 mg, 89%), m.p. 37 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 0.75 – 0.85 (t, J = 7.24, 3H), 1.10 – 1.70 (m, 41H), 2.17 (t, J = 6.92, 4H), 2.30 – 2.45 (m, 5H), 12.00 – 12.45 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.13, 14.45, 19.18, 19.20, 22.69, 24.50, 27.99, 28.29, 28.36, 28.74, 28.86, 28.91, 29.06, 29.11, 29.35, 29.49, 29.62, 29.64, 29.65, 31.92, 37.38, 65.25, 65.34, 77.35, 77.54, 81.17, 160.97, 171.32, 171.49. **$C_{32}H_{54}N_2O_3S$: MS** (LC-MS-I): *m/z*(%) [t_R = 15.3 min]: 547(100, MH+).

***tert*-Butyl (hexadecanamido)methylthiomethylenecarbamate (4c)**

Column chromatography: (PE/EA 9:1 R_f = 0.5). The product is a white solid (342 mg, 80%), m.p. 62 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 0.82 (t, J = 6.68, 3H), 1.18 – 1.28 (m, 24 H), 1.46 (s, 9H), 1.60 (m, 2H), 2.33 (s, 3H), 2.37 (m, 2H), 12.40 (bs, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.1, 14.5, 28.0, 22.7, 29.1 – 29.7, 31.9, 80.2. **$C_{23}H_{44}N_2O_3S$: MS** (CI, NH_3): m/z(%) 329(15), 429(100, MH+).

***tert*-Butyl-[4-(pyren-1-yl)butanamido]methylthiomethylenecarbamate (4d)**

Column chromatography (PE/EA 9:1 -> PE/EA 3:1, R_f = 0.4 [PE/EA 3:1]). The product was obtained as a white solid (299 mg, 65%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.53 (s, 9H), 2.18 – 2.32 (m, 2H), 2.32 – 2.43 (m, 3H), 2.45 – 2.70 (m, 2H), 3.41 (t, J = 7.46, 2H), 7.80 – 7.90 (m, 1H), 7.95 – 8.05 (m, 3H), 8.05 – 8.20 (m, 4H), 8.25 – 8.35 (m, 1H), 12.15 – 12.55 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.6, 26.1, 28.1, 32.5, 36.7, 81.3, 123.3, 124.86, 124.97, 125.01, 125.10, 125.88, 126.76, 127.32, 127.43, 127.47, 127.53, 128.74, 130.02, 130.03, 131.43, 135.26, 150.98, 161.04, 171.24. **$C_{27}H_{28}N_2O_3S$: MS** (LC-MS-I): *m/z*(%) [t_R = 15.5 min]: 461(100, MH+).

***tert*-Butyl-(pyren-1-carbonylamino)methylthiomethylenecarbamate (4e)**

Column chromatography (PE/EA 9:1, R_f = 0.1). The product was obtained as a bright yellow solid (190 mg, 45%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.54 – 1.63 (m, 9H), 2.53 – 2.67 (m, 3H), 7.95 – 8.40 (m, 7H), 8.90 – 9.00 (m, 1H), 9.40 – 9.50 (m, 1H), 12.60 – 13.40 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 15.1, 28.1, 83.6, 124.03, 124.36, 124.99, 125.55, 126.07, 126.21, 127.27, 127.38, 129.09, 129.54, 129.67, 130.42, 130.47, 131.05, 131.25, 134.25, 151.25, 171.15, 178.73. **$C_{24}H_{22}N_2O_3S$: MS** (LC-MS-I): *m/z*(%) [t_R = 16.3 min]: 419(100, MH+), 837(10).

***tert*-Butyl 5,15,20-trioxo-20-(pyren-1-yl)-7,10,13-trioxa-2-thia-4,16,19-triazaicosan-3-ylidenecarbamate (4f)**

Column chromatography (EA -> EA/MeOH 9:1, R_f = 0.3 [EA/MeOH 9:1]). The product was obtained as a light yellow solid (485 mg, 73%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.49 (s, 9H), 2.20 – 2.40 (m, 3H), 3.50 – 3.75 (m, 10H), 3.75 – 3.85 (m, 2H), 3.90 – 4.05 (m, 4H), 7.10 – 7.90 (m, 2H), 7.95 – 8.15 (m, 6H), 8.15 – 8.25 (m, 2H), 8.55 – 8.65 (m, 1H), 11.80 – 12.90 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.4, 28.0, 39.0, 40.9, 70.4, 70.5, 70.6, 70.8, 70.9, 71.5, 77.2, 124.4, 124.6, 124.7, 125.7, 125.8, 126.3, 127.1, 128.6, 128.7, 130.7, 131.2, 132.5, 165.9, 169.1, 170.4. **$C_{34}H_{40}N_4O_8S$: MS** (ES): m/z(%) 565(50), 665(100, MH+), 687(15).

Benzyl 3-{8-[(Boc-amino)-methylthio-methyleneamino]-8-oxooctanamido} propanoate (4g = 4o)[a]

Column chromatography (PE/EA 1:1 -> PE/EA 3:7, R_f = 0.15[PE/ EA 1:1]). The product is a viscous oil (364 mg, 72%). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.25 – 1.40 (m, 4H), 1.51 (s, 9H),

1.55 – 1.70 (m, 4H), 2.05 – 2.15 (m, 2H), 2.35 – 2.50 (m, 2H), 2.38 (s, 3H), 2.55 – 2.60 (m, 2H), 3.45 – 3.55 (m, 2H), 5.13 (s, 2H), 6.02 (s, 1H), 7.30 – 7.40 (m, 5H), 12.45 (bs, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.5, 25.4, 28.0, 28.7, 34.1, 34.8, 36.6, 66.6, 77.3, 128.3, 128.4, 128.7, 135.6, 172.6, 172.9. **$C_{25}H_{37}N_3O_6S$: MS** (ES): *m/z*(%) 408(25), 508(100, MH+).

[a] This compound was used for preparation of **1g** and **1o**.

(2*R*,3*R*,4*S*,5*R*)-6-[(Boc-amino)-methylthio-methyleneamino]-6-oxohexane-1,2,3,4,5-pentayl pentaacetate (4i)

Column chromatography (PE/EA 3:1, R_f = 0.1). Compound **4i** (252 mg, 44%) was obtained as a viscous oil. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.48 (s, 9H), 2.00 – 2.10 (m, 12H), 2.23 (s, 3H), 2.42 (s, 3H), 4.19 (dd, J1 = 5.84, J2 = 12.33, 1H), 4.34 (dd, J1 = 3.53, J2 = 12.33, 1H), 5.05 (dt, J1 = 3.53, J2 = 5.84, 1H), 5.40 (d, J = 3.08, 1H), 5.52 (dd, J = 5.84, 1H), 5.90 (dd, J1 = 3.08, J2 = 5.84, 1H), 11.88 (bs, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.8, 20.5, 20.6, 20.7, 20.8, 21.1, 27.9, 61.5, 69.1, 69.3, 70.1, 74.5, 84.1, 150.5, 169.5, 169.7, 169.8, 170.2, 170.5, 174.5, 176.8. **$C_{23}H_{34}N_2O_{13}S$: MS** (ES): *m/z*(%) 579(100, MH+).

Methyl 3-{8-[boc-amino(methylthio)methyleneamino]-8-oxooctanamido} propanoate (4k)

Column chromatography (PE/EA 1:1 -> EA, R_f = 0.4 [EA]). The product is a colorless oil (337 mg, 78%). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.33 (m, 4H), 1.51 (s, 9H), 1.63 (m, 4H), 2.14 (m, 2H), 2.35 – 2.50 (m, 5H), 2.53 (m, 2H), 3.47 – 3.54 (dd, J1 = 6.07, J2 = 11.94, 2H), 3.70 (s, 3H), 6.04 (bs, 1H), 12.00 – 12.70 (bs, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.5, 24.3, 25.4, 28.0, 28.7, 28.8, 33.9, 34.7, 36.6, 37.3, 51.8, 77.2, 81.3, 171.3, 172.9, 173.3. **$C_{19}H_{33}N_3O_6S$: MS** (CI, NH_3): m/z(%) 332(14), 432(100, MH+).

***tert*-Butyl-{4-[4-(*tert*-butoxymethyl)-1H-1,2,3-triazol-1-yl]butanamido} methylthio-methylenecarbamate (4l)**

Column chromatography (PE/EA 3:1 -> PE/ EA 4:6, R_f = 0.4[PE/EA 4:6]). Compound **4l** (254 mg, 62%) was obtained as a white solid, m.p. 93 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.26 (s, 9H), 1.49 (s, 9H), 2.15 – 2.30 (m, 2H), 2.37 (s, 3H), 2.40 – 2.60 (m, 2H), 4.40 (t, J = 6.33, 2H), 4.57 (s, 2H), 7.52 (s, 1H), 12.10 – 12.50 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.6, 25.2, 27.6, 28.0, 35.3, 49.1, 56.5, 73.8, 81.5, 83.6, 122.1, 147.0, 171.0, 171.4. **$C_{18}H_{31}N_5O_4S$: MS** (CI): *m/z*(%) 314(18), 414(100, MH+).

***tert*-Butyl {4-[4-(Boc-aminomethyl)-1H-1,2,3-triazol-1-yl]butanamido} methylthio-methylenecarbamate (4m)**

Column chromatography (PE/EA 3:1 -> PE/ EA 4:6, R_f = 0.3[PE/EA 4:6]). Compound **4m** (341 mg, 75%) was obtained as a colorless, viscous oil. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.34 (s, 9H), 1.41 (s, 9H), 2.10 – 2.25 (m, 2H), 2.29 (s, 3H), 2.30 – 2.50 (m, 2H), 4.25 – 4.40 (m, 4H), 5.20 – 5.40 (bs, 1H), 7.49 (s, 1H), 11.70 – 12.50 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.5, 24.9, 27.9, 28.3, 33.9, 37.6, 49.4, 79.5, 122.0, 145.6, 155.9, 160.7, 169.9, 184.5. **$C_{19}H_{32}N_6O_5S$: MS** (LC-MS-II): *m/z*(%) [t_R = 7.3 min]: 457(100, MH+), 913(40).

((3a*R*,5*R*,5a*S*,8a*S*,8b*R*)-2,2,7,7-Tetramethyltetrahydro-3a*H*-bis[1,3]dioxolo[4,5-b:4',5'-d]pyran-5-yl)methyl 4-((boc-amino)-methylthio-methyleneamino)-4-oxobutanoate (4n)

Column chromatography (PE/EA 9:1 -> PE/EA 3:1, R_f = 0.2 [PE/EA 9:1]). Compound **4n** (396 mg, 74%) was obtained as a colorless, viscous oil. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.29 (d, J = 2.47, 6H), 1.40 (s, 3H), 1.42 – 1.50 (m, 12H), 2.34 (s, 3H), 2.60 – 2.85 (m, 4H), 3.90 – 4.00 (m, 1H), 4.19 (dd, J1 = 1.55, J2 = 7.85, 2H), 4.24 (d, J = 4.96, 1H), 4.28 (dd, J1 = 2.50, J2 = 4.99, 1H), 4.57 (dd, J1 = 2.46, J2 = 7.88, 1H), 5.48 (d, J = 4.98, 1H), 12.05- 12.49 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.5, 24.5, 24.9, 25.9, 26.0, 27.9, 28.3, 29.3, 31.8, 35.8, 63.5, 63.8, 65.9, 70.4, 70.7, 71.0, 81.3, 83.4, 96.3, 108.7, 109.6, 150.9, 169.8, 170.9, 171.8, 172.8, 184.3. **$C_{23}H_{36}N_2O_{10}S$: MS** (ES): *m/z*(%) 533(100, MH+).

***tert*-Butyl [17,17-dimethyl-13-(methylthio)-4,11,15-trioxo-16-oxa-3,12,14-triazaoctadec-13-en-1-yl]carbamate (4p)**

Column chromatography (PE/EA 1:1 -> PE/EA 3:7, R_f = 0.1 [PE/EA 1:1]). The product was obtained as a white solid (368 mg, 77%), m.p. 95 °C **^{1}H NMR** (300 MHz, $CDCl_3$): 1.25 – 1.35 (m, 4H), 1.37 (s, 9H), 1.40 – 1.50 (m, 9H), 1.50 – 1.65 (m, 4H), 2.05 – 2.15 (m, 2H), 2.32 (s, 3H), 2.32 – 2.50 (m, 2H), 3.15 – 3.35 (m, 4H), 4.80 – 4.95 (bs, 1H), 6.00 – 6.20 (bs, 1H), 12.00 – 12.50 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.5, 24.3, 25.4, 28.0, 28.4, 28.6, 28.8, 36.5, 37.2, 40.3, 40.8, 79.7, 81.3, 157.0, 161.0, 171.3, 171.5, 173.7. **$C_{22}H_{40}N_4O_6S$: MS** (ES): *m/z*(%) 389(18), 489(100, MH+).

***tert*-Butyl-16,16-dimethyl-5,14-dioxo-15-oxa-2-thia-4,11,13-triazaheptadecane-3,12-diylidenedicarbamate (4q)**

Column chromatography (PE/EA 3:1, R_f = 0.35) Compound **4q** (413 mg, 76%) was obtained as a white solid, m.p. 116-120 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.30 – 1.45 (m, 2H), 1.48 (s,

9H), 1.49 (s, 9H), 1.52 (s, 9H), 1.55 – 1.75 (m, 4H), 2.30 – 2.55 (m, 5H), 3.35 – 3.45 (m, 2H), 8.30 (bs, 1H), 11.49 (bs, 1H), 12.16 – 12.48 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.5, 24.2, 26.3, 28.0, 28.1, 28.3, 28.8, 37.1, 40.6, 79.2, 81.3, 83.1, 153.3, 156.1, 161.0, 163.6, 171.2, 171.3. **$C_{24}H_{43}N_5O_7S$: MS** (LC-MS-I): *m/z*(%) [t_R = 14.2 min]: 546(100, MH+), 1092(10).

Di-*tert*-butyl-17,17-dimethyl-5,8,15-trioxo-16-oxa-2-thia-4,9,12,14-tetraaza-octadecane-3,13-diylidenedicarbamate (4t)

Column chromatography (PE/EA 4:6, R_f = 0.2). The product was obtained as a colorless, viscous oil (180 mg, 31%). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.43 – 1.52 (m, 27H), 2.34 (s, 3H), 2.50 – 2.90 (m, 4H), 3.35 – 3.75 (m, 4H), 7.60 – 7.90 (m, 1H), 8.40 – 8.70 (m, 1H), 11.37 (bs, 1H), 11.75 – 12.50 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.5, 27.99, 28.04, 28.25, 38.6, 38.8, 40.4, 41.6, 79.6, 83.2, 83.6, 153.0, 153.1, 156.9, 157.4, 162.8, 177.5. **$C_{24}H_{42}N_6O_8S$: MS** (LC-MS-I): *m/z*(%) [t_R = 12.9 min]: 575(100, MH+), 1149(15).

***tert*-Butyl 14-boc-amino-12-boc-5,8-dioxo-2-thia-4,9,12-triazatetradecan-3-ylidenecarbamate (4u)**

Column chromatography (PE/EA 1:1 -> EA, R_f = 0.15 [PE/EA 1:1]). The product was obtained as a colorless, viscous oil (386 mg, 67%). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.40 (s, 9H), 1.45 (s, 9H), 1.48 (s, 9H), 2.35 (s, 3H), 2.40 – 2.90 (m, 4H), 3.10 – 3.50 (m, 8H), 4.85 – 5.10 (m, 1H), 6.50 – 7.00 (m, 1H), 11.70 – 12.60 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.5, 28.0, 28.2, 28.3, 28.4, 39.0, 39.6, 47.2, 47.9, 79.4, 80.5, 156.1, 156.7, 170.5, 171.2. **$C_{25}H_{45}N_5O_8S$: MS** (ES): *m/z*(%) 576(100, MH+).

***tert*-Butyl 11-boc-amino-5,8-dioxo-2-thia-4,9-diazaundecan-3-ylidenecarbamate (4s)**

DIPEA (0.37 g, 2.84 mmol) and TBTU (0.96 g, 3.0 mmol) were added to a solution of **22** (0.74 g, 2.84 mmol) in DMF (5 mL). The mixture was stirred under argon for 15 min prior to the addition of **3** (0.595 g, 3.13 mmol) and DIPEA (0.74 g, 2.84 mmol). After stirring over night glacial acetic acid (162 µL, 2.84 mmol) was added and the solvent was removed under reduced pressure (1 mbar) at a temperature of 45 °C. Purification with column chromatography (dichloromethane/EA 3:1 **->** 1:2, R_f = 0.45[dichloromethane/EA 3:1]) yielded the product as a white solid (1.17 g, 95%). **^{1}H NMR** (300 MHz, DMSO-d6): 1.37 (s, 9H), 1.42 (s, 9H), 2.27(2.25) (s, 3H), 2.30 – 2.38 (m, 2H), 2.53 – 2.59 (m, 2H), 2.09 – 2.99 (m, 2H), 2.99 – 3.10 (m, 2H), 6.75 – 6.90 (m, 1H), 7.86 – 7.99 (m, 1H), 11.24(10.98) (s, 1H). **$C_{18}H_{32}N_4O_6S$:** (LC-MS-II): *m/z*(%) [t_R = 6.87 min]: 433(100, MH+).

General procedure for the preparation of compounds 1b-1g, 1i, 1k-1q and 1s-1u

The appropriate *S*-methylisothiourea derivative **4** (0.2 mmol), compound **5** (0.2 mmol) and $HgCl_2$ (0.2 mmol) were dissolved separately in small amounts of DMF (1-2 mL). The solutions of the compounds **4** and **5** were combined in a small flask under nitrogen atmosphere. NEt_3 (2 mmol) was added under stirring, then the $HgCl_2$ solution was added and the mixture was stirred overnight at room temperature. Next day DMF was removed completely and the residue was dissolved in DCM. The precipitate of mercury salts was filtered off and the organic phase was concentrated. The crude products were purified by column chromatography (PE/EA mixtures). The Boc- and ^{t}Bu-protected target molecules were deprotected to the corresponding target compounds **1b**-**1g**, **1i**, **1k**-**1q** and **1s**-**1u** without further characterisation: The corresponding protected compound was dissolved in a dichloromethane/TFA 1:1 mixture (5 mL) and stirred for two hours. After that time the solvent was removed completely under reduced pressure and the oily residue was repeatedly dissolved in dichloromethane and the solvent evaporated to remove TFA. Except for **1b** and **1s** (**1b**: purified by column chromatography, **1s**: no purification after deprotection) the deprotected compounds were purified with preparative HPLC. The given yield corresponds to the coupling step in the first part of this procedure, the deprotection step was quantitative.

(*R*)-1-[4-(2,2-Diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentyl]-2-pentacosa-10,12-diynoylguanidinium 2,2,2-trifluoroacetate (1b)

The product is a light yellow solid (170 mg, 72%), m.p. > 190 °C (decomp). **^{1}H NMR** (300 MHz, DMSO-d6): 0.85 (t, J = 6.51, 3H), 1.00 – 1.80 (m, 36H), 2.26 (t, J = 6.67, 4H), 2.41 (t, J = 7.19, 2H), 3.15 – 3.30 (m, 2H), 4.00 – 4.25 (m, 2H), 4.30 – 4.40 (m, 1H), 5.12 (s, 1H), 6.67 (d, J = 8.43, 2H), 7.00 (d, J = 8.41, 2H), 7.15 – 7.35 (m, 10H), 8.00 – 9.70 (m, 6H), 11.50 – 11.80 (m, 1H). **^{13}C NMR** (75 MHz, DMSO-d6): 13.8, 18.2, 22.0, 23.73, 27.57, 27.59, 28.05, 28.06, 28.15, 28.27, 28.45, 28.60, 28.75, 28.83, 28.90, 31.19, 36.02, 52.15, 55.80, 65.23, 77.84, 77.90, 114.87, 126.47, 128.04, 128.07, 128.29, 128.38, 128.40, 129.00, 140.15, 140.34, 152.71, 156.15, 170.86, 170.91, 175.17. **$C_{52}H_{71}N_5O_4$: MS** (LC-MS-I): *m/z*(%) [t_R = 12.3 min]: 830(100, MH+).

(*R*)-1-[4-(2,2-Diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentyl]-2-hexadecanoylguanidinium 2,2,2-trifluoracetate (1c)

The product is a white solid (48 mg, 53%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 0.88 (t, 3H), 1.20 – 1.35 (m, 26H), 1.40 – 1.70 (m, 4H), 2.42 (t, 2H), 2.70 - 3.10 (m, 2H), 3.90 – 4.10 (m, 1H), 4.15 – 4-35 (m, 2H), 4.99 (s, 1H), 5.80 – 6.50 (bs, 1H), 6.60 (d, 2H), 6.90 (d, 2H), 7.15 – 7.30 (m, 10H), 7.40 – 7.90 (m, 3H), 8.87 (bs, 1H), 9.45 (bs, 1H), 12.13 (bs, 1H), **$C_{43}H_{61}N_5O_4$: MS** (ES): m/z(%) 712(100, MH+). **HPLC**: 100% (ELSD, t_R = 23.8 min).

(*R*)-1-[4-(2,2-Diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentyl]-2-[4-(pyren-1-yl)butanoyl]guanidinium 2,2,2-trifluoroacetate (1d)

The product is a white solid (82 mg, 74%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 2.00 – 2.15 (m, 2H), 2.55 – 2.65 (t, J = 6.98, 2H), 3.15 – 3.25 (m, 2H), 3.35 – 3.45 (m, 2H), 4.05 – 4.20 (m, 2H), 4.25 – 4.40 (m, 1H), 5.12 (s, 1H), 6.67 (d, J = 8.48, 2H), 7.00 (d, J = 8.47, 2H), 7.10 – 7.30 (m, 10H), 7.90 – 8.00 (m, 1H), 8.00 – 8.10 (m, 1H), 8.15 (bs, 2H), 8.20 – 8.35 (m, 4H), 8.35 – 8.65 (m, 5H), 8.80 – 8.95 (m, 1H), 9.31 (bs, 1H), 11.32 (bs, 1H). **$C_{47}H_{45}N_5O_4$: MS** (LC-MS-I): *m/z*(%) [t_R = 10.2 min]: 744(100, MH+). **HPLC**: 100% (ELSD, t_R = 19.1 min). **UV** (MeCN): λ(ε) 264 ($20\cdot10^3$), 275 ($31\cdot10^3$), 312 ($10\cdot10^3$), 326 ($17\cdot10^3$), 342 ($23\cdot10^3$). **Fluorescence** ($\lambda_{ex.}$ = 340 nm, MeCN): 397 nm (monomer), 419 nm (monomer).

(*R*)-1-[4-(2,2-Diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentyl]-2-(pyrene-1-carbonyl)guanidinium 2,2,2-trifluoroacetate (1e)

The product is a yellow solid (106 mg, 68%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, CD_3CN): 1.50 – 1.90 (m, 4H), 3.20 – 3.40 (m, 2H), 4.10 – 4.30 (m, 2H), 4.35 – 4.45 (m, 1H), 5.05 (s, 1H), 6.72 (d, J = 8.51, 2H), 7.04 (d, J = 8.47, 2H), 7.15 – 7.40 (m, 13H), 8.05 – 8.40 (m, 8H), 8.50 – 8.60 (m, 1H), 9.25 – 10.30 (m, 2H), 13.05 (s, 1H). **$C_{44}H_{39}N_5O_4$: MS** (LC-MS-I): *m/z*(%) [t_R = 9.7 min]: 702(100, MH+). **HPLC**: 99% (ELSD, t_R = 17.8 min). **UV** (MeCN): λ(ε) 242 ($45\cdot10^3$), 277 ($33\cdot10^3$), 348 ($28\cdot10^3$). **Fluorescence** ($\lambda_{ex.}$ = 340 nm, MeCN): 406 nm (very broad).

(*R*)-23-(4-Hydroxybenzylcarbamoyl)-1,6,16,25-tetraoxo-26,26-diphenyl-1-(pyren-1-yl)-8,11,14-trioxa-2,5,17,19,24-pentaazahexacosan-18-iminium 2,2,2-trifluoroacetate (1f)

The product is a white solid (52 mg, 46%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 3.10 – 3.80 (m, 14H), 3.94 (s, 2H), 4.05 – 4.20 (m, 2H), 4.19 (s, 2H), 4.28 – 4.38 (m, 1H), 5.12 (s, 1H), 6.67 (d, J = 8.45, 2H), 7.00 (d, J = 8.45, 2H), 7.15 – 7.30 (m, 10H), 7.98 (t, J = 5.65, 1H), 8.08 – 8.45 (m, 9H), 8.45 – 8.55 (m, 2H), 8.55 – 8.85 (m, 3H), 8.95 – 9.05 (m, 1H), 9.32 (bs, 1H), 10.61 (bs, 1H). **$C_{54}H_{57}N_7O_9$: MS** (ES): m/z(%) 474(88), 948(100, MH+). **HPLC**: 99% (ELSD, t_R = 16.3 min). **UV** (H_2O): λ(ε) 267 ($26\cdot10^3$), 277 ($35\cdot10^3$), 330 ($24\cdot10^3$), 343 ($30\cdot10^3$). **Fluorescence** ($\lambda_{ex.}$= 340 nm, H_2O): λ(type) 401(monomer).

(*R*)-2-{8-[3-(Benzyloxy)-3-oxopropylamino]-8-oxooctanoyl}-1-[4-(2,2-diphenyl-acetamido)-5-(4-hydroxybenzylamino)-5-oxopentyl]guanidinium 2,2,2-trifluoroacetate (1g)

The product is a white solid (58 mg, 78%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.00 –1.80 (m, 12H), 1.95 –3.50 (m, 10H), 4.05 – 4.20 (m, 2H), 4.25 – 4.40 (m, 1H), 5.08 (s, 2H), 5.12 (s, 1H), 6.67 (d, J = 8.36, 2H), 7.00 (d, J = 8.40, 2H), 7.20 –7.40 (m, 15H), 7.85 – 7.95 (m, 1H), 8.30 – 8.60 (m, 4H), 8.70 – 8.90 (bs, 1H), 9.31 (bs, 1H), 11.20 (bs, 1H). **$C_{45}H_{54}N_6O_7$: MS** (ES): *m/z*(%) 791(100, MH+), 1582(1). **HPLC**: 100% (ELSD, t_R = 16.2 min).

(4*R*,12*R*,13*S*,14*R*,15*R*)-12,13,14,15-Tetraacetoxy-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,18-trioxo-17-oxa-2,8,10-triazanonadecan-9-iminium 2,2,2-trifluoroacetate (1i)

The product is a white solid (197 mg, 66%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.70 (m, 4H), 1.90 – 2.20 (m, 15H), 3.20 – 3.30 (m, 2H), 4.00 – 4.40 (m, 5H), 5.00 – 5.10 (bs, 1H), 5.12 (s, 1H), 5.24 (bs, 1H), 5.35 – 5.45 (m, 1H), 5.45 – 5.55 (m, 1H), 6.67 (d, J = 8.45, 2H), 7.00 (d, J = 8.45, 2H), 7.15 – 7.35 (m, 10H), 8.36 (t, J = 5.58, 1H), 8.47 (d, J = 8.14, 1H), 8.50 – 8.90 (m, 2H), 9.30 (bs, 1H), 11.80 (bs, 1H). **$C_{43}H_{51}N_5O_{14}$: MS** (ES): *m/z*(%) 862(100, MH+), 884(60). **HPLC**: 100% (ELSD, t_R = 16.2 min).

(*R*)-21-(4-Hydroxybenzylcarbamoyl)-3,7,14,23-tetraoxo-24,24-diphenyl-2-oxa-6,15,17,22-tetraazatetracosan-16-iminium 2,2,2-trifluoroacetate (1k)

The product is a white solid (149 mg, 64%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.10 – 1.70 (m, 12H), 2.16 (t, J = 7.28, 2H), 2.35 (t, J = 6.51, 2H), 2.54 (t, J = 5.66, 2H), 2.95 (bs, 1H), 3.15 (bs, 1H), 3.50 (m, 2H), 3.69 (s, 3H), 4.00 – 4.35 (m, 3H), 4.98 (s, 1H), 6.48 (t, J = 5.40, 1H), 6.70 (d, J = 7.86, 2H), 6.94 (d, J = 7.82, 2H), 7.15 – 7.30 (m, 10H), 7.40 – 7.70 (m, 3H), 8.89 (bs, 1H), 9.70 (bs, 1H), 12.19 (bs, 1H). **$C_{39}H_{50}N_6O_7$: MS** (ES): m/z(%) 715(100, MH+), 737(5). **HPLC:** 100% (ELSD, t_R = 14.1 min).

(*R*)-1-[4-(2,2-Diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentyl]-2-{4-[4-(hydroxymethyl)-1H-1,2,3-triazol-1-yl]butanoyl}guanidinium 2,2,2-trifluoroacetate (1l)

The product is a white solid (90 mg, 66%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.25 – 1.80 (m, 4H), 2.00 – 2.15 (m, 2H), 2.40 – 2.50 (m, 2H), 3.15 – 3.30 (m, 2H), 4.00 – 4.50 (m, 5H), 4.51 (s, 2H), 5.12 (s, 1H), 6.67 (d, J = 8.38, 2H), 7.00 (d, J = 8.38, 2H), 7.15 – 7.35 (m, 10H), 7.98 (s, 1H), 8.37 (t, J = 5.69, 1H), 8.40 – 8.70 (m, 3H), 8.80 – 10.50 (m, 2H), 11.48 (bs, 1H). **$C_{34}H_{40}N_8O_5$: MS** (ES): *m/z*(%) 641(100, MH+). **HPLC:** 99% (ELSD, t_R = 12.6 min).

(*R*)-2-{4-[4-(Ammoniomethyl)-1H-1,2,3-triazol-1-yl]butanoyl}-1-[4-(2,2-diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentyl]guanidinium 2,2,2-trifluoroacetate (1m)

The product is a white solid (139 mg, 73%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 2.09 (p, J = 7.20, 2H), 2.45 – 2.50 (m, 2H), 3.15 – 3.30 (m, 2H), 4.05 – 4.20 (m, 4H), 4.25 – 4.40 (m, 1H), 4.45 (t, J = 7.08, 2H), 5.12 (s, 1H), 6.67 (d, J = 8.45, 2H), 7.00 (d, J = 8.45, 2H), 7.15 – 7.35 (m, 10H), 8.15 (s, 1H), 8.20 – 8.35 (bs, 2H), 8.37 (t, J = 5.79, 1H), 8.50 (d, J = 8.02, 1H), 8.55 – 9.10 (m, 2H), 9.33 (bs, 1H), 12.11 (s, 1H). **$C_{34}H_{41}N_9O_4$: MS** (LC-MS-I): *m/z*(%) [t_R = 7.5 min]: 320(100), 341(35), 640(50, MH+). **HPLC:** 100% (ELSD, t_R = 10.7 min).

(13*R*)-13-(4-Hydroxybenzylcarbamoyl)-3,6,15-trioxo-16,16-diphenyl-1-((2*R*,3*R*,4*S*,5*R*)-3,4,5,6-tetrahydroxytetrahydro-2*H*-pyran-2-yl)-2-oxa-7,9,14-triazahexadecan-8-iminium 2,2,2-trifluoroacetate (1n)

The product is a white solid (35 mg, 76%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 2.55 – 2.80 (m, 4H), 3.20 – 3.90 (m, 5H), 3.90 – 4.80 (m,

8H), 4.85 – 5.00 (m, 2H), 5.12 (s, 1H), 5.20 – 6.50 (m, 1H), 6.67 (d, J = 8.41, 2H), 7.00 (d, J = 8.41, 2H), 7.15 – 7.35 (m, 10H), 8.38 (t, J = 5.63, 1H), 8.50 (d, J = 8.06, 1H), 8.50 – 8.70 (bs, 2H), 8.86 (bs, 1H), 9.30 (bs, 1H), 11.49 (bs, 1H). **$C_{37}H_{45}N_5O_{11}$: MS** (ES): *m/z*(%) 736(100, MH+), 758(20). **HPLC:** 100% (ELSD, t_R = 12.1 min).

(*R*)-21-Carboxy-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,18-trioxo-2,8,10,19-tetraazahenicosan-9-iminium 2,2,2-trifluoroacetate (1o)

1.) Removal of the benzyl-group: The Boc- and ^{t}Bu-protected precursor of compound **1o** (35 mg, 0.04 mmol) was dissolved in 2-propanol (5 mL). Pd/C (10 mg) was added and the mixture was stirred at 15 bar hydrogen pressure in an autoclave. After two hours TLC control confirmed complete conversion. Pd/C was filtered off over celite and the solvent was evaporated. The product was obtained quantitative.

2.) The N-Boc and O-^{t}Bu protected compound was deprotected as described in general procedure 4.4.

The product is a white solid (30 mg, 78%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.10 – 1.80 (m, 12H), 2.03 (t, J = 7.28, 2H), 2.30 – 2.45 (m, 4H), 3.15 – 3.30 (m, 4H), 4.05 – 4.25 (m, 2H), 4.25 – 4.40 (m, 1H), 5.12 (s, 1H), 6.67 (d, J = 8.41, 2H), 7.00 (d, J = 8.38, 2H), 7.15 – 7.35 (m, 10H), 7.86 (t, 1H), 8.30 – 8.70 (m, 4H), 8.80 – 9.00 (m, 1H), 9.20 – 9.40 (bs, 1H), 11.30 – 11.40 (bs, 1H). **$C_{38}H_{48}N_6O_7$: MS** (ES): *m/z*(%) 351(11), 701(100, MH+). **HPLC**: 99% (ELSD, t_R = 13.7 min).

(*R*)-4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-9-iminio-3,11,18-trioxo-2,8,10,19-tetraazahenicosan-21-aminium 2,2,2-trifluoroacetate (1p)

The product is a white solid (125 mg, 70%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.10 – 1.70 (m, 12H), 2.00 – 2.15 (m, 2H), 2.35 – 2.45 (m, 2H), 2.75 – 2.90 (m, 2H), 3.15 – 3.30 (m, 4H), 4.10 – 4.20 (m, 2H), 4.25 – 4.40 (m, 1H), 5.12 (s, 1H), 6.67 (d, J = 8.36, 2H), 7.00 (d, J = 8.36, 2H), 7.15 – 7.30 (m, 10H), 7.78 (bs, 3H), 8.00 (t, J = 5.52, 1H), 8.38 (t, J = 5.72, 1H), 8.51 (d, J = 8.02, 1H), 8.65 – 8.80 (bs, 2H), 9.12 (bs, 1H), 9.32 (bs, 1H), 11.73 (bs, 1H). **$C_{37}H_{49}N_7O_5$: MS** (ES): *m/z*(%) 336(100), 672(25, MH+). **HPLC**: 97% (ELSD, t_R = 11.2 min).

(*R*)-18-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11-dioxo-2,8,10,17-tetraazaoctadecane-9,18-diiminium 2,2,2-trifluoroacetate (1q)

The product is a white solid (93 mg, 71%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, CD_3CN): 1.25 –1.40 (m, 2H), 1.45 – 1.85 (m, 8H), 2.40 – 2.48 (t, J = 7.27, 2H), 3.00 – 3.10 (dd, J1 = 6.68, J2 = 12.84, 2H), 3.10 – 3.30 (m, 2H), 4.10 – 4.25 (m, 2H), 4.30 – 4.40 (m, 1H), 5.02 (s, 1H), 6.40 (bs, 4H), 6.71 (d, J = 8.50, 2H), 6.90 – 7.40 (m, 5H), 7.03 (d, J = 8.42, 2H), 7.20 – 7.35 (m, 10H), 9.45 (bs, 1H), 9.85 (bs, 1H), 12.73 (bs, 1H). **^{13}C NMR** (75 MHz, CD_3CN): 23.2, 23.4, 25.0, 27.4, 28.6, 35.9, 40.1, 40.8, 41.8, 52.4, 57.0, 114.8, 126.67, 126.73, 128.14, 128.17, 128.34, 128.40, 129.6, 154.1, 155.7, 171.9, 176.3. **$C_{34}H_{44}N_8O_4$: MS** (LC-MS-I): *m/z*(%) [t_R = 7.6 min]: 315(95), 335(100), 629(85, MH+), 675(30), 743(5). **HPLC:** 100% (ELSD, t_R = 11.2 min).

(*R*)-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-9-iminio-3,11,14-trioxo-2,8,10,15-tetraazaheptadecan-17-aminium 2,2,2-trifluoroacetate (1s)

The product is a white solid (0.74 g, 68%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.32 –1.60 (m, 3H), 1.60 – 1.76 (m, 1H), 2.46 (t, J = 6.71, 2H), 2.66 (t, J = 6.48, 2H), 2.80 – 2.88 (m, 2H), 3.18 – 3.31 (m, 4H), 4.11 (dd, J1 = 15.02, J2 = 5.76, 1H), 4.18 (dd, J1 = 15.05, J2 = 5.93, 1H), 4.28 – 4.38 (m, 1H), 5.12 (s, 1H), 6.67 (d, J = 8.53, 2H), 7.00 (d, J = 8.54, 2H), 7.18 – 7.32 (m, 10H), 7.76 (bs, 3H), 8.14 (t, J = 5.62, 1H), 8.38 (t, J = 5.74, 1H), 8.50 (d, J = 8.06, 1H), 8.71 (bs, 2H), 9.16 (t, non-res., 1H), 9.31 (bs, 1H), 11.88 (s, 1H). **$C_{33}H_{41}N_7O_5$: MS** (LC-MS-I): *m/z*(%) [t_R = 7.44 min]: 308.5(100), 616(27, MH+). **HPLC**: 96% (220 nm, t_R = 16.5 min).

(*R*)-1-Amino-16-(4-hydroxybenzylcarbamoyl)-6,9,18-trioxo-19,19-diphenyl-2,5,10,12,17-pentaazanonadecane-1,11-diiminium 2,2,2-trifluoroacetate (1t)

The product is a white solid (35 mg, 49%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, CD_3CN): 1.40 –1.80 (m, 4H), 2.49 (t, J = 6.59, 2H), 2.66 (t, J = 6.55, 2H), 3.10 – 3.30 (m, 6H), 4.10 – 4.25 (m, 2H), 4.25 – 4.40 (m, 1H), 5.02 (s, 1H), 6.10 – 7.50 (m, 10H), 6.70 (d, J = 8.50, 2H), 7.03 (d, J = 8.43, 2H), 7.20 – 7.35 (m, 10H), 9.39 (bs, 1H), 9.79 (bs, 1H), 12.82 (bs, 1H). **$C_{34}H_{43}N_9O_5$: MS** (LC-MS-I): *m/z*(%) [t_R = 7.5 min]: 329(100), 658(20, MH+). **HPLC**: 99% (ELSD, t_R = 10.7 min).

(*R*)-N^1-[4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-9-iminio-3,11,14-trioxo-2,8,10,15-tetraazaheptadecan-17-yl]ethane-1,2-diaminium 2,2,2-trifluoroacetate (1u)

The product is a white solid (62 mg, 39%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 2.62 – 2.70 (t, J = 6.29, 2H), 3.00 – 3.40 (m, 12H), 4.00 – 4.20 (m, 2H), 4.25 – 4.40 (m, 1H), 5.12 (s, 1H), 6.70 (d, J = 8.45, 2H), 7.00 (d, J = 8.45, 2H), 7.15 – 7.35 (m, 10H), 7.90 – 9.40 (m, 12H), 12.03 (bs, 1H). **$C_{35}H_{46}N_8O_5$: MS** (ES): m/z(%) 330(100), 659(20, MH+). **HPLC**: 99% (ELSD, t_R = 9.1 min).

(*R*)-1-(4-Fluorophenyl)-16-(4-hydroxybenzylcarbamoyl)-1,6,9,18-tetraoxo-19,19-diphenyl-2,5,10,12,17-pentaazanonadecan-11-iminium 2,2,2-trifluoroacetate (1j)

Compound **1s** (24 mg, 28.4 µmol) and NEt_3 (21 µL, 152 µmol) were dissolved in DMF (200 µL) followed by the addition of active ester **37** (4.5 mg, 19 µmol). The reaction was stopped by addition of 10% aq. TFA (corresponding to 60 µmol of TFA) after an incubation period of 3.5 h at rt. Purification with preparative HPLC (column: Eurospher-100 C18 (250 × 32 mm, 5 µm), Knauer, Berlin, Germany) and lyophilisation afforded the product as white fluffy solid (10.55 mg, 65%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.31 –1.60 (m, 3H), 1.60 – 1.73 (m, 1H), 2.45 (t, J = 6.60, 2H), 2.64 (t, J = 6.38, 2H), 3.16 – 3.34 (m, 6H), 4.10 (dd, J1 = 14.80, J2 = 5.66, 1H), 4.18 (dd, J1 = 15.06, J2 = 5.76, 1H), 4.28 – 4.38 (m, 1H), 5.12 (s, 1H), 6.67 (d, J = 8.52, 2H), 7.00 (d, J = 8.52, 2H), 7.18 – 7.33 (m, 12H), 7.86 – 7.93 (m, 2H), 8.11 (t, J = 5.51, 1H), 8.38 (t, J = 5.78, 1H), 8.47 – 8.62 (m, 4H), 8.87 (t, non-res., 1H), 9.30 (bs, 1H), 11.44 (s, 1H). **$C_{40}H_{44}FN_7O_6$:** (LC-MS-I): *m/z*(%) [t_R = 8.69 min]: 738(100, MH+). **HPLC**: 98% (220 nm, t_R = 23.7 min).

(*R*)-4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-9-iminio-3,11,14-trioxo-*N*-(2-propionamidoethyl)-2,8,10,15-tetraazaheptadecan-17-aminium 2,2,2-trifluoroacetate (1r)

Compound **1u** (35 mg, 35 µmol) and NEt_3 (23 µL, 186 µmol) were dissolved in DMF (200 µL) followed by the addition of active ester **38** (2.4 mg, 14 µmol). The reaction was stopped by addition of 10% aq. TFA (corresponding to 80 µmol of TFA) after an incubation period of 3 h at rt. Purification with preparative HPLC (column: Eurospher-100 C18 (250 × 32 mm, 5 µm), Knauer, Berlin, Germany) and lyophilisation afforded the product as white fluffy solid (2.71 mg, 21%), m.p. > 190 °C (decomp.). **^{1}H NMR** (600 MHz, DMSO-d6): 0.99 (t, J = 7.59, 3H), 1.35 –1.47 (m, 2H), 1.48 – 1.56 (m, 1H), 1.62 – 1.69 (m, 1H), 2.11 (q, J = 7.58, 2H), 2.45 (t, J = 6.73, 2H), 2.49 – 2.52 (m, 4H) 2.65 (t, J = 6.53, 2H), 2.95 – 3.01 (m, 2H), 3.19 –

3.24 (m, 2H), 3.27 – 3.33 (m, 2H), 4.10 (dd, J1 = 14.76, J2 = 5.66, 1H), 4.17 (dd, J1 = 14.83, J2 = 6.03, 1H), 4.30 – 4.35 (m, 1H), 5.11 (s, 1H), 6.66 (d, J = 8.52, 2H), 6.99 (d, J = 8.53, 2H), 7.19 – 7.31 (m, 10H), 8.00 (t, J = 5.64, 1H), 8.17 (t, J = 5.73, 1H), 8.35 (t, J = 5.82, 1H), 8.44 (bs, 2H), 8.47 (d, J = 8.15, 1H), 8.59 (bs, 2H), 8.93 (t, non-res., 1H), 9.28 (bs, 1H), 11.56 (s, 1H). **$C_{38}H_{50}N_8O_6$:** (LC-MS-I): *m/z*(%) [t_R = 7.54 min]: 358(100), 715(18, MH+). **HPLC**: 97% (220 nm, t_R = 17.3 min).

1.4.3 Experimental determination of logD values with HPLC

The logD values of compounds **1a, 1d, 1f, 1g, 1i, 1l, 1n, 1p, 1q** and **1u** were determined by RP-HPLC. A Luna C18 column (3.0 µm, 150 x 2 mm, Phenomenex, Aschaffenburg, Germany) with a flow of 0.30 mL/min was used. An ammonium acetate buffer (10 mM, pH 7.4) was used as solvent A. Solvent B was acetonitrile. The gradient was: 0 min [A/B 95/5], 30 min [A/B 2/98]. A volume of 2 µL of a solution of the analytes, dissolved in DMSO (1 mM), was injected. Column temperature was 25 °C. The compounds were detected with a diode array detector. The logD value was calibrated to the retention time (t_R) by running a mixture of 6 reference compounds and plotting the t_R vs. logD (supporting information).

1.4.4 Investigation of stability

Decomposition of the N^G-acylated argininamides to **1a** was investigated at neutral pH in the buffer used for radioligand binding[4] (10 mM HEPES, pH 7.4, filtered: 0.45 µm). Incubation was started by addition of 15 µL of a 1 mM solution of the compounds in DMSO to 285 µL of buffer to give a final concentration of 50 µM (in case of **1c** 60 µL of a 0.25 mM solution were added to 240 µL of buffer). After 20 and 90 min a 125 µL aliquot was taken and diluted with a mixture of MeCN, H_2O and 1% aq. TFA (for compounds **1c** – **1j**: 5:1:4, 125 µL; for compounds **1k** – **1u**: 1:1:1.33, 125 µL). 100 µL of the resulting solution (pH ≈ 2) were analysed with analytical HPLC on a RP-column (Eurospher-100 C18, 250 × 4 mm, 5 µm; Knauer, Berlin, Germany) using a system from Thermo Separation Products (composed of a SN400 controller, a P4000 pump, a degasser (Degassex DG-4400, phenomenex), an AS3000 autosampler and a Spectra Focus UV-VIS detector). Mixtures of 0.05 % aq. TFA (A) and acetonitrile (B) were used as mobile phase. The flow rate was set to 0.80 mL/min and the column temperature to 30 °C. Due to the wide range of logD values of the compounds two

different gradients were used for HPLC analysis (compounds **1c** – **1j**: 0 min [A/B 75/25], 28 min [A/B 80/20], 30 min [A/B 5/95], 38 min [A/B 5/95]; compounds **1k** – **1u**: 0 min [A/B 85/15], 28 min [A/B 55/45], 32 min [A/B 5/95], 40 min [A/B 5/95]). A five-point calibration curve (0.3, 1.5, 4, 10 and 25 µM) of BIBP 3226 (**1a**) was acquired for quantification of the decomposition product **1a**.

To study the decomposition products of **1j** with LC-MS, **1j** was incubated in 10 mM HEPES buffer as described above, but at a concentration of 30 µM (30 µL of a 0.5 mM solution of **1j** in MeCN/0.05% aq. TFA (20:80) to 470 µL of buffer). After 20 and 90 min a 125 µL aliquot was taken and diluted with a mixture of MeCN, H_2O and 1% aq. TFA (1:1:1.33, 125 µL). 10 µL of the resulting solution were injected into the LC-MS system and analysed with LC-MS method II (cf. general experimental conditions) (source type: ESI (capillary temperature: 250 °C, spray voltage: 4.0 kV, sheath and auxiliary gas: on).

1.4.5 Radioligand competition binding assay

Radioligand competition experiments at SK-N-MC neuroblastoma cells using the radioligand [^{3}H]UR-MK114 (1.5 nM) were performed as described elsewhere.[4]

1.4.6 Fura-2 assay on HEL cells

The Fura assay was performed with HEL cells as previously described using a Perkin-Elmer LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany).[19]

1.4.7 Data processing

Data from radioligand competition experiments were analyzed by 4 parameter sigmoidal fits (SigmaPlot 9.0, Systat Software). IC_{50} values from radioligand competition studies were converted to K_i values according to the Cheng-Prusoff equation[34] using the respective K_D value of the radioligand. Three data points (between 20 and 80 % inhibition, from fura-2 assays) served for the calculation of IC_{50} values after logit-log transformation. IC_{50} values were converted to K_b values according to the Cheng-Prusoff equation[33] using an EC_{50} value of 1.8 nM for pNPY (mean value from 4 independently determined concentration-effect curves on HEL cells).

1.5 References

1. Keller, M.; Teng, S.; Bernhardt, G.; Buschauer, A. *ChemMedChem* **2009**, *4*, 1733.
2. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; Konig, B. *Bioorg. Med. Chem.* **2008**, *16*, 9858.
3. Hutzler, C.; Kracht, J.; Mayer, M.; Graichen, F.; Bauer, B.; Schreiber, E.; Bollwein, S.; Bernhardt, G.; Dove, S.; Fricker, G.; Buschauer, A. *Arch. Pharm. (Weinheim, Ger.)* **2001**, *334*, 17.
4. Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. *J. Med. Chem.* **2008**, *51*, 8168.
5. Igel, P.; Schnell, D.; Bernhardt, G.; Seifert, R.; Buschauer, A. *ChemMedChem* **2009**, *4*, 225.
6. Schneider, E.; Keller, M.; Brennauer, A.; Hoefelschweiger, B. K.; Gross, D.; Wolfbeis, O. S.; Bernhardt, G.; Buschauer, A. *Chembiochem* **2007**, *8*, 1981.
7. Brennauer, A.; Dove, S.; Buschauer, A. *Structure-Activity Relationships of Nonpeptide Neuropeptide Y Receptor Antagonists. In Handbook of Experimental Pharmacology. Vol. 162. Michel, M.C. (ed)*, **2004**.
8. Dove, S.; Elz, S.; Seifert, R.; Buschauer, A. *Mini. Rev. Med. Chem.* **2004**, *4*, 941.
9. Ghorai, P.; Kraus, A.; Keller, M.; Gotte, C.; Igel, P.; Schneider, E.; Schnell, D.; Bernhardt, G.; Dove, S.; Zabel, M.; Elz, S.; Seifert, R.; Buschauer, A. *J. Med. Chem.* **2008**, *51*, 7193.
10. Igel, P.; Schneider, E.; Schnell, D.; Elz, S.; Seifert, R.; Buschauer, A. *J. Med. Chem.* **2009**, *52*, 2623.
11. Igel, P.; Geyer, R.; Strasser, A.; Dove, S.; Seifert, R.; Buschauer, A. *J. Med. Chem.* **2009**, *52*, 6297.
12. Kraus, A.; Ghorai, P.; Birnkammer, T.; Schnell, D.; Elz, S.; Seifert, R.; Dove, S.; Bernhardt, G.; Buschauer, A. *ChemMedChem* **2009**, *4*, 232.
13. Brennauer, A. Dissertation, **2006**.
14. Schneider, E.; Mayer, M.; Ziemek, R.; Li, L.; Hutzler, C.; Bernhardt, G.; Buschauer, A. *Chembiochem* **2006**, *7*, 1400.
15. Michel, M. C.; Beck-Sickinger, A.; Cox, H.; Doods, H. N.; Herzog, H.; Larhammar, D.; Quirion, R.; Schwartz, T.; Westfall, T. *Pharmacol. Rev.* **1998**, *50*, 143.
16. Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Beck-Sickinger, A. G.; Doods, H. N. *Eur. J. Pharmacol.* **1994**, *271*, R11.

17. Brennauer, A.; Keller, M.; Freund, M.; Bernhardt, G.; Buschauer, A. *Tetrahedron Lett.* **2007**, *48*, 6996.
18. Kerns, E. H.; Di, L.; Petusky, S.; Kleintop, T.; Huryn, D.; McConnell, O.; Carter, G. *J. Chromatogr. B* **2003**, *791*, 381.
19. Müller, M.; Knieps, S.; Gessele, K.; Dove, S.; Bernhardt, G.; Buschauer, A. *Arch. Pharm. (Weinheim).* **1997**, *330*, 333.
20. Knieps, S.; Dove, S.; Michel, M. C.; Rottmeier, K.; Werner, W.; Bernhardt, G.; Buschauer, A. *Pharm. Pharmacol. Lett.* **1996**, *6*, 27.
21. Beck-Sickinger, A. G.; Wieland, H. A.; Wittneben, H.; Willim, K. D.; Rudolf, K.; Jung, G. *Eur. J. Biochem.* **1994**, *225*, 947.
22. Khoukhi, N.; Vaultier, M.; Carrie, R. *Tetrahedron* **1987**, *43*, 1811.
23. Fan, X.; Potluri, V. K.; McLeod, M. C.; Wang, Y.; Liu, J.; Enick, R. M.; Hamilton, A. D.; Roberts, C. B.; Johnson, J. K.; Beckman, E. J. *J. Am. Chem. Soc.* **2005**, *127*, 11754.
24. Bartoli, G.; Bosco, M.; Locatelli, M.; Marcantoni, E.; Melchiorre, P.; Sambri, L. *Org. Lett.* **2005**, *7*, 427.
25. Qiao, C.; Jeon, H.-B.; Sayre, L. M. *J. Am. Chem. Soc.* **2004**, *126*, 8038.
26. Kartha, K. P. R. *Tetrahedron Lett.* **1986**, *27*, 3415.
27. Bartalucci, G.; Bianchini, R.; Catelani, G.; D'Andrea, F.; Guazzelli, L. *Eur. J. Org. Chem.* **2007**, 588.
28. Drake, B.; Patek, M.; Lebl, M. *Synthesis* **1994**, 579.
29. Carmignani, M.; Volpe, A. R.; Botta, B.; Espinal, R.; De Bonnevaux, S. C.; De Luca, C.; Botta, M.; Corelli, F.; Tafi, A.; Sacco, R.; Delle Monache, G. *J. Med. Chem.* **2001**, *44*, 2950.
30. Moura, C.; Vitor, R. F.; Maria, L.; Paulo, A.; Santos, I. C.; Santos, I. *Dalton Trans.* **2006**, 5630.
31. New, O. M.; Dolphin, D. *Eur. J. Org. Chem.* **2009**, 2675.
32. English, A. R.; Girard, D.; Jasys, V. J.; Martingano, R. J.; Kellogg, M. S. *J. Med. Chem.* **1990**, *33*, 344.
33. Vollmann, H.; Becker, H.; Corell, M.; Streeck, H.; Langbein, G. *Justus Liebigs Ann. Chem.* **1937**, *531*, 1.
34. Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.

CHAPTER 2

Pyrene Labeled Neuropeptide Y_1 Receptor Antagonists for the Detection of Y_1 Receptor Homodimers

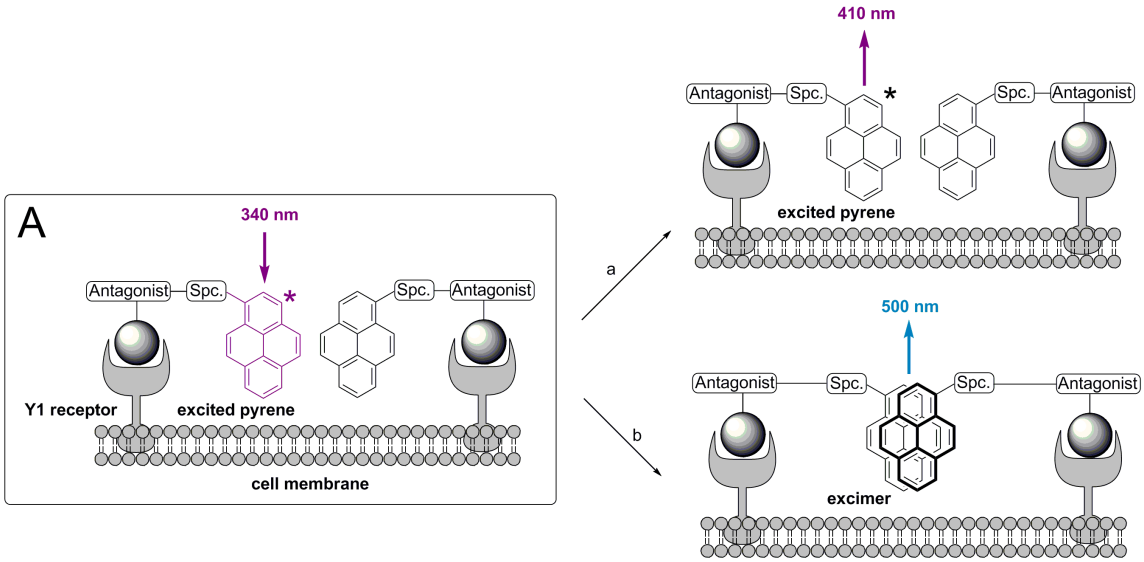
2.1 Introduction

Dimerisation and oligomerisation are a common phenomenon for the important class of G protein-coupled receptors (GPCR).[1] In many cases these trans-membrane receptors are not separated in the cell membrane, but form larger aggregates, as it is known for the clustering of the rhodopsine receptors.[2] The oligomerisation of GPCR´s has an influence on the modulation of signaling in the cell. For example the dimerisation of two different subtypes of receptors (heterodimers) as reported for the neuropeptide Y_1/Y_5 receptors[3] can alter the affinity of the receptors to agonists and antagonists and influence the internalisation behavior. Also the aggregation of two equal receptors (homodimers) can influence function and signal transduction of the receptors.[4] Homodimerisation is known for many GPCR´s, for example the β_2-adrenergic receptor and the dopamine receptors. There is also some evidence for the existence of NPY Y_1 receptor homodimers.[5]

The detection of the aggregation state of GPCR´s is therefore of great interest for pharmaceutical research. Fluorescence resonance energy transfer (FRET) experiments with fluorescent labeled neuropeptide Y (NPY) receptors[5] and bioluminescence resonance energy transfer (BRET) experiments with opioid receptors[6] were performed to observe GPCR oligomerisation, but there is some concern that the modification of the receptor protein could influence the binding of agonists and antagonists and alter the aggregation behavior.

The use of labeled small NPY Y_1 receptor antagonists should not alter the aggregation state of the receptors. The high affinity Y_1 receptor antagonist BIBP 3226 (BIBP)[7] was therefore derivatized with the fluorescent label pyrene. The pyrene moiety can form excited dimers (excimers) when in a high local concentration.[8, 9] The fluorescence of the excimer (500 nm) is red-shifted compared to the pyrene monomer fluorescence (410 nm). This effect was successfully applied for chemosensors,[10] for the investigation of the structure of micelles[11] and the hybridization of DNA.[12] The binding of two pyrene labeled antagonists to a hypothetical Y_1 receptor dimer in close proximity (high local pyrene-derivative concentration) should enable us to observe excimer fluorescence in case of a spacer with sufficient length. The advantage of this method compared to FRET is that only one type of probe molecule is

required. The application of two differently labeled antagonists like in FRET experiments can cause problems, because of their different physico-chemical properties and binding affinities. We therefore synthesized pyrene labeled Y_1 receptor antagonists that exhibit a nanomolar affinity to the Y_1 receptor and investigated their fluorescent properties and their binding to MCF-7 cells.



Scheme 1. Excimer formation at a hypothetical Y_1 receptor dimer. In picture **A** two pyrene labeled antagonists bind to two Y_1 receptors. One of the pyrene moieties (left) is excited with UV light. In case a) the excited pyrene moiety emits light before it could combine with another pyrene moiety (in ground state). In case b) the excited pyrene forms an excimer with the second pyrene moiety and the excimer emits light with a longer wavelength.

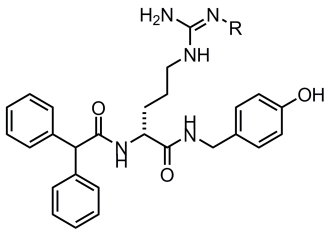
2.2 Results and Discussion

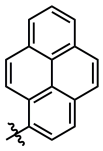
2.2.1 Synthesis and binding affinity of the pyrene-labeled Y_1R antagonists

The synthesis of compounds **1a**, **1b** and **1e** is described elsewhere.[13] The first step in our approach was to connect the high potent Y_1R antagonist BIBP 3226 **1** to the pyrene moiety. The synthesis started with pyrene that was converted to pyrene-1-carboxylic acid **4** in two steps. The target compounds **1c** and **1d** were prepared from their corresponding amines **5** and **6** respectively. The amines were treated with NHS ester **7** in THF and compounds **1c** and **1d** were obtained in moderate yield. Compound **1f** was obtained via a one pot reaction of the dicarboxylic acid **8** with the amines **9** and **10**. The yield was low, because of the statistical product distribution, but still a sufficient amount of product **11** was obtained to continue synthesis. This compound was guanidinylated with amine **12** and target compound **1f** was

obtained after deprotection with DCM/TFA. Compound **1g** was synthesized starting from precursor **4** that was converted with diethylenetriamine **13** to the double pyrene labeled amine **14**. Acylation of the primary amines of compound **13** is much faster than the acylation of the secondary amine, so product **14** was obtained in good yield. The secondary amine was then acylated with succinic acid anhydride **15** and acid **16** was obtained. Guanidinylation with amine **12** and subsequent deprotection resulted in compound **1g**. Compounds **18** and **19** were synthesized as control compounds for the unspecific binding.

The binding affinities differ significantly. Direct attachment of the pyrene moiety to the BIBP 3226 scaffold caused a complete loss of affinity. Also the very bulky compound **1g** with two pyrene units has no affinity to the Y_1R. An increase in spacer length restored some affinity. However, compounds **1b** – **1d** exhibit only moderate affinity to the Y_1R due to their high lipophilicity and low water solubility. The two antagonists **1e** and **1f** that contain a gylcol chain in their spacer were the antagonists with the best affinities; compound **1e** showed a K_i of about 60 nM.



Pyr = 

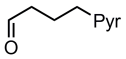
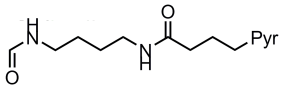
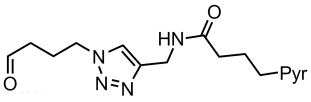
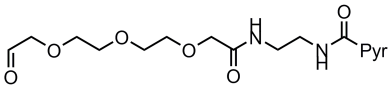
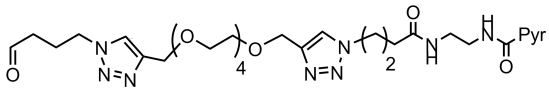
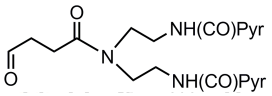
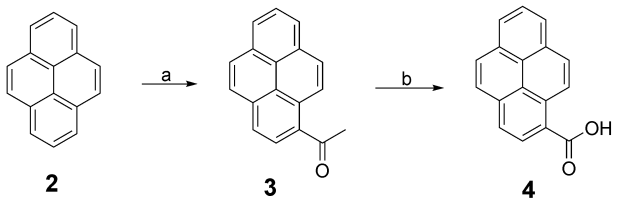
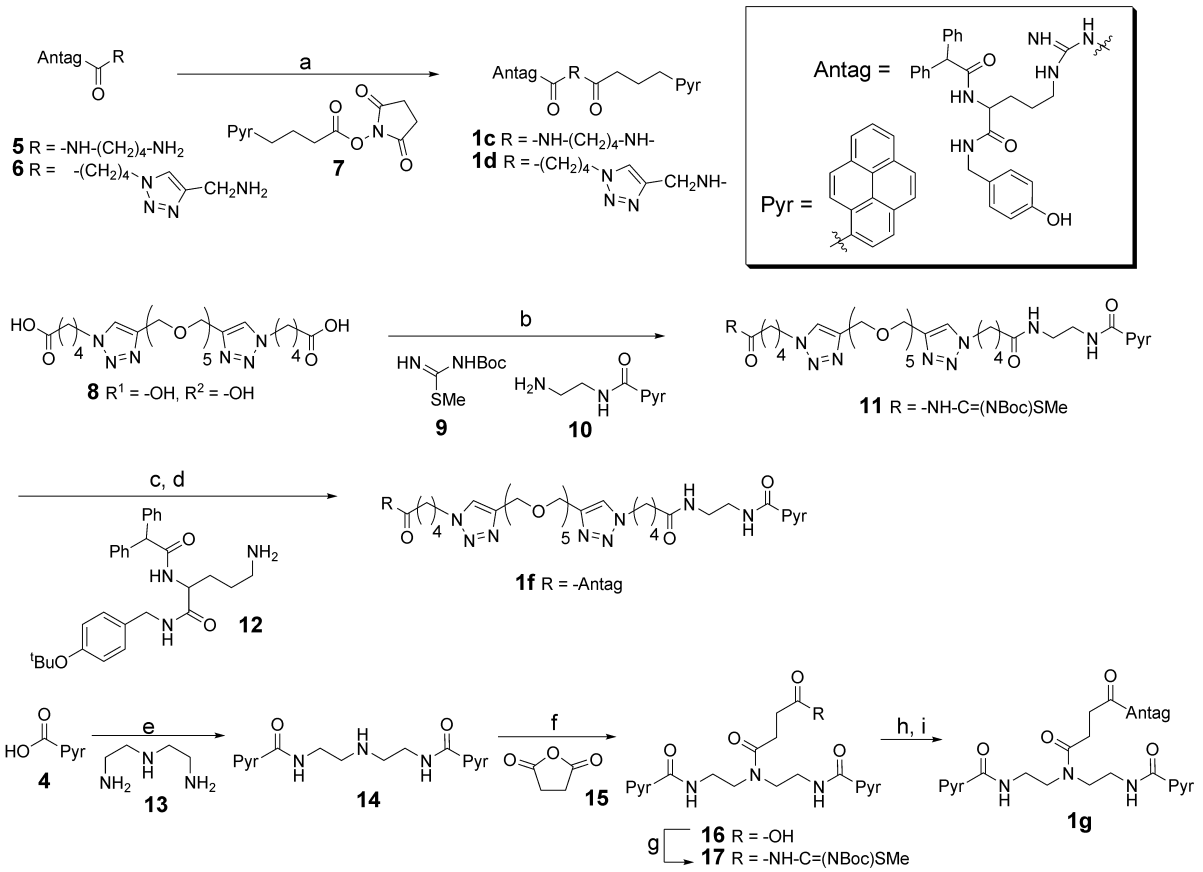
no.	R	K_i [nM]
1	H (BIBP 3226)	1.3 ± 0.2
1a		no affinity
1b		270 ± 70
1c		250
1d		170
1e		59 ± 16
1f		97 ± 8
1g		no affinity

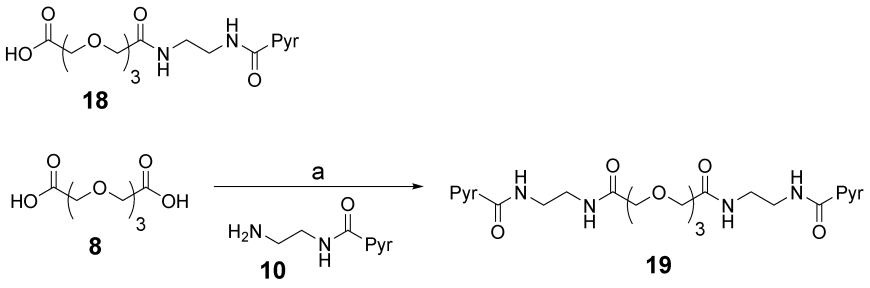
Figure 1. Structures and Y_1R affinities of the pyrene labeled Y_1R antagonists **1a-1g**. K_i values determined from the displacement of 1.5 nM [^{3}H]-UR-MK114[14] on SK-N-MC cells; all mean values ± SEM from two or three independent experiments (**1c**, **1d**: one single determination).



Scheme 2. Synthesis of the precursor pyrene-1-carboxylic acid. a) $ZnCl_2$, AcOH, Ac_2O, 55%; b) H_2O, THF, NaOCl, NaOH, 63%.



Scheme 3. Synthesis of the Y_1R antagonists **1c – 1g**. a) THF, NEt_3, 56% (**1c**), 50% (**1d**); b) EDC, HOBt, DIPEA, DMF, 11%; c) DMF, $HgCl_2$, NEt_3, compound **12**, 63%; d) TFA/DCM 1:1, quantitative; e) EDC, HOBt, DIPEA, DCM, 64%; f) DMF, NEt_3, 55%; g) EDC, HOBt, DIPEA, DCM, compound **9**, 60%; h) DMF, $HgCl_2$, NEt_3, compound **12**, 55%; i) TFA/DCM 1:1, quantitative.



Scheme 4. Control compounds **18** and **19** for the detection of unspecific binding; synthesis of compound **19**. a) DMF, EDC, HOBt, DIPEA, 75%.

2.2.2 Optical properties of the pyrene-labeled compounds

For the detection of the fluorescent labeled Y_1R anatgonists the quantum yield is a crucial factor. A disadvantage of the pyrene fluorophore compared with cyanine dyes is the excitation in the UV region, so there will be background fluorescence in the cell assay. A good quantum yield improves the signal to noise ratio and could therefore reduce the detection limit. The quantum yield of the pyrene labeled compounds **1b** – **1g** was determined in buffered aqueous solution and in acetonitrile. The mode of connection to the pyrene moiety has an influence on the quantum yield. Compounds **1b** – **1d** that were connected via the 1-pyrene butanoic acid spacer have only moderate quantum yields in acetonitrile and buffer. Addition of 1% bovine serum albumine (BSA) increased the quantum yields of these compounds. The situation is different for the compounds **1e** – **1g** containing the 1-pyrene carboxylic acid substructure. In this case the quantum yields are good in acetonitrile and moderate in buffered solution. An exception is the carboxylic acid **18**. Its quantum yield was highly increased in aqueous buffered solution. This effect can be caused by a strong self organisation (e.g. micelles or large hydrated anions) as more rigid systems can not loose their excitation energy via rotational deactivation. This effect disappears when BSA is present and the pre-organised structure is destroyed.

The absorption and emission spectra were also dependent on the substituent of the pyrene moiety. In case of the 1-pyrene butanoic acid spacer the maxima of the peaks in the absorption spectrum are slightly red shifted compared to pyrene. The first maxima of the compounds **1b** – **1d** is at 342 nm in contrast to pyrene with 334 nm. The emission maximum is also slightly red shifted from 390 nm (pyrene) to 394 nm. In case of compounds **1e** – **1f** containing the 1-pyrene carboxylic acid substructure the peak maxima in the absorption spectrum are equal to compounds **1b** – **1d**, but there is a broadening of the absorption bands. The fluorescence maximum is further red shifted to 400 nm. Compound **1g** substituted with two pyrenes is similar in its absorption properties to compounds **1e** – **1f**, but has an additional strong excimer band. The effect of the substituent is striking for compound **1a**. The direct connection of the pyrene moiety to the acyl-guanidine caused a strong broadening of the absorption and fluorescence maxima and diminished the emission intensity.

Another important factor for the understanding of the fluorescence properties of the pyrene labeled compounds is the monomer to excimer fluorescence ratio. This ratio is dependent on

the local concentration, the rotational degrees of freedom and the self aggregation behavior. In acetonitrile only the compounds **1g** and **19** that contain both two pyrene moieties in a single molecule emit a significant amount of excimer radiation, because of the high local concentration of pyrene units. The concentration of the dyes was 0.5 µM and is therefore too low for intermolecular excimer formation that is normally observed for concentrations above 100 µM. The situation in aqueous solution is different. Compounds **1b**, **1d** and **1g** are hardly water soluble. A high degree of excimer fluorescence was observed for the compounds due to self-aggregation of the pyrene rings. For the better water soluble compounds **1c**, **1e**, **1f** and **18** this effect was less pronounced. Addition of BSA had a significant effect on the ratio of monomer and excimer fluorescence: The aggregation of the hydrophobic molecules **1b**, **1d** and **1g** in aqueous solution was reduced upon binding to BSA and the excimer fluorescence was therefore diminished.

Table 1. Pyrene dye substructure and quantum yields of BIBP 3226 derivatives **1b** – **1g** and control compounds **18** and **19**.

No.	Dye substructure	Acetonitrile		Buffer[b]		Buffer[b] + 1% BSA	
		Φ [%]	(m/e)[a]	Φ [%]	(m/e)[a]	Φ [%]	(m/e)[a]
1b	Pyr-$(CH_2)_3$-CO-	14	95/5	11	36/64	19	68/32
1c	Pyr-$(CH_2)_3$-CO-	20	92/8	18	85/15	28	80/20
1d	Pyr-$(CH_2)_3$-CO-	12	96/4	16	41/59	40	85/15
1e	Pyr-CO-	43	96/4	16	87/13	13	85/15
1f	Pyr-CO-	41	97/3	37	91/9	8	81/19
1g	Pyr-CO-	38	50/50	15	17/83	17	26/74
18	Pyr-CO-	29	95/5	85	94/6	28	91/9
19	Pyr-CO-	31	22/78	27	23/77	12	80/20

[a] ratio monomer fluorescence (360 nm – 450 nm) to excimer fluorescence (450 nm – 600 nm); the cut off at 450 nm was chosen for the best separation of the two emission bands, small values in the excimer rate (< 10) do not necessarily imply the presence of excimers. The dye concentration was 0.5 µM in the fluorescence measurements.

[b] HEPES buffer, pH 7.4 (25 mM HEPES, 1.0 mM $MgCl_2$, 2.5 mM $CaCl_2$)

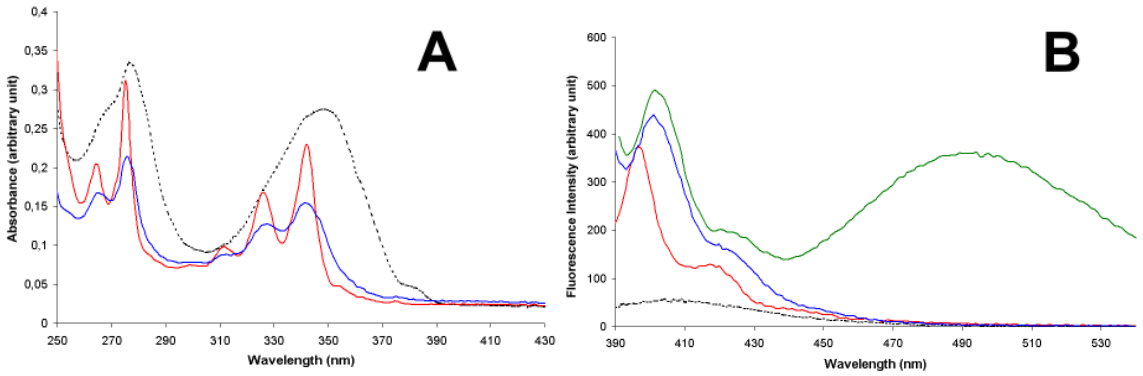


Figure 2. Absorbance (**A**) and Fluorescence spectra (λ_{ex} = 342 nm, **B**) of different types of pyrene derivatives in acetonitrile. The concentration of the compounds was 10 µM. Picture **A**: absorbance of compound **1b** (red), **1e** (blue) and **1a** (black, dashed line). Picture **B**: fluorescence spectra of compound **1b** (red), **1e** (blue), **1a** (black, dashed line) and compound **1g** (green; forms an internal excimer).

2.2.3 Fluorescence microscopy on MCF-7 cells

The pyrene-labeled compounds were investigated for the detection of NPY Y_1 receptor dimers on MCF-7 cells with fluorescence microscopy. The cells were therefore grown on 8-well slides and incubated with the pyrene-labeled compounds. Unspecific binding was determined with BIBP 3226. The first attempt using a Leica DM RBE microscope was performed to compare specific and unspecific binding (monomer and excimer channel not separated). Unfortunately the auto-fluorescence (picture **A**) was as bright as the stained cells (picture **B**). There was also no difference between total (**B**) and unspecific binding (**D**) visible. The staining of the MCF-7 cell nuclei with DAPI (**C**) was possible. There is no fluorescence in the cell nuclei without DAPI, so the fluorescent ligands and other fluorescent bio-molecules are in the cytoplasm or at the cell membrane. In a second attempt the cells were investigated with a 2-photon laser. With this technique only a small segment of the cells was excited at 680 nm and therefore the background fluorescence was diminished compared to the standard fluorescence microscopy. But the autofluorescence was still a major problem. The addition of the fluorescent ligand **1e** did not increase the fluorescence signal significantly. There was also no difference between total and unspecific binding.

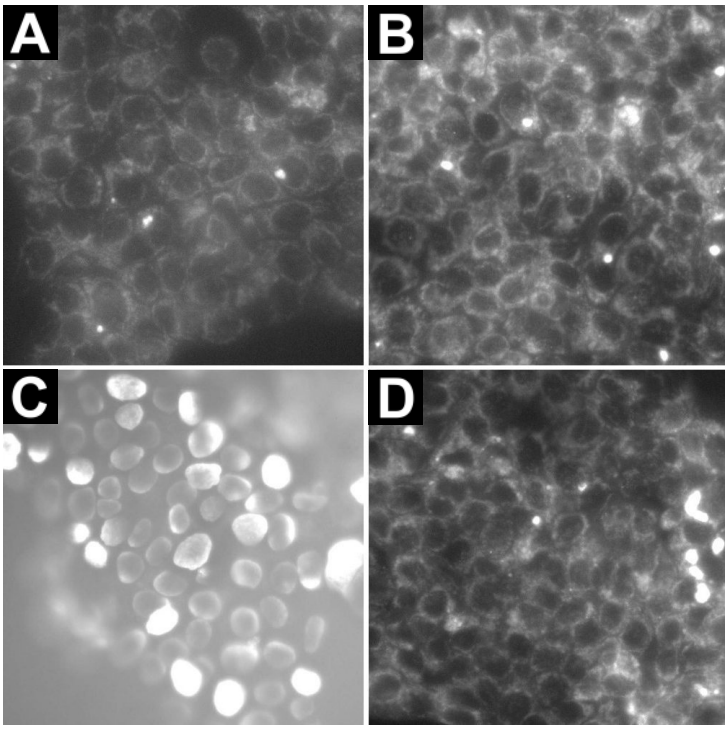


Figure 3. MCF-7 cells and fluorescent ligands (excitation: 340 – 380 nm, emission filter: 425 nm longpass), incubation time 20 minutes. Picture **A**: autofluorescence; **B**: 100 nM **1f**; **C**: DAPI (1.5 mg/mL); **D**: 100 nM **1f** + 10 µM BIBP.

2.2.4 FACS analysis of MCF-7 cells

An alternative strategy for the detection of Y_1R dimers using the pyrene dyes was the investigation of MCF-7 cells with FACS measurements. The cells were incubated with the pyrene labeled antagonists for 20 minutes and then analyzed. The result was exemplarily shown for compound **1f** in Figure 4 (pictures **A** and **B**). A right shift of the signal maximum is correlated with a binding of the fluorescent ligand to the cell. There was no difference in the monomer channel (395 nm – 415 nm; **A**) between unlabeled cells (black), total binding (blue) and unspecific binding (red). The excimer channel (515 nm – 545 nm) also shows no significant right-shift. The other pyrene labeled compounds behave equal. Only the red fluorescent compound **20**[14] that can be excited at 488 nm has a strong right shift compared to the unlabeled cells, but there is also some unspecific binding. In summary, the strong autofluorescence of the MCF-7 cells under UV light and the weak fluorescence of the pyrene derivatives do not permit the determination of total and unspecific binding of the pyrene labeled antagonists.

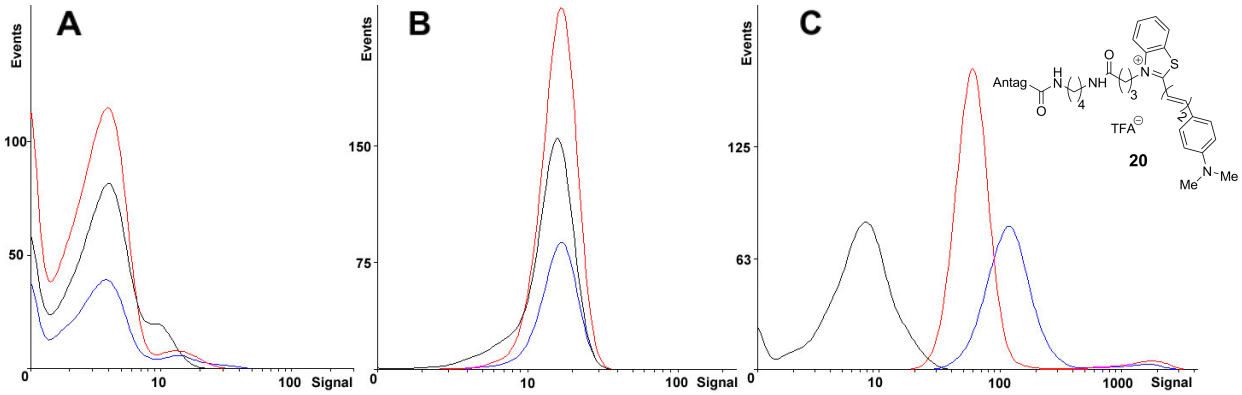
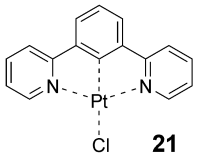


Figure 4. Histogram: Binding of compound **1f** to MCF-7 cells, monomer channel (**A**) and excimer channel (**B**); unlabeled cells (black), total binding (blue) and unspecific binding (red). Picture **C** shows the binding of the red fluorescent Y_1R antagonist **20** (505 nm longpass filter) for comparision. Specific binding was only detected for compound **20** (right shift of the blue curve).

2.3 Conclusion and Outlook

In this study we successfully prepared pyrene labeled derivatives of the Y_1 receptor antagonist BIBP 3226 that exhibited nanomolar affinity to the NPY Y_1 receptor. The new derivatives have a moderate quantum yield between 11% and 37% in aqueous buffered solution. Despite the good properties of the pyrene moiety (long lifetime of the excited state, fast excimer formation combined with a strong red-shift; small molecule with less sterical demand as other dyes) the strong autofluorescence of the MCF-7 cells decreased the signal to noise ratio and inhibits the detection of the specific antagonist binding at the Y_1 receptor. There are two ways to overcome this problem. One option is the investigation of the pyrene dyes with a time-resolved confocal microscope that has an UV laser (no standard equipment). The more convenient and more applicable solution for this problem is the investigation of dyes that absorb light in the visible part of the spectra (488 nm argon laser or 633 nm He-laser, standard equipment) and exhibit a long lifetime of the excited state, so that they can form excimers. Platinum-(II)-complexes like compound **21** as described in literature[15] (excimer: λ_{max} = 690 nm, τ = 7 µs) can be useful candidates. In summary, the study shows that pyrene-labeled NPY Y_1 antagonists are not suitable to proof the proposed excimer approach for the detection of GPCR dimers, but other excimer dyes may do.



21

Figure 5. A platinum-(II)-complex that forms excimers with an emission maximum of ca. 700 nm.

2.4 Experimental

2.4.1 General experimental conditions

Unless otherwise noted, solvents (analytical grade) were purchased from commercial suppliers and used without further purification. Ethyl acetate (EA), petrol ether (PE, 60 – 70 °C), methanol and dichloromethane were obtained in technical grade and distilled before application. Acetonitrile (MeCN) for HPLC was obtained from Merck (Darmstadt, Germany). Pyrene (Aldrich, Sigma-Aldrich Chemie GmbH, Munich, Germany), 1-pyrene butyric acid (Aldrich, Sigma-Aldrich Chemie GmbH, Munich, Germany) and diethylenetriamine (Acros, Fisher Scientific GmbH, Schwerte) were purchased. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18 (250 × 21 mm, 5 µm; Macherey-Nagel, Germany) and a Eurospher-100 C18 (250 × 32 mm, 5 µm; Knauer, Germany) served as RP-columns at flow rates of 20 and 38 mL/min, respectively. Mixtures of MeCN and diluted aqueous TFA (0.1 %) were used as mobile phase. ^{1}H-NMR spectra were recorded at 300 MHz on a Bruker Avance 300 spectrometer or at 600 MHz on a Bruker Avance III 600 with cryogenic probehead (Bruker, Karlsruhe, Germany). ^{13}C-NMR spectra were recorded at 75 MHz on a Bruker Avance 300 spectrometer. All chemical shifts values are reported in ppm. UV/VIS spectra were recorded with a Varian Cary BIO 50 UV/VIS/NIR spectrophotometer (Varian Inc., CA, USA). Fluorescence spectra were recorded with a Cary Eclipse spectrofluorimeter (Varian Inc., CA, USA). Mass spectra: Finnigan SSQ 710A (EI), Finnigan MAT 95 (CI), Finnigan MAT TSQ 7000 (Thermo FINNIGAN, USA) (ES/LC-MS). LC-system for LC-MS: Agilent 1100 (Palo Alto, USA). LC-MS method I (LC-MS-I): Column: Phenomex Luna C18, 3.0 µm, 100 x 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.30 mL/min; solvent A (water + 0.1% TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 1 min [A/B 95/5], 11 min [A/B 2/98], 18 min [A/B 2/98], 19 min [A/B 95/5], 24 min [A/B 95/5]. LC-MS method II (LC-MS): Column: Phenomex Luna C18, 2.5 µm, 50 x 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.40 mL/min; solvent A (water + 0.1% TFA), solvent B

(MeCN); gradient: 0 min [A/B 95/5], 8 min [A/B 2/98], 11 min [A/B 2/98], 12 min [A/B 95/5], 15 min [A/B 95/5]. Melting points were determined with a Lambda Photometrics Optimelt MPA100 apparatus (Lambda photometrics, Harpenden, UK), they are not corrected. Thin layer chromatography (TLC) was performed on alumina plates coated with silica gel (Merck silica gel 60 F_{245}, thickness 0.2 mm). Column chromatography (CC) was performed with Merck Geduran SI 60 silica gel as the stationary phase.
Compounds **1a**,[13] **1b**,[13] **1e**,[13] **3**,[16] **4**,[16] **5**,[14] **6**,[13] **7**,[17] **8**,[18] **9**,[18] **10**,[13] **12**[19] and **18**[13] were prepared according to literature procedures.

2.4.2 Synthetic protocols and analytical data

(*R*)-4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,18-trioxo-21-(pyren-1-yl)-2,8,10,12,17-pentaazahenicosan-9-iminium 2,2,2-trifluoroacetate (1c)

The amine **5** (7.6 mg, 9.3 µmol) was dissolved in THF (3 mL) and NEt_3 (9.3 mg, 93 µmol) was added. Then a solution of the NHS ester **7** (3.6 mg, 9.3 µmol) in THF (1 mL) was added slowly. Mixture was stirred until TLC control demonstrated complete conversion (1h). TFA was added dropwise until the pH was below 3. Then the mixture was concentrated and the crude material was purified with prep. HPLC. The product is a white solid (5.0 mg, 56%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.00 – 1.80 (m, 10H), 1.95 – 2.05 (m, 2H), 2.20 – 2.25 (m, 2H), 3.00 – 3.20 (m, 6H), 4.05 – 4.20 (m, 2H), 4.25 – 4.40 (m, 1H), 5.12 (s, 1H), 6.67 (d, J=8.45, 2H), 7.00 (d, J=8.45, 2H), 7.15 – 7.35 (m, 10H), 7.40 – 7.55 (bs, 1H), 7.80 – 8.60 (m, 14 H), 8.75 – 9.55 (m, 2H). **$C_{52}H_{55}N_7O_5$: MS** (LC-MS-I): *m/z*(%) [t_r=9.9 min.]: 858(100, M+).

(*R*)-(4-(2,2-Diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentylamino)(4-(4-((4-(pyren-1-yl)butanamido)methyl)-1H-1,2,3-triazol-1-yl)butanamido)methan-iminium 2,2,2-trifluoroacetate (1d)

The amine **6** (30.1 mg, 34.7 µmol) was dissolved in THF (3 mL) and NEt_3 (35 mg, 347 µmol) was added. A solution of the NHS ester **7** (12.2 mg, 35.6 µmol) in THF (2 mL) was added slowly. The mixture was stirred until TLC control demonstrated complete conversion (2h). TFA was added dropwise until the pH was below 3. Then the mixture was concentrated and the crude material was purified with prep. HPLC. The product is a white solid (18.0 mg, 50%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 1.95 – 2.10 (m, 4H), 2.20 – 2.35 (t, J=7.21, 2H), 2.35 – 2.45 (t, J=7.13, 2H), 3.15 – 3.25 (m, 2H),

3.25 – 3.35 (m, 2H), 4.05 – 4.20 (m, 2H), 4.25 – 4.40 (m, 5H), 5.12 (s, 1H), 6.67 (d, J=8.45, 2H), 7.00 (d, J=8.45, 2H), 7.15 – 7.35 (m, 10H), 7.90 – 7.95 (m, 2H), 8.00 – 8.45 (m, 10H), 8.45 – 9.80 (m, 4H), 11.41 (s, 1H). **$C_{54}H_{55}N_9O_5$: MS** (LC-MS-I): *m/z*(%) [t_r=9.8 min.]: 455(35), 910(100, M+).

***tert*-Butyl-methylthio(4-(4-(15-(1-(4-oxo-4-(2-(pyrene-1-carboxamido)-ethylamino)butyl)-1H-1,2,3-triazol-4-yl)-2,5,8,11,14-pentaoxapentadecyl)-1H-1,2,3-triazol-1-yl)butanamido)methylenecarbamate (11)**

Compound **8** (243 mg, 0.46 mmol), DIPEA (271 mg, 2.10 mmol) and HOBT · H_2O (124 mg, 0.92 mmol) were dissolved in 15 mL DMF and the mixture was cooled in an icebath under nitrogen atmosphere. Under vigorous stirring EDC (130 mg, 0.84 mmol) was added. After 15 minutes a mixture of the compounds **9** (80 mg, 0.42 mmol) and **10** (135 mg, 0.42 mmol) in 5 mL DMF was added. After the addition the ice bath was removed and the mixture was stirred at ambient temperature overnight. The next day DMF was evaporated completely and the residue was dissolved in DCM. It was washed with diluted $NaHSO_4$ solution (10%, 1 x 30 mL) and the organic layer was separated and dried over $MgSO_4$. The solvent was removed and the crude material was purified with column chromatography (EA/EtOH 8:2 -> EtOH, R_f=0.2[EA/EtOH 1:1]). A white solid was obtained (43 mg, 11%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.49 (s, 9H), 2.05 - 2.45 (m, 11H), 3.45 – 3.75 (m, 20H), 4.20 – 4.35 (m, 4H), 4.40 – 4.60 (m, 4H), 7.40 – 7.70 (m, 4H), 7.90 – 8.20 (m, 8H), 8.45 – 8.55 (m, 1H), 11.50 – 13.00 (m, 1H). **$C_{48}H_{62}N_{10}O_{10}S$: MS** (LC-MS): *m/z*(%) [t_r=11.3 min.]: 486(100), 971(35, MH+).

(*R*)-(4-(2,2-Diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentylamino)(4-(4-(15-(1-(4-oxo-4-(2-(pyrene-1-carboxamido)ethylamino)butyl)-1H-1,2,3-triazol-4-yl)-2,5,8,11,14-pentaoxapentadecyl)-1H-1,2,3-triazol-1-yl)butanamido)methaniminium 2,2,2-trifluoroacetate (1f)

Compound **11** (43 mg, 44 µmol) and amine **12** (22 mg, 44 µmol) were dissolved separately in DMF (1 mL). The solutions were combined in a small flask under nitrogen atmosphere. NEt_3 (0.5 mL) was added under stirring, then a $HgCl_2$ solution (44 µmol) in 0.5 mL DMF was added and the mixture was stirred overnight at room temperature. The next day DMF was removed completely and the residue was dissolved in DCM. The precipitate of mercury salts was removed by filtering over celite, the organic phase was washed with citric acid (1 x 20 mL) and dried over magnesium sulphate.

The DCM solution was concentrated and a 1:1 mixture of TFA and DCM was added (4 mL). The mixture was stirred for two hours. Then it was again concentrated and the residue was purified with HPLC. A white solid was obtained (38 mg, 63%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.30 – 1.80 (m, 4H), 2.00 – 2.20 (m, 8H), 2.20 – 3.70 (m, 22H), 4.05 – 4.20 (m, 2H), 4.30 – 4.40 (m, 5H), 4.45 (s, 2H), 4.50 (s, 2H), 5.12 (s, 1H), 6.67 (d, J=8.49, 2H), 7.00 (d, J=8.46, 2H), 7.15 – 7.30 (m, 10H), 8.00 – 8.60 (m, 16H), 8.65 – 8.75 (m, 2H), 9.30 (bs, 1H), 11.11 (bs, 1H). **$C_{68}H_{80}N_{13}O_{11}$: MS** (LC-MS): *m/z*(%) [t_r=9.3 min.]: 628(100), 1255(50, MH+). **UV** (MeCN): λ(ε) 233 ($57\cdot10^3$), 242 ($71\cdot10^3$), 265 ($26\cdot10^3$), 275 ($37\cdot10^3$), 327 ($22\cdot10^3$), 341 ($28\cdot10^3$).

N,N'-(2,2'-Azanediylbis(ethane-2,1-diyl))dipyrene-1-carboxamide (14)

Compound **4** (492 mg, 2.0 mmol), DIPEA (516 mg, 4.0 mmol) and HOBT · H_2O (284 mg, 2.1 mmol) were dissolved in 10 mL DMF. The mixture was cooled in an ice bath and EDC (310 mg, 2.0 mmol) was added under nitrogen atmosphere. After 20 minutes the cold solution was put into a dropping funnel. The reactive ester solution was dropped slowly into an ice-cooled solution of the amine **13** (103 mg, 1.0 mmol) in DMF (10 mL) over a period of 1 h. The mixture was stirred overnight at ambient temperature. Next day the mixture was concentrated and the solution was dropped into 100 mL diluted $NaHSO_4$ solution (5%). A light yellow solid precipitated. The suspension was heated to 100°C for 5 minutes. The hot suspension was filtered and the precipitate was washed with H_2O. A yellow solid was obtained (360 mg, 64%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 2.85 – 3.00 (m, 4H), 3.50 – 3.65 (m, 4H), 8.00 – 8.40 (m, 16H), 8.45 – 8.60 (m, 2H), 8.60 – 8.75 (m, 2H). **^{13}C NMR** (75 MHz, DMSO-d6): 39.6, 48.3, 123.52, 123.63, 124.21, 124.67, 125.10, 125.40, 125.62, 126.40, 127.03, 127.63, 127.89, 128.08, 130.06, 130.59, 131.33, 132.04, 168.89. **$C_{38}H_{29}N_3O_2$: MS** (LC-MS): *m/z*(%) [t_r=9.6 min.]: 560(100, MH+).

4-(bis(2-(Pyrene-1-carboxamido)ethyl)amino)-4-oxobutanoic acid (16)

Compound **14** (360 mg, 0.64 mmol) was dissolved in 5 mL DMF and NEt_3 (130 mg, 1.28 mmol) was added. Under stirring a solution of the anhydride **15** (77 mg, 0.77 mmol) in 5 mL DMF was added. The mixture was heated to 80°C and stirred for 2 h. After that time the mixture was cooled to ambient temperature and NaOH (1M, 3 mL) was added and the mixture was stirred for one more hour. Diluted $NaHSO_4$ solution was added (30 mL, 5%w) and a white solid precipitated. To increase the particle size it was heated to 100°C for 5 minutes and cooled down slowly. Then the precipitate was filtered. The crude material was

recrystallised in EtOH (50 mL), insoluble particles were filtered off. Light yellow crystals were obtained (94 mg, 55%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 2.45 – 2.55 (m, 2H), 2.70 – 2.80 (t, J=6.59, 2H), 3.55 – 3.85 (m, 8H), 8.00 – 8.40 (m, 16H), 8.45 – 8.55 (m, 2H), 8.80 (t, J=5.35, 1H), 8.96 (t, J=5.03, 1H). **^{13}C NMR** (75 MHz, DMSO-d6): 27.77, 29.38, 37.59, 38.11, 45.07, 46.84, 125.55, 123.57, 123.76, 124.32, 124.35, 124.57, 124.68, 125.16, 125.21, 125.45, 125.52, 125.66, 125.73, 126.40, 126.44, 127.07, 127.79, 127.81, 127.98, 128.07, 128.18, 128.26, 130.07, 130.60, 131.43, 131.53, 131.63, 131.78, 169.07, 169.32, 171.12, 174.12. **$C_{42}H_{33}N_3O_5$: MS** (LC-MS): *m/z*(%) [t_r=11.9 min.]: 660(100, MH+), 1319(10).

***tert*-Butyl-5,8,13-trioxo-13-(pyren-1-yl)-9-(2-(pyrene-1-carboxamido)ethyl)-2-thia-4,9,12-triazatridecan-3-ylidenecarbamate (17)**

Acid **16** (100 mg, 0.15 mmol), DIPEA (58 mg, 0.45 mmol) and HOBt (23 mg, 0.17 mmol) were dissolved in DCM and EDC (26 mg, 0.17 mmol) was added under nitrogen atmosphere. After 15 minutes compound **9** was added and the mixture was stirred overnight. The next day the reaction mixture was washed with $NaHSO_4$ solution (5%, 1 x 30 mL). Organic phase was dried over $MgSO_4$ and the solvent was evaporated. The crude product was purified with column chromatography (EA, R_f=0.1) yielding compound **17** (493 mg, 60%) as a white solid, m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.25 – 1.50 (m, 9H), 1.80 – 2.05 (m, 3H), 2.50 – 2.65 (bs, 2H), 2.65 – 2.80 (bs, 2H), 3.60 – 3.90 (m, 8H), 7.00 – 7.20 (m, 1H), 7.50 – 8.20 (m, 17H), 8.35 – 8.55 (m, 2H), 11.30 – 12.30 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.20, 27.94, 35.11, 39.09, 46.20, 48.43, 77.27, 124.12, 124.23, 124.47, 124.53, 124.71, 125.61, 125.69, 125.76, 126.14, 126.17, 126.85, 126.98, 128.32, 128.42, 128.56, 128.68, 130.05, 130.43, 130.48, 130.98, 132.35, 132.49, 170.51, 170.79. **$C_{49}H_{45}N_5O_6S$: MS** (LC-MS): *m/z*(%) [t_r=13.9 min.]: 832(100, MH+).

(*R*)-16-(4-Hydroxybenzylcarbamoyl)-1,6,9,18-tetraoxo-19,19-diphenyl-1-(pyren-1-yl)-5-(2-(pyrene-1-carboxamido)ethyl)-2,5,10,12,17-pentaazanonadecan-11-iminium 2,2,2-trifluoroacetate (1g)

Compound **17** (45 mg, 54 µmol) and amine **12** (26 mg, 54 µmol) were dissolved separately in DMF (1 mL). The solutions were combined in a small flask under nitrogen atmosphere. NEt_3 (0.5 mL) was added under stirring, then a $HgCl_2$ solution (54 µmol) in 0.5 mL DMF was added and the mixture was stirred overnight at room temperature. The next day DMF was removed completely and the residue was dissolved in DCM. The precipitate of mercury salts

was removed by filtering over celite, the organic phase was washed with citric acid (1 x 20 mL) and dried over magnesium sulphate.

The DCM solution was concentrated and a 1:1 mixture of TFA and DCM was added (4 mL). The mixture was stirred for two hours. Then it was again concentrated and the residue was purified with HPLC. A white solid was obtained (36 mg, 55%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.70 (m, 4H), 2.65 – 2.80 (m, 2H), 2.85 – 3.00 (m, 2H), 3.10 – 3.30 (m, 2H), 3.55 – 3.85 (m, 8H), 4.00 – 4.25 (m, 2H), 4.25 – 4.40 (m, 1H), 5.11 (bs, 1H), 6.60 – 6.70 (m, 2H), 6.95 – 7.05 (m, 2H), 7.15 – 7.35 (m, 10H), 8.05 – 8.40 (m, 19H), 8.40 – 8.60 (m, 4H), 8.70 – 8.85 (bs, 2H), 8.90 – 9.05 (bs, 1H), 9.31 (bs, 1H), 10.50 – 11.40 (m, 1H). **$C_{69}H_{63}N_8O_7$: MS** (LC-MS): *m/z*(%) [t_r=10.2 min.]: 558(15), 1115(100, MH+). **UV** (MeCN): λ(ε) 265 ($57\cdot10^3$), 276 ($77\cdot10^3$), 328 ($47\cdot10^3$), 342 ($61\cdot10^3$). **Fluorescence** ($\lambda_{ex.}$ = 340 nm, MeCN): 401 (monomer), 494 (excimer) nm.

N,N'-(4,14-Dioxo-6,9,12-trioxa-3,15-diazaheptadecane-1,17-diyl)dipyrene-1-carboxamide (19)

Compound **8** (38 mg, 0.12 mmol), DIPEA (93 mg, 0.72 mmol) and HOBt (34 mg, 0.25 mmol) were dissolved in a 4 mL of DMF and EDC (39 mg, 0.25 mmol) was added. After 15 minutes amine **10** (78 mg, 0.24 mmol) was added. The next day DMF was evaporated completely and the residue was purified with column chromatography (EE/MeOH 4:1 R_f = 0.1). The product was obtained as a bright yellow solid (69 mg, 75%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 3.20 – 3.70 (m, 16H), 3.93 (s, 4H), 7.98 (t, 2H, J=5.55), 8.05 – 8.40 (m, 16H), 8.50 (d, 2H, 9.28), 8.76 (t, 2H, J=5.33). **^{13}C NMR** (75 MHz, DMSO-d6): 38.1, 39.2, 66.5, 69.9, 70.1, 123.5, 123.7, 124.3, 124.7, 125.2, 125.5, 125.7, 126.5, 127.1, 127.9, 128.2, 130.1, 130.6, 131.5, 131.8, 168.9. **MS** (ES-MS): m/z(%) 763(100, MH+), 785(25). **UV** (MeOH): λ(ε) 234($68\cdot10^3$), 243($92\cdot10^3$), 265($36\cdot10^3$), 276($53\cdot10^3$), 328($33\cdot10^3$), 341($43\cdot10^3$).

2.4.3 Determination of the quantum yield

The quantum yield of the compounds **1b** – **1g** was determined with quinine (Aldrich, Sigma-Aldrich Chemie GmbH, Munich, Germany) as a standard (quantum yield 58%).[20] Stock solutions (1 mM) of the compounds **1b** – **1g** in DMSO were prepared. The final concentrations of the compounds were $5\cdot10^{-6}$ M for UV absorption spectra and $5\cdot10^{-7}$ M for fluorescence spectra. Acetonitrile, HEPES-buffer (25 mM HEPES, 1.0 mM $MgCl_2$, 2.5 mM

$CaCl_2$; pH 7.4) and HEPES buffer containing 1% BSA were applied as solvents. The quinine standard was prepared in 0.1 M H_2SO_4 (10^{-5} M for absorption and 10^{-6} for fluorescence). For the excitation wavelength of compounds **1b – 1g** the first maximum of the pyrene excitation pattern (330 – 345 nm) was choosen. Fluorescence spectra were recorded using the following parameters: excitation slit 5 nm, emission slit 10 nm, photomultiplier voltage 550 V, scanspeed medium. All spectra were recorded in silica glass cuvettes.

For the determination of reference spectra the pure solvents with the same DMSO content were used. From every emission spectrum the corresponding reference spectrum was substracted and the resulting spectrum was integrated up to 600 nm. The quantum yield was calculated according to the equation:

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

where A_s is the absorbance and F_s is the integral of the corrected emission spectrum of the quinine standard solution. A_x and F_x stand for the absorbance and the integral of the corrected emission spectrum of the fluorescent ligand. The refraction indices of the solvents are n_x and n_s. $\Phi_{F(S)}$ is the reported quantum yield of quinine (58%).

2.4.4 MCF-7 Cell culture and fluorescence microscopy

MCF-7 cells were cultivated at 37 °C for two days in 8 well µ-slides (Ibidi GmbH, Munich) with EMEM medium (Sigma, Deisenhofen, Germany) containing 5% FCS (Biochrom AG, Berlin, Germany). Estradiol (1 nM) was added to the cultivation medium to stimulate Y_1R expression. After two days, shortly before the experiment the medium was replaced by Leibovitz L15 medium (LM). The pyrene labeled compounds were prepared as 10 µM stock solutions in DMSO. Shortly before the assay a 100 nM solution of the compounds in LM was prepared. The cells were then incubated 15 minutes with the pyrene labeled compounds and pictures were taken. The fluorescence microscope was a Leica DM RBE microscope (Leica Mikrosystems, Bensheim, Germany). The dyes were excited with at 340 – 380 nm and emission was detected after a 425 nm long-pass filter (excimer and monomer fluorescence). The cell nucleus was stained with DAPI (1.5 mg/mL; Vectashield Fluoromount).

The cells were also observed with a Zeiss LSM 710 microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a 2-photon laser and a Plan-Apochromat 20x/0.8 M27 objective. The dyes were excited with the 2-photon laser at 680 nm. Fluorescence was detected at 380 – 420 nm (monomer) and 450 – 700 nm (excimer).

2.4.5 FACS analysis of MCF-7 cells

The MCF-7 cells were cultivated in a big cell culture flask under the same conditions as described above. The cells were suspended in HEPES-buffer (25 mM HEPES, 1.0 mM $MgCl_2$, 2.5 mM $CaCl_2$; pH 7.4) shortly before the experiment. FACS analysis was performed with a BD LSR-II flow cytometer (BD Biosciences, San Jose, USA), equipped with a UV trigon laser (355 nm). The emission filter set was 395 – 415 nm (monomer channel) and 515 – 545 nm (excimer channel). The pyrene labeled compounds were prepared as 10 µM stock solutions in DMSO/H_2O 1:1. The cell suspension (10^6 cells/mL) was then separated in FACS tubes (495 µL/tube) and 5 µL of the corresponding stock solution was added in each tube (100 nM final concentration). BIBP 3226 was applied for the determination of the unspecific binding (10 µM). The compounds were incubated for 20 minutes and then the cells were analyzed. The red labeled compound **20** (for comparison) was used at a final concentration of 20 nM and was excitated with a 488 nm argon laser.

2.5 References

1. George, S. R.; O´Dowd, B. F.; Lee, S. P. *Nat. rev. drug. discov.* **2002**, *1*, 808
2. Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshime, H.; Fox, B. A.; Trong, I. L.; Teller, D. C.; Okada, T.; Stenkamp, R. E. *Science* **2000**, *289*, 739
3. Gehlert, D. R.; Schober, D. A.; Morin, M.; Berglund, M. M. *Biochem. Pharmacol.* **2007**, *74*, 1652
4. Milligan, G. *Mol. Pharmacol.* **2004**, *66*, 1
5. Dinger, M. C.; Bader, J. E.; Kóbor, A. D.; Kretzschmar, A. K.; Beck-Sickinger, A. G.*J. Biol. Chem.* **2003**, *278*, 10562
6. McVey, M.; Ramsay, D.; Kellett, E.; Rees, S.; Wilson, S.; Pope, A. J.; Milligan, G. *J. Biol. Chem.* **2001**, *276*, 14092
7. Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Beck-Sickinger, A. G.; Doods, H. N. *Eur. J. Pharmacol.* **1994**, *271*, R11.
8. Birks, J. B. *Rep. Prog. Phys.* **1975**, *38*, 903
9. Winnik, F. M. *Chem. Rev.* **1993**, *93*, 587
10. Cho, H. K.; Lee, D. H.; Hong, J. *Chem. Comm.* **2005**, 1690
11. Chaudhuri, A.; Haldar, S.; Chattopadhyay, A. *Biochem. Biophys. Res. Commun.* **2009**, *390*, 728

12. Conlon, P.; Yang, C. J.; Wu, Y.; Chen, Y.; Martinez, K.; Kim, Y.; Stevens, N.; Marti, A. A.; Jockusch, S.; Turro, N. J.; Tan, W. *J. Am. Chem. Soc.* **2008**, *130*, 336
13. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B. *Bioorg. Med. Chem.* **2010**, *18*, 6292
14. Keller, M. Dissertation Thesis, University of Regensburg, **2008**
15. Develay, S.; Williams, J. A. G. *Dalton Trans.* **2008**, 4562
16. Vollmann, H.; Becker, H.; Corell, M.; Streeck, H.; Langbein, G. *Justus Liebigs Annalen der Chemie* **1937**, *531*, 1.
17. Zhaoqiang, W. *J. Appl. Polym. Sci.* **2008**, *110*, 777
18. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; Konig, B. *Bioorg. Med. Chem.* **2008**, *16*, 9858.
19. Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. *J. Med. Chem.* **2008**, *51*, 8168.
20. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*: Springer, **1999**

CHAPTER 3

Towards Target Specific Fluorescent Liposomes for the Neuropeptide Y_1 Receptor

3.1 Introduction

The selective interaction of liposomes with cells is a very important task in modern medicinal chemistry. Liposomes are spherical vesicles that form lipid bilayers in aqueous solution and enclose a small aqueous volume in their interior.[1] They are widely-used as biocompatible and biodegradable drug carriers for several drugs like for example doxorubicin[1, 2] and as chemosensors in analytical chemistry.[3] Compared to the administration of the drug alone these preparations can increase the effectiveness of the drug via an enrichment in malignant or inflamed tissues.[2] In this case it is not a receptor mediated selectivity, but an effect of the more penetrable tissue. The selectivity of the liposomes can be further improved by attaching a receptor selective antagonist or antibody to the liposome bilayer. This was studied for example for neuroblastoma cells overexpressing the disialoganglioside GD2 structure on the cell surface.[4] A liposome preparation containing an antibody against GD2 exhibited a strong target specific liposome accumulation at the cancer cells.[4] This antibody-antigen interaction is further amplified by multiligand binding, when many antibodies that are located on the same liposome bind to many antigens simultaneously.[5]

The neuropeptide Y_1 receptor (Y_1R) has an incidence of 85% in breast carcinoma cells and 100% in lymph node metastases.[6] Therefore this receptor is an important target for the detection of cancer cells. Agonist and antagonist decorated quantum dots (QD) as diagnostic tools for this receptor were already prepared and demonstrated a high selectivity for the Y_1R.[7] Despite the strong fluorescence of the quantum dots and the good receptor selectivity the long term toxicity is a big problem. The core of the polyethylene glycol (PEG) covered QDs consists of cadmium selenide. A metabolic degradation of the particles can deliver cadmium ions that are highly toxic. So fluorescent labeled liposomes can be a promising non toxic alternative to the QDs. In this study we prepared liposomes containing a substructure of the Y_1R selective antagonist BIBP 3226 (BIBP)[8] and lipophilic fluorescein derivatives for the detection of the MCF-7 breast cancer cells. A further advantage for the application of liposomes is their fast accessibility and the high diversity by just changing the mixture of the lipid composition.

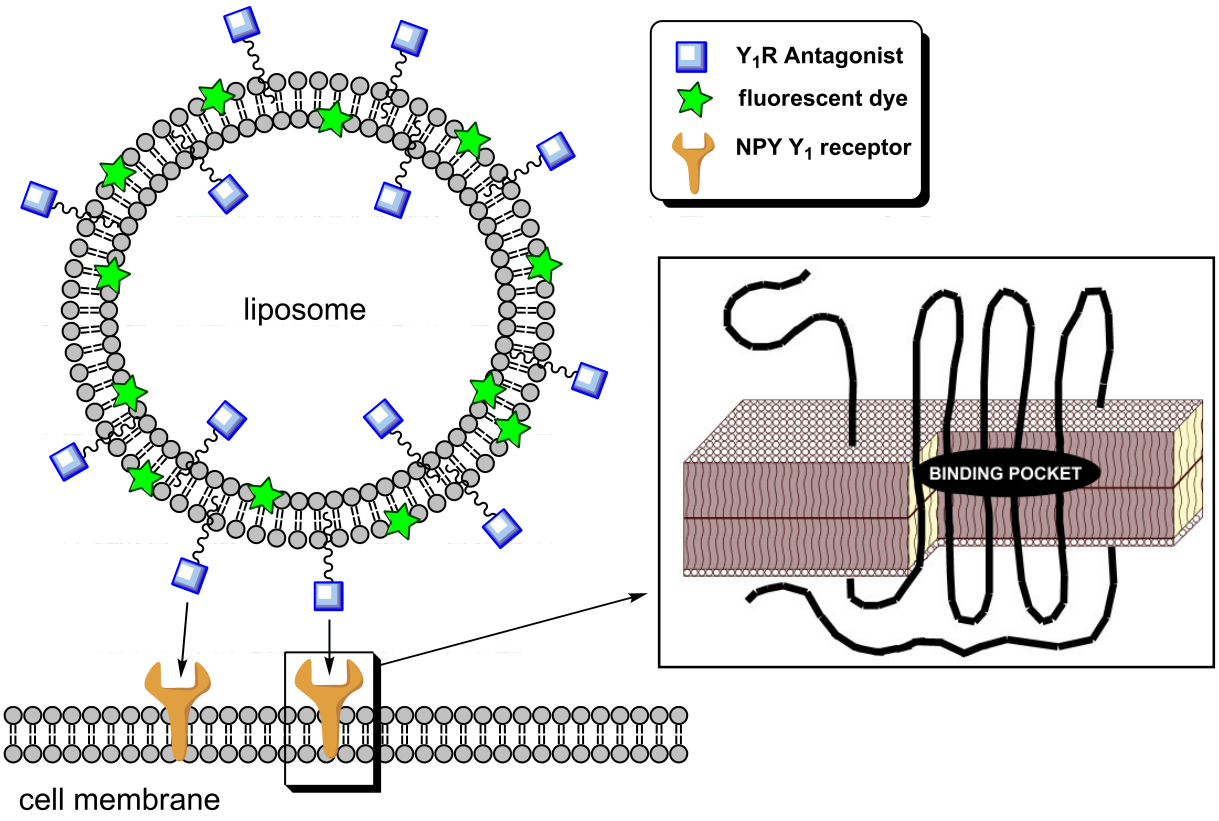


Figure 1. Antagonist mediated binding of a fluorescent labeled liposome to the NPY Y_1 receptors at the cell surface. The factual binding pocket for the antagonist is in the transmembrane domain.

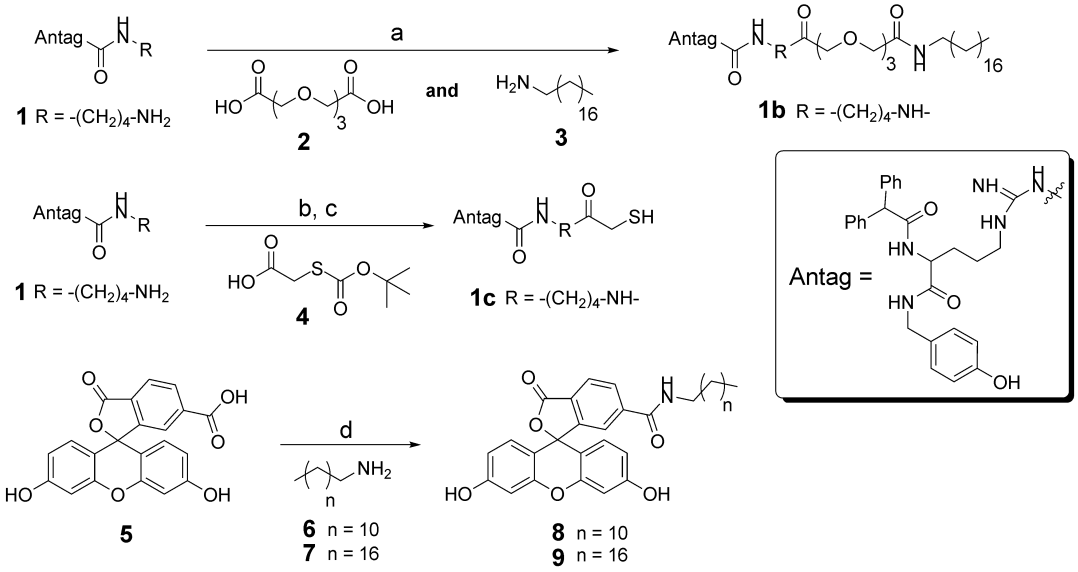
3.2 Results and Discussion

3.2.1 Synthesis of the lipophilic Y_1R antagonists and dyes

Lipophilic antagonists for the Y_1R were synthesized that can be included into the lipid bilayer as shown in Figure 1. Their structures are shown in Figure 2. The long hydrophobic tail of the compounds **1a** and **1b** is part of the lipid bilayer. Compound **1b** was prepared as an alternative to compound **1a** to gain a larger distance between the liposome bilayer and the antagonist moiety. The thiol modified antagonist **1c** was synthesized to obtain a molecule that can be "clicked" to a completed liposome via a Michael addition of the thiol to a maleinimide.[9] The synthesis of compound **1a** is described elsewhere.[10] The compounds **1b** and **1c** were synthesized starting with the antagonist precursor amine **1**. Compound **1b** was prepared via an acylation reaction of the dicarboxylic acid **2** and two amines simultaneously. Due to the statistical product distribution the yield was low. Compound **1c** was prepared from

Boc-protected 2-mercaptoacetic acid. After acylation with compound **1** the intermediate was deprotected with TFA and compound **1c** was obtained.

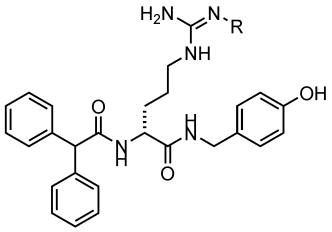
Carboxyfluorescein was chosen as scaffold for the preparation of lipophilic dyes due to its availability and good quantum yield. It was connected to dodecylamine and octadecylamine, respectively. The compounds **8** and **9** were obtained in moderate yield.



Scheme 1. Synthesis of the Y_1R antagonists **1b** and **1c** and the fluorescent dyes **8** and **9**. a) DMF, DIPEA, TBTU, 28%; b) DMF, DIPEA, TBTU; c) TFA, 25%; d) n=12: DMF, DIPEA, HOBt, DCC, 56%; n=16: DMF, NEt_3, TBTU, 55%.

3.2.2 Y_1R affinity of the lipophilic compounds

The affinities of the free ligands to the NPY Y_1R were about 200 to 500 times lower compared to the unsubstituted antagonist BIBP.



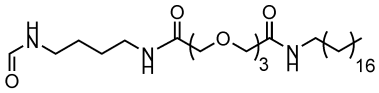
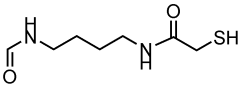
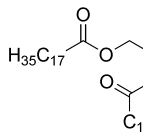
no.	R	K_i [nM]
1	H (BIBP 3226)	1.3 ± 0.2
1a		460 ± 19
1b		200
1c		390

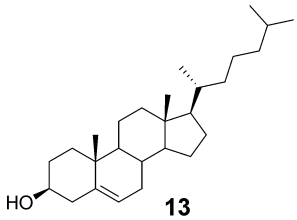
Figure 2. Structures and Y_1R affinities of the lipophilic antagonists **1a-1c**. K_i values determined from the displacement of 1.5 nM [^{3}H]-UR-MK114[11] on SK-N-MC cells; the mean values ± SEM are from two independent experiments (**1**, **1a**) or from one single experiment (**1b**, **1c**).

3.2.3 Formulation of the liposomes and studies on MCF-7 cells

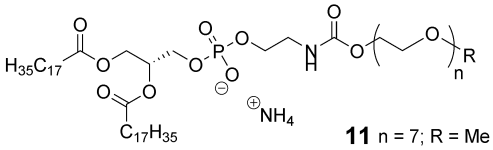
The liposomes were prepared from the bilayer forming lipids DSPC and DSPE (Figure 3) as main components (section 4.3). In some formulations cholesterol was added as a membrane stabilizing additive.[2]



10



13



11 n = 7; R = Me
12 n = 45; R = $(CH_2)_2$-NH-CO-$(CH_2)_2$-N

Figure 3. Building blocks for the liposomes: DPSC **10**, PEGylated DSPE derivatives **11** and **12** and cholesterol **13**.

Table 1. Composition of the liposome preparations.

entry	Antagonist (1a – 1c)		DSPC (comp. 10)	DSPE-(PEG)$_n$ (comp. 11 - 12)		Cholesterol (comp. 13)	Lipophilic dye (comp. 8 - 9)	
1	**1a**	10%	90%	-		-	**8**	0.5%
2	**1a**	10%	-	**11**	90%	-	**8**	0.5%
3	**1b**	0.5%	99%	-		-	**9**	0.5%
4	**1b**	0.5%	97%	**11**	2%	-	**9**	0.5%
5	**1b**	0.5%	94%	**11**	5%	-	**9**	0.5%
6	**1b**	0.5%	89%	**11**	10%	-	**9**	0.5%
7	**-**		94%	**11**	5%	-	**9**	0.5%
8	**1b**	1%	93%	**11**	5%	-	**9**	0.5%
9	**1b**	2%	92%	**11**	5%	-	**9**	0.5%
10	**1b**	0.5%	64%	**11**	5%	30%	**9**	0.5%
11	**1c**	3%	97%	**12**	3%	-	**9**	0.5%
12	**1c**	3%	68%	**12**	3%	29%	**9**	0.5%

The binding of the liposomes was investigated with confocal microscopy on MCF-7 breast cancer cells. This cell line has a high density of Y_1 receptors (40 000 – 300 000 receptors per cell).[12]

The best fluorescent properties of the liposomes were achieved with a dye fraction of 0.5%, because a higher concentration caused self-quenching of the fluoresceine moieties. So the dye concentration was constant for all liposome preparations in Table 1. There was no significant difference between the dyes **8** and **9** regarding fluorescence properties and inclusion in the lipid bilayer.

In entry 1 and 2 the liposomes were prepared with the lipophilic Y_1R antagonist **1a**. The total and unspecific binding were determined after 20 minutes. In entry 1 there was a very strong receptor independent binding of the liposomes to the cell membrane (high fluorescence intensity). The replacement of DSPC with the glycol modified DSPE derivative **11** in entry 2 reduced the unspecific binding significantly, but there was no difference between total binding and the unspecific binding determined with BIBP. A possible reason for this behavior is the insufficient distance of the BIBP moiety to the lipid bilayer. The binding pocket of the Y_1R is in the transmembrane region of the receptor. To bridge the distance between the liposome surface and the receptor binding pocket we prepared and investigated liposomes with the antagonist **1b** that has a glycol spacer between the hydrophobic part and the BIBP moiety.

In entry 3 – 6 the concentration of DSPE-PEG **11** was varied to investigate the effect of the PEG protection against unspecific binding interactions. The unspecific staining of the cell membrane was very strong for entry 3 (0% PEG) and was reduced with increasing concentration of PEG modified lipid (2% - 10% PEG). Entry 5 and 6 exhibit only a weak unspecific binding to the cells. This is shown in Figure 4. According to literature a content of about 10% of the PEG derivative **11** or 2% of derivative **12** is able to completely protect a liposome with a diameter of 100 nm.[2] The fluorescence intensity of the membrane is also time dependent. A longer incubation time of the liposomes with the cells increased the fluorescence of the membrane due to an increased accumulation of liposomes on the cell surface. After 40 to 50 minutes saturation in fluorescence was reached.
In entry 7 – 9 the concentration of antagonist **1b** was varied while the amount of PEG-lipid **11** (5%) was constant. This PEG content should reduce unspecific binding and still allow the BIBP moieties to bind to the Y_1R. The increased density of BIBP moieties resulted in no advancement in receptor specific binding of the liposomes. Entry 8 and 9 behave equally to the blank sample in entry 7. In entry 10 cholesterol was added as a membrane stabilizing compound, but there was no improvement compared with entry 5 without cholesterol.[2]

The general strategy of the liposome preparation was changed in entry 11 and 12. Here the DSPE-maleinimide derivative **12** was included into the liposome membrane together with DSPC and the dye. After extrusion the completed unilamellar liposomes were incubated with the thiol derivative **1c** overnight (Figure 5). Thiols attack maleinimides via a Michael addition under mild conditions and form a thioether bond. The thiol reactivity under these conditions (aqueous buffered solution, pH 7.4, room temperature) was verified with a test reaction between compound **1c** and maleinimide-N-propionic acid. Complete conversion in this similar system was observed after 24 hours. This post-modification technique placed the BIBP moiety in a large distance to the liposome surface (about 45 PEG-units) and leads to a much higher flexibility of the antagonist. So compared to the strategy in entry 1 – 10 the BIBP moiety should now be able to reach the transmembrane binding pocket of the receptor and elicit Y_1 receptor specific binding.
Unfortunately there was also no specific binding observed. A possible reason for this is the slightly lipophilic BIBP moiety that can possibly be included into the surface of the lipid bilayer. In this case the BIBP moieties would not be available for a specific interaction with the Y_1 receptor and the liposome preparations lose their selectivity.

3.3 Conclusion and Outlook

In this study we prepared liposomes containing a NPY Y_1 receptor selective BIBP moiety. Although a receptor selective binding of the liposomes to MCF-7 cells was not achieved the beneficial effect of PEG-modified liposomes was demonstrated. The PEG chains reduced the unspecific interactions of the liposome with the cell membrane significantly. A further problem that must be solved is the possible insertion of the BIBP moiety in the hydrophobic bilayer. An exchange of the BIBP moiety with a Y_1 receptor selective antibody or a thiol modified NPY derivative can avoid this problem, because of the lower lipophilicity of the proteins. In summary some further improvements of the liposome composition and surface modifications can lead to Y_1 receptor selective probes.

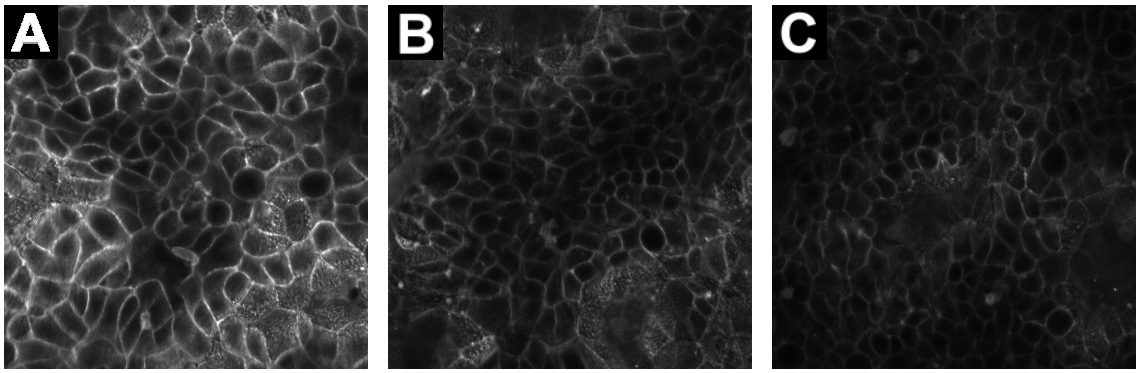


Figure 4. Fluorescence of the cell membranes after 20 minutes incubation time with liposomes containing different amounts of PEG. Pictures **A**: entry 3 (0% PEG), **B**: entry 5 (5% PEG) and **C**: entry 6 (10% PEG).

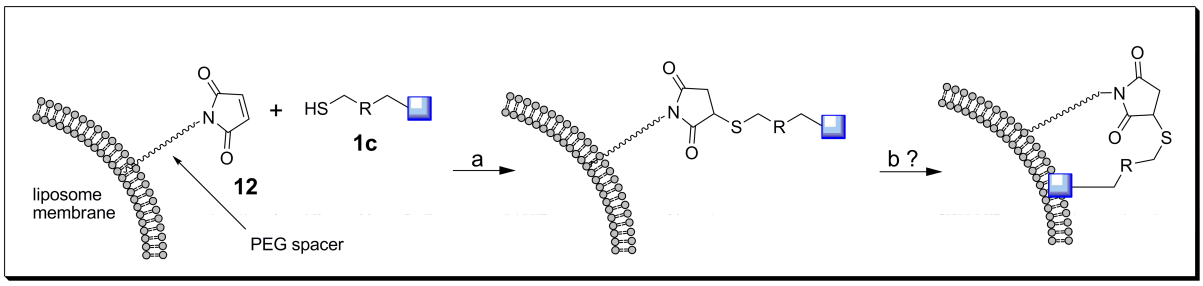


Figure 5. The antagonist **1c** is attached to the liposome over the PEG-maleinimide functional group (a). The PEG spacer separates the liposome surface from the receptor selective BIBP moiety (blue). Step b illustrates the possibly occurring undesired insertion of the BIBP moiety into the bilayer.

3.4 Experimental

3.4.1 General experimental conditions

Unless otherwise noted, solvents (analytical grade) were purchased from commercial suppliers and used without further purification. Ethyl acetate (EA), petrol ether (PE, 60 – 70 °C), methanol and dichloromethane were obtained in technical grade and distilled before application. Acetonitrile (MeCN) for HPLC was obtained from Merck (Darmstadt, Germany). Dodecylamine (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany), octadecylamine (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany), DSPC (Avanti Polar Lipids Inc., Alabaster, USA) and compounds **11** – **12** (Avanti Polar Lipids Inc., Alabaster, USA) were purchased. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18 (250 × 21 mm, 5 µm; Macherey-Nagel, Germany) and a Eurospher-100 C18 (250 × 32 mm, 5 µm; Knauer, Germany) served as RP-columns at flow rates of 20 and 38 mL/min, respectively. Mixtures of MeCN and diluted aqueous TFA (0.1 %) were used as mobile phase. ^{1}H-NMR spectra were recorded at 300 MHz on a Bruker Avance 300 spectrometer or at 600 MHz on a Bruker Avance III 600 with cryogenic probehead (Bruker, Karlsruhe, Germany). ^{13}C-NMR spectra were recorded at 75 MHz on a Bruker Avance 300 spectrometer. All chemical shifts values are reported in ppm. UV/VIS spectra were recorded with a Varian Cary BIO 50 UV/VIS/NIR spectrophotometer (Varian Inc., CA, USA). Fluorescence spectra were recorded with a Cary Eclipse spectrofluorimeter (Varian Inc., CA, USA). Mass spectra: Finnigan SSQ 710A (EI), Finnigan MAT 95 (CI), Finnigan MAT TSQ 7000 (Thermo FINNIGAN, USA) (ES/LC-MS). LC-system for LC-MS: Agilent 1100 (Palo Alto, USA). LC-MS method (LC-MS): Column: Phenomex Luna C18, 2.5 µm, 50 x 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.40 mL/min; solvent A (water + 0.1% TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 8 min [A/B 2/98], 11 min [A/B 2/98], 12 min [A/B 95/5], 15 min [A/B 95/5]. Melting points were determined with a Lambda Photometrics Optimelt MPA100 apparatus (Lambda photometrics, Harpenden, UK), they are not corrected. Thin layer chromatography (TLC) was performed on alumina plates coated with silica gel (Merck silica gel 60 F_{245}, thickness 0.2 mm). Column chromatography (CC) was performed with Merck Geduran SI 60 silica gel as the stationary phase.

Compounds **1**,[13] **4**,[14] and **8**[3] were prepared according to literature procedures.

3.4.2 Synthetic protocols and analytical data

(R)-4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,18,28-tetraoxo-20,23,26-trioxa-2,8,10,12,17,29-hexaazaheptatetracontan-9-iminium 2,2,2-trifluoroacetate (1b)

The dicarboxylic acid **2** (5.3 mg, 23.9 µmol) was dissolved in 2 mL of DMF and DIPEA (28 mg, 217 µmol) and TBTU (15.2 mg, 47.4 µmol) were added. After 10 minutes the reaction was started by the addition of the amines **1** (20 mg, 21.5 µmol) and **3** (5.8 mg, 21.5 µmol). The next day DMF was removed completely and the oily residue was purified with HPLC. A white solid was obtained (7.0 mg, 28%), m.p. > 190°C. **^{1}H NMR** (300 MHz, CD_3CN): 0.88 (t, J=6.66, 3H), 1.20 – 1.35 (m, 30H), 1.35 – 1-60 (m, 10H), 3.00 – 3.30 (m, 8H), 3.55 – 3.65 (m, 8H), 3.88 (s, 2H), 3.89 (s, 2H), 4.10 – 4.30 (m, 2H), 4.30 – 4.30 (m, 1H), 5.02 (s, 1H), 6.60 – 7.50 (m, 8H), 6.67 (d, J=8.45, 2H), 7.00 (d, J=8.45, 2H), 7.20 – 7.35 (m, 10H), 9.14 (bs, 1H). **$C_{58}H_{90}N_8O_9$: MS** (LC-MS): *m/z*(%) [t_r= 8.1min.]: 522(25), 1043(100, M+).

(R)-4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-19-mercapto-3,11,18-trioxo-2,8,10,12,17-pentaazanonadecan-9-iminium 2,2,2-trifluoroacetate (1c)

Boc-protected 2-mercaptoacetic acid (4.5 mg, 23.7 µmol) was dissolved in 2 mL of DMF and DIPEA (28 mg, 215 µmol) and TBTU (7.6 mg, 23.7 µmol) dissolved in 125 µL of MeCN were added. After 5 minutes compound **1** (20 mg, 21.5 µmol) was added and the mixture was stirred overnight. The next day DMF was evaporated completely and TFA was added (3 mL) and the mixture was stirred for 4 hours at room temperature. TFA was removed and the residue was purified with HPLC. A white solid was obtained (4.2 mg, 25%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 8H), 2.72 (t, J=7.93, 1H), 3.07 (d, J=7.93, 2H), 3.00 – 3.25 (m, 6H), 4.05 – 4.25 (m, 2H), 4.30 – 4.40 (m, 1H), 5.13 (s, 1H), 6.67 (d, J=8.47, 2H), 7.00 (d, J=8.47, 2H), 7.15 – 7.35 (m, 10H), 7.49 (bs, 1H), 8.02 (t, J=5.35, 1H), 8.39 (t, J=5.50, 3H), 8.50 (d, J=8.24, 1H), 8.87 (bs, 1H), 9.30 (bs, 1H), 9.49 (bs, 1H). **$C_{34}H_{43}N_7O_5S$: MS** (LC-MS): *m/z*(%) [t_r=5.48]: 662(100, MH+), 1324(5).

3',6'-Dihydroxy-N-octadecyl-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxamide (10, mixture of 5' and 6' isomer) (9)

Carboxyfluorescein (50 mg, 0.13 mmol, mixture of 5' and 6' isomer) was dissolved in 2 mL of DMF and a mixture of NEt_3 (50 mg, 0.50 mmol) and TBTU (48 mg, 0.15 mmol) in 1 mL of MeCN was added dropwise. After 15 minutes octadecylamine (35 mg, 0.13 mmol) dissolved in 1 mL DCM was added slowly. The reaction mixture was stirred 1 hour at 60°C. Then DMF was removed completely and the residue was dissolved in 10 mL DCM. Aqueous $NaHSO_4$

solution (20 mL, 5%w) was added and an orange solid precipitated. It was filtered off and dried. The crude material was purified with HPLC. A light yellow solid was obtained (45 mg, 55%), m.p. 236°C (decomp.). **^{1}H NMR** (300 MHz, MeOD): 0.89 (t, J=6.64, 3H), 1.00 – 1.80 (m, 32H), 3.20 – 3.50 (m, 2H), 6.50 – 6.80 (m, 5H), 6.85 – 8.80 (m, 4H). **$C_{39}H_{49}NO_6$: MS** (LC-MS): *m/z*(%) [t_{r1}=11.05 min.; t_{r2}=11.16]: 628(100, MH+), 1256(5). **Fluorescence** ($\lambda_{ex.}$ = 488 nm, MeOH + 10% NaOH(1M)): 527 nm (broad).

3.4.3 Vesicle preparation

In small glass reaction vessels DSPC was dissolved in chloroform and optionally the compounds **1a** or **1b** and **11** - **13** were added (about 1 µmol final lipid concentration). The solvent was completely removed under reduced pressure and an appropriate amount of buffer (HEPES 25 mM, pH 7.4) was added to obtain a final lipid concentration of 450 µM. Heating to 75 °C and vigorous shaking for 5 – 10 minutes yielded a turbid multi-lamellar vesicle suspension. Small uni-lamellar vesicles (SUV) were obtained by extrusion through 100 nm-pore size polycarbonate membranes with a LiposoFast liposome extruder from Avestin (Mannheim, Germany).[15] PCS measurements were performed on a Malvern Zetasizer 3000 at 25 °C using 1 cm disposable polystyrene fluorescence cuvettes (VWR). Three subsequent measurements of 60 seconds each were performed for each sample. Data analysis was performed using the Malvern PCS software.
For the post-modification of the liposome preparations entry 11 and 12 the thiol **1c** (1.0 eq.) was added after the extrusion of the liposomes and the mixture was incubated for 24 hours prior to testing.

3.4.4 Size exclusion chromatography

Vesicle dispersions were separated from low molecular weight solutes on minicolumns of Sephadex LH-20 gel filtration media by a previously described procedure.[16]

3.4.5 Cell culture and confocal microscopy

MCF-7 cells were cultivated at 37 °C for two days in 8 well µ-slides (Ibidi GmbH, Munich) with EMEM medium (Sigma, Deisenhofen, Germany) containing 5% FCS (Biochrom AG, Berlin, Germany). Estradiol (1 nM) was added to the cultivation medium to stimulate Y_1R

expression.[12] After two days, shortly before the experiment the medium was replaced by Leibovitz L15 medium (LM).

Confocal microscopy was performed with a Zeiss Axiovert 200M microscope, equipped with a LSM 510 laser scanner. The carboxyfluorescein label was excited with a 488 nm laser. The emission was detected after a 505 nm long pass filter. The objective was a Plan-Neofluar 40x/1.3 with oil immersion.

3.5 References

1. Lian, T.; Ho, R. J. Y. *J. Pharm. Sci.* **2001**, *90*, 667
2. Lasic, D. D.; Martin, F. J. *Stealth Liposomes*: CRC Press, **1995**
3. Gruber, B.; Stadlbauer, S.; Späth, A.; Weiss, S.; Kalinina, M.; König, B. *Angew. Chem.* **2010**, *122*, 7280
4. Gantert, M.; Lewrick, F.; Adrian, J. E.; Rössler, J.; Steenpaß, T.; Schubert, R.; Peschka-Süss, R. *Pharm. Res.* **2009**, *26*, 529
5. Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. *Angew. Chem. Int. Ed. Engl.* **2006,** *45*, 2348
6. Reubi, J. C.; Gugger, M.; Waser, B.; Schaer, J. *Cancer Res.* **2001**, *61*, 4636
7. Hild, W.; Pollinger, K.; Caporale, A.; Cabrele, C.; Keller, M.; Pluym, N.; Buschauer, A.; Rachel, R.; Tessmar, J.; Breunig, M.; Goepferich, A. *P. Natl. A. Sci.* **2010**, *107*, 10667
8. Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Beck-Sickinger, A. G.; Doods, H. N. *Eur. J. Pharmacol.* **1994**, *271*, R11.
9. Schelté, P.; Boeckler, C.; Frisch, B.; Schuber, F. *Bioconjugate. Chem.* **2000**, *11*, 118
10. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B. *Bioorg. Med. Chem.* **2010**, *18*, 6292
11. Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. *Journal of Medicinal Chemistry* **2008**, *51*, 8168.
12. Memminger, M. Dissertation Thesis, University of Regensburg, **2009**
13. Keller, M. Dissertation Thesis, University of Regensburg, **2008**
14. Xiang, Y.; Liao, Y.; Zhang, J.; Zhang, D.; Chen, S.; Lu, Q.; Zhang, Y.; Lin, H.; Yu, X. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3458
15. MacDonald, R. C.; MacDonald, R. I.; Menco, B. P. M.; Takeshita, K.; Subbarao, N. K.; Hu, L. *Biochim. Biophys. Acta* **1991**, *1061*, 297
16. Fry, D. W.; White, J. C.; Goldman, I. D. *Anal. Biochem.* **1978**, *90*, 809

CHAPTER 4

Towards the Catalytic Staining of NPY Y_1 Receptors on Living MCF-7 Cells with DMAP Modified BIBP 3226 Derivatives

4.1 Introduction

G protein-coupled receptors (GPCR) like the neuropeptide Y_1 receptor (Y_1R) belong to one of the major classes of biological targets in modern medicinal chemistry.[1] There are four subtypes of the NPY receptor known to date (Y_1, Y_2, Y_4 and Y_5).

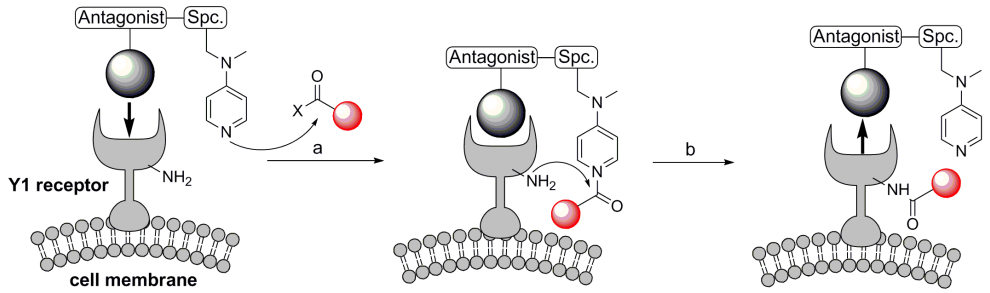
Recent investigations concerning the signalling of GPCR´s revealed the importance of receptor-protein interactions, e. g. the interaction with β-arrestins[2] that can cause receptor internalisation or initiate signalling cascades independent of the G protein (biased agonism).[2, 3] For the investigation of these interactions a fluorescent label at the receptor protein is necessary. Fluorescent labeled antagonists were successfully applied for the investigation of the NPY Y_1 receptor.[4] However, these ligands have two disadvantages concerning the investigation of receptor-protein interactions: (1) They do not form a covalent bond and can dissociate from the receptor. (2) The ligands bind to the same binding site as the agonist NPY and inhibit the binding of NPY to the Y_1R.

A covalent labeling of the receptor protein aside the NPY binding site can circumvent this problem. Our strategy was to label the Y_1R covalently using a catalyzed acyl-transfer reaction. This would still allow binding of agonists and antagonists to the labeled Y_1R, as the binding site of the receptor is not blocked by an Y_1R ligand after a washing step. DMAP is a well known nucleophilic acyl-transfer catalyst that was successfully applied for the target-specific acylation of lectins.[5, 6] In our investigations DMAP was connected to the potent Y_1R antagonist BIBP 3226[7] to obtain catalytically active Y_1R ligands. The BIBP 3226 skeleton tolerates acylation at the guanidine N-terminus with moderate loss or gain of affinity.[8-11]

A fluorescent active ester should then perform the catalytic Y_1R specific staining reaction (Scheme 1).[6] MCF-7 cells were selected as biological target for our experiments, because they express a high density of Y_1 receptors (40 000 – 300 000 receptors per cell).[12]

In this study we synthesized catalytically active antagonists with nanomolar affinity to the Y_1R and investigated the catalytic staining reaction (acyl-transfer) with simple nucleophiles

and with living MCF-7 cells expressing the Y_1R. Acyl-transfer to nucleophiles was studied with LC-MS and staining of the receptors was monitored with confocal microscopy.



Scheme 1. Catalytic staining of the Y_1R. The Y_1R is simplified in this scheme, it is a transmembrane receptor with a transmembrane binding pocket (GPCR). In a) the DMAP moiety of a catalytic antagonist attacks a fluorescent active ester. A nucleophilic amino acid (e.g. lysine) of the receptor protein is acylated in step b).

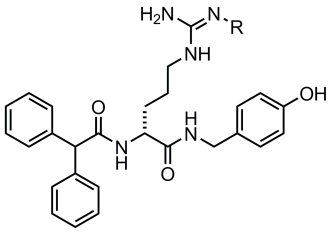
4.2 Results and Discussion

4.2.1 Synthesis and binding affinity of the catalytic active Y_1R antagonists

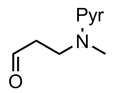
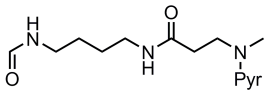
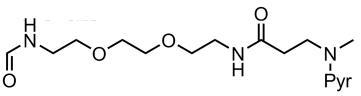
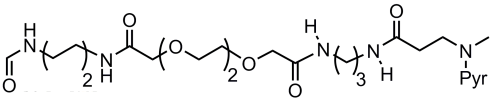
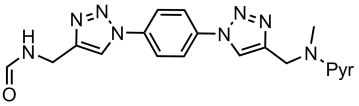
The first step in our approach was to covalently link the highly potent Y_1R antagonist BIBP 3226 **1** to the DMAP moiety. The DMAP substructure was derivatized at one of the N-methyl groups, because derivatives with longer alkyl chains at the N-amino nitrogen are as active as DMAP itself.[13] Synthesis started from 4-aminopyridine **2** that was converted to the catalytic precursor acid **5** in two steps. This acid was combined with spacers of different length and chemical structure to obtain catalytic compounds with adequate properties (high affinity to the Y_1R and good catalytic activity, table 1). Compound **1a** was synthesized according to scheme 3. Coupling procedures with *N*-Boc-*S*-methylisothiourea **6** and guanidinylation reactions with compound **8** are described in literature.[10, 11] Compounds **1b** and **1c** were obtained from a coupling reaction of the precursor amines **9** and **10** with the acid **5** in moderate yield. The compound with the longest spacer **1d** was also obtained from precursor amine **9**. It was coupled in a one pot reaction with the dicarboxylic acid **13** and amine **12**. Amine **12** was synthesized from acid **5** and the mono-Boc protected 1,3-propyldiamine. The rigid ligand **1e** was synthesized starting from 1,4-phenylenediamine. It was converted to the diazide **15** and followed by a copper catalysed "click-reaction" amine **16b** was obtained. Further coupling and guanidinylation resulted azide **18**. This was transformed in a cycloaddition with the propargyl derivative **20** to the rigid Y_1R antagonist **1e**.

The affinity was best for the antagonists **1c** and especially for **1d** as they were the most flexible and least sterically hindered compounds. A close proximity of the pyridine ring to the pharmacophore (**1a**) is unfavorable.

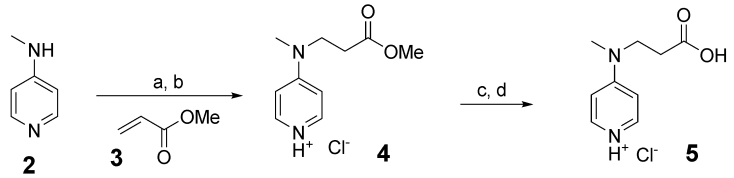
Table 1. Structures and Y_1R affinities of the catalytically active compounds **1a-1e**.



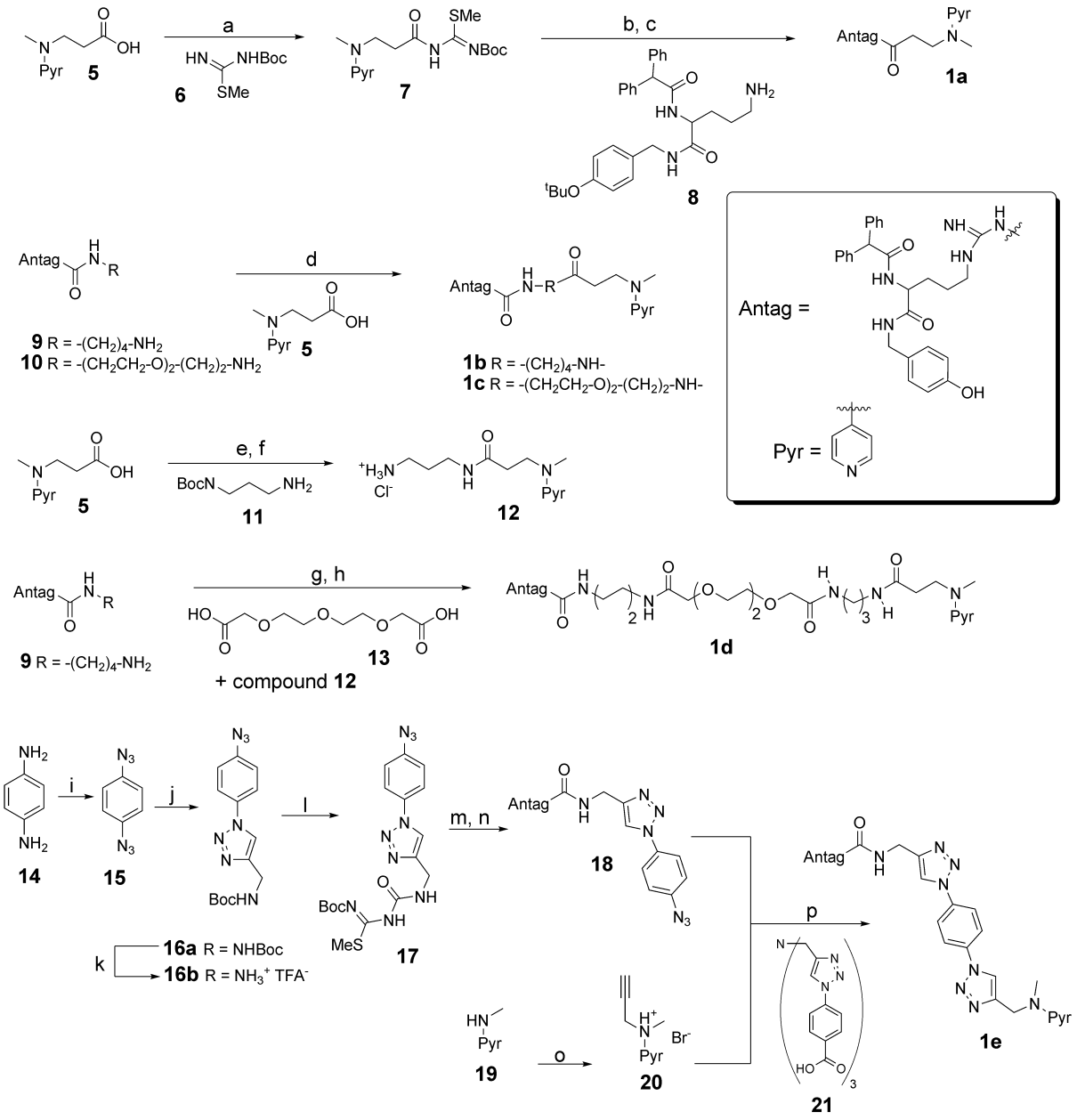
Pyr = 

no.	R	K_i [nM][a]
1	H (BIBP 3226)	1.3 ± 0.2
1a		53 ± 13
1b		96 ± 20
1c		90 ± 6
1d		78 ± 0.4
1e		400 ± 19

[a] K_i values determined from the displacement of 1.5 nM [^{3}H]-UR-MK114[14] on SK-N-MC cells; all mean values ± SEM from two or three independent experiments.



Scheme 2. Synthesis of the precursor **5** of the catalytic substructure. a) methyl acrylate, reflux; b) HCl, 76%; c) NaOH, MeOH; d) HCl, 30%.



Scheme 3. Synthesis of Y_1R antagonists **1a – 1e**. a) EDC, HOBt, DIPEA, DCM, 75%; b) DMF, $HgCl_2$, NEt_3, 36%; c) TFA/DCM 1:1, quantitative yield; d) DMF, DIPEA, TBTU, 67% (**1b**), 47% (**1c**); e) MeCN, DIPEA, DCC; f) MeOH/HCl 3:1, 36%; g) DMF, DIPEA, TBTU; h) MeCN, TFA, 16%. i) $NaNO_2$, H_2SO_4, NaN_3, 66%; j) Boc-propargylamine, $CHCl_3$/MeOH/H_2O, ascorbate, copper sulphate pentahydrate, 79%; k) DCM/TFA, quantitative; l) triphosgene, DCM, DIPEA; *N*-Boc-S-methyl-isothiourea, DCM, 46%; m) Compound **8**, DMF, NEt_3, $HgCl_2$, 57%; n) DCM/TFA, quantitative; o) THF, n-BuLi, propargylbromide, 10%; p) DMSO, MeOH, H_2O, ascorbate, copper sulphate pentahydrate, 51%.

4.2.2 Synthesis and investigation of active esters

For the catalytic staining reaction of the receptors we prepared active esters that can be activated with DMAP easily. Two types of fluorescent dyes were modified for their application as acyl-donors: the carboxyfluorescein and the cyanine scaffold (Figure 1). Compound **23** was prepared according to the procedure of Hamachi and coworkers.[6] The thiophenolester **26** and the p-nitrophenolester **27** were synthesized from the commercially available cyanine dye **24** in one coupling step.

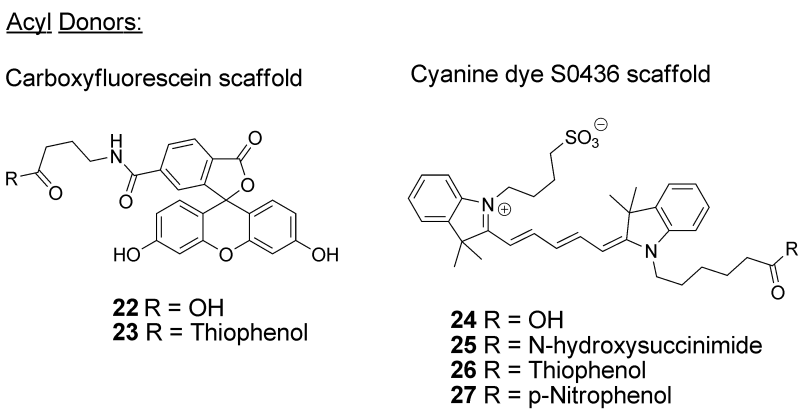


Figure 1. Structures of the acyl-donors based on the carboxyfluorescein and the cyanine dye scaffold

Before we started staining the MCF-7 cells, we investigated the hydrolysis kinetics of the active esters **22**, **25** and **27** and the acyl-transfer of the Y_1R ligands to simple nucleophiles in buffered aqueous solution at pH 7.4. For the measurements of the hydrolysis kinetics the dyes were incubated at 50 µM for 3 hours in phosphate buffer (30 mM) at pH 7.4. In a second series they were incubated with additional 50 µM DMAP. Samples were taken after 30, 90 and 150 minutes. The amount of decomposition was determined with HPLC (figure 2).

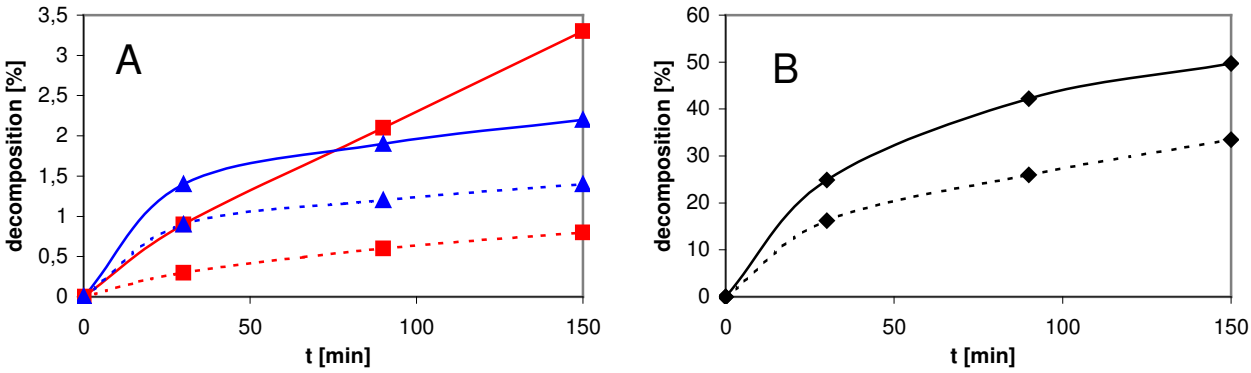


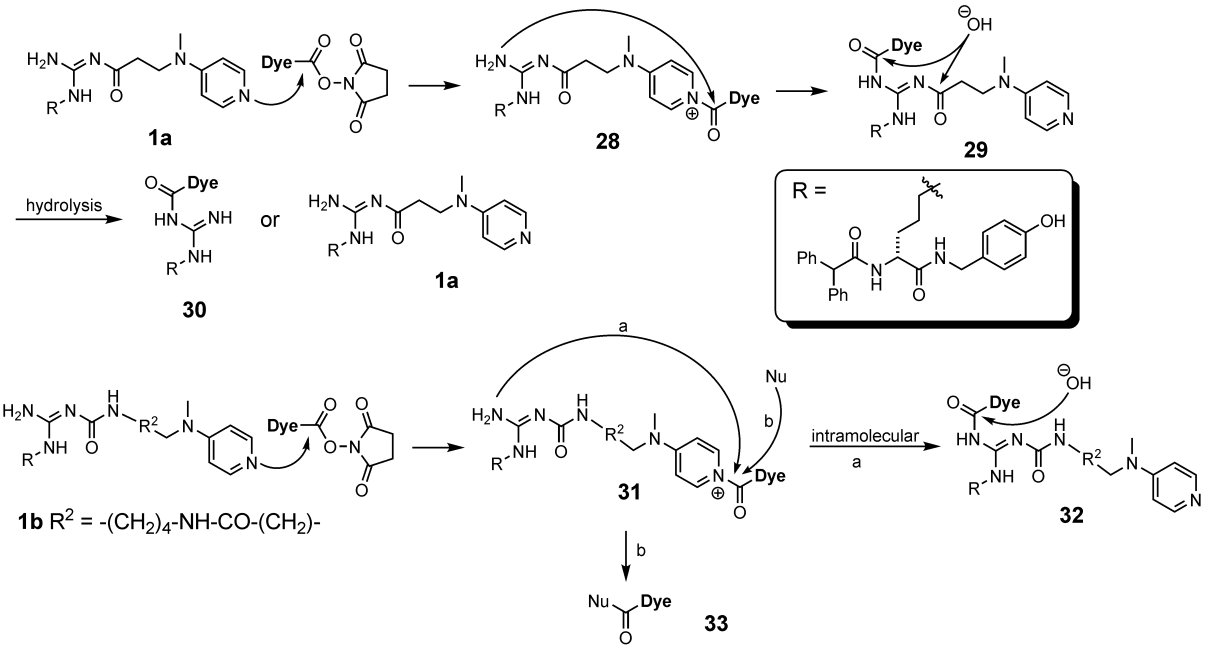
Figure 2. Diagram A: Hydrolysis of thiophenolester **22** (red) and p-nitropenolester **27** (blue) both 50 µM in presence (solid line) and absence (dashed line) of 50 µM DMAP. Diagram B: Hydrolysis graph of NHS ester **25** (50 µM) in presence (solid line) and absence (dashed line) of DMAP.

The acceleration of the hydrolysis with DMAP was most significant for thiophenolester **22** (about 400 % after 150 minutes) and only moderate for p-nitrophenolester **27** (60 %) and NHS ester **25** (48 %). Also the hydrolysis rates are not directly comparable to the acyl-transfer reaction rate to other nucleophiles, it indicates a superior selectivity of the thioester compared to the p-nitrophenol and the NHS ester. Active ester reactivity correlates with the acidity of the corresponding acid: pK_a(NHS): 6.0[15]; pK_a(p-nitrophenol): 7.4[15]; pK_a(thiophenol): 8.3[16].

4.2.3 Acyl-transfer studies

Intermolecular interactions at the cell surface are rather complex. Therefore we studied first the catalysed acyl-transfer to simple nucleophiles in buffered aqueous solution optimizing the reaction conditions. Incubation of the catalytic compound **1a** and the active ester **25** in solution and subsequent HPLC analysis demonstrated the complete decomposition of **1a** to compound **30** after an incubation time of 3 hours. As described in literature acyl-guanidines are labile against intra-molecular cleavage.[11, 17] We assume the formation of diacylguanidine **29** (Scheme 4) and fast hydrolysis of this labile intermediate.[18] In contrast the catalytic compound **1b** was not degraded after the incubation with the active ester. The guanidine-carbamoyl substructure prevents cleavage of the DMAP moiety, because of the electron donating effect of the second N-atom at the acyl-guanidine carbon. After the incubation period of 3 hours compound **1b** and compound **32** were detected by LC-MS. Therefore acyl-

guanidine **1a** was not applicable for the staining reaction, but the more stable carbamoyl-guanidines **1b** – **1e** are promising compounds. However, the fast intramolecular self-acylation of these compounds competes with the acyl-transfer to other nucleophiles and increases the hydrolysis rate of the active ester. The catalytic Y_1R antagonist **1e** was designed to circumvent this problem. The long and rigid spacer between the DMAP moiety and the BIBP 3226 scaffold prevents the intramolecular self-acylation.



Scheme 4. Self-acylation of the BIBP 3226 scaffold depicted for the Y_1R antagonists **1a** and **1b** with NHS ester derivative **25**. In the case of antagonist **1a** the catalytic self-acylation and following hydrolysis of the diacyl-guanidine leads to a fast formation of compound **30**. In case of compound **1b** the catalytic DMAP moiety was not cleaved.

Catalytic acyl-transfer to small nucleophiles was tested for the Y_1R antagonists **1b** – **1e**. 2-(Dimethylamino)ethylamine (DMAEA), n-butanol and BIBP 3226 (BIBP) were used as nucleophiles. They represent the most common nucleophilic residues in proteins (amine for lysine, butanol for serine and threonine, BIBP 3226 for arginine and tyrosine). The NHS ester **25** was tested exemplarily as acyl-donor. It was incubated 30 minutes with catalyst and nucleophile, the acylation products were detected with LC-MS measurements.

The hydrolysis rate of active ester **25** was accelerated in entry 2 – 6 (catalysed) compared with entry 1 (uncatalysed). Antagonists **1b** – **1e** were even more active as DMAP itself and

consumed NHS ester **25** completely (entry 3-5) or almost completely (entry 6). Acyl-transfer to BIBP 3226 was increased by adding the catalysts (entry 8 – 11) compared to the uncatalysed reaction (entry 7). The sterically hindered catalyst **1e** still forms an appreciable amount of the catalyst-dye byproduct (entry 6, 11, 13, 15). Due to the rigid structure we suggest the sideproduct is formed via an intermolecular attack of another molecule of **1e**. The primary alcohol n-butanol was not attacked by the active ester (entry 14, 15) due to its weak nucleophilic properties.

In summary the catalysts **1b** – **1e** were promising candidates for the catalytic staining of biological targets despite their self-acylation: the BIBP 3226 scaffold binds tightly to the Y_1R binding pocket and should prevent self-acylation during the nucleophilic attack of the DMAP moiety.

Table 2. Catalysed and uncatalysed acyl-transfer of NHS ester **25** to small nucleophiles (product yields were not quantified, the peak heights show only detected products and reaction tendencies)

				(% rel. peak height) after 30 minutes incubation time[a]			
entry	**active ester**	**catalyst**	**nucleophile**	**free acid (hydrolysis product)**	**active ester (educt)**	**catalyst-dye (byproduct) structure 32**	**nucleophile-dye (product) structure 33**
1	**25**	-	Water	79	21	-	-
2	**25**	DMAP	Water	92	8	-	-
3	**25**	**1b**	Water	78	0	22	-
4	**25**	**1c**	Water	83	0	17	-
5	**25**	**1d**	Water	83	0	17	-
6	**25**	**1e**	Water	75	4	21	-
7	**25**	-	BIBP	57	19	-	24
8	**25**	**1b**	BIBP	55	0	11	34
9	**25**	**1c**	BIBP	47	0	13	40
10	**25**	**1d**	BIBP	45	0	15	40
11	**25**	**1e**	BIBP	42	0	8	50
12	**25**	-	DMAEA	58	13	-	29
13	**25**	**1e**	DMAEA	74	0	13	13
14	**25**	-	n-butanol	77	23	-	-
15	**25**	**1e**	n-butanol	82	4	14	-

[a] relative fraction of the compounds in percent (the sum of all four peak heights = 100)

4.2.4 Catalytic Staining of MCF-7 cells

The active esters **23**, **25** - **27** were also attacked by cell-nucleophiles without catalyst, so we first monitored the uncatalysed staining of MCF-7 cells. The cells were incubated with 10 µM

of active ester and stained for 15 – 60 minutes in PBS (pH 7.4). The thioesters **23** and **26** were not applicable for the staining of the Y_1R of MCF-7 cells, because they pass the cell membrane and accumulate in the cell (exemplarily shown for thioester **26** in Figure 3). The p-nitropenol ester **27** penetrated the membrane to a significantly lower extent and the NHS ester **25** proved to be the most applicable active ester, as is can not pass the membrane.

For the catalytic staining reaction the cells were pre-incubated with the corresponding catalytic antagonist, washed with buffer and incubated with the NHS ester **25**. This is exemplarily shown for the antagonists **1c** and **1d**. Compared with the non-catalysed staining reaction there is no difference visible in the confocal microscopy pictures (Figure 4). The fraction of nucleophiles belonging to the Y_1R compared with the total amount of nucleophiles on the surface of a cell is very small, so the unspecific staining of the cell membranes is a problem.

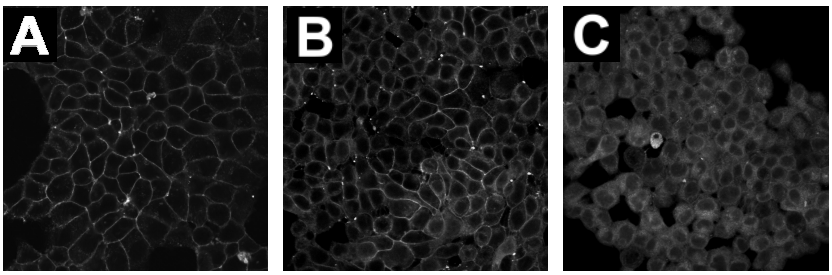


Figure 3. Picture A: cells were incubated with 20 µM NHS ester **25** for 30 min. Picture B: 10 µM p-nitrophenol ester **27**, 30 min. Picture C: 10 µM thiophenolester **26**, 30 min.

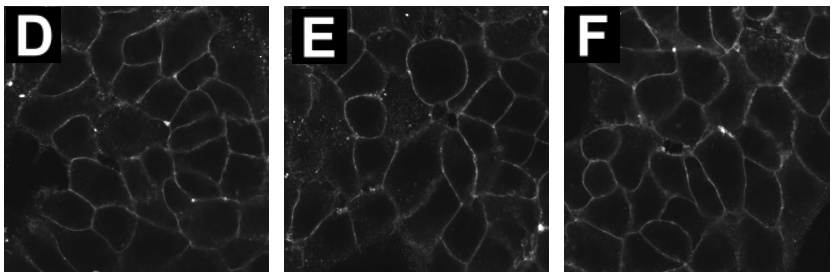


Figure 4. Pictures D and E: cells were preincubated with 200 nM **1c** (D) or **1d** (E). Subsequently the cells (pictures D – F) were washed and stained with 10 µM NHS ester **25** for 30 minutes. Pictures were taken after washing the cells with buffer. The fluorescent appearance of the catalytically stained cells (D, E) is similar to the non-specific stained cells (F).

4.3 Conclusion

In this study we successfully prepared catalytically active derivatives of the Y_1 receptor antagonist BIBP 3226 that exhibited a nanomolar affinity to the NPY Y_1 receptor. The acyl-transfer of active esters to nucleophiles was studied in absence and presence of the catalysts. A serious problem was the self-acylation at the catalysts guanidine moiety (**32**). This intramolecular reaction is very fast and is the main reaction in absence of good nucleophiles in aqueous solution. The self-acylation was also observed for the rigid compound **1e** due to an intermolecular reaction. In the cell studies the BIBP moiety is tightly bound to the binding pocket of the Y_1R and self-acylation is therefore probably reduced. The NHS ester **25** was the most applicable staining reagent for MCF-7 cells and was not able to enter the cells (Figure 3, A). However, there was no visible difference between the catalysed and uncatalysed staining reaction (Figure 4, D and E vs. F). The high reactivity of the NHS ester towards nucleophiles caused unspecific staining of the cell membrane. The investigation of more selective staining reagents and catalysts that do not interfere with other nucleophiles on the cell membrane is an aim for further studies. This approach is not restricted to the NPY field and with modification of the pharmacophore moiety a broad variety of catalytically active antagonists for G protein-coupled receptors can be envisaged.

4.4 Experimental

4.4.1 General experimental conditions

Unless otherwise noted, solvents (analytical grade) were purchased from commercial suppliers and used without further purification. Ethyl acetate (EA), petrol ether (PE, 60 – 70 °C), methanol and dichloromethane were obtained in technical grade and distilled before application. Acetonitrile (MeCN) for HPLC was obtained from Merck (Darmstadt, Germany). Cyanine dye S0436 and NHS ester S0536 (FEW Chemicals, Bitterfeld-Wolfen, Germany), 4-(methylamino)pyridine (Alfa Aesar, Karlsruhe, Germany), methylacrylate (Merck, Darmstadt, Germany), 3,6,9-trioxaundecanedioic acid (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany), 1,4-diaminobenzene (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany), butyllithium (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany) and propargylbromide (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany) were purchased. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18 (250 × 21 mm, 5 µm; Macherey-Nagel, Germany) and a Eurospher-100 C18 (250 × 32 mm, 5 µm; Knauer,

Germany) served as RP-columns at flow rates of 20 and 38 mL/min, respectively. Mixtures of MeCN and diluted aqueous TFA (0.1 %) were used as mobile phase. ^{1}H-NMR spectra were recorded at 300 MHz on a Bruker Avance 300 spectrometer or at 600 MHz on a Bruker Avance III 600 with cryogenic probehead (Bruker, Karlsruhe, Germany). ^{13}C-NMR spectra were recorded at 75 MHz on a Bruker Avance 300 spectrometer. All chemical shifts values are reported in ppm. UV/VIS spectra were recorded with a Varian Cary BIO 50 UV/VIS/NIR spectrophotometer (Varian Inc., CA, USA). Mass spectra: Finnigan SSQ 710A (EI), Finnigan MAT 95 (CI), Finnigan MAT TSQ 7000 (Thermo FINNIGAN, USA) (ES/LC-MS). LC-system for LC-MS: Agilent 1100 (Palo Alto, USA). LC-MS method I (LC-MS-I): Column: Phenomex Luna C18, 3.0 µm, 100 x 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.30 mL/min; solvent A (water + 0.1% TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 1 min [A/B 95/5], 11 min [A/B 2/98], 18 min [A/B 2/98], 19 min [A/B 95/5], 24 min [A/B 95/5]. LC-MS method II: Column: Phenomex Luna C18, 2.5 µm, 50 x 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.40 mL/min; solvent A (water + 0.1% TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 8 min [A/B 2/98], 11 min [A/B 2/98], 12 min [A/B 95/5], 15 min [A/B 95/5]. Melting points were determined with a Lambda Photometrics Optimelt MPA100 apparatus (Lambda photometrics, Harpenden, UK), they are not corrected. Thin layer chromatography (TLC) was performed on alumina plates coated with silica gel (Merck silica gel 60 F_{245}, thickness 0.2 mm). Column chromatography (CC) was performed with Merck Geduran SI 60 silica gel as the stationary phase.

Compounds **4**[13], **5**[13], **6**[10], **8**[14], **9**[18], **10**[18], **11**[19], **21**[20, 21], **22**[6], **23**[6] and Boc-propargylamine[22] were prepared according to literature procedures. The synthesis of literature known compound **15**[23] was improved.

4.4.2 Synthetic protocols and analytical data

tert-Butyl-{3-[methyl(pyridin-4-yl)amino]propanamido}(methylthio)methylene-carbamate (7)

Acid **5** (216 mg, 1.00 mmol), DIPEA (258 mg, 2.00 mmol) and HOBt (149 mg, 1.10 mmol) were dissolved in 10 mL cold DCM and EDC (171 mg, 1.10 mmol) was added. After 15 minutes compound **6** (190 mg, 1.00 mmol) was added. The mixture was stirred overnight. Next day the reaction mixture was concentrated and the crude product was purified with column chromatography (EA/EtOH 1:1, R_f=0.1). Compound **7** (323 mg, 75%) was obtained

as a colorless oil. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.48 (s, 9H), 2.36 (s, 3H), 2.79 (t, J=6.87, 2H), 3.14 (s, 3H), 3.83 (t, J=6.88, 2H), 6.65 – 6.75 (m, 2H), 8.10 – 8.20 (m, 2H), 9.00 – 13.00 (m, 2H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.7, 27.9, 31.7, 38.6, 48.0, 79.9, 106.8, 141.5, 141.8, 143.5, 156.0, 170.8. **$C_{16}H_{24}N_4O_3S$: MS** (LC-MS-I): *m/z*(%) [t_r=8.1 min.]: 353(100, MH+), 705(5).

(R)-4-((3-((4-(2,2-Diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentylamino)(iminio)methylamino)-3-oxopropyl)(methyl)amino)pyridinium 2,2,2-trifluoroacetate (1a)

Compound **7** (123 mg, 0.35 mmol) was dissolved in 4 mL DMF and mixed with compound **8** (170 mg, 0.35 mmol). NEt_3 (0.5 mL) was added and then a solution of $HgCl_2$ (95 mg, 0.35 mmol) in 1 mL DMF was dropped into the reaction mixture. The mixture was stirred overnight. The next day DMF was evaporated under high vacuum and DCM (5 mL) was added. The insoluble mercury salts were filtered off and the solution was concentrated. The crude material was purified with column chromatography (EA/EtOH 1:1).

The product was deprotected without further characterisation: a mixture of DCM/TFA (1:1; 4 mL) was added and the solution was stirred for 2 hours. Then it was concentrated and purified with HPLC. A white solid was obtained (109 mg, 36%), m.p. > 190° C (decomp.). **^{1}H NMR** (300 MHz, CD_3CN): 1.40 – 1.90 (m, 4H), 2.78 (t, J=7.05, 2H), 3.15 (s, 3H), 3.15 – 3.30 (m, 2H), 3.85 (t, J=7.06, 2H), 4.10 – 4.30 (m, 2H), 4.30 – 4.40 (m, 1H), 5.02 (s, 1H), 6.60 – 7.20 (m, 4H), 6.71 (d, J=8.54, 2H), 7.03 (d, J=8.50, 2H), 7.20 – 7.35 (m, 10H), 7.96 (bs, 2H), 9.47 (bs, 1H), 9.95 (bs, 1H), 12.13 (bs, 1H), 13.37 (bs, 1H). **$C_{36}H_{43}N_7O_4$: MS** (LC-MS-I): *m/z*(%) [t_r=7.6 min.]: 318(100), 339(35), 636(50, MH+).

(R)-4-((4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-9-iminio-3,11,18-trioxo-2,8,10,12,17-pentaazaicosan-20-yl)(methyl)amino)pyridinium 2,2,2-trifluoroacetate (1b)

The acid **5** (5.6 mg, 26 µmol) was dissolved in DMF (2 mL) and DIPEA (28 mg, 0.21 mmol) and TBTU (8.3 mg, 26 µmol) were added. After 10 minutes amine **9** (20 mg, 22 µmol) was added. The mixture was stirred overnight. The next day DMF was removed under high vacuum and the oily residue was purified with HPLC. A white solid was obtained (14 mg, 67%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, CD_3CN): 1.30 – 1.80 (m, 8H), 2.00 – 2.80 (m, 2H), 3.10 (s, 3H), 3.10 – 3.30 (m, 6H), 3.80 (t, J=6.45, 2H), 4.10 – 4.40 (m, 3H), 5.03 (s, 1H), 6.50 – 7.90 (m, 8H), 6.67 (d, J=8.45, 2H), 7.00 (d, J=8.45, 2H), 7.20 – 7.35 (m,

10H), 7.90 – 8.05 (m, 2H), 9.00 – 9.20 (bs, 1H), 11.90 – 13.00 (m, 1H). **$C_{41}H_{51}N_9O_5$: MS** (LC-MS-II): *m/z*(%) [t_r=4.9 min.]: 375(100), 750(30, M+).

(R)-4-((4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-9-iminio-3,11,22-trioxo-15,18-dioxa-2,8,10,12,21-pentaazatetracosan-24-yl)(methyl)amino)pyridinium 2,2,2-trifluoroacetate (1c)

Acid **5** (3.0 mg, 13.7 µmol) was dissolved in DMF (2 mL) and DIPEA (14.7 mg, 114 µmol) and TBTU (4.4 mg, 13.7 µmol; dissolved in 0.2 mL MeCN) were added. After 10 minutes amine **10** (10 mg, 11.4 µmol) was added. The solution was stirred overnight. Next day DMF was removed completely and the oily residue was suspended in some MeCN (1 mL). TFA was added until pH < 2. MeCN was evaporated and the crude material was purified with HPLC. A white solid was obtained (5.5 mg, 47%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, D_2O): 1.20 –1.80 (m, 4H), 2.38 (t, J=6.59, 2H), 2.93 (s, 3H), 3.03 (t, J=7.28, 2H), 3.13 (t, J=5.41, 2H), 3.25 (t, J=5.16, 2H), 3.34 (t, J=5.39, 2H), 3.40 – 3.55 (m, 6H), 3.64 (t, J=6.61, 2H), 4.00 – 4.10 (m, 1H), 4.10 – 4.25 (m, 2H), 5.01 (s, 1H), 6.55 – 6.85 (m, 6H), 6.92 – 7.00 (d, J=8.53, 2H), 7.00 – 7.10 (m, 2H), 7.10 – 7.30 (m, 8H), 7.82 (d, J=7.29, 2H). **$C_{43}H_{55}N_9O_7$: MS** (LC-MS-II): *m/z*(%) [t_r=4.98 min.]: 405(100), 810(10, MH+).

4-((3-(3-Ammoniopropylamino)-3-oxopropyl)(methyl)amino)pyridinium chloride (12)

Acid **5** (216 mg, 1.00 mmol) was dissolved in MeCN (10 mL) and DIPEA (271 mg, 2.10 mmol) was added. The mixture was cooled in an icebath and DCC (227 mg, 1.10 mmol) was added. After 15 minutes amine **11** (174 mg, 1.00 mmol) was added. The turbid reaction mixture was stirred at room temperature for five hours and then the mixture was heated to 50°C for 30 minutes. After that time MeCN was evaporated completely and the residue (viscous oil) was redissolved in DCM. The precipitate (DCU) was filtered off and the crude material was purified with column chromatography (EA/MeOH 9:1 + 1% NEt_3, R_f=0.05). The Boc-protected, purified white solid (134 mg, 40%) was deprotected with a 3:1 mixture of methanol and concentrated hydrochloric acid (4 mL). After complete deprotection (10 minutes, room temperature) the solvent was evaporated, water was added (2-3 mL) and the solution was lyophilized overnight. A white solid was obtained (110 mg, 36% overall), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, D_2O): 1.60 – 1.75 (m, 2H), 2.51 (t, J=6.61, 2H), 2.82 (t, J=7.62, 2H), 3.00 – 3.15 (m, 5H), 3.78 (t, J=6.61, 2H), 6.70 – 7.00 (bs, 2H), 7.93 (d, J=7.48, 2H). **$C_{12}H_{20}N_4O$: MS** (LC-MS-II): *m/z*(%) [t_r=0.31 min.]: 119(5), 139(65), 160(100), 237(65, MH+).

(R)-4-((4-(2,2-Diphenylacetamido)-1-(4-hydroxyphenyl)-9-iminio-3,11,18,28,34-pentaoxo-20,23,26-trioxa-2,8,10,12,17,29,33-heptaazahexatriacontan-36-yl)(methyl)amino)pyridinium 2,2,2-trifluoroacetate (1d)

Acid **5** (6.6 mg, 30 µmol) was dissolved in DMF (2 mL) and DIPEA (35 mg, 270 µmol) and TBTU (18.9 mg, 59 µmol) were added. After 10 minutes the reaction was started with the addition of the compounds **9** (25.0 mg, 27 µmol) and **12** (8.3 mg, 27 µmol). The reaction mixture was stirred overnight. Next day DMF was removed completely and the oily residue was suspended in some MeCN (1 mL). TFA was added until pH < 2. MeCN was evaporated and the crude mixture was purified with HPLC. A white solid was obtained (5.5 mg, 16%), m.p. > 190°C (decomp.). **^{1}H NMR** (300 MHz, D_2O): 1.25 – 1.90 (m, 10H), 2.44 (t, J=6.48, 2H), 2.90 – 3.20 (m, 13H), 3.50 – 3.60 (m, 8H), 3.72 (t, J=6.48, 2H), 3.89 (d, J=1.70, 4H), 4.05 – 4.15 (m, 1H), 4.15 – 4.30 (m, 2H), 5.05 (s, 1H), 6.60 – 6.90 (m, 4H), 6.95 – 7.30 (m, 12H), 7.87 (d, J=7.52, 2H). **$C_{52}H_{71}N_{11}O_{10}$: MS** (LC-MS-II): *m/z*(%) [t_r=4.99 min.]: 505(100), 1010(5, MH+).

1,4-Diazidobenzene (15)

$NaNO_2$ (1.45 g, 21 mmol) was suspended in 10 mL concentrated sulphuric acid at 10°C in small amounts. The mixture was stirred 10 minutes at 20°C and then heated to 70°C until a clear solution was obtained. The mixture was cooled to 10°C and 1,4-diaminobenzene (1.08 g, 10 mmol) dissolved in 10 mL glacial acetic acid was dropped slowly into the mixture. A yellow solid precipitated. The mixture was stirred one hour at 15°C. After that time the mixture was dropped in a 500 mL flask containing 150 mL icecold water (with ice). A clear, yellow solution was obtained. NaN_3 (1.43 g, 22 mmol) dissolved in 10 mL water was added dropwise at 5°C under vigorous stirring (caution: HN_3 gas can develop). Nitrogen was formed and a yellow solid precipitated. After 2 hours the product was extracted with EA (2 x 75 mL). The organic layer was separated and dried over $MgSO_4$. The solvent was concentrated and the crude material was recrystallized in PE. Yellow crystalls were obtained (1.06 g, 66%), m.p. 155°C (decomp.).

***tert*-Butyl [(1-(4-azidophenyl)-1H-1,2,3-triazol-4-yl)methyl]carbamate (16a)**

1,4-Diazidobenzene (500 mg, 3.13 mmol) was dissolved in 5 mL of $CHCl_3$ and was added to a solution of Boc- propargylamine (484 mg, 3.13 mmol) in 5 mL of MeOH. Then ascorbic acid (55 mg, 0.31 mmol, dissolved in 0.5 mL of H_2O) and $CuSO_4 \cdot 5\ H_2O$ (40 mg, 0.16 mmol;

dissolved in 0.5 mL of H_2O) were added. The mixture was stirred at 60 °C for 1 hour. EA (50 mL) and water (50 mL) were added and the organic layer was separated. The mixture was concentrated and a white solid precipitated (double coupled side product). The precipitate was filtered off and the remaining solution was concentrated and purified with column chromatography (PE/EA 2:3, R_f=0.5). The product was obtained as a white solid (782 mg, 79%), m.p. 155 °C (160 °C decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.45 (s, 9H), 4.47 (d, J=6.03, 2H), 5.20 (bs, 1H), 7.10 – 7.20 (m, 2H), 7.65 – 7.75 (m, 2H), 7.94 (s, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 28.4, 36.1, 79.9, 120.2, 122.0, 132.1, 133.8, 140.7, 146.4, 155.9. **$C_{14}H_{17}N_7O_2$: MS** (LC-MS-II): *m/z*(%) [t_r=3.57 min.]: 216(100, M^*H+), 257(20), 431(25). The compound was completely Boc-deprotected during LC-MS.

(1-(4-Azidophenyl)-1H-1,2,3-triazol-4-yl)methanaminium 2,2,2-trifluoroacetate (16b)

Compound **16a** (287 mg, 0.91 mmol) was deprotected with a 2:1 mixture of DCM and TFA (6 mL). The mixture was stirred for 1h and then the solvent was removed. Water was added (5 mL) and the solution was lyophilized. A white solid was obtained (300 mg, quantitative), m.p. 157 °C (decomp.). **^{1}H NMR** (300 MHz, D_2O): 4.36 (s, 2H), 7.14 – 7.17 (m, 2H), 7.63 – 7.66 (m, 2H), 8.44 (s, 1H). **^{13}C NMR** (75 MHz, D_2O): 33.9, 120.0, 122.1, 123.4, 132.6, 140.3, 140.9. **$C_9H_9N_7$: MS** (LC-MS-II): *m/z*(%) [t_r=3.57 min.]: 216(100, MH+), 257(20), 431(25). **IR** (ν [cm^{-1}]): 3166, 2757, 2136 (N_3), 2095 (N_3), 1711, 1618, 1555, 1514.

***tert*-Butyl (3-((1-(4-azidophenyl)-1H-1,2,3-triazol-4-yl)methyl)ureido) (methylthio) methylenecarbamate (17)**

Triphosgene (71 mg, 0.24 mmol) was dissolved in icecold DCM (10 mL) and a solution of compound **16b** (200 mg, 0.61 mmol) and DIPEA (285 mg, 2.21 mmol) was dropped slowly (20 minutes) at 0°C into the triphosgene solution. After complete addition the reaction was allowed to warm up to ambient temperature and a solution of Boc-S-methyl-isothiourea **6** (116 mg, 0.61 mmol) in DCM (10 mL) was added over a period of 10 minutes. The mixture was stirred overnight at ambient temperature. The next day the reaction mixture was concentrated and the crude material was purified with column chromatography (PE/EA 3:1 -> 2:3) [R_f=0.3 (PE/EA 3:2)]. A light yellow solid was obtained (122 mg, 46%), m.p. 126 °C (162 °C decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.48 (s, 9H), 2.29 (s, 3H), 4.58 (d, J=6.19, 2H), 6.23 (t, J=5.49, 1H), 7.10 – 7.20 (m, 2H), 7.75 – 7.75 (m, 2H), 7.97 (s, 1H), 12.19 (bs, 1H). **$C_{17}H_{21}N_9O_3S$: MS** (LC-MS-II): *m/z*(%) [t_r=7.83 min.]: 432(100, MH+), 863(40).

(R)-1-(1-(4-Azidophenyl)-1H-1,2,3-triazol-4-yl)-10-(4-hydroxybenzylcarbamoyl) -3,12-dioxo-13,13-diphenyl-2,4,6,11-tetraazatridecan-5-iminium 2,2,2-trifluoroacetate (18)

Compound **17** (101 mg, 0.23 mmol), compound **8** (114 mg, 0.23 mmol) and NEt_3 (230 mg, 2.3 mmol) were dissolved in 3 mL DMF. $HgCl_2$ (64 mg, 0.23 mmol) was dissolved in 0.5 mL of DMF and added to the mixture. The mixture was stirred overnight. Next day DMF was removed completely and the residue was dissolved in 5 mL DCM. Hg-salts were filtered off and the crude material was purified with column chromatography (PE/EA 7:3, R_f=0.4). The compound was deprotected without further characterization. DCM/TFA was added (1:1 mixture, 4 mL) and the mixture was stirred for 2 hours. Then it was concentrated and the crude material was purified with HPLC. A white solid was obtained (112 mg, 57%), m.p. > 190° C (decomp.). **^{1}H NMR** (600 MHz, DMSO-d6): 1.35 – 1.48 (m, 2H), 1.48 – 1.70 (m, 2H), 3.10 – 3.25 (m, 2H), 4.05 – 4.20 (m, 2H), 4.30 – 4.35 (m, 1H), 4.46 (d, J=5.29, 2H), 5.12 (s, 1H), 6.67 (d, J=8.45, 2H), 7.00 (d, J=8.45, 2H), 7.15 – 7.30 (m, 10H), 7.30 – 7.35 (m, 2H), 7.88 – 7.92 (m, 2H), 8.13 (bs, 1H), 8.36 (t, J=5.80, 1H), 8.37 – 8.46 (bs, 1H), 8.47 (d, J=8.11, 1H), 8.67 (s, 1H), 8.89 (bs, 1H), 9.28 (s, 1H), 9.73 (bs, 1H). **^{13}C NMR (HSQC, HMBC, ROESY)** (151 MHz, DMSO-d6): 24.55, 29.42, 34.82, 41.58, 52.23, 55.88, 114.97, 120.47, 121.10, 121.59, 126.56, 128.13, 128.17, 128.38, 128.46, 128.48, 129.11, 133.46, 139.70, 140.24, 140.42, 156.23, 170.93, 170.98. **$C_{37}H_{38}N_{12}O_4$: MS** (LC-MS-II): *m/z*(%) [t_r=6.01 min.]: 715(100, MH+). **UV** (MeCN): λ(ε) 272 ($20 \cdot 10^3$). **IR** (ν [cm^{-1}]): 3277, 2130 (N_3), 2100 (N_3), 1686, 1638, 1542, 1512, 1446, 1364.

4-(Methyl(prop-2-ynyl)amino)pyridinium chloride (20)

N-Methylpyridin-4-amine **19** (200 mg, 1.85 mmol) was dissolved in 5 mL of dry THF. Then a BuLi solution (1.6M in hexanes; 1.4 mL, 2.22 mmol) was added at room temperature under nitrogen. After 10 minutes, when no more gas was formed, a propargylbromide solution (80%w in toluene; 330 mg, 2.22 mmol) was added slowly at –5°C over a period of 1h. The mixture was stirred overnight. The next day water was added (5 mL) and then THF was removed completely under reduced pressure. NaOH (1M, 20 mL) and EA (20 mL) were added and the organic layer was collected. Organic phase was acidified with $NaHSO_4$ solution (20 mL, 5%w) and the aqueous layer was collected. NaOH (1M, 40 mL) and EA (40 mL) were added and the organic layer was collected. The organic layer was washed several times with water until not converted N-methylpyridin-4-amine **19** was removed completely. The organic layer was dried over $MgSO_4$ and the solvent was removed. A brown oil was obtained.

Diluted hydrochloric acid (10%) was added dropwise until pH < 2. A yellow precipitate was formed. It was collected and recrystallized with $CHCl_3$/PE. Light yellow-brown crystalls were obtained (33 mg, 10%), m.p. 193 °C (decomp.). **^{1}H NMR** (300 MHz, D_2O): 2.63 (t, J=2.47, 1H), 3.15 (s, 3H), 4.26 (d, J=2.47, 2H), 6.92 (d, J=7.26, 2H), 8.00 (d, J=7.67, 2H). **^{13}C NMR** (75 MHz, D_2O): 38.0, 41.5, 74.1, 77.0, 107.8, 138.9, 157.3. **$C_9H_{10}N_2$: MS** (LC-MS-II): *m/z*(%) [t_r=0.56 min.]: 147(100, MH+), 188(10), 293(10).

(R)-4-(((1-(4-(4-(10-(4-Hydroxybenzylcarbamoyl)-5-iminio-3,12-dioxo-13,13-diphenyl-2,4,6,11-tetraazatridecyl)-1H-1,2,3-triazol-1-yl)phenyl)-1H-1,2,3-triazol-4-yl)methyl)(methyl)amino)pyridinium 2,2,2-trifluoroacetate (1e)

Compound **18** (10 mg, 12.1 µmol) was dissolved in 0.5 mL of DMSO and alkyne **20** (2.2 mg, 12.1 µmol) was added. The solution was diluted with a of 3:1 mixture of MeOH and H_2O (0.5 mL). Then ascorbic acid (1.1 mg, 6.1 µmol [stock solution 100 mg/mL in 1M NaOH]) and $CuSO_4 \cdot 5\ H_2O$ (0.91 mg, 3.6 µmol [stock solution 100 mg/mL in H_2O]) were dropped into the mixture. Copper (I) stabilizing ligand **21** (0.8 mg, 1.21 µmol) was added and the mixture was heated to 65 °C for 1h. After that time TLC control demonstrated complete conversion. TFA was added until pH < 2 and the crude material was purified with HPLC. A white solid was obtained (6.7 mg, 51%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.35 – 1.75 (m, 4H), 3.15 – 3.25 (m, 2H), 3.32 (s, 3H), 4.05 – 4.25 (m, 2H), 4.25 – 4.40 (m, 1H), 4.40 – 4.55 (m, 2H), 5.02 (s, 2H), 5.12 (s, 1H), 6.67 (d, J=8.45, 2H), 7.00 (d, J=8.45, 2H), 7.05 – 7.40 (m, 12H), 8.05 – 8.20 (m, 5H), 8.25 – 8.60 (m, 6H), 8.81 (s, 1H), 8.91 (bs, 1H), 8.96 (s, 1H), 9.30 (s, 1H), 9.86 (bs, 1H), 13.44 (bs, 1H). **$C_{46}H_{58}N_{14}O_4$: MS** (LC-MS-II): *m/z*(%) [t_r=5.18 min.]: 431(100), 861(5, MH+), 975(2).

S0436-thiophenolester (26)

Compound **24** (3.00 mg, 4.96 µmol) was mixed with DIPEA (1.92 mg, 14.9 µmol), DMAP (0.06 mg, 0.49 µmol) and TBTU (1.91 mg, 5.95 µmol). After 10 minutes the reaction was started with the addition of thiophenole (0.65 mg, 5.95 µmol). Next day DMF was removed completely and the crude material was purified with HPLC. A blue solid was obtained (2.40 mg, 69%). **$C_{41}H_{49}N_2O_4S_2$: MS** (LC-MS-II): *m/z*(%) [t_r=7.87]: 697(100, M+), 1395(15).

S0436-p-nitrophenolester (27)

Compound **24** (2.70 mg, 4.46 µmol) was dissolved in in 1 mL of DMF and DIPEA (2.7 mg, 21 µmol) was added. Then bis(4-nitrophenyl) carbonate (3.1 mg, 10.20 µmol) was added. The

mixture was stirred overnight at room temperature. The next day DMF was removed completely and the residue was purified with HPLC. A blue solid was obtained (2.90 mg, 91%). **$C_{41}H_{48}N_3O_7S$: MS** (LC-MS-II): *m/z*(%) [t_r=7.61]: 726(100, M+).

4.4.3 Radioligand competition binding assay

Radioligand competition experiments at SK-N-MC neuroblastoma cells using the radioligand [^{3}H]UR-MK114 (1.5 nM) were performed as described elsewhere.[14]

4.4.4 Fura-2 assay on HEL cells

The Fura assay was performed with HEL cells as previously described using a Perkin-Elmer LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany).[24]

4.4.5 Active ester kinetics

The acyl-transfer studies were performed in 2 mL Eppendorf Cups. 285 µL phosphate buffer (30 mM, pH 7.4) was put into the cups. Stock solutions of the active esters (1 mM) and DMAP (10 mM) in DMSO were prepared. Then 1.5 µL of the DMAP solution was added to the corresponding cups. Reaction was started with the addition of 15 µL of the corresponding active ester solution (vortexed). Active ester concentration was 50 µM. After 30, 90 and 150 minutes 75 µL aliquots were taken and put into 75 µL quenching solution [25%$_{vol}$ MeCN, 62.5%$_{vol}$ H_2O, 37.5%$_{vol.}$ TFA_{aq}(1%)]. 5 µL of internal standard (1 mM in MeCN) was added to every cup and the samples were analyzed with HPLC. The integrals of active ester and free acid were determined and the total amount of active ester was calculated (calibration with different concentrations of the free acids from 0.5 µM to 25 µM).

4.4.6 Acyl-transfer studies

The acyl-transfer studies were performed in 2 mL Eppendorf Cups. 200 µL phosphate buffer (30 mM, pH 7.4) was put into the cups. Stock solutions of the catalysts, nucleophiles and active esters in DMSO were prepared (10 mM). According to the particular experiment 1 µL of catalyst and 1 µL of nucleophile were added to the buffer and the mixture was vortexed. The acyl-transfer was started with the addition of 1 µL of the active ester (vortexed). Then it

was incubated for 30 minutes. After that time the reaction was quenched with the addition of 10 µL AcOH. 1 µL of a 10 mM solution of an internal standard was added and the samples were analyzed with LC-MS. The internal standard peak height was set as 100% and the relative peak intensities were determined.

4.4.7 Cell culture and confocal microscopy

MCF-7 cells were cultivated at 37 °C for two days in 8 well µ-slides (Ibidi GmbH, Munich) with EMEM medium (Sigma, Deisenhofen, Germany) containing 5% FCS (Biochrom AG, Berlin, Germany). Estradiol (1 nM) was added to the cultivation medium to stimulate Y_1R expression[12]. After two days, shortly before the experiment the medium was replaced by Leibovitz L15 medium (LM). Cells were incubated with 200 nM catalyst (in LM, 200 µL per well) for 15 minutes at room temperature. Then the medium was removed and the cells were washed once with PBS. Cells without catalyst were also washed once with PBS. Then a 10 µM solution of NHS ester **25** in PBS was added (150 µL per well) and the cells were incubated without light for 30 minutes at room temperature. After that time the cells were washed carefully three times with LM to remove all not covalent bound NHS ester and the free acid **24** from the membranes. Then 100 nM pNPY (in LM, 200 µL) was added to the corresponding wells and cells were incubated at room temperature. Pictures were taken after 90 minutes pNPY incubation.

Confocal microscopy was performed with a Zeiss Axiovert 200M microscope, equipped with a LSM 510 laser scanner. The dye S0436 was excited with a 633 nm laser, carboxyfluorescein was excitated at 488 nm. The emission of S0436 dyes was detected after a 650 nm long pass filter and the emission of carboxyfluorescein after a 505 nm long pass filter. The objective was a Plan-Neofluar 40x/1.3 with oil immersion.

4.4.8 Data processing

Data from radioligand competition experiments were analyzed by 4 parameter sigmoidal fits (SigmaPlot 9.0, Systat Software). IC_{50} values from radioligand competition studies were converted to K_i values according to the Cheng-Prusoff equation[25] using the respective K_D value of the radioligand.

4.5 References

1. Filmore, D. *Modern Drug Discovery* **2004,** *7*, 24
2. Rajagopal, S.; Rajagopal, K.; Lefkowitz, J. *Nat. Rev. Drug Discov.* **2010,** *9*, 373
3. Luttrell, L. M.; Gesty-Palmer, D. *Pharmacol. Rev.* **2010,** *62*, 305
4. Schneider, E.; Keller, M.; Brennauer, A.; Hoefelschweiger, B. K.; Gross, D.; Wolfbeis, O. S.; Bernhardt, G.; Buschauer, A. *Chembiochem* **2007,** *8*, 1981.
5. Höfle, G.; Steglich, W.; Vorbrüggen, H. *Angew. Chem. Int. Ed.* **1978,** *17*, 569
6. Koshi, Y.; Nakata, E.; Miyagawa, M.; Tsukiji, S.; Ogawa, T.; Hamachi, I. *J. Am. Chem. Soc.* **2008,** *130*, 245
7. Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Beck-Sickinger, A. G.; Doods, H. N. *Eur. J. Pharmacol.* **1994,** *271*, R11.
8. Hutzler, C.; Kracht, J.; Mayer, M.; Graichen, F.; Bauer, B.; Schreiber, E.; Bollwein, S.; Bernhardt, G.; Dove, S.; Fricker, G.; Buschauer, A. *Archiv der Pharmazie (Weinheim, Germany)* **2001,** *334*, 17.
9. Keller, M.; Teng, S.; Bernhardt, G.; Buschauer, A. *ChemMedChem* **2009,** *4*, 1733.
10. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; Konig, B. *Bioorg. Med. Chem.* **2008,** *16*, 9858.
11. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B. *Bioorg. Med. Chem.* **2010,** *18*, 6292
12. Memminger, M. Dissertation, **2009**.
13. Delaney, E. J.; Wood, L. E.; Klotz, I. M. *J. Am. Chem. Soc.* **1982,** *104*, 799
14. Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. *Journal of Medicinal Chemistry* **2008,** *51*, 8168.
15. Cline, G. W.; Hanna, S. B. *J. Org. Chem.* **1988,** *53*, 3583
16. Kleeberg, H. *J. Mol. Struct.* **1988,** *177*, 157
17. Brennauer, A.; Keller, M.; Freund, M.; Bernhardt, G.; Buschauer, A. *Tetrahedron Letters* **2007,** *48*, 6996.
18. Keller, M. Dissertation, **2008**.
19. Cinelli, M. A.; Cordero, B.; Dexheimer, T. S.; Pommier, Y.; Cushman, M. *Bioorg. Med. Chem.* **2009,** *17*, 7145
20. Ritter, S.; König, B. *Chem. Commun.* **2006,** *45*, 4694
21. Chan, T. R.; Hildegraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004,** *6*, 2853
22. Qiao, C.; Jeon, H.; Sayre, L. M. *J. Am. Chem. Soc.* **2004,** *126*, 8038

23. Herring, D. L. *J. Org. Chem.* **1961,** *26*, 3998
24. Müller, M.; Knieps, S.; Gessele, K.; Dove, S.; Bernhardt, G.; Buschauer, A. *Arch. Pharm. (Weinheim).* **1997,** *330*, 333.
25. Cheng, Y.; Prusoff, W. H. *Biochem Pharmacol* **1973,** *22*, 3099.

CHAPTER 5

Ligand Dependent Irreversible Blockade of the Neuropeptide Y_1 Receptor

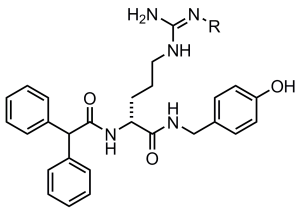
5.1 Introduction

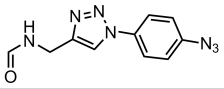
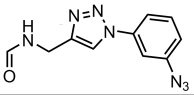
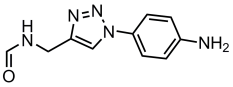
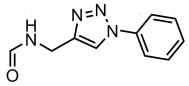
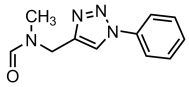
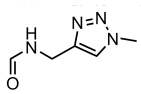
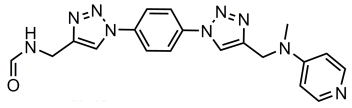
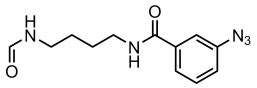
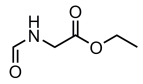
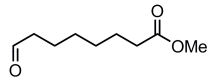
G protein-coupled receptors (GPCR) are one of the most important classes of targets in modern medicinal chemistry.[1] Approximately 40 – 50% of all currently marketed drugs target GPCRs, so an understanding of the molecular functionality of these receptors is highly clinically and commercially relevant. Most of these drugs bind to their receptors in a competitive, reversible manner and can be displaced by the natural agonist or receptor specific synthetic agonists or antagonists. However, there is a growing number of antagonists that bind to GPCRs and can not be displaced anymore.[2, 3] Two modes of irreversible binding of antagonists are conceivable: (1) The ligand is covalently bound to the receptor protein as described, e. g., for the alkylation of α-adrenergic receptors and histamine H_1 receptors with phenoxybenzamine and dibenamine, respectively.[4, 5] (2) The dissociation of the ligand is extremely slow (pseudo-irreversible binding) or the receptor is stabilized in a conformation with reduced affinity for the natural ligand.[3] Examples of such non-covalently binding insurmountable antagonists are described in the literature, e. g., certain ligands of histamine H_2 and neuropeptide (NPY) Y_5 receptors, respectively.[6, 7] Irrespective of the mode of irreversible binding, such ligands diminish the number of free receptors accessible to an agonist, resulting in concentration- and/or time-dependent decrease in the maximum response in functional assays, unless there is a sufficient number of spare receptors. Irreversible antagonists can be useful as drugs with long-lasting biological effects and as pharmacological tools, for instance, for the determination of a receptor reserve.[8-10]

The NPY Y_1 receptor (Y_1R) is a GPCR and is highly conserved in mammals. It is for example present in the central nervous system and regulates mood, anxiety and food intake.[11] BIBP 3226 (BIBP) is a very potent, fully reversibly binding antagonist and a useful selective tool for the investigation of the NPY Y_1 receptor.[12] Derivatization of the BIBP scaffold at the N^G-terminus is tolerated with moderate loss or gain of affinity.[13-15] With respect to the development of irreversible antagonists, two prototypical derivatives **1a** and **1h** substituted with an aromatic azide moiety were designed that should covalently bind to the receptor after irradiation with UV light via a nitrene intermediate.[16] Aromatic azides were successfully

applied as photo affinity labels for many targets in medicinal chemistry.[17, 18] Investigation of compound **1a** in microscopy studies with MCF-7 breast cancer cells revealed ligand dependent irreversible Y_1 receptor blockade without irradiation and decomposition of the azide group. In contrast compound **1h** is only irreversible after UV irradiation. To elucidate the molecular mechanism of the non-covalent ligand dependent irreversible binding of compound **1a** a series of structural analogues, most of them lacking the light-sensitive azide group, was synthesized and investigated for Y_1 receptor binding. Previously described derivatives such as **1i** and **1j**[14] and a complete series of acylguanidines with different logD values[15] from earlier investigations were included in this study. Some of these compounds blocked the Y_1R irreversibly with respect to NPY and a fluorescent Y_1R ligand, although a covalent bond was not formed.

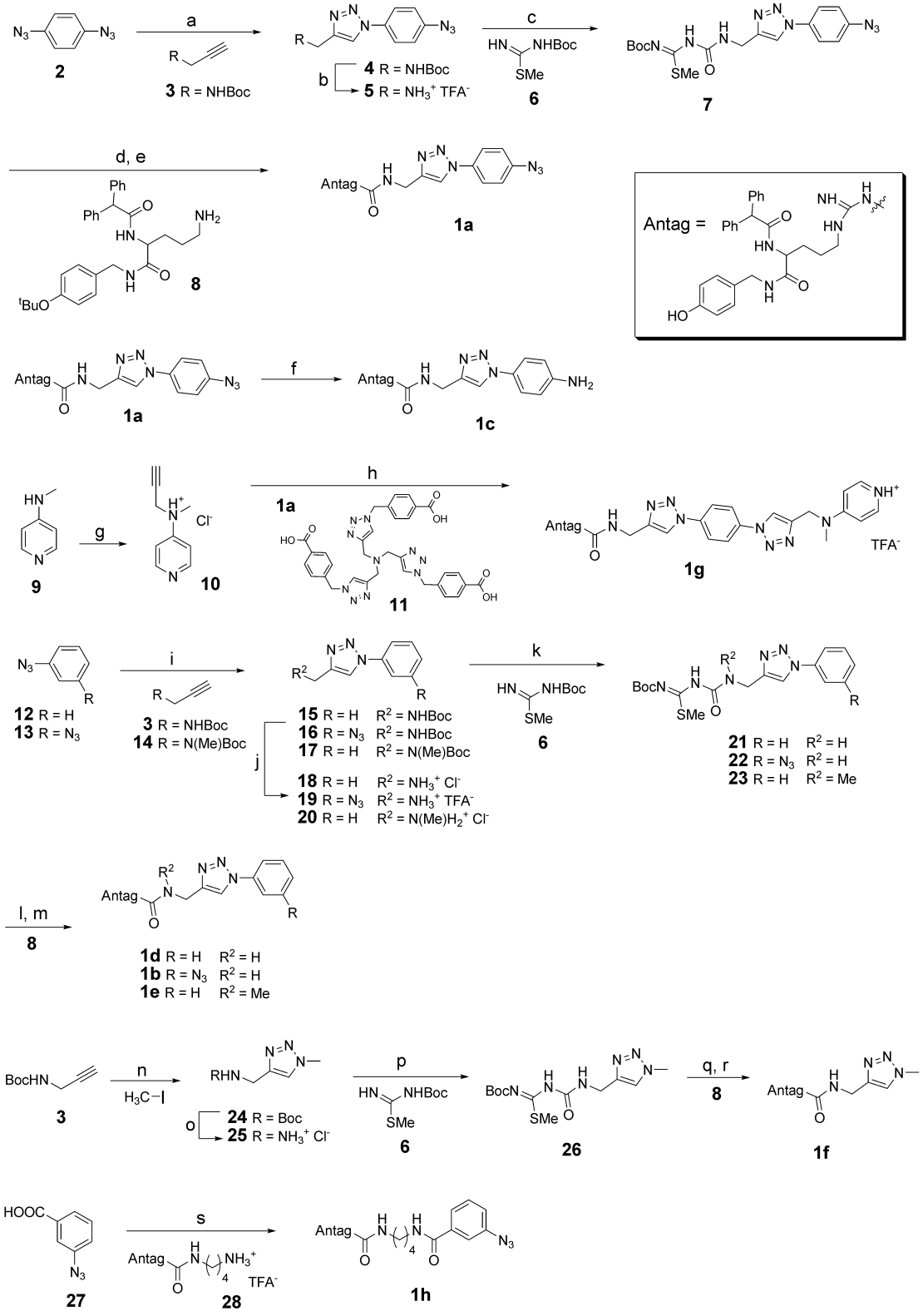
Table 1. Structures and Y_1R binding data of the compounds **1a-1k**.



no.	R	K_i [nM][a]
1	H (BIBP 3226)	1.3 ± 0.2
1a		4.6 ± 0.6
1b		6.9 ± 1.4
1c		4.0 ± 0.2
1d		3.1 ± 0.6
1e		41 ± 1.7
1f		0.6 ± 0.1
1g		400 ± 19
1h		68 ± 6.3
1i		0.1[b]
1j		0.1[b]
1k		0.9 ± 0.1[c]

[a] K_i values were determined from the displacement of 1.5 nM [^{3}H]-UR-MK114 on SK-N-MC cells (simultaneous incubation of radioligand and antagonist);[14] all mean values ± SEM from two independent experiments. Except entity **1** all depicted compounds exhibit irreversible binding properties at the Y_1R with respect to NPY and the fluorescent Y_1R antagonist **29** (**1h** only with UV irradiation).

[b, c] K_i values were taken from literature.[14, 19]



Scheme 1. Synthesis of the Y_1R antagonists **1a** – **1h**. a) $CHCl_3$, MeOH, H_2O, ascorbic acid, $CuSO_4$, 29%; b) DCM/TFA, quantitative; c) DCM, DIPEA, triphosgene, compound **6**, 46%; d) DMF, NEt_3, $HgCl_2$, 57%; e) DCM/TFA, quantitative; f) Pd/C, MeOH, quantitative; g) THF, BuLi, propargylbromide, 10%; h) Compound **1a**, DMSO, MeOH, H_2O, ascorbic acid, $CuSO_4$, 51%; i) MeOH, H_2O, ascorbic acid, $CuSO_4$, 38 - 86%; j) DCM/TFA

(**19**) or MeOH/HCl_{aq}, quantitative; k) DCM, DIPEA, triphosgene, 25 - 79%; l) DMF, NEt_3, $HgCl_2$, 21 – 76%; m) DCM/TFA, quantitative; n) NaN_3, MeI, $TBA^+BF_4^-$, Et_2O, H_2O; 2.) $CuSO_4$, MeOH, Et_2O, H_2O, ascorbic acid, 72%; o) MeOH, HCl, quantitative; p) DCM, DIPEA, triphosgene, 38%; q) DMF, NEt_3, $HgCl_2$, 55%; r) DCM/TFA, quantitative; s) DMF, DIPEA, TBTU, 61%.

5.2 Results and Discussion

5.2.1 Synthesis

The synthesis of compound **1a** started with a single "click-reaction" of 1,4-diazidobenzene and *N*-Boc-propargylamine. The cycloaddition product **4** was obtained in moderate yield, because of the undesired formation of the double cycloaddition byproduct. After Boc-deprotection with TFA the amine was converted into the isocyanate with triphosgene and this was converted with *N*-Boc-*S*-methylisothiourea **6** to compound **7**. A guanidinylation reaction with compound **8** and subsequent deprotection with TFA gave compound **1a** in moderate yield. Compound **1c** was prepared from **1a** via a simple Pd-catalyzed hydrogenation reaction in quantitative yield. Derivative **1g** was obtained by a cycloaddition reaction of compound **1a** and alkyne **10**, catalyzed with Cu^I and the Cu^I stabilizing ligand **11**, in moderate yield. Compounds **1b**, **1d** and **1e** were prepared according to the synthesis strategy for compound **1a**. The yields were dependent on the substitution pattern. The best yields were obtained for the unsubstituted phenylazide **12** and Boc-protected propargylamine **3**. Methylazide, required for the preparation of compound **1f,** was prepared from methyliodide and sodium azide in a water/diethylether mixture with phase transfer catalyst. To circumvent the isolation of the explosive methylazide, the ethereal methylazide solution was allowed to react with compound **3** yielding the cycloaddition product **24**. After Boc-deprotection the free amine was converted into the antagonist **1f** following the same route as for compound **1a**.

5.2.2 Fluorescence based ligand binding studies

Ligand **1a** was detected to inhibit the binding of the fluorescent labeled Y_1R antagonist **29** (K_i = 26 nM)[20] to the Y_1R on MCF-7 or adherent HEL cells without UV irradiation during confocal microscopy experiments. This Y_1R blockade with respect to ligand **29** was dependent on the concentration and the preincubation time of compound **1a** prior to the washing step and the following incubation with ligand **29**. A concentration of 50 nM and an

incubation time of 15 minutes was sufficient to inhibit binding of compound **29** and resulted in a complete loss of fluorescence compared with untreated cells. The binding of fluorescent labeled Cy5-NPY to MCF-7 cells was also inhibited under the same experimental conditions and even after a incubation time of 50 minutes no binding of Cy5-NPY was observed. In further experiments MCF-7 cells were preincubated with other potentially irreversible Y_1R blockers from earlier investigations[14, 15] and from this study (compounds **1a** – **1h**). The compounds were incubated at a concentration that was the tenfold K_i value from Table 1 (receptor saturation) for 15 minutes, washed and incubated with fluorescent labeled compound **29**. A complete loss of fluorescence intensity (picture B in Figure 1) compared to the fluorescence of untreated cells (picture A) indicated an irreversible blockade of the Y_1R with respect to ligand **29**. This was the case for all compounds listed in Table 1. However, the majority of N^G-substituted BIBP derivatives bind reversible to the Y_1R. For the investigation whether the irreversible ligands bind in a competitive manner to the Y_1R on the same binding site as the reversible ligands and NPY co-incubation experiments were performed. Antagonist **1a** (50 nM) was incubated with an excess of BIBP (100 nM) simultaneously and the Y_1 receptors were partially protected against irreversible blockade with respect to antagonist **29**. This is a proof of the competition for the same binding site (Figure 1) and excludes an allosteric receptor blockade of ligand **1a**. Additionally the (S)-enantiomer (distomer) of compound **1k** has no affinity to the BIBP binding pocket of the Y_1R and did not affect the binding of compound **29** to the receptor.

Further displacement studies of ligand **1a** were performed: MCF-7 cells were preincubated with compound **1a** as described above, washed twice with buffer, treated with fluorescent ligand **29** and investigated by flow cytometry, but there was no binding observed on a timescale of hours.

The irreversible blockade of a receptor can be the result of an alkylation of the receptor protein resulting in a covalently blocked receptor. This was not observed in these studies and is rather implausible, as the ligand dependent irreversible Y_1R blockers **1d**, **1f** or **1i** for example possess no azido or other reactive functional group. Further incubation studies of antagonist **1a** with different amino acids in buffered solution (pH 7.4) never demonstrated any decomposition of **1a** in absence of UV light. Therefore the ligand dependent irreversible receptor blockade is most likely a result of a very slow dissociation of the ligand from the receptor binding pocket (K_{off}-value is very low) or the conformation of the receptor R is changed to a ligand specific conformation R´ (R -> R´) that has a drastically reduced affinity to ligand **29** and Cy5-NPY after binding of such a ligand.

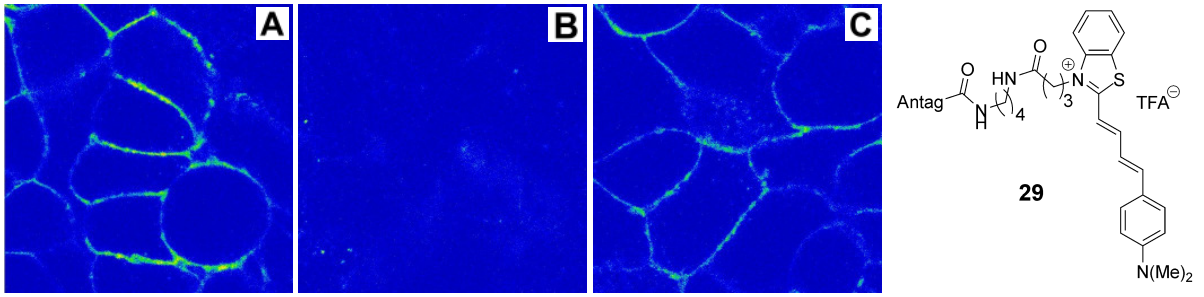


Figure 1. MCF-7 cells were incubated with 30 nM of the fluorescent antagonist **29** for 20 minutes. **A**: Cells were incubated with compound **29** (total binding). **B**: Cells were 15 min. pre-incubated with 50 nM **1a**, washed and incubated with compound **29**. **C**: Cells were 15 min. pre-incubated with 50 nM **1a** and 100 nM BIBP simultaneously, washed and incubated with compound **29**. Co-incubation of compound **1a** and BIBP in picture **C** preserved some receptors, while sole incubation of the cells with compound **1a** in picture **B** blocked the receptors completely.

5.2.3 Structure and irreversible binding properties

The structure of the N^G-substituent on the BIBP scaffold is crucial for ligand dependent irreversible binding, as the majority of N^G-substituted BIBP derivatives were in contrast reversible, competitive antagonists for the Y_1R.[15, 19-22] The azido group and the phenyl substituent like in compound **1a** were not necessary. The N-methylated compound **1e** showed reduced affinity compared with **1d** and was irreversible regarding antagonist **29** at higher concentration. A methyltriazolyl-carbamoyl substituent as in compound **1f** was sufficient to turn the reversible into irreversible binding, nevertheless, irreversible binding ligands were obtained with various N^G-substituents as demonstrated by compounds **1i** - **1k**. Even very bulky substituents like in compound **1g** were tolerated.

5.2.4 Radioligand binding studies

After the detection of the irreversible binding of compound **1a** to the Y_1R regarding ligand **29** and Cy5-NPY we investigated if the binding of radioligand **30** ([^{3}H]-UR-MK114) was also inhibited. Therefore SK-N-MC cells were preincubated with 50 nM of compound **1a** for 15 minutes (tenfold higher concentration compared to K_i value, saturation of the receptor), washed twice and then incubated with 1.5 nM radioligand **30**. The amount of specifically bound radioligand was determined at different points in time. Interestingly, the binding of

radioligand **30** was still possible and the binding of ligand **1a** was not irreversible with respect to the radioligand. The association speed was reduced compared to untreated cells (Figure 2) and the maximum number of accessible binding sites (B_{max}) was reduced slightly compared to untreated cells (B_{max} was 84% of the untreated cells). As the displacement of **1a** was possible within a relatively short period of time, slow dissociation of ligand **1a** cannot explain the irreversible receptor blockade regarding ligand **29** and Cy5-NPY.

This different behavior of the pretreated cells towards the radioligand and the fluorescent labeled compounds lead to the assumption that the conformation of the receptor has changed (R -> R´) after binding of ligand **1a**. It can be speculated that compound **30** still has high affinity for the receptor in a ligand specific conformation R´ due to the small propionyl substituent, i. e. the lack of bulk, at the guanidine group in radioligand **30.** By contrast, the sterically demanding N^G-substituted ligand **29** and the labeled peptide possess no or strongly reduced affinity for the receptor in state R´.

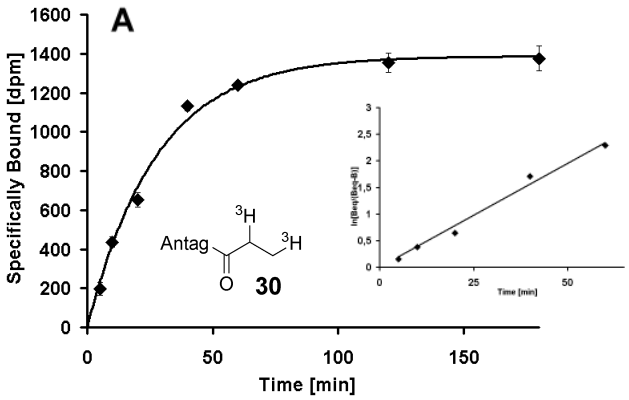


Figure 2. Association kinetics for the specific Y_1R binding of radioligand **30** on SK-N-MC cells after treatment with 50 nM ligand **1a** for 15 minutes. As the association of radioligand **30** is very fast concerning untreated cells (K_{on} = 0.19 min^{-1} nM^{-1})[14] the reduced association speed of compound **30** in graph **A** (K_{ob} = 0.039 min^{-1}) is due to a reduced affinity of the ligand to the Y_1R in conformation R´ or the slow dissociation of ligand **1a**. Radioligand concentration was 1.5 nM and the experiment was performed at room temperature.

5.2.5 Functional studies on HEL cells

Functional studies (Ca^{2+}-response) were performed on HEL cells expressing the NPY Y_1 receptor. Therefore the cells were preincubated with compound **1a** at different concentrations

in the dark for 15 minutes. After washing of the cells, the agonist pNPY was added at increasing concentrations (Figure 3). There was a rightward shift of the concentration-response curves with increasing concentrations of compound **1a**, indicating that a portion of the receptors remained (irreversibly) blocked against pNPY by antagonist **1a** under these conditions. Preincubation with compound **1a** at a concentration as low as 3 nM resulted in a decrease in the maximal NPY-induced Ca^{2+}-response by 50%. Increasing the concentrations of **1a** up to 100 nM did not further decrease the maximum Ca^{2+}-response. A partial agonistic behavior of compound **1a** was excluded, as there was no calcium response observed without pNPY even at the highest preincubation concentration of 100 nM. These findings lead to the assumption that there is a strong signal amplification in HEL cells caused by the occupancy of the small fraction of remaining free receptors with pNPY. Therefore a high concentration of pNPY is necessary. Compound **1a** shows partially insurmountable antagonism[3, 23] regarding pNPY, as the maximum calcium response can not be elicited anymore after preincubation with concentrations above 1 nM.

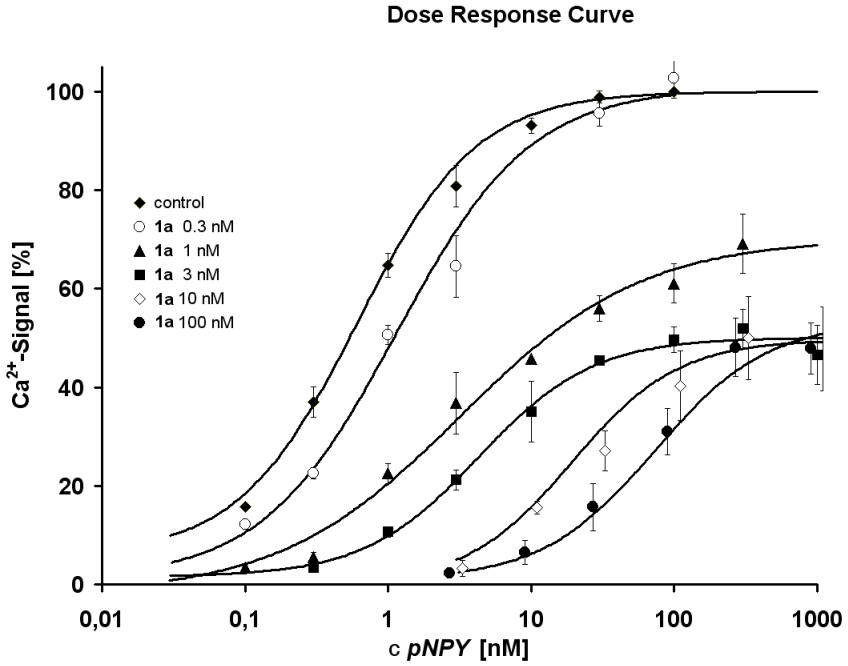


Figure 3. Concentration-response curves of pNPY on HEL cells preincubated for 15 minutes with different concentrations of the irreversible (with respect to pNPY) Y_1R blocker ligand **1a**. The cells were washed and centrifuged twice before addition of pNPY.

5.3 Conclusion

A series of ligand dependent irreversible receptor blockers for the NPY Y_1R was detected and investigated on the basis of ligand **1a**. The irreversible binding with respect to ligand **29** and NPY is most probably due to a conformational change (R -> R´) of the Y_1R after binding of these ligands, because the displacement of compound **1a** with the radioligand **30** was still possible on a timescale of minutes. The fluorescent antagonist **29** and Cy5-NPY were in contrast not able to bind to the receptor anymore. This supports the hypothesis that the NPY Y_1 receptor can adopt different ligand specific conformations R´ that possess altered affinities for agonists and antagonists.[3] Compound **1a** is a partially insurmountable antagonist on HEL cells regarding pNPY, this is most probably due to a strong signal amplification in this cell line. The principle of ligands that act as insurmountable antagonists is not restricted to the field of NPY receptors, but is a frequent phenomenon concerning GPCR´s, an example is the recent detection of an insurmountable histamine H_2 receptor radioligand.[24] The exact molecular mechanism of the ligand dependent irreversible binding and the reason for partially insurmountable antagonism is an aim for further studies.

5.4 Experimental

5.4.1 General experimental conditions

Unless otherwise noted, solvents (analytical grade) were purchased from commercial suppliers and used without further purification. Ethyl acetate (EA), petrol ether (PE, 60 – 70 °C), methanol and dichloromethane were obtained in technical grade and distilled before application. Acetonitrile (MeCN) for HPLC was obtained from Merck (Darmstadt, Germany). 4-(methylamino)pyridine (Alfa Aesar, Karlsruhe, Germany), 1,4-diaminobenzene (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany), butyllithium (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany) and propargylbromide (Fluka, Sigma-Aldrich Chemie GmbH, Munich, Germany) were purchased. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18 (250 × 21 mm, 5 µm; Macherey-Nagel, Germany) and a Eurospher-100 C18 (250 × 32 mm, 5 µm; Knauer, Germany) served as RP-columns at flow rates of 20 and 38 mL/min, respectively. Mixtures of MeCN and diluted aqueous TFA (0.1 %) were used as

mobile phase. ^{1}H-NMR spectra were recorded at 300 MHz on a Bruker Avance 300 spectrometer or at 600 MHz on a Bruker Avance III 600 with cryogenic probehead (Bruker, Karlsruhe, Germany). ^{13}C-NMR spectra were recorded at 75 MHz on a Bruker Avance 300 spectrometer or at 151 MHz on a Bruker Avance III 600. All chemical shifts values are reported in ppm. UV/VIS spectra were recorded with a Varian Cary BIO 50 UV/VIS/NIR spectrophotometer (Varian Inc., CA, USA). Mass spectra: Finnigan SSQ 710A (EI), Finnigan MAT 95 (CI), Finnigan MAT TSQ 7000 (Thermo FINNIGAN, USA) (ES/LC-MS). LC-system for LC-MS: Agilent 1100 (Palo Alto, USA). LC-MS method: Column: Phenomex Luna C18, 2.5 µm, 50 x 2 mm HST (Phenomenex, Aschaffenburg, Germany); flow: 0.40 mL/min; solvent A (water + 0.1% TFA), solvent B (MeCN); gradient: 0 min [A/B 95/5], 8 min [A/B 2/98], 11 min [A/B 2/98], 12 min [A/B 95/5], 15 min [A/B 95/5]. Melting points were determined with a Lambda Photometrics Optimelt MPA100 apparatus (Lambda photometrics, Harpenden, UK), they are not corrected. Thin layer chromatography (TLC) was performed on alumina plates coated with silica gel (Merck silica gel 60 F_{245}, thickness 0.2 mm). Column chromatography (CC) was performed with Merck Geduran SI 60 silica gel as the stationary phase.

Compounds **3**,[25] **6**,[22] **8**,[14] **11**,[26] **12**,[27] **13**,[28] **14**,[29] **27**[30] and **28**[21] were prepared according to literature procedures. The synthesis of literature known compound **2**[31] was improved.

5.4.2 Synthetic protocols and analytical data

General procedure A:

Triphosgene (116 mg, 0.39 mmol) was dissolved in 10 mL of ice-cold DCM or MeCN (**26)** and a solution of the appropriate amine (1.00 mmol) in the same solvent and DIPEA (396 mg, 3.07 mmol) was added slowly for 20 minutes at 0 °C under vigorous stirring. After that time the mixture was warmed up to ambient temperature and a solution of compound **6** (200 mg, 1.00 mmol) in 10 mL of DCM was added over a period of 10 minutes. The mixture was stirred overnight at ambient temperature. The next day it was concentrated and purified with column chromatography.

General procedure B:

The appropriate *S*-methylisothiourea derivative (0.2 mmol), compound **8** (0.2 mmol) and $HgCl_2$ (0.2 mmol) were dissolved separately in small amounts of DMF (1-2 mL). The solutions were combined in a small flask under nitrogen atmosphere. NEt_3 (2 mmol) was

added under stirring, then the $HgCl_2$ solution was added and the mixture was stirred overnight at room temperature. The next day DMF was removed completely and the residue was dissolved in DCM. The precipitate of mercury salts was filtered off and the organic phase was concentrated. The crude products were purified by column chromatography (PE/EA mixtures). The Boc- and ^{t}Bu-protected target molecules were deprotected to the corresponding target compounds without further characterisation: The corresponding protected compound was dissolved in a DCM/TFA 1:1 mixture (5 mL) and stirred for two hours. After that time the solvent was removed completely under reduced pressure and the oily residue was repeatedly dissolved in dichloromethane and the solvent evaporated to remove TFA. The deprotected compounds were purified with preparative HPLC except compound **1e**. The given yield corresponds to the coupling step in the first part of this procedure, the deprotection step was quantitative.

General procedure C:

The appropriate Boc-protected alkyne derivative **3** or **14** (2.0 mmol) and azide **12** or **13** (2.0 mmol) were dissolved in 5 mL of MeOH. Then ascorbic acid (35 mg, 0.2 mmol) dissolved in 0.5 mL H_2O was added. Then $CuSO_4 \cdot 5\ H_2O$ (25 mg, 0.1 mmol) dissolved in 0.5 mL H_2O was added. The mixture was stirred at 60 °C for 1h. Then it was concentrated and the residue was purified with column chromatography.

General procedure D:

The appropriate Boc-protected compound was dissolved in 4 mL MeOH/$HCl_{aq.}$ 3:1 or in 4 mL DCM/TFA 2:1 (**19**). After 30 minutes the reaction was finished and the mixture was concentrated. The products were obtained in quantitative yield.

1,4-Diazidobenzene (2)

$NaNO_2$ (1.45 g, 21 mmol) was suspended in 10 mL concentrated sulphuric acid at 10 °C in small amounts. The mixture was stirred 10 minutes at 20 °C and then heated to 70 °C until a clear solution was obtained. The mixture was cooled to 10 °C and 1,4-diaminobenzene (1.08 g, 10 mmol) dissolved in 10 mL glacial acetic acid was dropped slowly into the mixture. A yellow solid precipitated. The mixture was stirred one hour at 15 °C. After that time the mixture was dropped in a 500 mL flask containing 150 mL ice-cold water (with ice). A clear, yellow solution was obtained. NaN_3 (1.43 g, 22 mmol) dissolved in 10 mL water was added dropwise at 5 °C under vigorous stirring (caution: HN_3 gas can develop). Nitrogen was

formed and a yellow solid precipitated. After 2 hours the product was extracted with EA (2 x 75 mL). The organic layer was separated and dried over $MgSO_4$. The solvent was concentrated and the crude material was recrystallized in PE. Yellow crystals were obtained (1.06 g, 66%), m.p. 155°C (decomp.).

***tert*-Butyl [(1-(4-azidophenyl)-1H-1,2,3-triazol-4-yl)methyl]carbamate (4)**

1,4-Diazidobenzene (500 mg, 3.13 mmol) was dissolved in 5 mL of $CHCl_3$ and was added to a solution of Boc-propargylamine (484 mg, 3.13 mmol) in 5 mL of MeOH. Then ascorbic acid (55 mg, 0.31 mmol, dissolved in 0.5 mL of H_2O) and $CuSO_4 \cdot 5\ H_2O$ (40 mg, 0.16 mmol; dissolved in 0.5 mL of H_2O) were added. The mixture was stirred at 60 °C for 1 hour. EA (50 mL) and water (50 mL) were added and the organic layer was separated. The mixture was concentrated and a white solid precipitated (double coupled side product). The precipitate was filtered off and the remaining solution was concentrated and purified with column chromatography (PE/EA 2:3, R_f=0.5). The product was obtained as a white solid (782 mg, 79%), m.p. 155 °C (160 °C decomp.). **^{1}H NMR** (300 MHz, $CDCl_3$): 1.45 (s, 9H), 4.47 (d, J=6.03, 2H), 5.20 (bs, 1H), 7.10 – 7.20 (m, 2H), 7.65 – 7.75 (m, 2H), 7.94 (s, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 28.4, 36.1, 79.9, 120.2, 122.0, 132.1, 133.8, 140.7, 146.4, 155.9. **$C_{14}H_{17}N_7O_2$: MS** (LC-MS): *m/z*(%) [t_r=3.57 min.]: 216(100, M^*H+), 257(20), 431(25). The compound was completely Boc-deprotected during LC-MS.

(1-(4-Azidophenyl)-1H-1,2,3-triazol-4-yl)methanaminium 2,2,2-trifluoroacetate (5)

Compound **4** (287 mg, 0.91 mmol) was deprotected with a 2:1 mixture of DCM and TFA (6 mL). The mixture was stirred for 1h at room temperature and then the solvent was removed. Water was added (5 mL) and the solution was lyophilized. A white solid was obtained (300 mg, quantitative), m.p. 157 °C (decomp.). **^{1}H NMR** (300 MHz, D_2O): 4.36 (s, 2H), 7.14 – 7.17 (m, 2H), 7.63 – 7.66 (m, 2H), 8.44 (s, 1H). **^{13}C NMR** (75 MHz, D_2O): 33.9, 120.0, 122.1, 123.4, 132.6, 140.3, 140.9. **$C_9H_9N_7$: MS** (LC-MS): *m/z*(%) [t_r=3.57 min.]: 216(100, MH+), 257(20), 431(25). **IR** (ν [cm^{-1}]): 3166, 2757, 2136 (N_3), 2095 (N_3), 1711, 1618, 1555, 1514.

***tert*-Butyl (3-((1-(4-azidophenyl)-1H-1,2,3-triazol-4-yl)methyl)ureido)(methylthio) methylenecarbamate (7)**

The compound was prepared according to general procedure A. Column chromatography: (PE/EA 3:1 -> 2:3) [Rf=0.3 (PE/EA 3:2)]. A light yellow solid was obtained (200 mg, 46%), m.p. 126 °C (162 °C decomp.). **^{1}H NMR** (300 MHz, CDCl3): 1.48 (s, 9H), 2.29 (s, 3H), 4.58 (d, J=6.19, 2H), 6.23 (t, J=5.49, 1H), 7.10 – 7.20 (m, 2H), 7.75 – 7.75 (m, 2H), 7.97 (s, 1H), 12.19 (bs, 1H). **$C_{17}H_{21}N_9O_3S$: MS** (LC-MS): m/z(%) [tr=7.83 min.]: 432(100, MH+), 863(40).

(R)-1-(1-(4-Azidophenyl)-1H-1,2,3-triazol-4-yl)-10-(4-hydroxybenzylcarbamoyl)-3,12-dioxo-13,13-diphenyl-2,4,6,11-tetraazatridecan-5-iminium 2,2,2-trifluoroacetate (1a)

The compound was prepared according to general procedure B. A white solid was obtained (97 mg, 57%), m.p. > 190° C (decomp.). **^{1}H NMR** (600 MHz, DMSO-d6): 1.35 – 1.48 (m, 2H), 1.48 – 1.70 (m, 2H), 3.10 – 3.25 (m, 2H), 4.05 – 4.20 (m, 2H), 4.30 – 4.35 (m, 1H), 4.46 (d, J=5.29, 2H), 5.12 (s, 1H), 6.67 (d, J=8.45, 2H), 7.00 (d, J=8.45, 2H), 7.15 – 7.30 (m, 10H), 7.30 – 7.35 (m, 2H), 7.88 – 7.92 (m, 2H), 8.13 (bs, 1H), 8.36 (t, J=5.80, 1H), 8.37 – 8.46 (bs, 1H), 8.47 (d, J=8.11, 1H), 8.67 (s, 1H), 8.89 (bs, 1H), 9.28 (s, 1H), 9.73 (bs, 1H). **^{13}C NMR (HSQC, HMBC, ROESY)** (151 MHz, DMSO-d6): 24.55, 29.42, 34.82, 41.58, 52.23, 55.88, 114.97, 120.47, 121.10, 121.59, 126.56, 128.13, 128.17, 128.38, 128.46, 128.48, 129.11, 133.46, 139.70, 140.24, 140.42, 156.23, 170.93, 170.98. **$C_{37}H_{38}N_{12}O_4$: MS** (LC-MS): *m/z*(%) [t_r=6.01 min.]: 715(100, MH+). **UV** (MeCN): λ(ε) 272 ($20\cdot10^3$). **IR** (ν [cm^{-1}]): 3277, 2130 (N_3), 2100 (N_3), 1686, 1638, 1542, 1512, 1446, 1364.

1-(1-(4-Aminophenyl)-1H-1,2,3-triazol-4-yl)-10-(4-hydroxybenzylcarbamoyl)-3,12-dioxo-13,13-diphenyl-2,4,6,11-tetraazatridecan-5-iminium 2,2,2-trifluoroacetate (1c)

Azide **1a** (6.2 mg, 7.5 µg) was dissolved in 5 mL MeOH and 5 mg Pd/C were added. The mixture was stirred under 1 bar hydrogen pressure for 2 hours. Then the mixture was filtered over cellite and concentrated. The crude material was purified with HPLC. A white solid was obtained (6.0 mg, quantitative), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.40 – 1.70 (m, 4H), 3.15 – 3.25 (m, 2H), 4.05 – 4.25 (m, 2H), 4.30 – 4.40 (m, 1H), 4.40 – 4.45 (d, J=5.21, 2H), 5.12 (s, 1H), 6.63 – 6.75 (m, 4H), 7.00 (d, J=8.47, 2H), 7.15 – 7.35 (m, 11H), 7.45 (d, J=8.80, 2H), 8.08 (s, 1H), 8.30 – 8.60 (m, 5H), 8.70 – 9.40 (m, 2H), 9.62 (s, 1H). **$C_{37}H_{40}N_{10}O_4$: MS** (LC-MS): *m/z*(%) [t_r=5.27 min.]: 345(80), 386(50), 689(100, MH+). **UV** (MeCN): λ(ε) 276 ($13\cdot10^3$).

4-(Methyl(prop-2-ynyl)amino)pyridinium chloride (10)

N-Methylpyridin-4-amine **9** (200 mg, 1.85 mmol) was dissolved in 5 mL of dry THF. Then BuLi solution (1.6M in hexanes; 1.4 mL, 2.22 mmol) was added at room temperature under nitrogen. After 10 minutes, when no more gas was formed, a propargylbromide solution (80%w in toluene; 330 mg, 2.22 mmol) was added slowly at –5 °C over a period of 1h. The mixture was stirred overnight. The next day water was added (5 mL) and then THF was removed completely under reduced pressure. NaOH (1M, 20 mL) and EA (20 mL) were added and the organic layer was collected. The organic phase was acidified with $NaHSO_4$ solution (20 mL, 5%w) and the aqueous layer was collected. NaOH (1M, 40 mL) and EA (40 mL) were added and the organic layer was collected. The organic layer was washed several times with water until not converted compound **9** was removed completely. The organic layer was dried over $MgSO_4$ and the solvent was removed. A brown oil was obtained. Diluted hydrochloric acid (10%) was added dropwise until pH < 2. A yellow precipitate was formed. It was collected and recrystallized with $CHCl_3$/PE. Light yellow-brown crystalls were obtained (33 mg, 10%), m.p. 193 °C (decomp.). **^{1}H NMR** (300 MHz, D_2O): 2.63 (t, J=2.47, 1H), 3.15 (s, 3H), 4.26 (d, J=2.47, 2H), 6.92 (d, J=7.26, 2H), 8.00 (d, J=7.67, 2H). **^{13}C NMR** (75 MHz, D_2O): 38.0, 41.5, 74.1, 77.0, 107.8, 138.9, 157.3. **$C_9H_{10}N_2$: MS** (LC-MS): *m/z*(%) [t_r=0.56 min.]: 147(100, MH+), 188(10), 293(10).

(R)-4-(((1-(4-(4-(10-(4-Hydroxybenzylcarbamoyl)-5-iminio-3,12-dioxo-13,13-diphenyl-2,4,6,11-tetraazatridecyl)-1H-1,2,3-triazol-1-yl)phenyl)-1H-1,2,3-triazol-4-yl)methyl)(methyl)amino)pyridinium 2,2,2-trifluoroacetate (1g)

Compound **1a** (10 mg, 12.1 µmol) was dissolved in 0.5 mL of DMSO and alkyne **10** (2.2 mg, 12.1 µmol) was added. The solution was diluted with a of 3:1 mixture of MeOH and H_2O (0.5 mL). Then ascorbic acid (1.1 mg, 6.1 µmol [stock solution 100 mg/mL in 1M NaOH]) and $CuSO_4 \cdot 5\ H_2O$ (0.91 mg, 3.6 µmol [stock solution 100 mg/mL in H_2O]) were dropped into the mixture. Copper (I) stabilizing ligand **11** (0.8 mg, 1.21 µmol) was added and the mixture was heated to 65 °C for 1h. After that time TLC control demonstrated complete conversion. TFA was added until pH < 2 and the crude material was purified with HPLC. A white solid was obtained (6.7 mg, 51%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.35 – 1.75 (m, 4H), 3.15 – 3.25 (m, 2H), 3.32 (s, 3H), 4.05 – 4.25 (m, 2H), 4.25 – 4.40 (m, 1H), 4.40 – 4.55 (m, 2H), 5.02 (s, 2H), 5.12 (s, 1H), 6.67 (d, J=8.45, 2H), 7.00 (d, J=8.45, 2H), 7.05 – 7.40 (m, 12H), 8.05 – 8.20 (m, 5H), 8.25 – 8.60 (m, 6H), 8.81 (s, 1H), 8.91 (bs, 1H),

8.96 (s, 1H), 9.30 (s, 1H), 9.86 (bs, 1H), 13.44 (bs, 1H). **$C_{46}H_{58}N_{14}O_4$: MS** (LC-MS): *m/z*(%) [t_r=5.18 min.]: 431(100), 861(5, MH+), 975(2).

***tert*-Butyl (1-phenyl-1H-1,2,3-triazol-4-yl)methylcarbamate (15)**

The compound was prepared according to general procedure C. Column chromatography: (PE/EA 2:3, R_f=0.45). A white solid was obtained (471 mg, 86%). The compound was deprotected without further characterisation.

***tert*-Butyl (1-(3-azidophenyl)-1H-1,2,3-triazol-4-yl)methylcarbamate (16)**

The compound was prepared according to general procedure C. Column chromatography: (PE/EA 2:3, R_f=0.6). A yellow solid was obtained (237 mg, 38%). The compound was deprotected without further characterisation.

***tert*-Butyl methyl((1-phenyl-1H-1,2,3-triazol-4-yl)methyl)carbamate (17)**

The compound was prepared according to general procedure C. Column chromatography: (PE/EE 7:3, R_f=0.45). A white solid was obtained (469 mg, 81%). The compound was deprotected without further characterisation.

(1-Phenyl-1H-1,2,3-triazol-4-yl)methanaminium chloride (18)

The compound was prepared according to general procedure D. A white solid was obtained, m.p. 244 °C. **^{1}H NMR** (300 MHz, D_2O): 4.40 (s, 2H), 7.50 – 7.65 (m, 3H), 7.65 – 7.80 (m, 2H), 8.52 (s, 1H). **^{13}C NMR** (75 MHz, D_2O): 34.0, 121.0, 123.8, 129.7, 129.9, 136.0, 140.3. **$C_9H_{10}N_4$:** MS (LC-MS): *m/z*(%) [t_r=0.88 min.]: 175(100, MH+), 349(30).

(1-(3-Azidophenyl)-1H-1,2,3-triazol-4-yl)methanaminium 2,2,2-trifluoroacetate (19)

The compound was prepared according to general procedure D. A yellow oil was obtained. **^{1}H NMR** (300 MHz, D_2O): 4.27 (s, 2H), 7.00 – 7.10 (m, 1H), 7.20 – 7.26 (m, 1H), 7.30 – 7.45 (m, 2H), 8.38 (s, 1H). **^{13}C NMR** (75 MHz, D_2O): 33.8, 110.5, 116.1, 118.9, 122.9, 130.8, 136.8, 140.4, 141.1. **$C_9H_9N_7$:** MS (LC-MS): *m/z*(%) [t_r=2.93 min.]: 216(100, MH+), 431 (100).

N-Methyl-1-(1-phenyl-1H-1,2,3-triazol-4-yl)methanaminium chloride (20)

The compound was prepared according to general procedure D. A white solid was obtained, m.p. 228 °C. **^{1}H NMR** (300 MHz, D_2O): 2.73 (s, 3H), 4.38 (s, 2H), 7.40 – 7.58 (m, 3H), 7.60

– 7.70 (m, 2H), 8.50 (s, 1H). **^{13}C NMR** (75 MHz, D_2O): 32.1, 42.6, 121.1, 124.8, 129.8, 130.0, 136.0, 138.5. **$C_{10}H_{12}N_4$:** MS (LC-MS): *m/z*(%) [t_r=1.04 min.]: 189(100, MH+), 377(35).

***tert*-Butyl methylthio(3-((1-phenyl-1H-1,2,3-triazol-4-yl)methyl)ureido)-methylene-carbamate (21)**

The compound was prepared according to general procedure A. Column chromatography (PE/EA 3:1 -> 2:3, R_f=0.3(PE/EA 3:2)). A white solid was obtained (308 mg, 79%), m.p. 157 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.48 (s, 9H), 2.30 (s, 3H), 4.55 – 4.65 (d, J=6.15, 2H), 6.24 (s, 1H), 7.35 – 7.60 (m, 3H), 7.60 – 7.80 (m, 2H), 7.80 (s, 1H), 12.21 (bs, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.4, 28.0, 35.6, 82.8, 120.4, 120.6, 128.8, 129.8, 137.0, 145.6, 160.0, 161.8, 168.3. **$C_{17}H_{22}N_6O_3S$:** MS (LC-MS): *m/z*(%) [t_r=7.51 min.]: 391(100, MH+), 781(60).

***tert*-Butyl (3-((1-(3-azidophenyl)-1H-1,2,3-triazol-4-yl)methyl)ureido)(methylthio)-methylenecarbamate (22)**

The compound was prepared according to general procedure A. Column chromatography (PE/EA 3:1 -> 2:3, R_f=0.3(PE/EA 3:2)). A white solid was obtained (164 mg, 38%), m.p. 128 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.48 (s, 9H), 2.30 (s, 3H), 4.55 – 4.65 (d, J=6.15, 2H), 6.24 (s, 1H), 7.00 – 7.10 (m, 1H), 7.20 – 7.26 (m, 1H), 7.30 – 7.45 (m, 2H), 7.70 (s, 1H), 12.18 (bs, 1H). **$C_{17}H_{22}N_6O_3S$:** MS (LC-MS): *m/z*(%) [t_r=7.88 min.]: 432(100), 863(60).

***tert*-Butyl (3-methyl-3-((1-phenyl-1H-1,2,3-triazol-4-yl)methyl)ureido)(methyl-thio)methylenecarbamate (23)**

The compound was prepared according to general procedure A. Column chromatography (PE/EA 3:1 -> 2:3, R_f=0.3(PE/EA 3:2)). A white solid was obtained (144 mg, 25%), m.p. 145 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.49 (s, 9H), 2.33 (d, J=4.75, 3H), 3.00 – 3.30 (m, 3H), 4.65 – 5.30 (m, 2H), 7.35 – 7.60 (m, 3H), 7.65 – 7.75 (m, 2H), 7.75 – 8.05 (m, 1H), 12.30 – 12.55 (m, 1H). **$C_{18}H_{24}N_6O_3S$:** MS (LC-MS): *m/z*(%) [t_r=7.95 min.]: 405 (100, MH+), 809(20).

10-(4-Hydroxybenzylcarbamoyl)-3,12-dioxo-13,13-diphenyl-1-(1-phenyl-1H-1,2,3-triazol-4-yl)-2,4,6,11-tetraazatridecan-5-iminium 2,2,2-trifluoroacetate (1d)

The compound was prepared according to general procedure B. The product is a white solid (158 mg, 57%), m.p. > 190 °C (decomp). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 3.15 – 3.30 (m, 2H), 4.05 – 4.25 (m, 2H), 4.25 – 4.40 (m, 1H), 4.40 – 4.55 (d, J=5.31,

2H), 5.12 (s, 1H), 6.68 (d, J=8.44, 2H), 7.00 (d, J=8.44, 2H), 7.15 – 7.35 (m, 10H), 7.45 – 7.55 (m, 1H), 7.55 – 7.65 (m, 2H), 7.85 – 7.95 (m, 2H), 8.11 (m, 1H), 8.30 – 8.65 (m, 4H), 8.70 (s, 1H), 8.96 (bs, 1H), 9.30 (bs, 1H), 10.08 (s, 1H). **$C_{37}H_{39}N_9O_4$: MS** (LC-MS): *m/z*(%) [t_r=5.63 min.]: 337(10), 674(100, MH+). **UV** (MeCN): λ(ε) 248 ($9 \cdot 10^3$).

1-(1-(3-Azidophenyl)-1H-1,2,3-triazol-4-yl)-10-(4-hydroxybenzylcarbamoyl)-3,12-dioxo-13,13-diphenyl-2,4,6,11-tetraazatridecan-5-iminium 2,2,2-trifluoroacetate (1b)

The compound was prepared according to general procedure B. The product is a white solid (166 mg, 21%), m.p. > 190 °C (decomp). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 3.15 – 3.30 (m, 2H), 4.05 – 4.25 (m, 2H), 4.25 – 4.40 (m, 1H), 4.40 – 4.55 (d, J=5.31, 2H), 5.12 (s, 1H), 6.68 (d, J=8.45, 2H), 7.00 (d, J=8.45, 2H), 7.15 – 7.35 (m, 11H), 7.58 – 7.68 (m, 2H), 7.68 – 7.78 (m, 1H), 8.12 (bs, 1H), 8.30 – 8.55 (m, 4H), 8.79 (s, 1H), 8.91 (bs, 1H), 9.30 (s, 1H), 9.91 (s, 1H). **$C_{37}H_{38}N_{12}O_4$: MS** (LC-MS): *m/z*(%) [t_r=5.78 min.]: 715(100, MH+). **UV** (MeCN): λ(ε) 244 ($19 \cdot 10^3$).

10-(4-Hydroxybenzylcarbamoyl)-2-methyl-3,12-dioxo-13,13-diphenyl-1-(1-phenyl-1H-1,2,3-triazol-4-yl)-2,4,6,11-tetraazatridecan-5-iminium 2,2,2-trifluoroacetate (1e)

The compound was prepared according to general procedure B. The product is a white solid (160 mg, 72%), m.p. > 190 °C (decomp). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 3.06 (s, 3H), 3.20 – 3.30 (m, 2H), 4.05 – 4.25 (m, 2H), 4.25 – 4.40 (m, 1H), 4.70 (s, 2H), 5.13 (s, 1H), 6.67 (d, J=8.42, 2H), 7.00 (d, J=8.42, 2H), 7.15 – 7.35 (m, 10H), 7.45 – 7.55 (m, 1H), 7.55 – 7.65 (m, 2H), 7.85 – 7.95 (m, 2H), 8.33 – 8.43 (m, 1H), 8.45 – 8.65 (m, 3H), 8.77 (s, 1H), 8.90 – 9.05 (bs, 1H), 9.31 (bs, 1H), 9.79 (bs, 1H). **$C_{38}H_{41}N_9O_4$: MS** (LC-MS): *m/z*(%) [t_r=5.70 min.]: 688(100, MH+). **UV** (MeCN): λ(ε) 248 ($12 \cdot 10^3$).

***tert*-Butyl (1-methyl-1H-1,2,3-triazol-4-yl)methylcarbamate (24)**

NaN_3 (714 mg, 11.0 mmol) was dissolved in 2 mL H_2O in a 10 mL flask and 4 mL Et_2O were added. Then the phase-transfer catalyst tetrabutylammonium tetrafluoroborate (90 mg, 0.27 mmol) was added and then MeI (780 mg, 5.49 mmol) was added. The mixture was stirred vigorously for two days at room temperature. Then the organic layer was separated and the aqueous phase was washed with Et_2O (2 x 2 mL). Then the organic fractions were mixed and a solution of the Boc-protected propargylamine (510 mg, 3.29 mmol) in 3 mL of MeOH was added. Then ascorbic acid (95 mg, 0.54 mmol, dissolved in 0.8 mL H_2O) and $CuSO_4 \cdot 5\ H_2O$ (68 mg, 0.27 mmol, dissolved in 0.8 mL H_2O) were added. The mixture was stirred

vigorously at room temperature overnight. The next day the solvent was evaporated completely and the residue was dissolved in 1 mL of ($CHCl_3$/EtOH 19:1). The crude material was purified with column chromatography ($CHCl_3$/EtOH 19:1 -> 9:1, R_f=0.3 [$CHCl_3$/EtOH 9:1]). A white solid was obtained (500 mg, 72%). The compound was deprotected without further characterisation.

(1-Methyl-1H-1,2,3-triazol-4-yl)methanaminium chloride (25)

The compound was deprotected according to general procedure D. A light yellow solid was obtained (351 mg, quantitative), m.p. 172 °C. **^{1}H NMR** (300 MHz, D_2O): 4.10 (s, 3H), 4.30 (s, 2H), 8.02 (s, 1H). **^{13}C NMR** (75 MHz, D_2O): 34.0, 36.7, 126.2. **$C_4H_8N_4$: MS** (LC-MS): *m/z*(%) [t_r=0.32 min.]: 113(60, MH+), 225(100).

***tert*-Butyl (3-((1-methyl-1H-1,2,3-triazol-4-yl)methyl)ureido)(methylthio)-methylenecarbamate (26)**

The compound was prepared according to general procedure A. Column chromatography (EA, R_f=0.26). A white solid was obtained (735 mg, 78%), m.p. 138 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): 1.49 (s, 9H), 2.33 (s, 3H), 4.08 (s, 3H), 4.40 – 4.80 (m, 2H), 6.40 (bs, 1H), 7.58 (bs, 1H), 12.28 (bs, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.3, 28.0, 35.5, 36.7, 82.7, 123.0, 145.1, 151.0, 161.8, 168.0. **$C_{12}H_{20}N_6O_3S$: MS** (LC-MS): *m/z*(%) [t_r=6.27 min.]: 328(75, MH+), 657(100).

10-(4-Hydroxybenzylcarbamoyl)-1-(1-methyl-1H-1,2,3-triazol-4-yl)-3,12-dioxo-13,13-diphenyl-2,4,6,11-tetraazatridecan-5-iminium 2,2,2-trifluoroacetate (1f)

The compound was prepared according to general procedure B. A white solid was obtained (80 mg, 55%), m.p. > 190 °C (decomp.). **^{1}H NMR** (300 MHz, DMSO-d6): 1.30 – 1.80 (m, 4H), 3.10 – 3.30 (m, 2H), 4.01 (s, 3H), 4.05 – 4.25 (m, 2H), 4.25 – 4.40 (m, 3H), 5.13 (s, 1H), 6.67 (d, J=8.42, 2H), 7.00 (d, J=8.42, 2H), 7.15 – 7.35 (m, 11H), 7.93 (s, 1H), 7.95 – 8.05 (m, 1H), 8.30 – 8.60 (m, 4H), 8.98 (s, 1H), 10.17 (s, 1H). **$C_{32}H_{37}N_9O_4$: MS** (LC-MS): *m/z*(%) [t_r=5.15 min.]: 612(100, MH+), 1223(5).

(R)-1-(3-Azidophenyl)-15-(4-hydroxybenzylcarbamoyl)-1,8,17-trioxo-18,18-diphenyl-2,7,9,11,16-pentaazaoctadecan-10-iminium 2,2,2-trifluoroacetate (1h)

Compound **27** (3.9 mg, 23.7 µmol) was dissolved in 1.5 mL of DMF. DIPEA (28 mg, 215 µmol) and TBTU (8.3 mg, 25.8 µmol) dissolved in 500 µL of MeCN was added. After 10

minutes compound **28** (20.0 mg, 21.5 µmol) was added and the mixture was stirred overnight. Next day DMF was removed completely and the crude material was redissolved in 1 mL of MeCN/H_2O (1:9) containing 0.1% TFA. It was purified with HPLC. A white solid was obtained (11.1 mg, 61%), m.p. 158 °C (decomp.). **^{1}H NMR** (600 MHz, DMSO-d6): 1.30 – 1.70 (m, 8H), 3.05 – 3.35 (m, 6H), 4.05 – 4.20 (m, 2H), 4.33 (m, 1H), 5.12 (s, 1H), 6.67 (d, J=8.46, 2H), 7.00 (d, J=8.46, 2H), 7.15 – 7.30 (m, 11H), 7.35 – 7.80 (m, 5H), 8.20 – 8.50 (bs, 1H), 8.35 (t, J=5.78, 1H), 8.47 (d, J=8.09, 1H), 8.58 (t, J=5.57, 1H), 8.87 (bs, 1H), 9.27 (bs, 1H), 9.60 (bs, 1H). **^{13}C NMR (HSQC, HMBC)** (151 MHz, DMSO-d6): 24.53, 26.43, 26.50, 29.40, 40.04, 40.31, 41.58, 52.21, 55.88, 114.97, 117.60, 121.70, 123.87, 130.01, 126.55, 128.14, 128.17, 128.39, 128.47, 128.48, 129.10, 136.25, 139.63, 140.24, 140.43, 156.23, 165.06, 170.92, 170.98. **$C_{39}H_{44}N_{10}O_5$: MS** (LC-MS): *m/z*(%) [t_r=5.83 min.]: 733(100, MH+). **UV** (MeCN): λ(ε) 249 ($16\cdot10^3$). **UV** (MeCN): λ(ε) 248 ($16\cdot10^3$). **IR** (ν [cm^{-1}]): 3303, 2114 (N_3), 1638, 1538, 1515, 1450.

5.4.3 Radioligand competition binding assay

Radioligand competition experiments at SK-N-MC neuroblastoma cells using the radioligand [^{3}H]UR-MK114 (1.5 nM) were performed as described elsewhere.[14]

5.4.4 Fura-2 assay on HEL cells

The Fura assay was performed with HEL cells as previously described using a Perkin-Elmer LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany).[32]

5.4.5 Cell culture and confocal microscopy

MCF-7 cells were cultivated at 37 °C for two days in 8 well µ-slides (Ibidi GmbH, Munich) with EMEM medium (Sigma, Deisenhofen, Germany) containing 5% FCS (Biochrom AG, Berlin, Germany). Estradiol (1 nM) was added to the cultivation medium to stimulate Y_1R expression.[33] After two days, shortly before the experiment the medium was replaced by Leibovitz L15 medium (LM). All microscopy experiments were performed in LM. Stock solutions of the ligands were prepared in DMSO at a concentration that is at least hundredfold higher as the concentration in the experiment. The corresponding dilutions in LM were prepared shortly before the experiment, the DMSO concentration in the cell experiment was equal or below 1%.

Confocal microscopy was performed with a Zeiss Axiovert 200M microscope, equipped with a LSM 510 laser scanner. The fluorescent dye **29** was excited with a HeNe-laser at 633 nm and fluorescence was detected with a 650 nm long-pass filter. The objective was a Plan-Neofluar 40x/1.3 or a Plan-Apochromat 63x/1.4 with oil immersion.

5.4.6 Data processing

Data from radioligand competition experiments were analyzed by 4 parameter sigmoidal fits (SigmaPlot 9.0, Systat Software). IC_{50} values from radioligand competition studies were converted to K_i values according to the Cheng-Prusoff equation[34] using the respective K_D value of the radioligand.

5.4.7 References

1. Filmore, D. *Modern Drug Discovery* **2004,** *7*, 24
2. Kenakin, T.; Jenkinson, S.; Watson, C. *J. Pharm. Exp. Ther.* **2006,** *319*, 710
3. Vauquelin, G.; Liefde, I. V.; Birzbier, B. B.; Vanderheyden, P. M. L. *Fund. Clin. Pharm.* **2002,** *16*, 263
4. Christophe, B.; Carlier, B.; Gillard, M.; Chatelain, P.; Peck, M.; Massingham, R. *Eur. J. Pharmacol.* **2003,** *470*, 87
5. Henkel, J. G.; Portoghese, P. S. *J. Med. Chem.* **1976,** *19*, 6
6. Torchiana, M. L.; Pendleton, R. G.; Cook, P. G.; Hanson, C. A.; Clineschmidt, B. V. *Pharmacol. Exp. Ther.* **1983,** *224*, 514
7. Mullins, D.; Adham, N.; Hesk, D.; Wu, Y.; Kelly, J.; Huang, Y.; Guzzi, M.; Zhang, X.; McCombie, S.; Stamford, A.; Parker, E. *Eur. J. Pharmacol.* **2008,** *601*, 1
8. Shryock, J. C.; Snowdy, S.; Baraldi, P. G.; Cacciari, B.; Spalluto, G.; Monopoli, A.; Ongini, E.; Baker, S. P.; Belardinelle, L. *Circulation* **1998,** *98*, 711
9. Gifford, A. N.; Bruneus, M.; Gatley, S. J.; Lan, R.; Makriyannis, A.; Volkow, N. D. *J. Pharm. Exp. Ther.* **1999,** *288*, 478
10. Meller, E.; Helmer-Matyjek, E.; Bohmaker, K.; Adler, C. H.; Friedhoff, A. J.; Goldstein, M. *Eur. J. Pharmacol.* **1986,** *123*, 311
11. Sajdyk, T. J. *Drug Dev. Res.* **2005,** *65*, 301
12. Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Beck-Sickinger, A. G.; Doods, H. N. *Eur. J. Pharmacol.* **1994,** *271*, R11.

13. Brennauer, A.; Dove, S.; Buschauer, A. *Structure-Activity Relationships of Nonpeptide Neuropeptide Y Receptor Antagonists. In Handbook of Experimental Pharmacology. Vol. 162. Michel, M.C. (ed)*, 2004.
14. Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. *Journal of Medicinal Chemistry* **2008,** *51*, 8168.
15. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B. *Bioorg. Med. Chem.* **2010,** *18*, 6292
16. Budyka, M. F. *Russ. Chem. Rev.* **2008,** *77*, 709
17. Kym, P. R.; Carlson, K. E.; Katzenellenbogen, J. A. *Bioconjug. Chem.* **1995,** *6*, 115
18. Burgermeister, W.; Hekman, M.; Helmreich, E. J. M. *J. Biol. Chem.* **1982,** *257*, 5306
19. Keller, M.; Teng, S.; Bernhardt, G.; Buschauer, A. *ChemMedChem* **2009,** *4*, 1733.
20. Schneider, E.; Keller, M.; Brennauer, A.; Hoefelschweiger, B. K.; Gross, D.; Wolfbeis, O. S.; Bernhardt, G.; Buschauer, A. *Chembiochem* **2007,** *8*, 1981.
21. Keller, M. Dissertation, **2008**.
22. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B. *Bioorg. Med. Chem.* **2008,** *16*, 9858.
23. Vauquelin, G.; Van Liedfde, I.; Vanderheyden, P. *Trends Pharmacol. Sci.* **2002,** *23*, 514
24. Erdmann, D., University of Regensburg2010.
25. Qiao, C.; Jeon, H.-B.; Sayre, L. M. *Journal of the American Chemical Society* **2004,** *126*, 8038.
26. Ritter, S. C.; König, B. *Chem. Commun.* **2006**, 4694
27. Ciocoiu, C. C.; Nikolic, N.; Nguyen, H. H.; Thoresen, G. H.; Aasen, A. J.; Hansen, T. V. *Eur. J. Med. Chem.* **2010,** *45*, 3047
28. Latyshev, G. V.; Baranov, M. S.; Kazantsev, A. V.; Averin, A. D.; Lukashev, N. V.; Beletskaya, I. P. *Synthesis* **2009,** *15*, 2605
29. Bradbury, B. J.; Baumgold, J.; Jacobson, K. A. *J. Med. Chem.* **1990,** *33*, 741
30. Xiong, Y.; Bernardi, D.; Bratton, S.; Ward, M. D.; Battaglia, E.; Finel, M.; Drake, R. R.; Radominska-Pandya, A. *Biochem.* **2006,** *45*, 2322
31. Herring, D. L. *J. Org. Chem.* **1961,** *26*, 3998
32. Müller, M.; Knieps, S.; Gessele, K.; Dove, S.; Bernhardt, G.; Buschauer, A. *Arch Pharm (Weinheim)* **1997,** *330*, 333.
33. Amlal, H.; Faroqui, S.; Balasubramaniam, A.; Sheriff, S. *Cancer Res.* **2006,** *66*, 3706
34. Cheng, Y.; Prusoff, W. H. *Biochem Pharmacol* **1973,** *22*, 3099.

Appendix

A.1 Abbreviations

aq.	Aqueous
B_{max}	maximum number of binding sites
Bn	benzyl
Boc	*tert*-butoxycarbonyl
Boc_2O	di-*tert*-butyl dicarbonate
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
c	concentration
Cbz	benzyloxycarbonyl
Cy5	red fluorescent cyanine dye
DAD	diode array detector
DAPI	4′,6-Diamidin-2-phenylindol
DCC	*N*,*N*´-dicyclohexylcarbodiimide
DCM	dichloromethane
DIPEA	*N*,*N*-diisopropyl-ethylamine
DMAEA	2-(Dimethylamino)ethylamine
DMAP	4-(dimethylamino)-pyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
EA	ethylacetate
EC_{50}	agonist concentration that elicits 50 % of the max. response
EDC	3-(ethyliminomethyleneamino)-N,N-dimethyl-propan-1-amine
EtOH	ethanol

ELSD	evaporative light scattering detection
Excimer	excited dimer
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GPCR	G protein-coupled receptor
HEL cells	human erythroleukemia cells
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure
HMBC	heteronuclear multiple bond coherence
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
IC_{50}	antagonist concentration that suppresses 50 % of an agonist induced effect or displaces 50 % of a labeled ligand from the binding site
IR	infrared spectroscopy
K_d	dissociation constant derived from a saturation experiment
K_i	dissociation constant derived from a competition binding assay
K_{ob}	observed association rate constant
K_{off}	dissociation rate constant
K_{on}	association rate constant
logD	logarithm of the pH dependent n-octanol/water partition coefficient
logP	logarithm of the n-octanol/water partition coefficient
MCF-7 cells	human breast adenocarcinoma cells
MeCN	acetonitrile
MeOH	methanol

mp	melting point
MS	mass spectroscopy
NEt_3	triethylamine
N^G	guanidine nitrogen
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
NPY	neuropeptide Y
PBS	phosphate buffered saline
Pd-C	palladium on activated charcoal (5 – 10 % w)
PE	petrol ether
PEG	polyethylene glycol
pNPY	porcine neuropeptide Y
R_f	retention factor
RT	room temperature
SEM	standard error of the mean
SK-N-MC cells	human neuroblastoma cell line
TBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
^{t}Bu	*tert*-butyl
TFA	trifluoroacetic acid
THF	tetrahydrofurane
TLC	thin layer chromatography
UV	ultraviolet
VIS	visible
Y_1R	neuropeptide Y_1 receptor subtype

A.2 Curriculum Vitae

Personal data

Name	Stefan Weiß
Date of birth	06. August 1981
Place of birth	Straubing
Nationality	German
Marital status	Single
Email	stefan1.weiss@chemie.uni-regensburg.de

Education

1988 – 1992	Grundschule St.Josef, Straubing
1992 – 2001	Ludwigsgymnasium, Straubing
2001	Abitur

Professional training

2002 – 2007	University of Regensburg
2007	Diplom (M. Sc.) in Chemistry
11/2007 – 12/2010	PhD program at the department of organic chemistry, University of Regensburg Advisor: Prof. Dr. Burkhard König
Since 04/2008	Associate member of the research training group (GRK 760): *Medicinal Chemistry: Molecular recognition – ligand-receptor interactions*

A.3 Publications, Posters and Professional Training

Publications:

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

Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B. "N^G-Acyl-argininamides as NPY Y_1 Receptor Antagonists: Influence of Stucturally Diverse Acyl Substituents on Stability and Affinity", *Bioorg. Med. Chem.* **2010,** *18*, 6292.

Gruber, B.; Stadlbauer, S.; Späth, A.; Weiss, S.; Kalinina, M.; König, B. "Modulare Chemosensoren auf Basis selbstorganisierter Vesikelmembranen mit künstlichen Rezeptoren und fluoreszierenden Reportergruppen", *Angew. Chem.* **2010,** *122*, 7280

Raster, P.; Weiss, S.; Hilt, G.; König, B. "Synthesis and Photoisomerization of Diarylcyclobutenes", *Synthesis* **2011**, *accepted*

Poster Presentations:

Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B., General modular synthesis of nonpeptidic bivalent NPY receptor antagonists, **„Frontiers in Medicinal Chemistry", Regensburg, March 2008**

Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B., General modular synthesis of nonpeptidic bivalent NPY receptor antagonists and detection of Y1 receptor dimers, **4th International Summer School "Medicinal Chemistry", Regensburg, September 2008** and **2nd EuCheMS Chemistry Congress, Turin, September 2008**

Weiss, S.; Bernhardt, G.; Buschauer, A.; König, B., Catalytic staining of a G protein-coupled receptor, **239th ACS National Meeting, San Francisco, March 2010**

Weiss, S.; Bernhardt, G.; Buschauer, A.; König, B., Catalytic staining of NPY Y1 receptors on living MCF-7 cells with DMAP modified BIBP 3226 derivatives, **5th International Summer School "Medicinal Chemistry", Regensburg, September 2010**

Short Lectures:

Catalytic staining of a G protein-coupled receptor and internalisation studies, **3rd EuCheMS Chemistry Congress, Nürnberg, August 2010**

Professional Training:

Since April 2007 member of the Research Training Group (**GRK 760**) "Medicinal Chemistry: **Molecular Recognition – Ligand Receptor Interactions**"

A.4 Summary in German and English

Summary in German

In dieser Arbeit wurden Antagonisten für den Neuropeptid Y_1 Rezeptor synthetisiert. Das Ziel dabei war einen bekannten Antagonisten (BIBP 3226) mit unterschiedlichen Substituenten zu versehen, deren spezielle Eigenschaften einen Einblick in die Struktur und Funktionalität des Rezeptors gewähren sollen.

Dazu wurde im ersten Kapitel die Stabilität und Affinität verschiedener substituierter BIBP 3226 Derivate untersucht, die eine große Diversität in Polarität und Struktur aufwiesen. Das Ergebnis war, dass der Y_1 Rezeptor eine große Vielfalt an verschieden substituierten BIBP 3226 Derivaten mit relativ hoher Affinität binden kann und dass polare Acylguanidine zum Teil eine starke Tendenz zur Hydrolyse aufweisen.

Im zweiten Kapitel wurde Pyren als fluoreszierende Sonde über verschieden lange Spacer an das BIBP 3226 Grundgerüst angebaut, um eine eventuelle Ausbildung

von Rezeptordimeren beobachten zu können. Pyren bildet bei hohen lokalen Konzentrationen Excimere, die Licht mit einer längeren Wellenlänge emittieren als das Pyren Monomer. Es wurden Moleküle mit nanomolaren Affinitäten erhalten, die aber leider im eingesetzten Konzentrationsbereich zu schwach fluoreszierten um eine Detektion von Rezeptordimeren zu ermöglichen.
Im dritten Kapitel wurden lipophile BIBP 3226 Derivate hergestellt und zusammen mit lipophilen Carboxyfluorescein Derivaten in Vesikel eingebaut um Y_1 Rezeptor spezifische Vesikel zu erhalten. Als weiterer Bestandteil wurde PEG verschiedener Kettenlänge in die Vesikel mit eingebaut. Die Spezifität der Vesikel konnte zwar nicht erreicht werden, jedoch konnte die starke Bindung der PEG freien Vesikel an die Zellmembran mit geringen PEG-Konzentrationen stark reduziert werden.
Im vierten Teil wurde der Acylierungskatalysator DMAP an BIBP 3226 angebaut. Die so erhaltenen Derivate sollten den Y_1 Rezeptor spezifisch in Gegenwart eines fluoreszierenden Aktivesters färben. Dazu wurden Testreaktionen durchgeführt um den besten Aktivester zu ermitteln und die Reaktion wurde dann an lebenden Zellen getestet.
Das fünfte Kapitel behandelt (pseudo)-irreversible Antagonisten für den Y_1 Rezeptor. Es wurde eine Serie von Substanzen gefunden, welche den Rezeptor nach Bindung irreversibel gegenüber NPY und einem fluoreszierenden Y_1 Antagonisten blockieren können.

Summary in English

In this thesis antagonists for the neuropeptide Y_1 receptor were synthesized. The goal was to modify the known Y_1 receptor antagonist BIBP 3266 with different substituents to obtain an insight in structure and functionality of the receptor.
Therefore the stability and the affinity of the diverse substituted BIBP 3226 derivatives was investigated in the first chapter. The result was that the Y_1 receptor has a relative high affinity to a broad variety of different substituted BIBP 3226 derivatives and that some polar acylguanidines exhibit a strong tendency for hydrolysis.

In the second chapter the fluorescent probe pyrene was attached to the BIBP 3226 scaffold with spacers of different length to investigate a possible formation of receptor dimers. Pyrene forms excimers at a high local concentration that emit light at a longer wavelength as the pyrene monomer. Molecules with nanomolar affinity were obtained, but their fluorescence intensity was too weak to use them for the detection of receptordimers.

In the third chapter lipophilic BIBP 3226 derivatives were synthesized and embeded in vesicles together with lipophilic carboxyfluorescein derivatives to obtain Y_1 receptor specific vesicles. PEG of different chain length was added as a further component. The specifity of the vesicles was not achieved, but the strong binding of the PEG free vesicles to the cell membrane could be reduced significantly with small amounts of PEG.

In the fourth part the acyl-transfer catalyst DMAP was attached to the BIBP 3226 scaffold. The resulting derivatives should specifically stain the Y_1 receptor in presence of a fluorescent active ester. Therefore test reactions were performed to identify the best active ester and then the reaction was performed on living cells.

The fifth chapter deals with (pseudo)-irreversible antagonists for the Y_1 receptor. A series of compounds was investigated that block the receptor irreversible against NPY and a fluorescent Y_1 receptor antagonist.