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# Red-fluorescent argininamide-type NPY $Y_1$ receptor antagonists as pharmacological tools

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ABSTRACT

Fluorescently labelled NPY $Y_1$ receptor ($Y_1$R) ligands were synthesized by connecting pyrylium and cyanine dyes with the argininamide-type $Y_1$R antagonist core structure by linkers, covering a wide variety in length and chemical nature, attached to the guanidine group. The most promising fluorescent probes had $Y_1$R affinities (radioligand binding) and antagonistic activities (calcium assay) in the one- to two-digit nanomolar range. These compounds turned out to be stable under assay conditions and to be appropriate for the detection of $Y_1$Rs by confocal microscopy in live cells. To improve the signal-to-noise ratio by shifting the emission into the near infrared, a new benzothiazolium-type fluorescent cyanine dye (UR-DE99) was synthesized and attached to the parent antagonist via a carbamoyl linker yielding UR-MK131, a highly potent fluorescent $Y_1$R probe, which was also successfully applied in flow cytometry.



## 1. Introduction

In the last two decades fluorescence-based techniques became highly attractive alternatives to radiometric assays for the investigation of ligand–receptor interactions. Fluorescent compounds are preferred over radioligands in terms of safety precautions, and are, in contrast to radiotracers, applicable to powerful techniques, which have become routine in many laboratories, such as fluorescence microscopy and flow cytometry. In the field of peptidergic G-protein coupled receptors (GPCRs), such as neuropeptide Y (NPY) receptors, a straightforward and frequently successful approach to obtain potent fluorescent ligands is the labelling of peptides.[1–4] The application of endogenous peptides, which are usually agonists, may be compromised by insufficient receptor subtype selectivity, slow binding kinetics, enzymatic degradation as well as induction of receptor desensitisation and internalisation. Therefore, we focused on the development of non-peptidic fluorescent NPY receptor ligands.[2,5] A major challenge in the preparation of low molecular weight fluorescent ligands is to retain the binding affinity, when a bulky fluorophore is attached to a small ligand. Exploring and exploiting the concept of guanidine/acylguanidine bioisosterism we recently reported an $N^G$-acylated argininamide (**2**, Fig. 1) as the first highly potent fluorescent NPY $Y_1$R antagonist.[5] The same bioisosteric approach was also shown to be a successful strategy to develop receptor subtype-selective radioligands, bivalent ligands and prodrugs.[6–10]

In continuation of our work on fluorescent GPCR ligands the present study is aiming at pharmacological tools with improved physicochemical and optical properties to broaden the range of potential applications, for example, with respect to optical in vivo imaging. The $Y_1$R was selected as a representative model of a peptidergic GPCR, for instance, as this receptor subtype was recently associated with diagnosis and treatment of tumours.[11–19] For the synthesis of non-peptidic fluorescent ligands, the argininamide-type $Y_1$R selective antagonists BIBP 3226[20] and BIBO 3304[21] (Fig. 1) were considered appropriate parent molecules to construct high affinity fluorescent probes with reduced propensity to induce internalisation compared to (peptidic) agonists. The present work was focused on the variation of chemical nature and length of the linkers connecting the pharmacophoric entity and the fluorophore.

## 2. Results and discussion

### 2.1. Chemistry

With respect to signal-to-noise ratios in cellular assays red-emitting fluorophores (emission wavelength >590 nm) were considered for the design of the fluorescent $Y_1$R ligands (Fig. 1).

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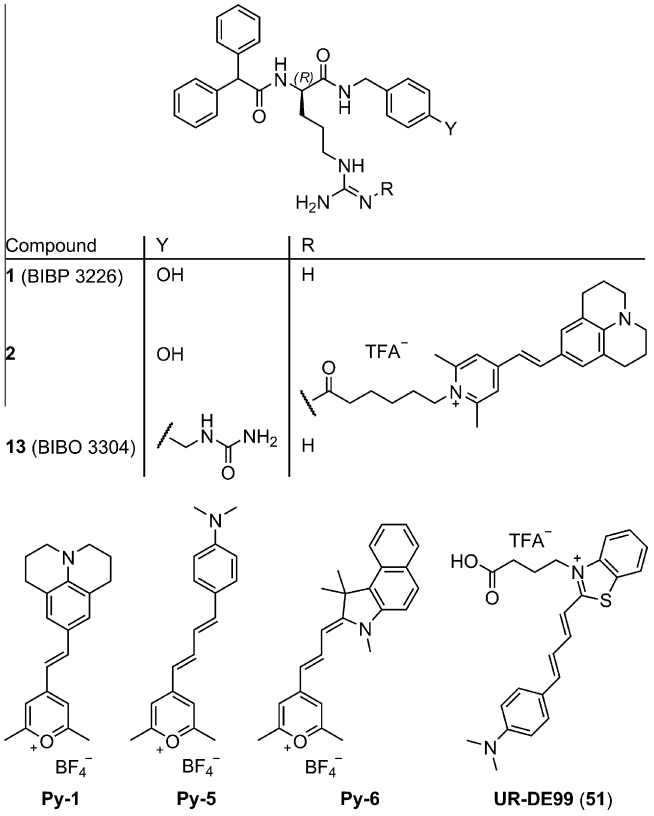
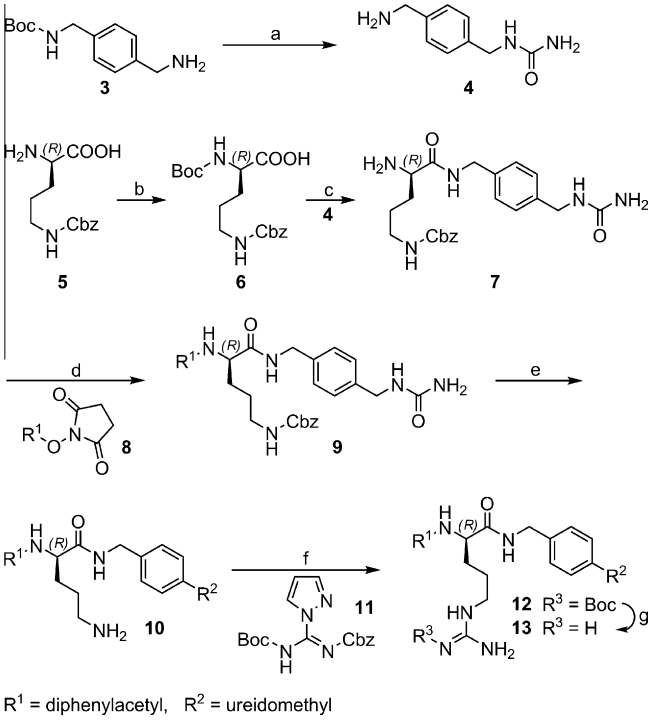



**Figure 1.** Chemical structures of the $Y_1R$ antagonists BIBP 3226 (**1**), the recently described BIBP 3226 derived fluorescent ligand **2**, BIBO 3304 and of the dyes used for the preparation of the herein described fluorescent $Y_1R$ ligands.

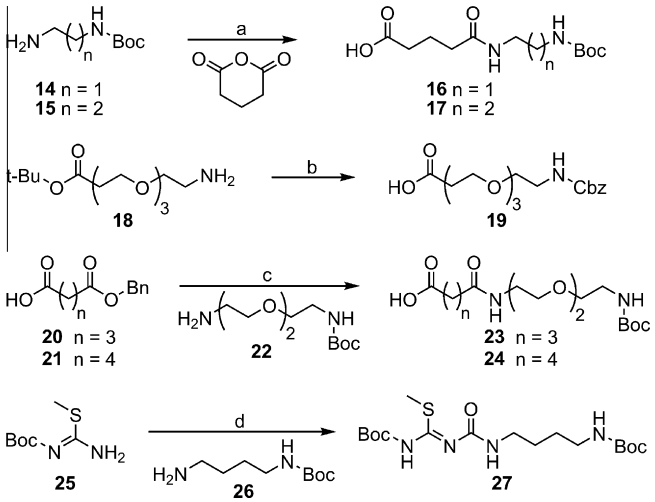



**Scheme 1.** Synthesis of the parent argininamide BIBO 3304 (**13**). Reagents: (a) (1) potassium cyanate, $H_2O$, EtOH, 1 M aq HCl; (2) acetyl chloride (AcCl), MeOH, 88%; (b) $K_2CO_3$, di-*tert*-butyl dicarbonate, 1,4-dioxane, $H_2O$, 92%; (c) (1) CDI, $NEt_3$, 4-(dimethylamino)pyridine (DMAP), DMF; (2) AcCl, MeOH, 85%; (d) $NEt_3$, DMAP, DMF, 83%; (e) Pd/C, $H_2$, $CH_3COOH$, MeOH, 99%; (f) (1) $NEt_3$, MeOH, DCM; (2) Pd/C, $H_2$, $CH_3COOH$, MeOH, 73%; (g) TFA, MeOH, 95%.

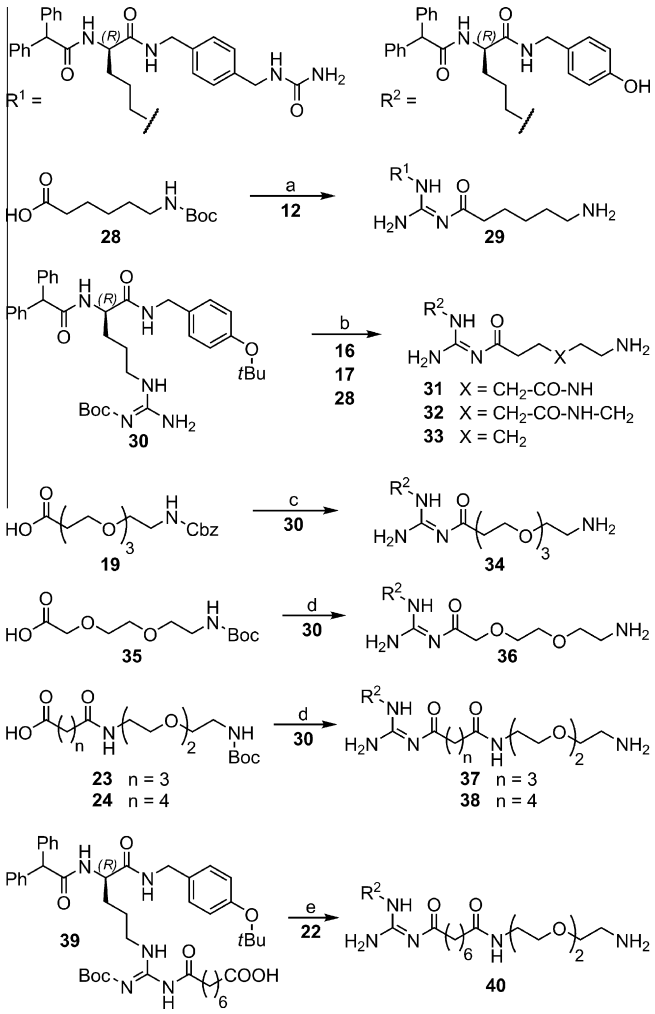
Pyrylium dyes (Py-1, Py-5 and Py-6)[22,23] were preferred over cyanine dyes such as S0436 and Dy-635 due to positive results from previous studies.[5] The pyrylium dyes Py-1, Py-5 and Py-6 belong to the class of 'chameleon labels', which has been developed for the staining and quantification of proteins at picomolar concentrations.[22] In addition, we synthesized UR-DE99 (**51**) (Fig. 1, Scheme 5), a red fluorescent dye derived from reported push–pull conjugated benzothiazolium compounds.[24] The used dyes are relatively low molecular weight compounds (300–400 g/mol), and are therefore anticipated to be well suited for the construction of fluorescent antagonists from small GPCR ligands. The resulting fluorescent pyridinium compounds can be excited with the 488 nm argon laser and UR-DE99 derivatives are suited for excitation with a red diode laser (635 nm). Both lasers are standard components of flow cytometers and confocal laser scanning microscopes.

The previously described synthesis of the parent argininamide **13** (BIBO 3304) starts with $N^{\omega}$-nitro protected D-arginine, which was first treated with diphenylacetyl chloride for acylation of the N-terminus, followed by amidation of the C-terminus with *N*-(4-aminomethylbenzyl)urea (**4**) using TBTU as coupling reagent.[25] Final cleavage of the $N^{\omega}$-nitro group (palladium catalyst, hydrogen at 5 bar, 80% aq acetic acid, 40 °C) yielded **13**. Here we describe a different strategy for the preparation of argininamide **13**, which starts with $N^{\alpha}$ Boc-protection of $N^{\delta}$ benzyloxycarbonylated (Cbz protected) D-ornithine **5** to obtain D-ornithine derivative **6** (Scheme 1). The urea derivative **4**[25] was obtained from mono Boc-protected 1,4-bis(aminomethyl)benzene (**3**)[26] and potassium cyanate.[27–30] The synthesis of **3** through treatment of 1,4-bis(aminomethyl)benzene with Boc anhydride yielded a considerable amount of the di Boc-protected by-product, which was not separated from **3**. Instead, the mixture of **3** and di Boc-protected 1,4-bis(aminomethyl)benzene was used for the preparation of **4**, which was subsequently separated from the di Boc compound. Amine **4** was used to amidate compound **6**. Subsequent removal of the $N^{\alpha}$-Boc group gave intermediate **7** (Scheme 1). Acylation of the α-amino group of **7** using succinimidyl diphenylacetate **8** (synthesis described elsewhere)[6] yielded compound **9**,[25] which was Cbz-deprotected through hydrogenation to give amine **10**. Treatment of **10** with the guanidinylating reagent **11**[6] and hydrogenation in the presence of a palladium-on-charcoal catalyst (Cbz-deprotection) gave the $N^{\omega}$-Boc protected argininamide **12**, a versatile building block for the preparation of $N^{\omega}$-acylated BIBO 3304 derivatives (cf. Scheme 3). The parent compound **13** was obtained through treatment of **12** with trifluoroacetic acid. Along with the incorporation of the urea moiety into the ornithine scaffold (compound **7**) synthesis of building block **12** was hampered by a dramatic decrease in solubility. Intermediate **9** (Scheme 1) exhibited lowest solubility, that is, the compound was only slightly soluble in methanol, dimethylformamide (DMF) and dimethylsulfoxide, but insoluble in dichloromethane (DCM), acetonitrile, 1,2-dimethoxyethane, THF, 1,4-dioxane, 2-propanol, ethanol and in mixtures of water with water-miscible solvents. Nevertheless, compound **12** was obtained in good yield (Scheme 1) as a building block for fluorescence labelling.

The length of the aminoalkanoyl and aminoalkylcarbamoyl linkers between the guanidine group and the fluorescent entity was varied in the range of 6–17 atoms. With respect to improved water solubility, linkers containing glycol ether or amide groups were designed. The synthesis of commercially unavailable linkers is shown in Scheme 2. Glutaric acid derivatives **16**[31] and **17** were obtained through treatment of mono Boc-protected ethane-1,2-diamine (**14**) and propane-1,3-diamine (**15**), respectively. The *N*-Cbz protected ω-amino carboxylic acid **19** was prepared from



**Scheme 2.** Synthesis of the carboxylic acids **16**, **17**, **19**, **23**, **24** and guanidinylating reagent **27**. Reagents: (a) DCM, 97%; (b) (1) Cbz-Cl, $NEt_3$, DCM; (2) DCM/TFA 4:1 (v/v), 88%; (c) (1) EDC, DCM; (2) Pd/C, $H_2$, MeOH, 63–66%; (d) triphosgene, diisopropylethylamine, DCM, 75%.



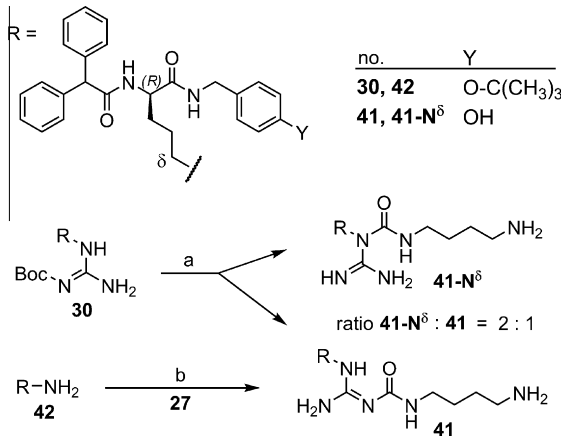
**Scheme 3.** Synthesis of the amine precursors **29**, **31–34**, **36–38** and **40**. Reagents: (a) (1) CDI, $NEt_3$, acetonitrile; (2) acetonitrile, TFA, 36%; (b) (1) CDI, $NEt_3$, DCM; (2) DCM/TFA/$H_2O$ 18:6:1 (v/v/v), 64–94%; (c) (1) CDI, $NEt_3$, DCM; (2) Pd/C, $H_2$, MeOH; (3) DCM/TFA 10:1 (v/v), 36%; (d) (1) CDI, $NEt_3$, DCM; (2) DCM/TFA, 44–64%; (e) (1) EDC, DCM; (2) DCM/TFA, 43%.

the *tert*-butyl ester of ω-amino carboxylic acid **18** via preparation of the benzylcarbamate (Cbz-protection) followed by cleavage of the *tert*-butyl ester. Glutaric acid derivative **23** and adipic acid derivative **24** were prepared through amidation of the monobenzyl esters **20** and **21** with mono Boc protected diamine **22** followed by hydrogenolytic debenzylation (Scheme 2). The carbamoylated isothiourea **27** was synthesized from the mono Boc protected building blocks **25** and **26** by coupling with triphosgene.

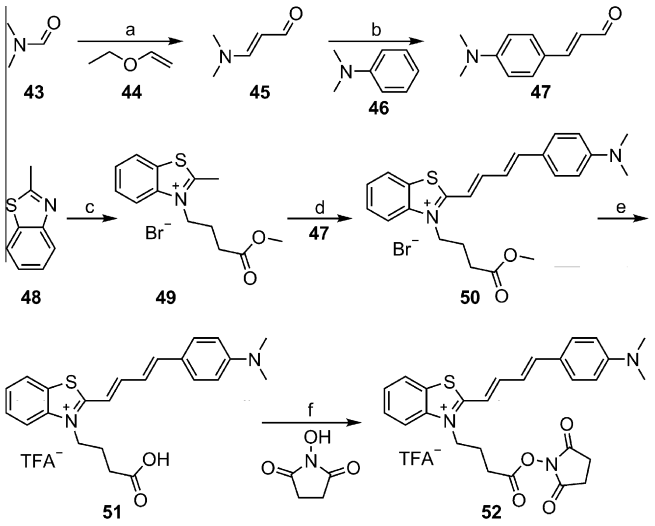
The synthesis of amines **29**, **31–34**, **36–38** and **40** as precursors of fluorescent ligands is depicted in Scheme 3. Except for **40** these compounds were prepared by coupling of the corresponding N-protected (Boc, Cbz) ω-aminocarboxylic acids (**16**, **17**, **19**, **23**, **24**, **28**, **35**) to building block **12** or **30** (synthesis described previously)[6] with the aid of 1,1′-carbonyldiimidazole (CDI) (Scheme 3). The protected intermediates were purified by column chromatography prior to Boc- or Cbz-deprotection yielding the amines. Compound **40** was prepared through amidation of the $N^{\omega}$-(7-carboxyheptanoyl), $N^{\omega\prime}$-Boc, *O*-*tert*-butyl protected argininamide **39** (synthesis reported elsewhere)[8] with mono Boc protected diamine **22**. The carboxy group of **39** was activated with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC).

Compound **33**, containing a $N^{\omega}$-6-aminohexanoyl substituent, proved to be sufficiently stable under basic conditions in contrast to its lower homologue, the $N^{\omega}$-5-aminopentanoyl derivative (synthesis not shown), which is cleaved by intramolecular aminolysis into **1** (Fig. 1) and valerolactam. This decomposition reaction takes place within seconds as demonstrated by kinetic investigations on aminopentanoyl-guanidine as a model compound.[32] By contrast, the formation of a seven-membered ring, namely ε-lactam, by elimination of the 6-aminohexanoyl substituent in **33** is considerably less favoured.

In a first attempt the $N^{\omega}$-carbamoylated precursor **41** was prepared from *N*-Boc-protected butane-1,4-diamine and building block **30** (Scheme 4) following a reported protocol for the transformation of amines into isocyanates with the aid of triphosgene.[33] Unfortunately, this procedure preferentially gave the $N^{\delta}$-substituted compound (**41-N$^{\delta}$**) instead of the $N^{\omega}$-substituted argininamide derivative (Scheme 4, Fig. 2). This is presumably facilitated by the small size of the electrophilic isocyanate entity, but other reasons such as different nucleophilicity of the δ-nitrogen and the ω-nitrogen, seem to play a role as well. Therefore, amine



**Scheme 4.** Synthesis of amine precursor **41** via direct carbamoylation of the $N^{\omega}$-Boc, *O*-*tert*-butyl protected building block **30** and through guanidinylation of amine **42** with reagent **27**. Reagents: (a) (1) *N*-Boc-butane-1,4-diamine, triphosgene, DIEA, DCM; (2) DCM/TFA 1:1 (v/v), total yield: 50%; (b) (1) $HgCl_2$, DIEA, DMF; (2) DCM/TFA 1:1 (v/v), 74%. **41** and **41-N$^{\delta}$** differ in the $^1$H NMR signal of the δ-$CH_2$ group (**41**: $\delta$ (DMSO-$d_6$) = 3.19 ppm, **41-N$^{\delta}$**: $\delta$ = 3.57 ppm).



**Scheme 5.** Synthesis of the fluorescent dye UR-DE99 (**51**) and its succinimidyl ester **52**. Reagents: (a) $POCl_3$, DMF, 40%; (b) (1) $POCl_3$, chloroform; (2) sodium hydroxide (20% in water), 33%; (c) methyl 4-bromobutanoate, 27%; (d) pyridine, MeOH, 65%; (e) LiOH, ethanol/THF/water 1:1:1 (v/v/v), 28%; (f) DCC, DMF, 57%.

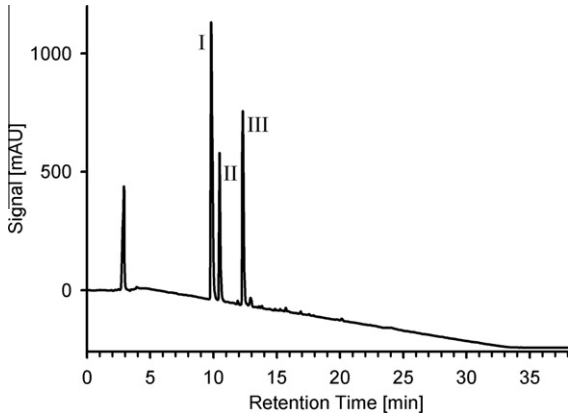
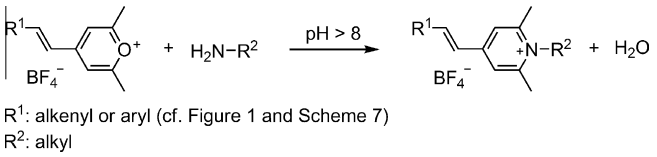



**Figure 2.** Synthesis of amine precursor **41** from *N*-Boc-butane-1,4-diamine (**26**) and building block **30** (cf. Scheme 4): HPLC analysis of the reaction mixture (after deprotection). Conditions: column: Eurospher-100 C18 (250 × 4 mm, 5 μm), eluent: mixtures of acetonitrile (A) and 0.05% aq TFA (B), gradient: 0–30 min: A/B 20:80–95:5, 30–40 min: 95:5, flow rate: 0.8 mL/min, UV-detection: 210 nm. I: **41**-$N^{\delta}$ ($t_R$ = 9.8 min), II: **41** ($t_R$ = 10.5 min), III: BIBP 3226 ($t_R$ = 12.3 min).

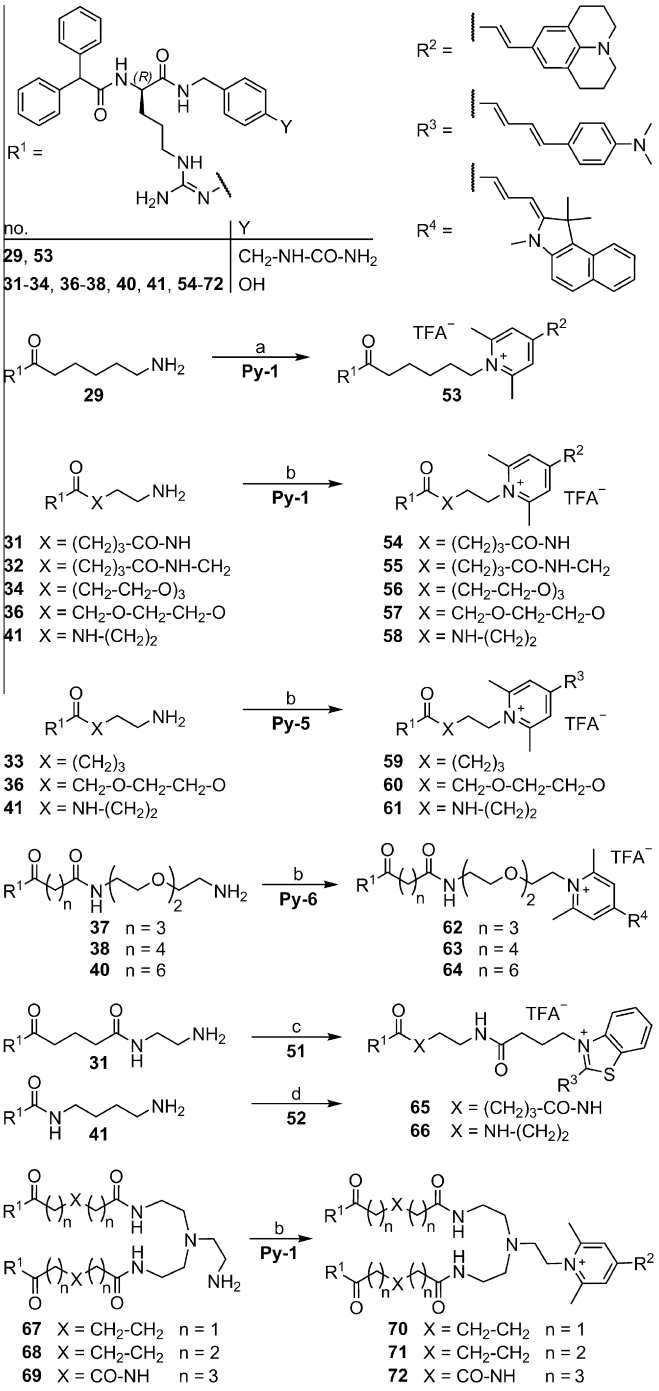
precursor **41** was prepared from the guanidinylating reagent **27** (Scheme 2) and amine **42** as depicted in Scheme 4.

For the synthesis of fluorescent dye UR-DE99 (**51**) dimethylformamide (**43**) and ethoxyethene (**44**) were converted to dimethylaminoacrylic aldehyde **45** (Vilsmeier formylation), which was subjected to Vilsmeier–Haack reaction with *N*,*N*-dimethylaniline (**46**) to give acrylic aldehyde **47** (Scheme 5).[24] N-Alkylation of benzothiazole **48** with methyl 4-bromobutanoate using the latter as solvent gave N-alkylated benzothiazolium derivative **49**. Condensation of acrylic aldehyde **47** with benzothiazolium derivative **49** yielded methyl ester **50**,[24] which was hydrolysed to the carboxylic acid **51** using lithium hydroxide. Final conversion of **51** into the active ester **52** was performed with dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide in anhydrous DMF (Scheme 5).

All fluorescent ligands were prepared by direct coupling of the fluorescent dyes (Py-1, Py-5, Py-6, UR-DE99) to the respective amines (**29**, **31–34**, **36–38**, **40**, **41**, **67–69**) (Scheme 7). Pyrylium dyes react very rapidly with primary amines at pH 8–9 at room temperature to form positively charged N-substituted pyridinium




**Scheme 6.** Conversion of pyrylium to pyridinium entities through reaction with primary amines.




**Scheme 7.** Synthesis of the fluorescent $Y_1R$ ligands (**53–66**, **70–72**). Reagents: (a) $NEt_3$, acetonitrile, $H_2O$, 33%; (b) $NEt_3$, acetonitrile, DMF, 13–56%; (c) CDI, $NEt_3$, DMF, 23%; (d) $NEt_3$, DMF, 24%.

adducts (Scheme 6).[34] The ring transformation is accompanied by a hypsochromic shift of the absorption maximum by more than

100 nm (change in colour from blue to red; 'chameleon labels')[22] as an indicator of the coupling reaction.

UR-DE99 (**51**) was coupled through its carboxylic group to the amine precursors either pre-activated as succinimidyl ester **52** or with the aid of coupling reagents (CDI, EDC) (Scheme 7). Activation of **51** with CDI in DMF and subsequent treatment with **31** in the presence of triethylamine gave the fluorescent ligand **65** in 23% yield (Scheme 7), whereas the synthesis of **66** from the carbamoyl precursor **41** failed under these conditions (yield: 0.3%). The same result with the latter was obtained when EDC was used instead of CDI. Interestingly, this was due to decomposition of precursor **41** yielding BIBP 3226 (**1**, cf. Fig. 1) as detected by HPLC analysis (data not shown). By contrast, labelling of amine **41** using succinimidyl ester **52** afforded **66** in 24% yield (Scheme 7). Labelling of the bivalent $Y_1R$ antagonists **67–69** (synthesis described elsewhere)[8] with Py-1 afforded the fluorescent bivalent ligands **70–72** in satisfactory yields, but efforts to label the bivalent $Y_1R$ ligand **69** with either **51** (activation with EDC or CDI) or its succinimidyl ester **52** failed.

### 2.2. Fluorescence properties of the labelled $Y_1R$ antagonists

The fluorescence quantum yields (Table 1) were determined (reference: cresyl violet perchlorate) in phosphate buffered saline (PBS) at pH 7.0 and in PBS containing 1% bovine serum albumin (BSA) to simulate assay conditions and to study the influence of proteins on the fluorescence properties. Additionally, the quantum yields of most compounds were determined in ethanol to examine the influence of the polarity of the solvent.

Generally, the $Y_1R$ antagonist core structures (BIBP 3226 or BIBO 3304) and the chemical structure of the linkers, connecting pharmacophore and fluorophore, have almost no effect on the fluorescence properties. Merely the Py-1 labelled bivalent ligands **70–72** showed a significant bathochromic shift of the excitation maximum in PBS ($\lambda_{ex} \approx 543$ nm) compared to the monovalent fluorescent ligands labelled with Py-1 ($\lambda_{ex} \approx 507$ nm).

All fluorescent ligands exhibit highest quantum yields (up to 52%) in PBS in the presence of 1% BSA, whereas the quantum yields were low in PBS alone (for all compounds less than 8%). In some cases the quantum yield increased up to 100-fold after addition of BSA (**58**, **72**, Table 1). Reasons for this phenomenon are obviously intermolecular hydrophobic and electrostatic interactions of the fluorophores with the protein. Moreover, binding of the fluorescent ligands to proteins can be regarded as a kind of rigidisation, which generally leads to an increase in quantum yield. Therefore, when these fluorescent ligands are used in BSA free buffers, the fluorescence could increase upon receptor binding. However, an increase in fluorescence intensity could also result from non-specific interactions of the ligand with other proteins or with the cell membrane. It is noticeable that the quantum yields in ethanol are quite high (≈22%) for the Py-5 and Py-6 labelled ligands (**59–64**) compared to the compounds labelled with other fluorescent dyes (Py-1, UR-DE99; quantum yields in ethanol <7%, Table 1). Consequently, ligands labelled with Py-5 or Py-6 (**59–64**) are more sensitive to hydrophobic interactions than those labelled with Py-1 or UR-DE99 (**53–58**, **65**, **66**, **70–72**).

The excitation and corrected emission spectra of a Py-1, Py-5, Py-6 and UR-DE99 fluorescently labelled ligand in PBS containing 1% BSA are depicted in Figure 3. The Stoke's shift is most pronounced for the Py-5 labelled compound **60**. The UR-DE99 labelled fluorescent ligand **66** can be excited with the red diode laser at 635 nm and additionally, albeit with lower efficiency, with the argon laser at 488 nm. Py-1, Py-5 and Py-6 labelled compounds (e.g. **57**, **60** and **62** in Fig. 3) can be excited at 488 nm (highest efficiency for Py-5). Thus, the fluorescence properties of all synthesized fluorescent ligands are compatible with an application to flow cytometric equilibrium binding studies (Section 2.7) and confocal microscopy (see Section 2.5).

**Table 1**
Fluorescence properties of the $Y_1R$ antagonists **53–66** and **70–72**: influence of the polarity of the solvent (PBS pH 7.0 vs ethanol) and protein (BSA) on the fluorescent properties (quantum yield $\Phi$ (reference: cresyl violet perchlorate) and excitation and emission maxima)

| Compd | Dye | PBS | | PBS + 1% BSA | | EtOH | |
|---|---|---|---|---|---|---|---|
| | | $\lambda_{ex}/\lambda_{em}$ | $\Phi$ (%) | $\lambda_{ex}/\lambda_{em}$ | $\Phi$ (%) | $\lambda_{ex}/\lambda_{em}$ | $\Phi$ (%) |
| **53** | Py-1 | 509/646 | 0.6 | 527/614 | 21 | nd | nd |
| **54** | Py-1 | 514/643 | 1.6 | 524/610 | 48 | 520/633 | 2.4 |
| **55** | Py-1 | 505/642 | 0.8 | 526/612 | 49 | 522/632 | 2.1 |
| **56** | Py-1 | 508/640 | 1.1 | 525/613 | 45 | 521/633 | 2.5 |
| **57** | Py-1 | 506/640 | 1.4 | 518/610 | 50 | 519/633 | 2.3 |
| **58** | Py-1 | 504/640 | 0.5 | 517/604 | 52 | 517/630 | 2.4 |
| **59** | Py-5 | 458/709 | 2.0 | 488/644 | 34 | 504/706 | 21 |
| **60** | Py-5 | 470/708 | 4.6 | 488/643 | 38 | 509/708 | 20 |
| **61** | Py-5 | 456/707 | 2.4 | 489/643 | 34 | 497/705 | 24 |
| **62** | Py-6 | 533/607 | 7.6 | 543 / 596 | 48 | 541/609 | 23 |
| **63** | Py-6 | 534/607 | 7.7 | 541/596 | 47 | 542/609 | 21 |
| **64** | Py-6 | 534/607 | 4.9 | 542/596 | 48 | 540/609 | 23 |
| **65** | **51** | 583/704 | 1.8 | 600/680 | 25 | 595/706 | 6.6 |
| **66** | **51** | 580/705 | 1.1 | 608/676 | 29 | 595/705 | 6.9 |
| **70** | Py-1 | 545/644 | 0.7 | 527/612 | 37 | 525/634 | 2.8 |
| **71** | Py-1 | 540/644 | 0.6 | 526/610 | 23 | 525/635 | 2.7 |
| **72** | Py-1 | 543/642 | 0.5 | 526/611 | 50 | 524/633 | 3.0 |

### 2.3. $Y_1$ receptor antagonism, affinity and selectivity

The fluorescent $Y_1R$ antagonists as well as the precursors **33** and **41** were investigated for $Y_1R$ antagonism in a spectrofluorimetric $Ca^{2+}$-assay (Fura-2 assay) on human erythroleukemia (HEL) cells[35] and for $Y_1R$ affinity in a binding assay on SK-N-MC neuroblastoma cells using [$^3$H]-UR-MK114[6] as radioligand (Table 2). All fluorescent ligands were able to antagonise the effect of the agonist pNPY on HEL cells ($K_b$ values in the one- to two-digit nanomolar range). Radioligand competition experiments on SK-N-MC cells revealed that most of the fluorescent $Y_1R$ antagonists are high affinity $Y_1R$ ligands with binding constants in the same range (Table 2). Lowest affinities ($K_i$ values: 100–150 nM) were determined for the fluorescent ligands **58** and **61–64**.

The reported picomolar affinity of the argininamide BIBO 3304 (**13**, Fig. 1)[21] gave reason to prepare a prototypical fluorescently labelled derivative (**53**). Whereas the high affinity of **13** was confirmed ($K_i$ = 0.25 nM, Table 2), attachment of a fluorescent dye through an acyl linker to the guanidine of **13** resulted in a considerable decrease in $Y_1R$ affinity (compound **53** (Scheme 7): $K_i$ = 23 nM, Table 2). By contrast, attachment of the same acyl substituent as in **53** to the guanidine group of BIBP 3226 (**1**, Fig. 1) afforded a fluorescent ligand with retained affinity, the recently reported $Y_1R$ antagonist **2** ($K_i$ = 29 nM, structure depicted in Fig. 1).[5] Thus, with regard to the lower affinity of **1** compared to **13**, the guanidine–acylguanidine bioisosteric approach is more efficient for BIBP 3226 (**1**), and it may be speculated about a slightly different orientation of the parent compounds BIBO 3304 (**13**) and BIBP 3226 at the $Y_1$ receptor. Due to lack of advantages in terms of potency and unexpectedly low solubility (org.solvents) of compounds derived from **13**, the preparation of fluorescent $Y_1R$ antagonists was carried on using BIBP 3226 as the preferred building block.

Amine precursor **41** was prepared because BIBP 3226 derived $N^{\omega}$-carbamoylated arginine derivatives turned out to have higher affinities than the parent compound.[36] Indeed, compound **41**, the carbamoyl analogue of **33**, is more potent than **33** by a factor of about four ($K_i$ values: 4.3 nM and 19 nM, Table 2). However, in terms of $Y_1R$ affinity $N^{\omega}$-carbamoylated arginine derivatives are not always superior to their $N^{\omega}$-acylated congeners. For instance, the Py-1 and Py-5 labelled ligands **58** and **61** ($K_i$ values: 140 nM

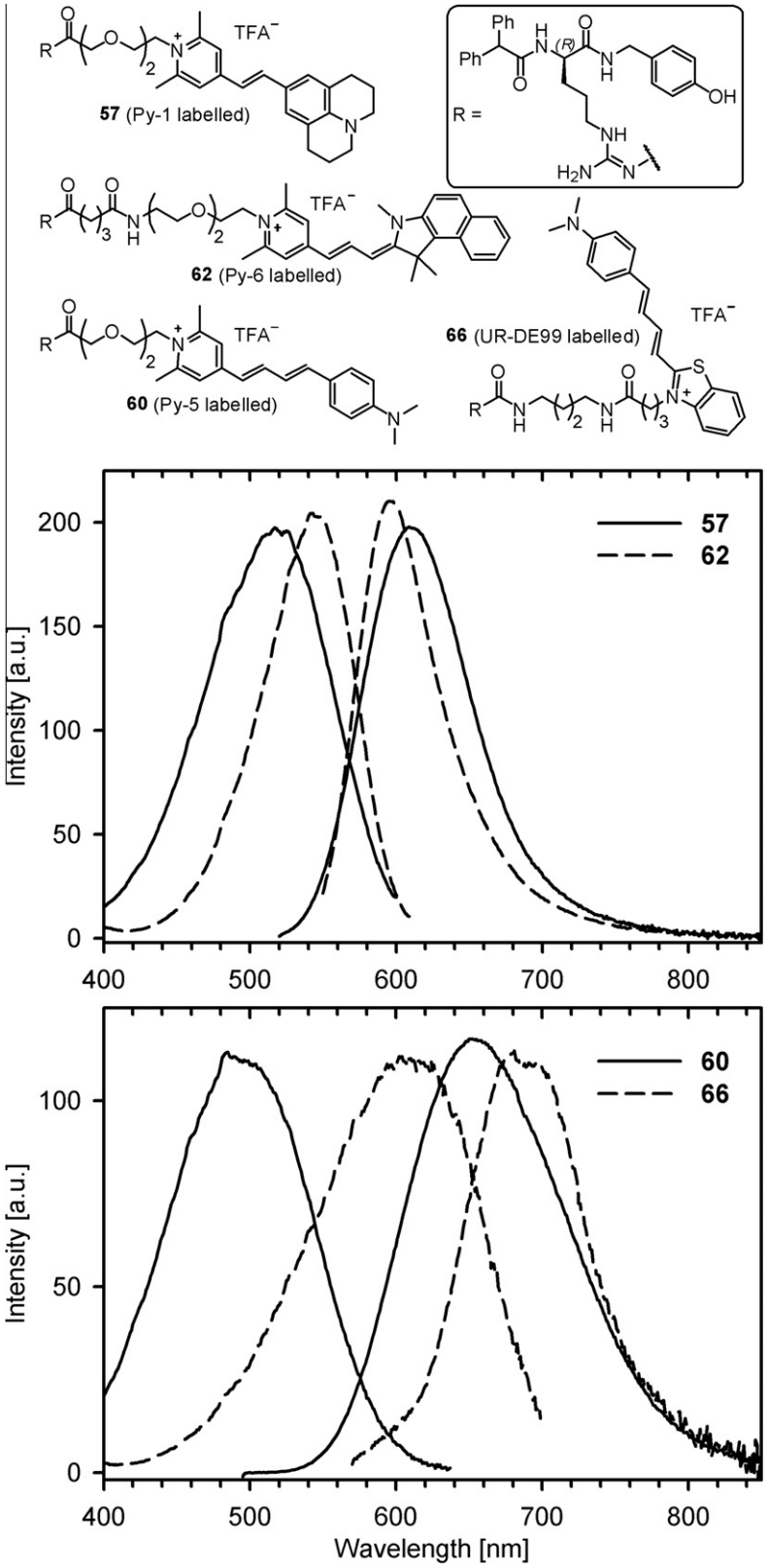



**Figure 3.** Excitation and corrected emission spectra of a Py-1 (**57**), Py-5 (**60**), Py-6 (**62**) and UR-DE99 labelled (**66**) $Y_1R$ antagonist dissolved in PBS supplemented with 1% BSA (recorded at 22 °C).

and 150 nM, respectively), have lower affinities than compound **59** ($K_i$ = 50 nM), the 'carba analogue' of **61**. Therefore, it is possible that the presence of the carbamoyl NH-group implicates a different orientation of the $N^{\omega}$-substituent which is less compatible with the attachment of bulky moieties such as fluorophores. However, labelling of **41** with UR-DE99 resulted in fluorescent ligand UR-MK131 (**66**), which proved to be one of the most potent fluorescent ligands ($K_i$ = 26 nM, $K_d$ = 5.0 nM). With respect to its high stability

at pH 7.0 (cf. Section 2.4) **66** is, therefore, considered as the most valuable compound of this series of fluorescent $Y_1R$ ligands.

The $K_i$ and $K_b$ values from binding and functional assays, respectively, are not identical. A possible reason for this discrepancy is that the Fura-2 $Ca^{2+}$-assay is performed in a time window (a few minutes) in which the antagonists, exhibiting a high on- and off-rate (compare association and dissociation kinetics of the argininamide [$^3$H]-UR-MK114[6]), can almost reach equilibrium, whereas the agonist pNPY is characterized by slow binding kinetics.[37] Moreover, also the structure of the compounds seems to play a role: The $K_b$ value of most BIBP 3226 derived fluorescent ligands is significantly lower than the $K_i$ value, but a good correlation between $K_b$ and $K_i$ value was found, for example, for the parent compounds **1** and **13** and the BIBO 3304 derived ligand **53**. In case of amine precursors **33**, **41** and **41-N$^\delta$** the $K_b$ value was even higher than the $K_i$ value.

The $Y_1R$ selectivity was exemplarily determined for compounds **54** and **66** in flow cytometric binding studies using cyanine labelled peptides (Cy5-pNPY, Dy-635-pNPY, Cy5-[K$^4$]hPP) and cells expressing human $Y_2$, $Y_4$ and $Y_5$ receptors (Table 3).[2,38,39] The data acquisition for the UR-DE99 labelled ligand **66** is compromised by the excitability of UR-DE99 with the red diode laser (635 nm). Nevertheless the subtraction of the fluorescence contributed by **66** at concentrations up to 1 μM, measured in the absence of the fluorescently labelled peptides, enabled the reliable estimation of the affinity of **66** for NPY receptor subtypes by flow cytometry.

**Table 2**
$Y_1R$ antagonism and affinity of BIBP 3226 (**1**), BIBO 3304 (**13**), amine precursors **33** and **41** as well as fluorescent ligands **53–66** and **70–72**

| Compound | Dye[a] | Linker length[b] | $K_b$[c] (nM) | $K_i$[d] (nM) |
|---|---|---|---|---|
| **1** | — | — | 1.5 ± 0.1 | 1.3 ± 0.2 |
| **13** | — | — | 0.7 ± 0.1 | 0.25 ± 0.01 |
| **33** | — | — | 95 ± 19 | 19 ± 5 |
| **41** | — | — | 16 ± 0.5 | 4.3 ± 0.5 |
| **41-N$^\delta$** | — | — | 510 ± 120 | 38 ± 3 |
| **53** | Py-1 | 6 | 12 ± 0.3 | 23 ± 3 |
| **54** | Py-1 | 8 | 6.0 ± 0.3 | 22 ± 5 |
| **55** | Py-1 | 9 | 11 ± 0.4 | 38 ± 2 |
| **56** | Py-1 | 12 | 4.1 ± 0.3 | 97 ± 6 |
| **57** | Py-1 | 8 | 26 ± 5 | 13 ± 4 |
| **58** | Py-1 | 6 | 7.2 ± 1.2 | 140 ± 10 |
| **59** | Py-5 | 6 | 3.7 ± 0.7 | 50 ± 4 |
| **60** | Py-5 | 8 | 11 ± 1 | 24 ± 3 |
| **61** | Py-5 | 6 | 7.6 ± 0.6 | 150 ± 15 |
| **62** | Py-6 | 14 | 6.8 ± 1.3 | 96 ± 16 |
| **63** | Py-6 | 15 | 8.0 ± 1 | 130 ± 40 |
| **64** | Py-6 | 17 | 7.8 ± 2.6 | 130 ± 27 |
| **65** | **51** | 9 | 2.4 ± 0.2 | 42 ± 5 |
| **66** | **51** | 7 | 0.7 ± 0.08 | 26 ± 2 |
| **70** | Py-1 | 12 | 1.2 ± 0.06 | 61 ± 8 |
| **71** | Py-1 | 14 | 0.6 ± 0.05 | 100 ± 10 |
| **72** | Py-1 | 16 | 1.0 ± 0.2 | 56 ± 4 |

[a] Fluorescent dye used for the preparation of the fluorescent ligand (cf. Fig. 1).
[b] Length of the linker between the argininamide and fluorescent dye given as number of atoms, which accounts for the spacing.
[c] Inhibition of 10 nM pNPY induced $[Ca^{2+}]_i$ mobilisation in HEL cells; mean values ± SEM from two independent experiments.
[d] Dissociation constant determined from the displacement of [$^3$H]-UR-MK114 ($K_d$ = 1.2 nM, $c$ = 1.5 nM) on SK-N-MC cells; mean values ± SEM from two independent experiments performed in triplicate.

**Table 3**
NPY receptor subtype selectivity of the fluorescent ligands **54** and **66**

| Compd | $Y_1R$, $K_i$[a] (nM) | $Y_2R$, $K_i$[b] (nM) | $Y_4R$, $K_i$[c] (nM) | $Y_5R$, $K_i$[b] (nM) |
|---|---|---|---|---|
| **54** | 22 ± 5 | >3000 | >3000 | >5000 |
| **66** | 26 ± 2 | >500 | >500 | >400 |

[a] $K_i$ values from radioligand competition assay with [$^3$H]-UR-MK114 ($c$ = 1.5 nM) on SK-N-MC neuroblastoma cells (cf. Table 2).
[b] Flow cytometric binding assay on CHO-$Y_2$ and HEC-1B-$Y_5$ cells using Dy-635-pNPY or Cy5-pNPY as labelled ligands.
[c] Flow cytometric binding assay on CHO-$Y_4$ cells with Cy5-[K$^4$]-hPP as fluorescent ligand. At $Y_2R$, $Y_4R$ and $Y_5R$ the displacement of the pertinent fluorescently labelled peptide by **54** and **66** did not exceed 50% at the highest concentrations used, that is, $IC_{50}$ values are supposed to be higher than 10 μM (**54**) and 1 μM (**66**), respectively.

## 2.4. Stability of the fluorescent ligands

As N$^\omega$-acylated argininamides were recently reported to be considered critical with respect to the stability of the acylguanidine moiety[32,40] a selection of the most potent fluorescent $Y_1R$ antagonists was investigated with respect to decomposition giving BIBP 3226 (**1**) under assay-like conditions (aqueous buffer, pH 7, 20 °C, time scale 20 and 90 min, respectively, cf. Table 4). A release of **1** under the conditions of the pharmacological assays has to be taken into account as **1** is a highly potent $Y_1R$ antagonist ($K_i$ = 1.3 nM), possibly interfering with the measured potency of the investigated compounds. Whereas in case of **53** and the bivalent ligand **70** only

**Table 4**
Chemical stability (pH 7.0, 20 °C) of the fluorescent ligands **53**, **54**, **57**, **66** and **70**

| Compd | % Decomposition (20 min) | % Decomposition (90 min) |
|---|---|---|
| **53** | 0.0 | <0.5 |
| **54** | 1.0 | 2.6 |
| **57** | <0.5 | 1.5 |
| **66** | 0.0 | 0.0 |
| **70** | 0.0 | <0.5 |

Cleavage of the acyl or carbamoyl substituent from the guanidine resulting in BIBP 3226 (**1**) and BIBO 3304 (**13**), respectively, was analysed by HPLC.

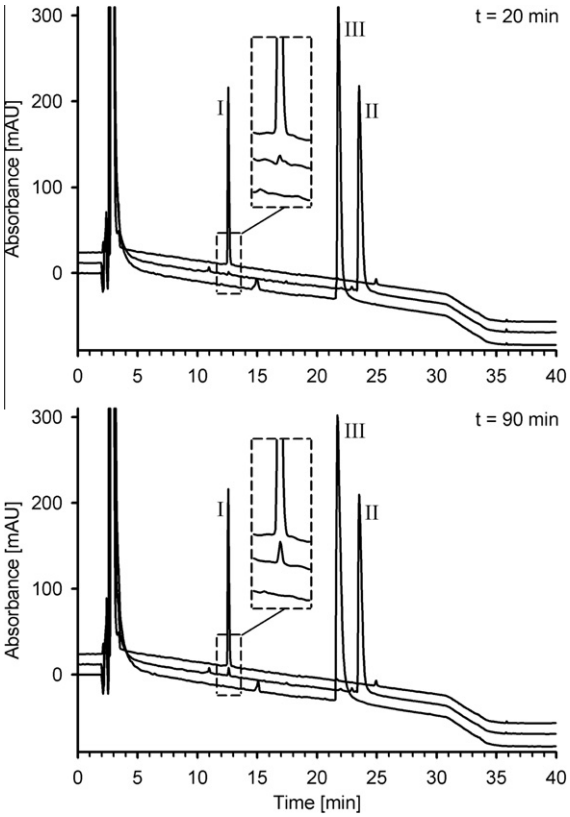



**Figure 4.** Reversed-phase HPLC analysis of the fluorescent ligands **57** (peak II) and **66** (peak III) after incubation in PBS, pH 7, at 20 °C for up to 90 min. While **66** shows no decomposition of the carbamoylguanidine entity over 90 min, about 1.5% of **57** decomposed to **1** (peak I) by cleavage of the acyl substituent from the guanidine.

traces of the corresponding parent compounds **13** and **1** were detected after 90 min, the decomposition of the $Y_1R$ antagonists **54** and **57** was more pronounced enabling a quantification of **1** (2.6% and 1.5%, respectively). However, with respect to the assay periods (<90 min from preparation of solutions to be read out) the high $Y_1R$ affinities of **54** and **57** ($K_i$ = 22 and 13 nM, respectively; Table 2) cannot be attributed to released **1**.

Fluorescent $Y_1R$ antagonist **66**, containing a carbamoylguanidine moiety, showed no decomposition (Fig. 4), demonstrating the superiority of carbamoylated compared to acylated guanidines in terms of stability in aqueous media at neutral pH. Solutions of all

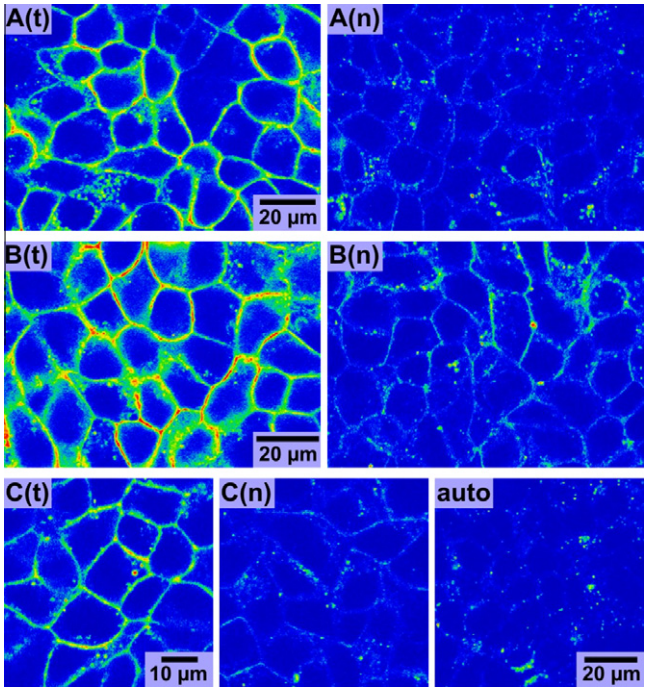



**Figure 5.** Binding of the fluorescent $Y_1R$ antagonists **53** (A), **54** (B) and **60** (C) to MCF-7-$Y_1$ mamma carcinoma cells, visualized by confocal microscopy after incubation periods of 5–8 min. All ligands were used at a concentration of 50 nM. 't' designates total binding, 'n' non-specific binding in the presence of BIBP 3226 (5 µM) and 'auto' autofluorescence for A, B and C. Images were acquired with a Zeiss Axiovert 200 M microscope.

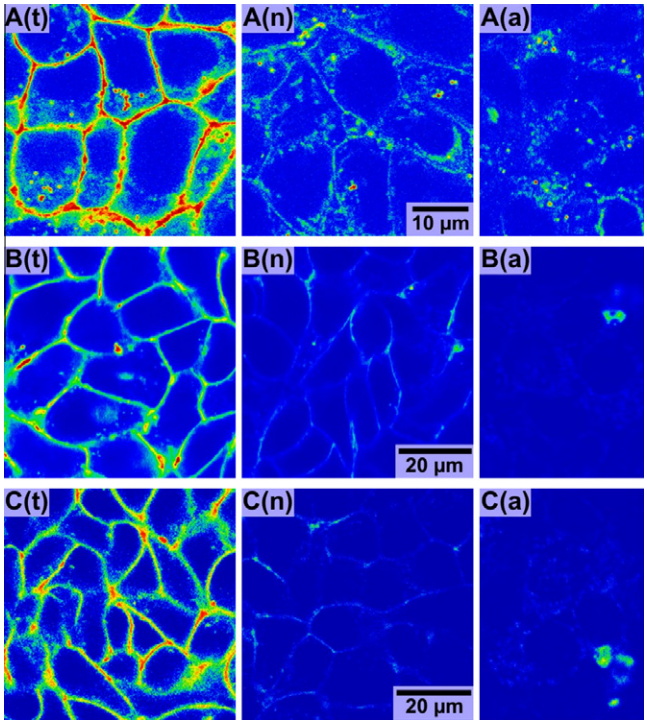



**Figure 6.** Binding of the fluorescent $Y_1R$ antagonists **57** ($c$ = 60 nM, panel A), **70** ($c$ = 80 nM, panel B) and **72** ($c$ = 80 nM, panel C) to MCF-7-$Y_1$ mamma carcinoma cells, visualized by confocal microscopy after incubation periods of 7, 21 and 23 min, respectively. 't' designates total binding, 'n' nonspecific binding (in the presence of BIBP 3226 at 80–125-fold higher concentrations), 'a' autofluorescence. Images were acquired with a Zeiss Axiovert 200 M microscope.

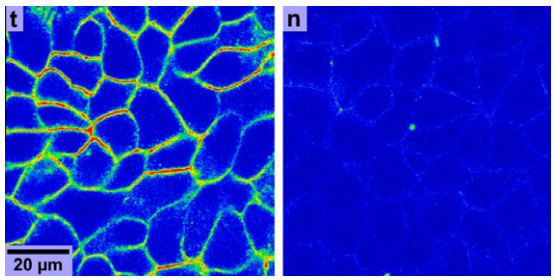



**Figure 7.** Binding of the fluorescent ligand **66** ($c$ = 50 nM) to $Y_1R$ expressing MCF-7-$Y_1$ tumour cells, visualized by confocal microscopy. t: total binding, n: non-specific binding in the presence of BIBP 3226 (5 µM). Images were acquired with a Zeiss Axiovert 200 M microscope after 12–15 min of incubation at rt in Leibowitz L15 culture medium.

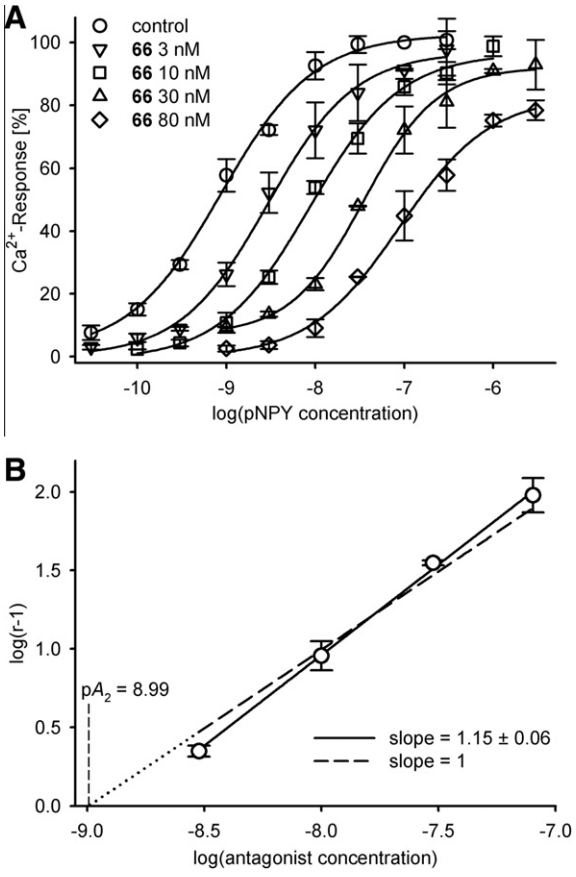



**Figure 8.** (A) Concentration–response curves (CRCs) of pNPY from a Fura-2 assay on HEL cells. The presence of **66** led to a parallel rightward shift of the curves. (B) Schild regression: log($r$−1) plotted against log[antagonist]; the concentration ratios $r$ ($r = 10^{\Delta pEC50}$) were calculated from the rightward shifts ($\Delta pEC_{50}$) of the CRCs in the presence of **66** (A). The slope of the line equals unity, therefore, the $pA_2$ value corresponds to $pK_b$: $pA_2 \approx pK_b = 9.0$ (slope forced to unity), $K_b$ = 1.0 nM (mean ± SEM, $n$ = 3).

compounds (TFA salts) in DMSO proved to be stable over long periods of time (12–24 months) when stored below 0 °C.

### 2.5. Application of fluorescent $Y_1R$ antagonists to confocal microscopy

For $Y_1R$ binding studies performed with confocal microscopy the fluorescent $Y_1R$ antagonists with $K_i$ values (SK-N-MC cells) of 50 nM and below were used. In view of a potential diagnostic value of fluorescent GPCR ligands in microscopy, steroid hormone-sensitive MCF-7 breast cancer cells were selected as a model, since these cells were reported to express the $Y_1R$.[11,41] As a MCF-7 subclone showing higher $Y_1$ receptor expression than the wild-type MCF-7 cells (ATCC number HTB 22) was recently established in our laboratory, this cell line (designated MCF-7-$Y_1$) was used for confocal microscopy experiments. Prior to the experiments cells were incubated with 1 nM 17β-estradiol for 48 or 72 h to increase the $Y_1R$ expression.[6,42]

As shown in Figures 5–7, a clear difference between total and non-specific binding was observed for all of the investigated compounds (**53**, **54**, **57**, **60**, **66**, **70** and **72**). Non-specific binding was determined in the presence of the non-fluorescent $Y_1R$ antagonist BIBP 3226.

### 2.6. Schild analysis of 66

As **66** exhibits highest stability and excellent fluorescent properties and proved to be one of the most potent $Y_1R$ antagonists in the $Ca^{2+}$-assay on HEL cells (cf. Section 2.3), concentration–response curves of pNPY were constructed in the absence and presence of **66** at different concentrations (Fig. 8A). The data were subjected to Schild analysis[43] (Fig. 8B). The p$K_b$ value of 9.0 (in this case comparable to p$A_2$) reflects the affinity of the antagonist. Since the slope in this linear plot nearly equals unity, a competitive antagonism of **66** is very likely.

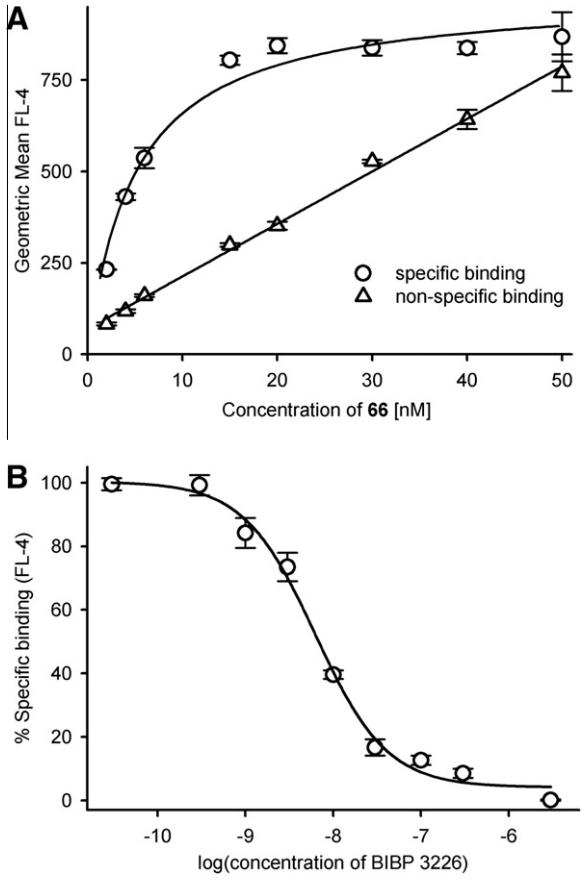



**Figure 9.** (A) Flow cytometric saturation binding experiment with fluorescent ligand **66** at human MCF-7-$Y_1$ breast cancer cells. Unspecific binding was determined in the presence of BIBP 3226 (200-fold excess). Determined $K_d$ value: 5.0 ± 0.6 nM (mean ± SEM, two independent experiments with $n = 2$). (B) Flow cytometric $Y_1R$ competition assay with **66** (10 nM) and BIBP 3226 (**1**) using MCF-7-$Y_1$ cells. Calculated $K_i$ value for BIBP 3226: 2.2 nM (mean ± SEM, $n = 3$).

### 2.7. Application of 66 in flow cytometry

As shown in Figure 9A, flow cytometric saturation binding experiments with **66** using MCF-7-$Y_1$ breast cancer cells yielded saturation curves with low non-specific binding and a $K_d$ value of 5.0 nM. Displacement of the fluorescent ligand **66** by the well described $Y_1R$ antagonist **1** yielded a $K_i$ value of 2.2 nM (calculated with the $K_d$ value of 5.0 nM) for the model compound **1**, which is in accordance with previously reported $K_i$ values of 1.5 nM,[6] 7 nM[20] and 5.1 nM.[44] Thus, the displacement of **66** in flow cytometry offers an alternative to radioligand binding assays to determine affinities of unlabelled $Y_1R$ ligands.

## 3. Conclusion

The design of fluorescent $Y_1R$ ligands followed the guanidine–acylguanidine bioisosteric approach: The guanidine group of the argininamide-type $Y_1R$ antagonists BIBP 3226 and BIBO 3304 was linked to fluorophores via ω-aminoacyl spacers of different lengths and chemical nature. Such derivatives proved to be potent and selective fluorescently labelled $Y_1R$ antagonists, even though in most cases a moderate decrease in activity compared to the parent argininamides was found (factor of 10–100). The impact of variation of the linker on the $Y_1R$ affinity turned out to be low. The attachment of the pyrylium dyes Py-1 or Py-5 through an eight-membered chain yielded compounds with high affinity (**54**, **57** and **60**: $K_i \approx 20$ nM). Labelling of the amine precursor **41** with the new benzothiazolium-type cyanine dye UR-DE99 (**51**), emitting in the near infrared, yielded the most interesting fluorescent probe (**66**) due to high affinity, quantum yield and chemical stability. Such fluorescent probes are versatile pharmacological tools for the screening and detailed analysis of $Y_1R$ ligands as well as for the detection of $Y_1R$ on living cells, as demonstrated by flow cytometry and confocal microscopy.

## 4. Experimental section

### 4.1. General experimental conditions

Unless otherwise stated, chemicals and solvents were purchased from commercial suppliers and used without further purification. 12-Amino-4,7,10-trioxadodecanoic acid *tert*-butyl ester (**18**), *N*-Boc-3,6-dioxaoctane-1,8-diamine (**22**), *N*-Boc-8-amino-3,6-dioxaoctanoic acid (**35**, as dicyclohexylamine salt), *N*-Boc-propane-1,3-diamine (**15**) and *N*-Boc-butane-1,4-diamine (**26**) were purchased from Fluka (Sigma–Aldrich Chemie GmbH, Munich, Germany). D-Ornithine hydrochloride (**5**) was obtained from Iris Biotech GmbH (Marktredwitz, Germany). Ethyl vinyl ether (**44**) was purchased from Aldrich (Sigma–Aldrich Chemie GmbH, Munich, Germany) and *N*,*N*-dimethylanilin (**46**) was obtained from Merck Schuchardt OHG (Hohenbrunn, Germany). Bovine serum albumin (BSA) was from Serva (Heidelberg, Germany). The tetrafluoroborate salts of the pyrylium dyes Py-1, Py-5 and Py-6 were kindly provided by Professor Dr. O. S. Wolfbeis (Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, Germany). The syntheses of succinimidyl diphenylacetate (**8**),[6] guanidinylating reagent **11**,[6] benzyl esters **20** and **21**,[8]

*S*-methyl-isothiourea (**25**),[45] building block **30**,[6] compound **39**,[8] amine **42**[6] and the bivalent amine precursors **67–69**[8] were described previously. The preparations of Dy-635-pNPY, Cy5-pNPY and Cy5-[K$^4$]-hPP were reported elsewhere.[2,39] Porcine NPY (pNPY) was prepared in the laboratory of Professor Dr. C. Cabrele (Ruhr University, Bochum, Germany). The Fura-2 AM (Calbiochem/Merck Biosciences, Beeston, UK) stock solution (1 mM) was prepared in DMSO. Pluronic F-127 (Calbiochem/Merck Biosciences, Beeston, UK) was dissolved in DMSO to obtain a concentration of 20%.

Millipore water was used throughout for the preparation of buffers and HPLC eluents. Petroleum ether (40–60 °C) was distilled before use. DMF (Acros Organics, Geel, Belgium) was stored over molecular sieves (3 Å). Anhydrous reactions were run under an atmosphere of argon. Thin layer chromatography was performed on Merck Silica Gel 60 $F_{254}$ TLC aluminium plates. For column chromatography silica gel Geduran 60 (Merck, 0.063–0.200 mm) was used. Optical rotations at 589 nm ($Na_D$ line) were determined on a Polarimeter 241 (PerkinElmer, Ueberlingen, Germany) using a quartz micro-cuvette (layer thickness: 100 mm, volume: 1 mL, thermostated at 20 °C) and acetonitrile/$H_2O$ (9:1 v/v) as solvent. IR spectra were measured on a FTS 3000 MX spectrometer (Excalibur Series) from Bio-Rad (Hercules, CA, USA) equipped with an attenuated total reflectance (ATR) unit (Specac Golden Gate Diamond Single Reflection ATR System). NMR spectra were recorded on a Bruker Avance 300 (1H: 300 MHz), a Bruker Avance 600 (1H: 600 MHz) and a Bruker Avance III 600 (cryogenic probe, Bruker, Karlsruhe, Germany) with TMS as external standard. Low-resolution mass spectrometry (MS) was performed in-house on a Finnigan ThermoQuest TSQ 7000 instrument (ES-MS, LC–MS) and a Finnigan SSQ 710A instrument (EI-MS 70 eV, CI-MS). The following LC method was used for LC–MS analysis: Column: Phenomex Luna C18, 3.0 μm, 100 × 2 mm HST (phenomenex, Aschaffenburg, Germany); flow: 0.30 mL/min; solvent A: MeCN, solvent B: water + 0.1% TFA; gradient: 0–1 min: A/B 5:95, 1–11 min: 5:95–98:2, 11–18 min: 98:2, 18–19 min: 98:2–5:95, 19–24 min: 5:95. High-resolution mass spectrometry (HRMS) for compounds **12**, **13**, **29**, **31–34**, **36–38**, **40**, **41** and **51** was performed on a Finnigan MAT 95 instrument. HRMS for compounds **53–66** and **72** was performed on a JMS-700 instrument (JOEL Ltd, Tokyo, Japan), and HRMS for compounds **70** and **71** was performed on a Finnigan LTQ FT instrument. Melting points were determined with a Büchi 530 (Büchi GmbH, Essen, Germany) and are uncorrected. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Eurospher-100 C18 (250 × 32 mm, 5 μm; Knauer, Germany) served as RP-column at a flow rate of 38 mL/min. Mixtures of acetonitrile and 0.1% aq TFA (for **52** 0.05% aq TFA) were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure (final pressure: 60 mbar) at 40 °C prior to lyophilisation (Christ alpha 2–4 LD lyophilisation apparatus equipped with a vacuubrand RZ 6 rotary vane vacuum pump). Analytical HPLC analysis was performed on a system from Thermo Separation Products (composed of a SN400 controller, a P4000 pump, a degasser (Degassex DG-4400, phenomenex), an AS3000 autosampler and a Spectra Focus UV–VIS detector). A Eurospher-100 C18 (250 × 4 mm, 5 μm, Knauer, Germany) served as RP-column. Mixtures of acetonitrile (A) and 0.05% aq TFA (B) were used as mobile phase. Helium degassing, an oven temperature of 30 °C and a flow rate of 0.8 mL/min were used throughout. Solutions for injection (concentrations in the two-digit μM range) were prepared in a mixture of A and B corresponding to the mixture at the start of the gradient. The following linear gradients were applied for analytical HPLC analysis.

Gradient 1: 0–30 min: A/B 20:80–95:5, 30–40 min: 95:5.

Gradient 2: 0–28 min: A/B 25:75–70:30, 28–31 min: 70:30–95:5, 31–40 min: 95:5.

## 4.2. Chemistry: experimental protocols and analytical data

### 4.2.1. *tert*-Butyl 4-(aminomethyl)benzylcarbamate (3)[26]

1,4-Bis(aminomethyl)benzene (30 g, 220.3 mmol) was dissolved/suspended in 1 M aq NaOH (220 mL, 220.3 mmol). 1,4-Dioxane was added (250 mL) and the solution was cooled in an ice-water bath. Di-*tert*-butyl dicarbonate (48.1 g, 220.3 mmol) was added dropwise in 1,4-dioxane (150 mL) over a period of 2 h. The ice-water bath was removed and the mixture was allowed to stand at rt overnight. The volume was reduced to about 250 mL under reduced pressure. The white solid was separated by filtration, washed twice with ice-cold water (2 × 100 mL) and dried in vacuo. White solid (47.6 g of a mixture of **3** and di-Boc-protected 1,4-bis(aminomethyl)benzene (≈55:45), 22 g (42% related to **3**); $^1$H NMR (300 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.44 (s, 24H, *tert*-butyl, **3** and 'di-Boc'), 3.84 (s, 2H, C$H_2$–$NH_2$), 4.19 (s, 3.3H, C$H_2$–NH, 'di-Boc'), 4.20 (s, 2H, C$H_2$–NH, **3**), 7.2 (s, 3.3H, C$H^{Ar}$, 'di-Boc'), 7.29 (m, 4H, C$H^{Ar}$, **3**); MS (CI, $NH_3$): *m/z* 254 [*M*+$NH_4$]$^+$, 354 ['di-Boc'+$NH_4$]$^+$; $C_{13}H_{20}N_2O_2$ (236.2).

### 4.2.2. *N*-(4-Aminomethylbenzyl)urea (4)[25]

Compound **3** (19.6 g, 83.1 mmol, 1 equiv; corresponding to 42.5 g of a mixture with di-Boc-protected 1,4-bis(aminomethyl)benzene, cf. preparation of **3**) was suspended in water (500 mL) and ethanol (400 mL). The suspension was heated to 60 °C and 1 M aq hydrochloric acid (100 mL) was added yielding a pH of about 3. Potassium cyanate (7 g, 86.3 mmol) was added and the mixture was refluxed for 75 min. 1 M aq hydrochloric acid (8 mL) was added to adjust the pH to 7, followed by the addition of 4.67 g potassium cyanate (4.67 g, 57.6 mmol). Reflux was continued for 2 h, then the mixture was stirred at rt overnight (final pH: ≈8) and concentrated under reduced pressure to a volume of about 200 mL. The white solid was separated by filtration, washed twice with water (2 × 150 mL) and dried in vacuo. A suspension was prepared in a mixture of MeOH and DCM (1:10, 2000 mL) and subjected to column chromatography (DCM/MeOH 20:1–7.5:1). The isolated Boc-protected intermediate (well soluble in MeOH, poorly soluble in water, acetonitrile, ethyl acetate and DCM) was dissolved in MeOH (300 mL) under mild heating. Acetyl chloride (30 mL) was added dropwise over a period of 2 h and stirring was continued for 30 min. Volatiles were removed under reduced pressure and the residue was suspended in water (150 mL). Lyophilisation afforded the product as a white solid (15.8 g, 88%). $^1$H NMR (300 MHz, MeOH-$d_4$/$D_2O$ 80:20): $\delta$ (ppm) 4.06 (s, 2H), 4.26 (s, 2H), 7.34 (m, 4H); MS (CI, $NH_3$): *m/z* 197 [*M*+$NH_4$]$^+$, 180 [*M*+H]$^+$; $C_{14}H_{21}N_3O_3 \times HCl$ (215.8).

### 4.2.3. (*R*)-$N^{\delta}$-Benzyloxycarbonyl-$N^{\alpha}$-(*tert*-butoxycarbonyl)-ornithine (6)[46]

Compound **5** (22 g, 82.6 mmol) was dissolved/suspended in an aqueous solution (250 mL) of potassium carbonate (12.56 g, 90.9 mmol) and 1,4-dioxane (100 mL) was added. Di-*tert*-butyl dicarbonate (18.93 g, 86.75 mmol) was added dropwise in 1,4-dioxane (200 mL) over a period of 60 min. The mixture was stirred at rt overnight, then concentrated under reduced pressure to a volume of about 150 mL. Water (50 mL) was added and the pH was adjusted to 2–3 by the addition of 1 M aq hydrochloric acid (about 150 mL). The product was extracted with ethyl acetate (400 mL and 3 × 300 mL) and the combined organic phases were treated with 10 mM aq hydrochloric acid (100 mL), saturated aq $NH_4Cl$ (200 mL), water (100 mL) as well as brine (250 mL) prior to drying over sodium sulfate. Filtration and evaporation of the solvent yielded a yellowish oil which turned into a foam that hardened to a solid during drying in vacuo (27.8 g, 92%). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.38 (s, 9H), 1.4–1.59 (m, 3H),

1.65 (m, 1H), 2.98 (m, 2H), 3.83 (m, 1H), 5.0 (s, 2H), 7.07 (d, 1H, $^3J$ = 8.0 Hz), 7.25 (t, 1H, $^3J$ = 5.5 Hz), 7.34 (m, 5H), 12.42 (s, 1H); $C_{18}H_{28}N_2O_6$ (366.4).

#### 4.2.4. (*R*)-$N^{\delta}$-Benzyloxycarbonyl-*N*-(4-ureidomethyl-benzyl)ornithinamide hydrochloride (7)

Compound **6** (9 g, 17.1 mmol) was dissolved in MeOH (250 mL) and acetyl chloride (30 mL) was added dropwise under water cooling (20 °C) over a period of 1.5 h. Stirring was continued for 30 min, volatiles were removed under reduced pressure and the residue was suspended in water (100 mL). Lyophilisation afforded the product as a white solid (7.9 g, 100%). $^1$H NMR (300 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.57 (m, 2H), 1.72–2.04 (br m, 2H), 2.98 (m, 1H), 3.14 (m, 2H), 4.34–4.42 (m, 4H), 5.05 (s, 2H), 7.26–7.36 (m, 9H); MS (ES, MeOH + 10 mM $NH_4OAc$): *m/z* 428 $[M+H]^+$; $C_{22}H_{29}N_5O_4 \times HCl$ (464.0).

#### 4.2.5. (*R*)-$N^{\delta}$-Benzyloxycarbonyl-$N^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-ureidomethyl-benzyl)ornithinamide (9)[25]

Compound **7** (7.8 g, 16.8 mmol) was dissolved in anhydrous DMF (100 mL). $NEt_3$ (2.6 g, 3.5 mL, 25.2 mmol), DMAP (1 g, 8.4 mmol) and **8** (6.2 g, 20.2 mmol) were added and the mixture was kept under stirring for 20 h at rt. Glacial acetic acid (3 mL) was added and DMF was removed under reduced pressure at 45 °C yielding a light yellow-brown solid which was dried in vacuo. The solid was insoluble or poorly soluble in MeOH, DCM, acetonitrile, dimethoxyethane, THF, 1,4-dioxane, propan-2-ol, ethanol and in mixtures with water of the water-miscible solvents. Therefore, the solid material was crushed mechanically to small pellets and suspended in acetonitrile (2500 mL). The solid turned into a white fluffy solid during heating (70 °C) and treatment with ultrasound (30 min). Separation by filtration and intensive washing with acetonitrile (2 × 250 mL) and water (3 × 150 mL) yielded the product as a white solid (8.7 g, 83%). Solubility: insoluble or poorly soluble in above listed solvents; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.37 (m, 2H), 1.54 (m, 1H), 1.64 (m, 1H), 2.95 (m, 2H), 4.14 (d, 2H, $^3J$ = 5.5 Hz), 4.23 (d, 2H, $^3J$ = 5.4 Hz), 4.31 (m, 1H), 5.00 (s, 2H), 5.12 (s, 1H), 5.53 (br s, 2H), 6.38 (t, 1H, $^3J$ = 5.7 Hz), 7.15 (m, 4H), 7.18–7.38 (m, 16H), 8.42 (t, 1H, $^3J$ = 5.8 Hz), 8.47 (d, 1H, $^3J$ = 8.1 Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ (ppm) 26.0, 29.7, 39.9, 41.8, 42.6, 52.5, 55.9, 65.2, 126.6, 127.0, 127.7, 127.8, 128.19, 128.21, 128.4, 128.5, 128.6, 137.3, 137.4, 139.4, 140.4, 140.6, 156.1, 158.7, 171.0, 171.4; MS (ES, acetonitrile/TFA): *m/z* 622 $[M+H]^+$; $C_{36}H_{39}N_5O_5$ (621.7).

#### 4.2.6. (*R*)-$N^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-ureidomethyl-benzyl)ornithinamide (10)[25]

Compound **9** (2.7 g, 4.3 mmol) was suspended in MeOH (300 mL). The amount of solid could be reduced by heating the suspension to 60 °C. Glacial acetic acid (1.2 mL) and a 10% Pd/C catalyst (430 mg) were added and hydrogen was led through the vigorously stirred mixture at 45 °C for 2.5 h until the white solid had disappeared. After 3 h the catalyst was removed by filtration, volatiles were removed under reduced pressure and the residue was suspended in water (50 mL) at 40 °C. Lyophilisation afforded the product as a white solid (2.1 g, 99%). $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.42–1.63 (m, 3H), 1.69 (m, 1H), 2.76 (m, 2H), 4.15 (m, 2H), 4.24 (m, 2H), 4.35 (m, 1H), 5.13 (s, 1H), 5.54 (br s, 2H), 6.45 (t, 1H, $^3J$ = 5.5 Hz), 7.15 (m, 4H), 7.20–7.33 (m, 10H), 7.74 (br s, 2.7H), 8.51 (t, 1H, $^3J$ = 5.8 Hz), 8.58 (d, 1H, $^3J$ = 8.1 Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ (ppm) 23.7, 29.2, 38.5, 41.8, 42.5, 52.3, 55.9, 126.7, 126.96, 126.98, 128.2, 128.49, 128.55, 137.3, 139.6, 140.3, 140.5, 158.7, 171.1; MS (ES, acetonitrile/TFA): *m/z* 488 $[M+H]^+$; $C_{28}H_{33}N_5O_3$ (487.6).

#### 4.2.7. (*R*)-$N^{\omega}$-*tert*-Butoxycarbonyl-$N^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-ureidomethyl-benzyl)argininamide hydroacetate (12)

Compound **10** (2.05 g, 3.74 mmol) was suspended in MeOH (30 mL). $NEt_3$ (0.57 g, 0.78 mL, 5.6 mmol) and **11** (1.55 g, 4.5 mmol) were added in DCM (10 mL), and the mixture was kept under stirring at rt for 20 h. The suspension turned into a cloudy solution during the first 30 min of the reaction. Volatiles were removed under reduced pressure and purification of the intermediate by column chromatography (DCM/MeOH 100:1–10:1) yielded a white solid which was dried in vacuo and dissolved in MeOH (180 mL). Glacial acetic acid (0.25 mL) and a 10% Pd/C catalyst (400 mg) were added and hydrogen was lead through the vigorously stirred mixture for 6.5 h. The catalyst was removed by filtration, volatiles were removed under reduced pressure and the residue was suspended in water (60 mL). Lyophilisation afforded the product as a white solid (1.8 g, 73%). $[\alpha]_D^{20}$ +13.8 cm$^3$ g$^{-1}$ dm$^{-1}$ (*c* 0.0099 in MeCN/$H_2O$ 9:1); IR (neat): 3330 br, 1635, 1545, 1495, 1150 cm$^{-1}$; $^1$H NMR (COSY, 600 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.30–1.44 (m, 11H), 1.54 (m, 1H), 1.67 (m, 1H), 1.90 (s, 1.5H), 3.06 (t, 2H, $^3J$ = 6.8 Hz), 4.14 (d, 2H, $^3J$ = 6.0 Hz), 4.24 (d, 2H, $^3J$ = 5.7 Hz), 4.33 (m, 1H), 5.13 (s, 1H), 5.53 (s, 2H), 6.40 (t, 1H, $^3J$ = 6.0 Hz), 7.15 (m, 4H), 7.20–7.25 (m, 2H), 7.27–7.32 (m, 8H), 8.46 (t, 1H, $^3J$ = 5.8 Hz), 8.50 (d, 1H, $^3J$ = 8.0 Hz); $^{13}$C NMR (150 MHz, DMSO-$d_6$): $\delta$ (ppm) 21.2 (AcOH), 28.2, 29.6, 40.1, 41.8, 42.6, 52.5, 55.9, 126.6, 127.0, 128.1, 128.2, 128.5, 128.6, 137.4, 139.4, 140.3, 140.5, 158.7, 171.0, 171.4, 172.3; RP-HPLC (gradient 1): 93% (210 nm, $t_R$ = 14.8 min, $k$ = 4.5); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 630 $[M+H]^+$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{34}H_{44}N_7O_3^+$: 630.3404, found: 630.3411; $C_{34}H_{43}N_7O_5 \times 0.5\text{-}C_2H_4O_2$ (659.8).

#### 4.2.8. (*R*)-$N^{\alpha}$-(2,2-Diphenylacetyl)-*N*-(4-ureidomethylbenzyl) argininamide hydrotrifluoroacetate (13)[25]

Compound **12** × 0.5 AcOH (166 mg, 0.24 mmol) was dissolved in MeOH (5 mL). TFA (1.5 mL) was added and the mixture was stirred at rt for 4 h. MeOH (25 mL) was added three times, each time followed by evaporation under reduced pressure. Purification with preparative HPLC (gradient: 0–25 min: MeCN/0.1% aq TFA 15:85–45:55, $t_R$ = 19.1 min) and lyophilisation afforded the product as a white fluffy solid (147 mg, 0.23 mmol, 95%); mp >60 °C (decomp.); $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.4 (m, 2H), 1.53 (m, 1H), 1.66 (m, 1H), 3.06 (m, 2H), 4.15 (s, 2H), 4.23 (d, 2H, $^3J$ = 5.7 Hz), 4.34 (m, 1H), 5.13 (s, 1H), 6.47 (s, 1H), 7.05 (br s, 2H), 7.15 (m, 4H), 7.26 (m, 10H), 7.59 (t, 1H, $^3J$ = 5.6 Hz), 8.52 (m, 2H); RP-HPLC (gradient 1): 99% (210 nm, $t_R$ = 10.8 min, $k$ = 3.0); HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{29}H_{36}N_7O_3^+$: 530.2874, found: 530.2867; $C_{29}H_{35}N_7O_3 \times C_2HF_3O_2$ (643.7).

#### 4.2.9. *tert*-Butyl 2-aminoethylcarbamate (14)[47]

Ethane-1,2-diamine (36.1 g, 0.6 mol) was dissolved in chloroform (450 mL) and the solution was cooled to −15 °C. A solution of di-*tert*-butyl dicarbonate (10.9 g, 50 mmol) in chloroform (40 mL) was added dropwise over a period of 4 h. Stirring was continued overnight and the mixture was allowed to slowly warm up to rt. The solution was washed three times with alkalified brine (3 × 130 mL of brine + 5 mL of 1 M aq NaOH), then with brine (130 mL) and water (100 mL). Drying over sodium sulfate, filtration and removal of the solvent under reduced pressure yielded the product as yellow oil (7.82 g, 97%). $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ (ppm) 1.42 (s, 9H), 2.77 (t, 2H, $^3J$ = 5.9 Hz), 3.14 (m, 2H); $C_7H_{16}N_2O_2$ (160.2).

#### 4.2.10. 4-(2-*tert*-Butoxycarbonylaminoethyl) aminocarbonylbutanoic acid (16)[31]

Glutaric anhydride (2.28 g, 20 mmol) was added to a solution of **14** (3.5 g, 22 mmol) in DCM (20 mL) and the mixture was stirred at

rt for 60 min. DCM (150 mL) was added prior to washing with aq $NH_4Cl$ solution (two times 30 mL of a saturated solution + 10 mL of water) and brine (40 mL). Drying over sodium sulfate, filtration and removal of the solvent under reduced pressure yielded the product as highly viscous yellowish oil (5.4 g, 98%). $^1H$ NMR (300 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.43 (s, 9H), 1.88 (p, 2H, $^3J$ = 7.2 Hz), 2.24 (t, 2H, $^3J$ = 7.5 Hz), 2.32 (t, 2H, $^3J$ = 7.4 Hz), 3.14 (m, 2H), 3.24 (m, 2H); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 273 $[M-H]^-$; $C_{12}H_{22}N_2O_5$ (274.3).

#### 4.2.11. 4-(3-*tert*-Butoxycarbonylaminopropyl) aminocarbonylbutanoic acid (17)

Glutaric anhydride (0.31 g, 2.73 mmol) in DCM (5 mL) was added dropwise to a solution of *N*-Boc-propane-1,3-diamine (**15**) (0.5 g, 2.87 mmol) in DCM (1.5 mL) over a period of 5 min. The mixture was stirred at rt for 60 min. Chloroform (40 mL) was added prior to washing with aq $NH_4Cl$ solution (two times 8 mL of a saturated solution + 2 mL of water) and brine (10 mL). Drying over sodium sulfate, filtration and removal of the solvent under reduced pressure yielded the product as highly viscous yellowish oil (0.8 g, 97%). $^1H$ NMR (300 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.43 (s, 9H), 1.63 (p, 2H, $^3J$ = 6.8 Hz), 1.88 (p, 2H, $^3J$ = 7.2 Hz), 2.24 (t, 2H, $^3J$ = 7.5 Hz), 2.32 (t, 2H, $^3J$ = 7.4 Hz), 3.06 (t, 2H, $^3J$ = 6.8 Hz), 3.19 (t, 2H, $^3J$ = 6.9 Hz); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 287 $[M-H]^-$; $C_{13}H_{24}N_2O_5$ (288.3).

#### 4.2.12. 12-(Benzyloxycarbonylamino)-4,7,10-trioxadodecanoic acid (19)

12-Amino-4,7,10-trioxadodecanoic acid *tert*-butyl ester (**18**) (1 g, 3.6 mmol) was dissolved in DCM (10 mL). $NEt_3$ (0.73 g, 1 mL, 7.2 mmol) was added and the solution was cooled to 0 °C. Benzyl chloroformate (0.68 g, 3.97 mmol) was added dropwise in DCM (10 mL) over a period of 30 min. The ice-water bath was removed and the mixture was allowed to stand at rt for 2 h. The intermediate was purified by column chromatography (eluent: DCM to DCM/EtOAc 1:2) prior to ester cleavage in DCM/TFA 4:1 (v/v, 25 mL) for 3 h. DCM (20 mL) was added three times, each time followed by evaporation under reduced pressure. Drying in vacuo yielded the product as reddish oil (1.13 g, 88%). $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 2.43 (t, 2H, $^3J$ = 6.4 Hz), 3.14 (m, 2H), 3.41 (t, 2H, $^3J$ = 5.9 Hz), 3.48 (m, 8H), 3.59 (t, 2H, $^3J$ = 6.4 Hz), 5.01 (s, 2H), 7.27 (m, 1H), 7.3–7.4 (m, 5H); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 356 $[M+H]^+$, 354 $[M-H]^-$; $C_{17}H_{25}NO_7$ (355.4).

#### 4.2.13. General procedure for the synthesis of carboxylic acids 23 and 24

*N*-Boc-3,6-dioxaoctane-1,8-diamine (**22**) (1 equiv) and the pertinent alkanedioic acid monobenzyl ester (1 equiv) were dissolved in anhydrous DCM. EDC (1.2 equiv) was added and the mixture was kept under stirring at rt for 20 h. The intermediate was purified by column chromatography (eluent: EtOAc/MeOH 10:1–5:1) prior to hydrogenation at rt and atmospheric pressure in MeOH using hydrogen and a 10% Pd/C catalyst.

**4.2.13.1. 4-(8-tert-Butoxycarbonylamino-3,6-dioxaoctyl)aminocarbonylbutanoic acid (23).** Prepared from glutaric acid monobenzyl ester **20** (448 mg, 2.01 mmol) and **22** (500 mg, 2.01 mmol); EDC: 463 mg, 2.42 mmol; 63% (460 mg); $^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ (ppm) 1.43 (s, 9H), 1.94 (m, 2H), 2.31 (m, 2H), 2.39 (m, 2H), 3.31 (m, 2H), 3.47 (m, 2H), 3.51–3.63 (m, 8H); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 363 $[M+H]^+$; $C_{16}H_{30}N_2O_7$ (362.4).

**4.2.13.2. 5-(8-tert-Butoxycarbonylamino-3,6-dioxaoctyl)aminocarbonylpentanoic acid (24).** Prepared from adipic acid monobenzyl ester **21** (247 mg, 1.05 mmol) and **22** (260 mg, 1.05 mmol); EDC: 241 mg, 1.26 mmol; 66% (260 mg); $^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ (ppm) 1.36 (s, 9H), 1.6 (m, 4H), 2.17 (m, 2H), 2.28 (m, 2H), 3.25 (m, 2H), 3.39 (m, 2H), 3.45–3.63 (m, 8H); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 377 $[M+H]^+$; $C_{17}H_{32}N_2O_7$ (376.5).

#### 4.2.14. *N*-*tert*-Butoxycarbonyl-*N'*-[*N*-(4-*tert*-butoxycarbonylaminobutyl)aminocarbonyl]-*S*-methylisothiourea (27)

The reaction was carried out in an argon purged 50-mL two-necked round bottom flask equipped with a pressure equalizing addition funnel. The flask and the funnel were evacuated and heated prior to the reaction. A solution of *N*-Boc-butane-1,4-diamine (**26**) (250 mg, 1.33 mmol) and diisopropylethylamine (480 mg, 3.7 mmol) in anhydrous DCM (10 mL) was added dropwise to a solution of triphosgene (197 mg, 0.66 mmol) in anhydrous DCM (5 mL) over a period of 30 min. *N*-Boc-*S*-methylisothiourea (**25**) (505 mg, 2.66 mmol) was added and stirring was continued for 2.5 h. The volume was reduced under reduced pressure to about 3 mL and the mixture was directly subjected to column chromatography (eluent: DCM/EtOAc 50:1–5:1). Although **27** and **25** showed identical $R_f$-values in TLC analysis ($R_f$ = 0.6 for DCM/EtOAc 5:1), separation of the product from the excess of **25** became possible by column chromatography (eluent see above). As an alternative, the use of 1 equiv of **25** is recommended. Highly viscous yellowish oil (403 mg, 75%). $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.27–1.53 (m, 22H), 2.28 (s, 3H), 2.89 (m, 2H), 3.01 (m, 2H), 6.80 (t, 1H, $^3J$ = 5.2 Hz), 7.79 (t, 1H, $^3J$ = 5.8 Hz), 12.44 (s, 1H); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 405 $[M+H]^+$; $C_{17}H_{34}N_4O_5S$ (406.5).

#### 4.2.15. 6-(*tert*-Butoxycarbonylamino)hexanoic acid (28)[48]

To a vigorously stirred solution of 6-aminohexanoic acid (2.93 g, 25.0 mmol) and $NaHCO_3$ (5.26 g, 62.5 mmol) in water (50 mL) was added di-*tert*-butyl dicarbonate (6.55 g, 30.0 mmol) in 1,4-dioxane (50 mL), and stirring was continued overnight. The resulting mixture was extracted with diethyl ether (50 mL, discarded) and carefully acidified (pH 2) with 2 M hydrochloric acid. The aqueous layer was extracted with ethyl acetate (3 × 30 mL), and the combined extracts were washed with brine and dried over anhydrous $Na_2SO_4$. The volatiles were removed under reduced pressure and the residue was crystallized from diethyl ether/*n*-pentane to afford a white solid (4.84 g, 84%); mp 35–37 °C; $^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ (ppm) = 1.44 (s, 9H), 1.32–1.55 (m, 4H), 1.75 (q, 2H, $^3J$ = 7.4 Hz), 2.36 (t, 2H, $^3J$ = 7.4 Hz), 3.12 (m, 2H), 4.55 (m, 1H); MS (CI, $NH_3$): *m/z* 249 $[M+NH_4]^+$, 232 $[M+H]^+$, 193 $[M-C_4H_8+NH_4]^+$; $C_{11}H_{21}NO_4$ (231.3).

#### 4.2.16. (*R*)-$N^{\omega}$-(6-Aminohexanoyl)-$N^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-ureidomethylbenzyl)argininamide bis(hydrotrifluoroacetate) (29)

Carbonyldiimidazole (56 mg, 0.35 mmol) was added to a solution of 6-(*tert*-butoxycarbonylamino)hexanoic acid (**28**) (71.3 mg, 0.31 mmol) in anhydrous acetonitrile (2 mL). The mixture was stirred for 40 min at rt and then added to a suspension of **12** (85 mg, 0.12 mmol) and $NEt_3$ (62.3 mg, 0.62 mmol) in acetonitrile (10 mL). The precipitate disappeared almost completely during heating to 40 °C for 2 h. The mixture was stirred at 35 °C overnight, then TFA (10 mL) was added and the mixture was allowed to stand at 50 °C for 1.5 h. MeOH (20 mL) was added three times, each time followed by evaporation under reduced pressure. Purification by preparative HPLC (gradient: 0–30 min: MeCN/0.1% aq TFA 15:85–40:60, $t_R$ = 23.6 min) afforded the product as a white fluffy, hygroscopic solid (39 mg, 36%). $[\alpha]_D^{20}$ +11.2 $cm^3\ g^{-1}\ dm^{-1}$ (*c* 0.0067 in $MeCN/H_2O$ 9:1); IR (neat): 1635, 1530, 1495, 1135 $cm^{-1}$; $^1H$ NMR (300 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.44 (m, 2H), 1.5–1.77 (br m, 7H), 1.84 (m, 1H), 2.50 (t, 2H, $^3J$ = 7.3 Hz), 2.91 (t, 2H, $^3J$ = 7.6 Hz), 3.25 (m, 2H), 4.26 (s, 2H), 4.32 (d, 2H, $^3J$ = 7.7 Hz), 4.42 (m, 1H), 5.08 (s, 1H), 7.15–7.34 (m, 14H); $^{13}C$ NMR (75 MHz, MeOH-$d_4$): $\delta$

(ppm) 24.7, 25.4, 26.7, 28.2, 30.2, 37.3, 40.5, 41.8, 43.8, 44.4, 54.3, 58.7, 128.1, 128.2, 128.4, 128.6, 129.5, 129.9, 138.4, 140.2, 140.8, 141.0, 155.1, 162.1, 173.5, 174.9, 177.0; MS (ES, TFA/acetonitrile): *m/z* 322 $[M+2H]^{2+}$, 643 $[M+H]^+$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{35}H_{47}N_8O_4^+$: 643.3720, found: 643.3715; $C_{35}H_{46}N_8O_4 \times C_4H_2F_6O_4$ (870.8).

### 4.2.17. General procedure for the synthesis of amines 31–33

CDI (1.6 equiv) was added to a solution of the pertinent carboxylic acid (1.5 equiv) in DCM (2–3 mL) and the mixture was allowed to stand at rt for 30–40 min. Compound **30**[6] (1 equiv) and $NEt_3$ (0.3 equiv) were added and the mixture was kept under stirring at rt for 20 h. The mixture was directly subjected to column chromatography for purification of the intermediate (**31** and **32**: DCM/EtOAc 2:1–EtOAc/MeOH 50:1, **33**: DCM/EtOAc 5:1–1:1; for column packing the starting eluent was supplemented with 1% $NEt_3$). For deprotection the intermediate was dissolved in DCM/TFA/water 18:6:1 (12.5 mL) and the mixture was stirred for 3 h. DCM (20 mL) was added three times, each time followed by evaporation under reduced pressure. The oily residue was dissolved in water (20 mL) and lyophilized to afford the product as a white fluffy, hygroscopic solid.

#### 4.2.17.1. (R)-$N^{\omega}$-[4-(2-Aminoethyl)aminocarbonylbutanoyl]-$N^{\alpha}$-(2,2-diphenylacetyl)-N-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) (31).

Prepared from **16** (216 mg, 0.79 mmol) and **30** (331 mg, 0.53 mmol); CDI: 136 mg, 0.84 mmol; $NEt_3$: 16 mg, 0.16 mmol; 71% (320 mg); IR (neat): 1640, 1515, 1495, 1170, 1130 $cm^{-1}$; $^1H$ NMR (COSY, 600 MHz, DMSO-$d_6$,): $\delta$ (ppm) 1.38–1.50 (m, 2H), 1.55 (m, 1H), 1.68 (m, 1H), 1.79 (p, 2H, $^3J$ = 7.4 Hz), 2.16 (t, 2H, $^3J$ = 7.4 Hz), 2.45 (t, 2H, $^3J$ = 7.4 Hz), 2.85 (m, 2H), 3.23 (m, 2H), 3.28 (m, 2H), 4.1 (dd, 1H, $^2J$ = 14.9 Hz, $^3J$ = 5.7 Hz), 4.17 (dd, 1H, $^2J$ = 14.8 Hz, $^3J$ = 5.9 Hz), 4.33 (m, 1H), 5.13 (s, 1H), 6.68 (m, 2H), 7.00 (m, 2H), 7.22 (m, 2H), 7.28 (m, 8H), 7.85 (br s, 3H), 8.05 (t, 1H, $^3J$ = 5.6 Hz), 8.35 (t, 1H, $^3J$ = 5.8 Hz), 8.48 (d, 1H, $^3J$ = 8.0 Hz), 8.64 (br s, 1H), 8.98 (br s, 1H), 9.35 (t, 1H, $^3J$ = 5.4 Hz), 12.05 (s, 1H); $^{13}C$ NMR (150 MHz, DMSO-$d_6$): $\delta$ (ppm) 19.7, 24.4, 29.4, 34.0, 35.4, 36.4, 38.7, 40.4, 41.6, 52.3, 56.0, 113.5 (TFA), 115.0, 115.4 (TFA), 117.4 (TFA), 119.3 (TFA), 126.6, 128.17, 128.21, 128.4, 128.51, 128.54, 129.1, 140.3, 140.5, 153.0, 156.3, 158.7 (TFA), 158.9 (TFA), 159.1 (TFA), 159.4 (TFA), 171.0, 171.1, 172.4, 175.1; MS (ES, acetonitrile/TFA): *m/z* 315.5 $[M+2H]^{2+}$, 630 $[M+H]^+$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{34}H_{44}N_7O_5^+$: 630.3404, found: 630.3408; $C_{34}H_{43}N_7O_5 \times C_4H_2F_6O_4$ (857.7).

#### 4.2.17.2. (R)-$N^{\omega}$-[4-(3-Aminopropyl)aminocarbonylbutanoyl]-$N^{\alpha}$-(2,2-diphenylacetyl)-N-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) (32).

Prepared from **17** (240 mg, 0.83 mmol) and **30** (350 mg, 0.56 mmol); CDI: 144 mg, 0.89 mmol; $NEt_3$: 17 mg, 0.17 mmol; 64% (310 mg); $[\alpha]_D^{20}$ +4.8 $cm^3\ g^{-1}\ dm^{-1}$ (*c* 0.0108 in MeCN/$H_2O$ 9:1); IR (neat): 1635, 1540, 1515, 1495, 1170, 1130 $cm^{-1}$; $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.32–1.6 (m, 3H), 1.65 (m, 3H), 1.77 (m, 2H), 2.14 (t, 2H, $^3J$ = 7.4 Hz), 2.43 (t, 2H, $^3J$ = 7.0 Hz), 2.76 (m, 2H), 3.1 (m, 2H), 3.24 (m, 2H), 4.1 (dd, 1H, $^2J$ = 15.2 Hz, $^3J$ = 6.0 Hz), 4.18 (dd, 1H, $^2J$ = 15.1 Hz, $^3J$ = 5.9 Hz), 4.34 (m, 1H), 5.12 (s, 1H), 6.67 (m, 2H), 7.0 (m, 2H), 7.18–7.33 (m, 10H), 7.65 (br s, 3H), 7.98 (t, 1H, $^3J$ = 5.5 Hz), 8.38 (t, 1H, $^3J$ = 5.7 Hz), 8.5 (d, 1H), 8.56 (s, 2H), 8.85 (m, 1H), 9.3 (s, 1H), 11.32 (s, 1H); $^{13}C$ NMR (150 MHz, MeOH-$d_4$): $\delta$ (ppm) 21.3, 25.4, 28.8, 30.2, 35.5, 36.8, 36.9, 38.2, 41.8, 43.7, 54.3, 58.7, 116.3, 116.9 (TFA), 118.9 (TFA), 128.1, 128.2, 129.48, 129.53, 129.8, 129.9, 130.0, 130.3, 140.8, 140.9, 155.0, 157.8, 162.4 (TFA), 162.6 (TFA), 162.8 (TFA), 163.1 (TFA), 173.3, 174.9, 176.0, 176.6; MS (ES, acetonitrile/TFA): *m/z* 322.5 $[M+2H]^{2+}$, 644 $[M+H]^+$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{35}H_{46}N_7O_5^+$: 644.3560, found: 644.3555; $C_{35}H_{45}N_7O_5 \times C_4H_2F_6O_4$ (871.7).

#### 4.2.17.3. (R)-$N^{\omega}$-(6-Aminohexanoyl)-$N^{\alpha}$-(2,2-diphenylacetyl)-N-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) (33).

Prepared from *N*-Boc-6-aminohexanoic acid **28** (222 mg, 0.96 mmol) and **30** (403 mg, 0.64 mmol); CDI: 166 mg, 1.02 mmol; $NEt_3$: 19 mg, 0.19 mmol; 94% (490 mg); $[\alpha]_D^{20}$ +8.8 $cm^3\ g^{-1}\ dm^{-1}$ (*c* 0.0093 in MeCN/$H_2O$ 9:1); IR (neat): 1670, 1630, 1535, 1515, 1495, 1190, 1130 $cm^{-1}$; $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.32 (m, 2H), 1.37–1.6 (m, 7H), 1.67 (m, 1H), 2.42 (t, 2H, $^3J$ = 7.3 Hz), 2.76 (m, 2H), 3.24 (m, 2H), 4.1 (dd, 1H, $^2J$ = 15.2 Hz, $^3J$ = 5.9 Hz), 4.18 (dd, 1H, $^2J$ = 15.1 Hz, $^3J$ = 6.1 Hz), 4.33 (m, 1H), 5.13 (s, 1H), 6.67 (m, 2H), 7.0 (m, 2H), 7.18–7.33 (m, 10H), 7.63 (br s, 3H), 8.38 (t, 1H, $^3J$ = 5.8 Hz), 8.5 (d, 1H, $^3J$ = 8.1 Hz), 8.6 (s, 2H), 8.95 (m, 1H), 9.3 (s, 1H), 11.4 (s, 1H); $^{13}C$ NMR (150 MHz, MeOH-$d_4$): $\delta$ (ppm) 24.7, 25.5, 26.7, 28.2, 30.2, 37.3, 40.5, 41.8, 43.7, 54.3, 58.7, 116.3, 117.0 (TFA), 118.9 (TFA), 128.1, 128.2, 129.47, 129.52, 129.8, 129.9, 130.0, 130.3, 140.8, 140.9, 155.1, 157.8, 162.5 (TFA), 162.8 (TFA), 163.0 (TFA), 163.2 (TFA), 173.4, 174.9, 177.0; MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 587 $[M+H]^+$, 294 $[M+2H]^{2+}$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{33}H_{43}N_6O_4^+$: 587.3346, found: 587.3357; $C_{33}H_{42}N_6O_4 \times C_4H_2F_6O_4$ (814.7).

### 4.2.18. (*R*)-$N^{\omega}$-(12-Amino-4,7,10-trioxadodecanoyl)-$N^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) (34)

A solution of **19** (300 mg, 0.84 mmol) and CDI (156 mg, 0.96 mmol) in DCM (10 mL) was stirred for 30 min. **30** (506 mg, 0.8 mmol) and $NEt_3$ (40.7 mg, 56 μL, 0.4 mmol) were added and the mixture was kept under stirring at rt for 20 h. Volatiles were removed under reduced pressure and the intermediate was purified by column chromatography (DCM/MeOH 50:1–10:1) prior to hydrogenation at rt and atmospheric pressure in MeOH (20 mL) using hydrogen and a 10% Pd/C catalyst (120 mg). The catalyst was removed by filtration; removal of the solvent in vacuo yielded a highly viscous colourless oil which was dissolved in DCM/TFA 10:1 (v/v). The mixture was allowed to stand at rt overnight and was subsequently concentrated under reduced pressure. Purification by preparative HPLC (gradient: 0–20 min: MeCN/0.1% aq TFA 15:85–38:62, 20–20.1 min: 38:62–67:33, 20.1–25 min: 67:33, $t_R$ = 19.8 min) afforded the product as a white fluffy, hygroscopic solid (264 mg, 36%). $[\alpha]_D^{20}$ +8.2 $cm^3\ g^{-1}\ dm^{-1}$ (*c* 0.0060 in MeCN/$H_2O$ 9:1); IR (neat): 1640, 1515, 1495, 1180, 1130 $cm^{-1}$; $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.32–1.6 (m, 3H), 1.66 (m, 1H), 2.68 (t, 2H, $^3J$ = 5.9 Hz), 2.96 (m, 2H), 3.24 (m, 2H), 3.54 (m, 10H), 3.68 (t, 2H, $^3J$ = 6.0 Hz), 4.1 (dd, 1H, $^2J$ = 15.1 Hz, $^3J$ = 5.9 Hz), 4.19 (dd, 1H, $^2J$ = 15.1 Hz, $^3J$ = 5.8 Hz), 4.34 (m, 1H), 5.12 (s, 1H), 6.67 (m, 2H), 7.0 (m, 2H), 7.18–7.35 (m, 10H), 7.75 (br s, 3H), 8.38 (t, 1H, $^3J$ = 5.7 Hz), 8.50 (d, 1H, $^3J$ = 8.1 Hz), 8.68 (s, 2H), 9.07 (m, 1H), 9.3 (s, 1H), 11.63 (s, 1H); $^{13}C$ NMR (75 MHz, DMSO-$d_6$): $\delta$ (ppm) 25.5, 30.2, 38.4, 40.6, 41.8, 43.7, 54.3, 58.7, 66.9, 67.8, 71.16, 71.25, 71.32, 71.44, 116.3, 128.1, 128.2, 129.5, 129.8, 129.9, 130.0, 130.3, 140.8, 140.9, 155.0, 157.8, 173.3, 174.9, 175.4; MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 677 $[M+H]^+$, 339 $[M+2H]^{2+}$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{36}H_{49}N_6O_7^+$: 677.3663, found: 677.3655; $C_{36}H_{48}N_6O_7 \times C_4H_2F_6O_4$ (904.8).

### 4.2.19. (*R*)-$N^{\omega}$-(8-Amino-3,6-dioxaoctanoyl)-$N^{\alpha}$-(2,2-diphenylacetyl)-*N*-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) (36)

*N*-Boc-8-amino-3,6-dioxaoctanoic acid × dicyclohexylamine (300 mg) was dissolved in dimethoxyethane (10 mL). The amine was precipitated by addition of 37% hydrochloric acid (≈60 μL) and removed by filtration. Removal of the solvent in vacuo yielded a yellow oil (200 mg, 0.76 mmol) which was dissolved in

anhydrous DCM (3 mL). CDI (148 mg, 0.91 mmol) was added and the mixture was allowed to stand at rt for 30 min. DCM (15 mL), **30** (478 mg, 0.76 mmol) and $NEt_3$ (77 mg, 105 μL, 0.76 mmol) were added and the mixture was kept under stirring at rt for 20 h. TFA (10 mL) was added, the mixture was allowed to stand at rt for 4 h before it was concentrated under reduced pressure. MeOH (20 mL) was added twice each time followed by evaporation under reduced pressure. Purification by preparative HPLC (isocratic: MeCN/0.1% aq TFA 32:68, $t_R$ = 14 min) afforded the product as a white fluffy, hygroscopic solid (300 mg, 47%). $[\alpha]_D^{20}$ +6.2 $cm^3\ g^{-1}\ dm^{-1}$ (*c* 0.010 in MeCN/$H_2O$ 9:1); IR (neat): 1670, 1635, 1515, 1495, 1200, 1130 $cm^{-1}$; $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.32–1.61 (m, 3H), 1.68 (m, 1H), 2.98 (m, 2H), 3.25 (m, 2H), 3.61 (m, 4H), 3.71 (m, 2H), 4.1 (dd, 1H, $^2J$ = 15.4 Hz, $^3J$ = 6.1 Hz), 4.18 (dd, 1H, $^2J$ = 15.4 Hz, $^3J$ = 6.2 Hz), 4.23 (s, 2H), 4.33 (m, 1H), 5.13 (s, 1H), 6.67 (m, 2H), 7.0 (m, 2H), 7.18–7.33 (m, 10H), 7.8 (br s, 3H), 8.39 (t, 1H, $^3J$ = 5.8 Hz), 8.51 (d, 1H, $^3J$ = 8.1 Hz), 8.81 (s, 2H), 9.12 (m, 1H), 9.32 (s, 1H), 10.98 (s, 1H); $^{13}C$ NMR (150 MHz, MeOH-$d_4$): $\delta$ (ppm) 25.4, 30.2, 40.6, 42.0, 43.7, 54.3, 58.7, 67.9, 71.1, 71.3, 72.1, 116.3, 117.3 (TFA), 119.2 (TFA), 128.1, 128.2, 129.49, 129.52, 129.8, 129.9, 130.0, 130.3, 140.8, 140.9, 154.7, 157.8, 162.7 (TFA), 163.0 (TFA), 163.2 (TFA), 163.4 (TFA), 173.3, 174.0, 174.9; MS (ES, acetonitrile/TFA): *m/z* 310 $[M+2H]^{2+}$, 619 $[M+H]^+$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{33}H_{43}N_6O_6^+$: 619.3244, found: 619.3257; $C_{33}H_{42}N_6O_6 \times C_4H_2F_6O_4$ (846.8).

#### 4.2.20. General procedure for the synthesis of amines 37 and 38

CDI (1.2 equiv) was added to a solution of acid **23** or **24** (1 equiv) in DCM and the mixture was stirred for 30 min at rt. Compound **30** (1 equiv) was added and stirring was continued at rt overnight. The intermediate was purified by column chromatography (eluent: DCM/EtOAc 2:1 to EtOAc/MeOH 25:1; for column packing the starting eluent was supplemented with 1% $NEt_3$) prior to deprotection in DCM/TFA 1:1 (v/v) for 2.5 h. DCM (5 vol parts) was added three times, each time followed by evaporation under reduced pressure. The product was purified using preparative HPLC.

**4.2.20.1. (R)-N$^{\omega}$-[4-(8-Amino-3,6-dioxaoctyl)aminocarbonylbutanoyl]-N$^{\alpha}$-(2,2-diphenylacetyl)-N-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) (37).** Prepared from **23** (115 mg, 0.32 mmol) and **30** (200 mg, 0.32 mmol); CDI: 62 mg, 0.38 mmol; gradient for preparative HPLC: 0–15 min: MeCN/0.1% aq TFA 15:85–45:55 ($t_R$ = 11.2 min); white, hygroscopic solid; 64% (192 mg); IR (neat): 1670, 1635, 1540, 1515, 1190, 1130 $cm^{-1}$; $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.34–1.6 (br m, 3H), 1.66 (m, 1H), 1.76 (p, 2H, $^3J$ = 7.3 Hz), 2.13 (t, 2H, $^3J$ = 7.3 Hz), 2.42 (t, 2H, $^3J$ = 7.3 Hz), 2.97 (m, 2H), 3.21 (m, 4H), 3.4 (t, 2H, $^3J$ = 6.1 Hz), 3.5–3.61 (m, 6H), 4.14 (m, 2H), 4.33 (m, 1H), 5.12 (s, 1H), 6.67 (m, 2H), 7.0 (m, 2H), 7.18–7.33 (m, 10H), 7.77 (br s, 2H), 7.92 (t, 1H, $^3J$ = 5.6 Hz), 8.39 (t, 1H, $^3J$ = 5.9 Hz), 8.5 (d, 1H, $^3J$ = 8.0 Hz), 8.64 (s, 2H), 9.03 (s, 1H), 9.31 (s, 1H), 11.54 (s, 1H); $^{13}C$ NMR (150 MHz, MeOH-$d_4$): $\delta$ (ppm) 21.4, 25.5, 30.3, 35.6, 36.8, 40.1, 40.7, 41.9, 43.7, 54.3, 58.7, 67.9, 70.6, 71.3, 71.4, 116.3, 128.1, 128.2, 129.50, 129.54, 129.8, 129.9, 130.0, 130.3, 140.8, 140.9, 155.0, 157.9, 162.9 (TFA), 163.1 (TFA), 173.3, 174.9, 175.3; MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 718 $[M+H]^+$, 359.5 $[M+2H]^{2+}$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{38}H_{52}N_7O_7^+$: 718.3928, found: 718.3942; $C_{38}H_{51}N_7O_7 \times C_4H_2F_6O_4$ (945.9).

**4.2.20.2. (R)-N$^{\omega}$-[5-(8-Amino-3,6-dioxaoctyl)aminocarbonylpentanoyl]-N$^{\alpha}$-(2,2-diphenylacetyl)-N-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) (38).** Prepared from **24** (120 mg, 0.32 mmol) and **30** (200 mg, 0.32 mmol); CDI: 62 mg, 0.38 mmol; gradient for preparative HPLC: 0–15 min: MeCN/0.1% aq TFA 15:85–40:60 ($t_R$ = 12.6 min); white, hygroscopic solid; 44% (135 mg); $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.34–1.6 (br m, 7H), 1.66 (m, 1H), 2.08 (t, 2H, $^3J$ = 6.2 Hz), 2.42 (t, 2H, $^3J$ = 6.1 Hz), 2.97 (m, 2H), 3.2 (m, 4H), 3.39 (t, 2H, $^3J$ = 6.1 Hz), 3.5–3.62 (m, 6H), 4.14 (m, 2H), 4.33 (m, 1H), 5.12 (s, 1H), 6.67 (m, 2H), 7.0 (m, 2H), 7.18–7.33 (m, 10H), 7.77 (br s, 2H), 7.88 (t, 1H, $^3J$ = 5.5 Hz), 8.38 (t, 1H, $^3J$ = 5.9 Hz), 8.5 (d, 1H, $^3J$ = 8.0 Hz), 8.66 (s, 2H), 9.08 (s, 1H), 9.31 (s, 1H), 11.6 (s, 1H); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 732 $[M+H]^+$, 366.5 $[M+2H]^{2+}$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{39}H_{54}N_7O_7^+$: 732.4085, found: 732.4106; $C_{39}H_{53}N_7O_7 \times C_4H_2F_6O_4$ (959.9).

**4.2.21. (*R*)-N$^{\omega}$-[7-(8-Amino-3,6-dioxaoctyl) aminocarbonylheptanoyl-N$^{\alpha}$-(2,2-diphenylacetyl)-N-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) (40)**

The carboxylic acid **39** (370 mg, 0.47 mmol) and amine **22** (140 mg, 0.56 mmol) were dissolved in DCM (5 mL). EDC (108 mg, 0.56 mmol) was added and the mixture was kept under stirring at rt for 20 h. TFA (4 mL) was added and stirring was continued for 2.5 h. DCM (30 mL) was added three times, each time followed by evaporation under reduced pressure. The product was purified using preparative HPLC (gradient: 0–30 min: MeCN/0.1% aq TFA 30:70–50:50, $t_R$ = 18.9 min). Brownish resin (200 mg, 43%); $^1H$ NMR (300 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.25 (m, 4H), 1.35–1.6 (m, 7H), 1.66 (m, 1H), 2.05 (t, 2H, $^3J$ = 7.4 Hz), 2.4 (t, 2H, $^3J$ = 7.3 Hz), 2.97 (m, 2H), 3.2 (m, 4H), 3.39 (t, 2H, $^3J$ = 6.1 Hz), 3.5–3.61 (m, 6H), 4.14 (m, 2H), 4.33 (m, 1H), 5.12 (s, 1H), 6.67 (m, 2H), 7.0 (m, 2H), 7.18–7.34 (m, 10H), 7.76 (br s, 2H), 7.84 (t, 1H, $^3J$ = 5.5 Hz), 8.39 (t, 1H, $^3J$ = 5.8 Hz), 8.5 (d, 1H, $^3J$ = 7.9 Hz), 8.62 (s, 2H), 8.99 (s, 1H), 9.31 (s, 1H), 11.45 (s, 1H); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 380.5 $[M+2H]^{2+}$, 760 $[M+H]^+$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{41}H_{58}N_7O_7^+$: 760.4398, found: 760.4382; $C_{41}H_{57}N_7O_7 \times C_4H_2F_6O_4$ (988.0).

**4.2.22. (*R*)-N$^{\omega}$-[(4-Aminobutyl)aminocarbonyl]-N$^{\alpha}$-(2,2-diphenylacetyl)-N-(4-hydroxybenzyl)argininamide bis(hydrotrifluoroacetate) (41)**

Compound **27** (178 mg, 0.44 mmol) and **42**[6] (215 mg, 0.44 mmol) were dissolved in anhydrous DMF (10 mL). $HgCl_2$ (197 mg, 0.66 mmol) and diisopropylethylamine (114 mg, 0.88 mmol) were added, the mixture was stirred at rt overnight and then concentrated under reduced pressure (final pressure: 1 mbar) at 43 °C. The intermediate was purified by column chromatography (DCM/EtOAc 4:1–1:2) prior to deprotection with DCM/TFA 1:1 (4 mL) and three drops of water (2 h). DCM (20 mL) was added three times, each time followed by evaporation under reduced pressure. Uptake of the oily residue in water (20 mL) and lyophilisation afforded the product as a white fluffy, hygroscopic solid (266 mg, 74%). $[\alpha]_D^{20}$ +9.1 $cm^3\ g^{-1}\ dm^{-1}$ (*c* 0.0045 in MeCN/$H_2O$ 9:1); IR (neat): 1670, 1535, 1515, 1495, 1200, 1130 $cm^{-1}$; $^1H$ NMR (COSY, 600 MHz, DMSO-$d_6$): $\delta$ (ppm) 1.34–1.56 (m, 7H), 1.66 (m, 1H), 2.78 (m, 2H), 3.11 (m, 2H), 3.19 (m, 2H), 4.11 (dd, 1H, $^2J$ = 14.8 Hz, $^3J$ = 5.6 Hz), 4.17 (dd, 1H, $^2J$ = 14.8 Hz, $^3J$ = 5.8 Hz), 4.33 (m, 1H), 5.11 (s, 1H), 6.66 (m, 2H), 6.99 (m, 2H), 7.21 (m, 2H), 7.28 (m, 8H), 7.54 (m, 1H), 7.66 (br s, 3H), 8.35 (m, 1H), 8.4 (br s, 2H), 8.47 (d, 1H, $^3J$ = 8.1 Hz), 8.9 (s, 1H), 9.27 (s, 1H), 9.96 (s, 1H); $^{13}C$ NMR (150 MHz, DMSO-$d_6$): $\delta$ (ppm) 24.4, 24.6, 26.0, 29.4, 38.5, 38.6, 40.3, 41.6, 52.3, 55.9, 115.0, 116.2 (TFA), 118.1 (TFA), 126.6, 128.16, 128.19, 128.4, 128.49, 128.51, 129.1, 140.3, 140.5, 153.6, 153.8, 156.3, 158.4 (TFA), 158.6 (TFA), 170.95, 171.02; MS (ES, acetonitrile/TFA): *m/z* 294.5 $[M+2H]^{2+}$, 588 $[M+H]^+$; HRMS: (LSI-MS): *m/z* $[M+H]^+$ calcd for $C_{32}H_{42}N_7O_4^+$: 588.3298, found: 588.3302; $C_{32}H_{41}N_7O_4 \times C_4H_2F_6O_4$ (815.7).

#### 4.2.23. (*E*)-3-(Dimethylamino)propenal (45)[49]

Synthesis of **45** was essentially performed as described from ethoxyethene (**44**) (5.7 mL, 0.059 mol), dimethylformamide (**43**) (13 mL, 0.17 mol) and $POCl_3$ (6.3 mL, 0.069 mol).[49] The product was obtained as a brown sticky oil (2.38 g, 40%) and used without further purification. $^1$H NMR (300 MHz, MeOH-$d_4$): $\delta$ (ppm) 2.91 (s, 3H), 3.18 (s, 3H), 5.16 (dd, 1H, $^3J$ = 9.0 Hz, $^3J$ = 12.2 Hz), 7.42 (d, 1H, $^3J$ = 12.3 Hz), 8.82 (d, 1H, $^3J$ = 9.0 Hz); $^{13}$C NMR: (75 MHz, MeOH-$d_4$): $\delta$ (ppm) 37.8, 45.4, 101.6, 164.3, 191.1; MS (CI, $NH_3$): *m/z* 100 [*M*+H]$^+$, 117 [*M*+$NH_4$]$^+$, 199 [2*M*+H]$^+$); $C_5H_9NO$ (99.13).

#### 4.2.24. (*E*)-3-[4-(Dimethylamino)phenyl]propenal (47)[24]

Compound **47** was prepared as described, but hexane/ethyl acetate (4:1 v/v) was used as eluent for purification by column chromatography on silica gel. The title compound was obtained as a yellow solid, which changed into green during storage (470 mg, 33%). Mp 132 °C (137–139 °C24**); $^1$H NMR (300 MHz, MeOH-$d_4$): $\delta$ (ppm) 3.04 (s, 6 H), 6.54 (dd, 1H, $^3J$ = 8.0 Hz, $^3J$ = 15.6 Hz), 6.75 (d, 2H, $^3J$ = 9.0 Hz), 7.50–7.53 (m, 2H), 7.54 (d, 1H, $^3J$ = 15.4 Hz), 9.48 (d, 1H, $^3J$ = 8.0 Hz); $^{13}$C NMR (75 MHz, MeOH-$d_4$): $\delta$ (ppm) 40.2, 112.9, 123.1, 124.0, 132.0, 154.4, 157.2, 196.2; MS (CI, $NH_3$): *m/z* 176 [*M*+H]$^+$, 193 [*M*+$NH_4$]$^+$; $C_{11}H_{13}NO$ (175.23).

#### 4.2.25. 3-(4-Methoxy-4-oxobutyl)-2-methylbenzo[*d*]thiazol-3-ium bromide (49)

A mixture of benzothiazole **48** (3.0 g, 0.02 mol) and methyl 4-bromobutanoate (2.0 g, 0.011 mol) was heated to 110 °C for 72 h. Every 24 h the grey precipitate was removed and the reaction mixture was refluxed for another 24 h. The product was washed with a mixture of ethyl acetate and ethanol (9:1 v/v) until the supernatant remained colourless upon washing. During this treatment the residue turned from dark grey to beige. Filtration and drying afforded the title compound as a beige solid (987 mg, 27%). The compound was used without further purification. Mp >132 °C (decomp); $^1$H NMR (300 MHz, MeOH-$d_4$): $\delta$ (ppm) 2.19–2.29 (m, 2H), 2.68 (t, 2H, $^3J$ = 6.45 Hz), 3.26 (s, 3H), 3.68 (s, 3H), 4.76–4.87 (m, 2H), 7.82 (t, 1H, $^3J$ = 7.7 Hz), 7.90–7.96 (m, 1H), 8.29–8.33 (m, 1H), 8.38 (d, 1H, $^3J$ = 8.5 Hz); $^{13}$C NMR (75 MHz, MeOH-$d_4$): $\delta$ (ppm) 17.2, 24.2, 30.8, 49.9, 52.4, 117.9, 125.4, 129.8, 131.1, 142.7, 174.7; MS (LC–MS): *m/z* [$t_R$ = 3.19 min]: 250 *M*$^+$; $C_{13}H_{16}NO_2S^+ \times Br^-$ (330.24).

#### 4.2.26. 2-((1*E*,3*E*)-4-(4-(Dimethylamino)phenyl)buta-1,3-dienyl)-3-(4-methoxy-4-oxobutyl)benzo[*d*]thiazol-3-ium bromide (50)

To a solution of **49** (0.25 g, 757 μmol) and **47** (0.146 g, 833 μmol) in methanol (10 mL) three drops of pyridine were added and the mixture was heated to reflux for 18 h. After evaporation of the solvent the residue was suspended in a small amount of methanol. Addition of diethyl ether led to the formation of blue-black crystals. The product was used without further purification (239 mg, 65%). Mp >139 °C (decomp); $^1$H NMR (600 MHz, MeOH-$d_4$): $\delta$ (ppm) 2.17–2.22 (m, 2H), 2.64 (t, 2H, $^3J$ = 6.6 Hz), 3.05–3.06 (m, 6H), 3.7 (s, 3H), 4.70–4.72 (m, 2H), 6.72–6.75 (m, 2H), 7.15–7.21 (m, 1H), 7.25 (d, 1H, $^3J$ = 14.4 Hz), 7.37 (d, 1H, $^3J$ = 14.5 Hz), 7.53–7.55 (m, 2H), 7.66–7.68 (m, 1H), 7.77–7.80 (m, 1H), 7.95–7.8 (m, 1H), 8.1–8.12 (m, 2H); $^{13}$C NMR (150 MHz, MeOH-$d_4$): $\delta$ (ppm) 24.5, 30.8, 40.2, 48.6, 52.4, 111.6, 113.2, 116.7, 123.2, 124.7, 129.1, 130.5, 132.2, 142.7, 151.0, 154.2, 172.4, 174.8; MS (LC–MS): *m/z* [$t_R$ = 9.26 min]: 407 *M*$^+$; $C_{24}H_{27}N_2O_2S^+ \times Br^-$ (487.79).

#### 4.2.27. 4-(2-((1*E*,3*E*)-4-(4-(Dimethylamino)phenyl)buta-1,3-dienyl)benzo[*d*]thiazol-3-ium-3-yl)butanoate trifluoroacetate (51)

Compound **50** (0.1 g, 204 μmol) and lithium hydroxide (0.0234 g, 978 μmol) were dissolved in a mixture of ethanol (400 μL), THF (400 μL) and $H_2O$ (400 μL) and stirred at rt for 3 h. 10% aqueous TFA (60 μL) was added and purification with preparative HPLC (gradient: 0–50 min: MeCN/0.1% aq TFA 20:80–75:25, $t_R$ = 27.3 min) afforded the product as a dark blue solid (30 mg, 28%). Mp 126–129 °C; $^1$H NMR (600 MHz, MeOH-$d_4$): $\delta$ (ppm) 2.16–2.21 (m, 2H), 2.60–2.64 (m, 2H), 3.08 (s, 3H), 3.15 (s, 3H), 4.71–4.78 (m, 2H), 6.83 (d, 1H, $^3J$ = 9.1 Hz), 6.77 (d, 1H, $^3J$ = 9.0 Hz), 7.17–7.20 (m, 1H), 7.29 (d, 1H, $^3J$ = 14.4 Hz), 7.38 (d, 1H, $^3J$ = 14.9 Hz), 7.56 (d, 1H, $^3J$ = 8.9 Hz), 7.66–7.68 (m, 1H), 7.76–7.80 (m, 1H), 7.86 (d, 1H, $^3J$ = 8.9 Hz), 8.06–8.08 (m, 1H), 8.1–8.14 (m, 2H); $^{13}$C NMR (150 MHz, MeOH-$d_4$): $\delta$ (ppm) 24.6, 30.7, 40.2, 49.6, 106.1, 113.2, 116.5, 123.5, 124.6, 128.8, 130.4, 132.2, 142.7, 152.9, 154.1, 173.1; MS (ES, MeOH + 10 mM $NH_4OAc$): *m/z* 393 *M*$^+$; HRMS: (LSI-MS): *m/z* *M*$^+$ calcd for $C_{23}H_{25}N_2O_2S^+$: 393.1637, found: 393.1649; $C_{23}H_{25}N_2O_2S^+ \times C_2F_3O_2^-$ (506.53).

#### 4.2.28. 2-(4-(4-(Dimethylamino)phenyl)buta-1,3-dienyl)-3-(4-(2,5-dioxopyrrolidin-1-yloxy)-4-oxobutyl)benzo[*d*]thiazol-3-ium trifluoroacetate (52, UR-DE99)

Compound **51** (0.028 g, 55 μmol) and NHS (0.0164 g, 142 μmol) were dissolved in anhydrous DMF (2 mL) and cooled to 0 °C. DCC (0.0294 g, 142 μmol) was added and the mixture was stirred at 0 °C for 10 h. Subsequently the solution was stirred at rt over night. As a considerable amount of **51** could still be detected by HPLC analysis, NHS (12.7 mg, 107 μmol) and DCC (50 mg, 242 μmol) were added and the solution was stirred at rt for 16 h. The reaction was performed under argon. The product was purified by preparative HPLC (gradient: 0–50 min: MeCN/0.05% aq TFA 20:80–75:25, $t_R$ = 29.0 min). Lyophilisation afforded the product as a dark blue solid (18.9 mg, 57%). $^1$H NMR (600 MHz, MeOH-$d_4$): $\delta$ (ppm) 2.16–2.22 (m, 2H), 2.61 (t, 2H, $^3J$ = 6.5 Hz), 2.68 (s, 4H), 3.10 (s, 6H), 4.71–4.75 (m, 2H), 6.78–6.81 (m, 2H), 7.18–7.22 (m, 1H), 7.28 (d, 1H, $^3J$ = 14.5 Hz), 7.39 (d, 1H, $^3J$ = 14.9 Hz), 7.56–7.59 (m, 2H), 7.67–7.71 (m, 1H), 7.78–7.82 (m, 1H), 7.99–8.04 (m, 1H), 8.11–8.15 (m, 2H); $^{13}$C NMR/HSQC (150 MHz, MeOH-$d_4$, no quarternary carbons): $\delta$ (ppm) 24.6, 26.3, 30.9, 40.2, 48.7, 111.6, 113.2, 116.7, 123.2, 124.7, 129.0, 130.6, 132.2, 151.2, 154,1; MS (ES, MeOH + 0.1% TFA): *m/z* 490 *M*$^+$; $C_{27}H_{28}N_3O_4S^+ \times C_2F_3O_2^-$ (603.59).

#### 4.2.29. 1-(6-(Amino((*R*)-4-(2,2-diphenylacetamido)-5-oxo-5-(4-(ureidomethyl)benzylamino)pentylamino)methyleneamino)-6-oxohexyl)-4-((*E*)-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolin-9-yl)ethenyl)-2,6-dimethylpyridinium hydrotrifluoroacetate trifluoroacetate (53)

Compound **29** (9.3 mg, 10.7 μmol) and $NEt_3$ (5.1 mg, 7.1 μL, 50.9 μmol) were dissolved in a mixture of acetonitrile (1 mL) and water (100 μL) followed by the addition of Py-1 × $1BF_4^-$ (2 mg, 5.1 μmol) in acetonitrile (200 μL). The reaction was stopped by addition of 10% aq TFA (corresponding to 10 equiv TFA) after an incubation period of 2 h at rt. The product was purified with preparative HPLC (gradient: 0–40 min: MeCN/0.1% aq TFA 15:85–70:30, 40–40.1 min: 70:30–90:10, 40.1–45 min: 90:10, $t_R$ = 42.8 min). 33% (1.75 mg); RP-HPLC (gradient 1): 82% (210 nm, $t_R$ = 19.7 min, $k$ = 6.3); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): *m/z* 465.5 [*M*$^+$+H]$^{2+}$, 930 *M*$^+$; HRMS: (FAB): *m/z* *M*$^+$ calcd for $C_{56}H_{68}N_9O_4^+$: 930.5389, found: 930.5377; $C_{56}H_{68}N_9O_4^+ \times C_4HF_6O_4^-$ (1158.2).

#### 4.2.30. General procedure for the synthesis of fluorescent ligands 54–64 and 70–72

The pertinent amine precursor (2–3 equiv) and $NEt_3$ (6–10 equiv) was dissolved in a mixture of acetonitrile and DMF (≈10–20% DMF v/v, total volume: 300–600 μL) followed by the addition of the pertinent pyrylium dye × $1BF_4^-$ (1 equiv) in DMF (≈25–50 μL). The reaction was stopped by addition of 10% aq TFA (corresponding to 6–10 equiv TFA) after an incubation period of 1–2 h at rt. The product was purified by preparative HPLC.

**4.2.30.1. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,15-trioxo-2,8,10,16-tetraazaoctadec-9-en-18-yl)-4-((E)-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-ij]quinolin-9-yl)ethenyl)-2,6-dimethylpyridinium hydrotrifluoroacetate trifluoroacetate (54).** Prepared from **31** and Py-1; gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–75:25 ($t_R$ = 33.4 min); 53% (3.07 mg); $^1$H NMR (COSY, 600 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.48–1.63 (m, 2H), 1.70 (m, 1H), 1.81–1.86 (m, 1H), 1.88 (p, 2H, $^3J$ = 7.3 Hz), 1.95 (p, 4H, $^3J$ = 6.0 Hz), 2.27 (t, 2H, $^3J$ = 7.4 Hz), 2.45 (t, 2H, $^3J$ = 7.3 Hz), 2.74 (t, 4H, $^3J$ = 6.2 Hz), 2.82 (s, 6H), 3.19–3.26 (m, 2H), 3.29 (t, 4H, $^3J$ = 5.8 Hz), 3.63 (t, 2H, $^3J$ = 6.9 Hz), 4.24 (m, 2H), 4.41 (m, 1H), 4.46 (t, 2H, $^3J$ = 7.0 Hz), 5.06 (s, 1H), 6.70 (m, 2H), 6.82 (d, 1H, $^3J$ = 15.8 Hz), 7.05 (m, 2H), 7.11 (s, 2H), 7.21–7.31 (m, 10H), 7.63 (d, 1H, $^3J$ = 15.9 Hz), 7.66 (s, 2H); RP-HPLC (gradient 1): 96% (210 nm, $t_R$ = 18.5 min, $k$ = 5.9); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): $m/z$ 459 $[M^+ + H]^{2+}$, 917 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{55}H_{65}N_8O_5^+$: 917.5072, found: 917.5064; $C_{55}H_{65}N_8O_5^+ \times C_4HF_6O_4^-$ (1145.2).

**4.2.30.2. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,15-trioxo-2,8,10,16-tetraazanonadec-9-en-19-yl)-4-((E)-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-ij]quinolin-9-yl)ethenyl)-2,6-dimethylpyridinium hydrotrifluoroacetate trifluoroacetate (55).** Prepared from **32** and Py-1; gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–75:25 ($t_R$ = 34.8 min); 36% (2.15 mg); RP-HPLC (gradient 1): 96% (210 nm, $t_R$ = 18.9 min, $k$ = 6.0); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): $m/z$ 466 $[M^+ + H]^{2+}$, 931 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{56}H_{67}N_8O_5^+$: 931.5234, found: 931.5250; $C_{56}H_{67}N_8O_5^+ \times C_4HF_6O_4^-$ (1159.2).

**4.2.30.3. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11-dioxo-14,17,20-trioxa-2,8,10-triazadocos-9-en-22-yl)-4-((E)-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-ij]quinolin-9-yl)ethenyl)-2,6-dimethylpyridinium hydrotrifluoroacetate trifluoroacetate (56).** Prepared from **34** and Py-1; gradient for preparative HPLC: 0–40 min: MeCN/0.1% aq TFA 15:85–73:27 ($t_R$ = 33.3 min); 47% (2.84 mg); RP-HPLC (gradient 1): 92% (210 nm, $t_R$ = 20.0 min, $k$ = 6.4); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): $m/z$ 482.5 $[M^+ + H]^{2+}$, 964 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{57}H_{70}N_7O_7^+$: 964.5331, found: 964.5357; $C_{57}H_{70}N_7O_7^+ \times C_4HF_6O_4^-$ (1192.2).

**4.2.30.4. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11-dioxo-13,16-dioxa-2,8,10-triazaoctadec-9-en-18-yl)-4-((E)-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-ij]quinolin-9-yl)ethenyl)-2,6-dimethylpyridinium hydrotrifluoroacetate trifluoroacetate (57).** Prepared from **36** and Py-1; gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–85:15 ($t_R$ = 31.9 min); 56% (1.63 mg); $^1$H NMR (COSY, 600 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.47–1.61 (m, 2H), 1.67 (m, 1H), 1.82 (m, 1H), 1.93 (p, 4H, $^3J$ = 6.0 Hz), 2.73 (t, 4H, $^3J$ = 6.3 Hz), 2.80 (s, 6H), 3.19 (m, 2H), 3.27 (t, 4H, $^3J$ = 5.6 Hz), 3.66 (m, 2H), 3.72 (m, 2H), 3.94 (t, 2H, $^3J$ = 5.1 Hz), 4.10 (s, 2H), 4.20–4.28 (m, 2H), 4.39 (m, 1H), 4.66 (t, 2H, $^3J$ = 5.1 Hz), 5.05 (s, 1H), 6.69 (m, 2H), 6.81 (d, 1H, $^3J$ = 15.9 Hz), 7.04 (m, 2H), 7.11 (s, 2H), 7.21–7.30 (m, 10H), 7.63 (d, 1H, $^3J$ = 15.9 Hz), 7.65 (s, 2H); RP-HPLC (gradient 1): 97% (210 nm, $t_R$ = 19.4 min, $k$ = 6.2); MS (ES, TFA/acetonitrile): $m/z$ 453.5 $[M^+ + H]^{2+}$, 906 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{54}H_{64}N_7O_6^+$: 906.4913, found: 906.4924; $C_{54}H_{64}N_7O_6^+ \times C_4HF_6O_4^-$ (1134.1).

**4.2.30.5. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11-dioxo-2,8,10,12-tetraazahexadec-9-en-16-yl)-4-((E)-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-ij]quinolin-9-yl)ethenyl)-2,6-dimethylpyridinium hydrotrifluoroacetate trifluoroacetate (58).** Prepared from **41** and Py-1; gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–85:15 ($t_R$ = 33.6 min); 26% (1.94 mg); $^1$H NMR (COSY, 600 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.50–1.63 (m, 2H), 1.68–1.78 (m, 3H), 1.86 (m, 3H), 1.96 (p, 4H, $^3J$ = 6.0 Hz), 2.74 (m, 4H), 2.76 (s, 6H), 3.21–3.28 (m, 2H), 3.28–3.36 (m, 6H), 4.20–4.29 (m, 2H), 4.36 (m, 2H), 4.43 (m, 1H), 5.06 (s, 1H), 6.70 (m, 2H), 6.81 (d, 1H, $^3J$ = 15.8 Hz), 7.05 (m, 2H), 7.11 (s, 2H), 7.21–7.30 (m, 10H), 7.62 (d, 1H, $^3J$ = 15.9 Hz), 7.65 (s, 2H); RP-HPLC (gradient 1): 97% (210 nm, $t_R$ = 20.3 min, $k$ = 6.5); MS (ES, TFA/acetonitrile): $m/z$ 438 $[M^+ + H]^{2+}$, 875 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{53}H_{63}N_8O_4^+$: 875.4967, found: 875.4981; $C_{53}H_{63}N_8O_4^+ \times C_4HF_6O_4^-$ (1103.1).

**4.2.30.6. 1-(6-(Amino((R)-4-(2,2-diphenylacetamido)-5-(4-hydroxybenzylamino)-5-oxopentylamino)methyleneamino)-6-oxohexyl)-4-((1E,3E)-4-(4-(dimethylamino)phenyl)buta-1,3-dienyl)-2,6-dimethylpyridinium hydrotrifluoroacetate trifluoroacetate (59).** Prepared from **33** and Py-5; gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–75:25 ($t_R$ = 31.3 min); 15% (1.3 mg); RP-HPLC (gradient 1): 95% (210 nm, $t_R$ = 18.1 min, $k$ = 5.7); MS (ES, $H_2O$/acetonitrile/MeOH + 10 mM $NH_4OAc$): $m/z$ 424.5 $[M^+ + H]^{2+}$, 848 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{52}H_{62}N_7O_4^+$: 848.4858, found: 848.4872; $C_{52}H_{62}N_7O_4^+ \times C_4HF_6O_4^-$ (1076.1).

**4.2.30.7. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11-dioxo-13,16-dioxa-2,8,10-triazaoctadec-9-en-18-yl)-4-((1E,3E)-4-(4-(dimethylamino)phenyl)buta-1,3-dienyl)-2,6-dimethylpyridinium hydrotrifluoroacetate trifluoroacetate (60).** Prepared from **36** and Py-5; gradient for preparative HPLC: 0–40 min: MeCN/0.1% aq TFA 15:85 to 75:25 ($t_R$ = 26.6 min); 23% (1.58 mg); $^1$H NMR (COSY, 600 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.49–1.63 (m, 2H), 1.68 (m, 1H), 1.83 (m, 1H), 2.83 (s, 6H), 3.02 (s, 6H), 3.26 (m, 2H), 3.66 (m, 2H), 3.72 (m, 2H), 3.96 (t, 2H, $^3J$ = 5.1 Hz), 4.1 (s, 2H), 4.24 (m, 2H), 4.40 (m, 1H), 4.70 (t, 2H, $^3J$ = 5.1 Hz), 5.05 (s, 1H), 6.55 (d, 1H, $^3J$ = 15.2 Hz), 6.69 (m, 2H), 6.75 (m, 2H), 6.91–6.96 (m, 1H), 7.01 (d, 1H, $^3J$ = 15.2 Hz), 7.05 (m, 2H), 7.22–7.30 (m, 10H), 7.43 (m, 2H), 7.63–7.73 (m, 3H); RP-HPLC (gradient 1): 96% (210 nm, $t_R$ = 16.8 min, $k$ = 5.2); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): $m/z$ 440.5 $[M^+ + H]^{2+}$, 880 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{52}H_{62}N_7O_6^+$: 880.4756, found: 880.4771; $C_{52}H_{62}N_7O_6^+ \times C_4HF_6O_4^-$ (1108.1).

**4.2.30.8. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11-dioxo-2,8,10,12-tetraazahexadec-9-en-16-yl)-4-((1E,3E)-4-(4-(dimethylamino)phenyl)buta-1,3-dienyl)-2,6-dimethylpyridinium hydrotrifluoroacetate trifluoroacetate (61).** Prepared from **41** and Py-5; gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–80:20 ($t_R$ = 27.2 min); 23% (0.94 mg); RP-HPLC (gradient 1): 99% (210 nm, $t_R$ = 17.4 min, $k$ = 5.4); MS (ES, TFA/acetonitrile): $m/z$ 425 $[M^+ + H]^{2+}$, 849 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{51}H_{61}N_8O_4^+$: 849.4810, found: 849.4796; $C_{51}H_{61}N_8O_4^+ \times C_4HF_6O_4^-$ (1077.1).

**4.2.30.9. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,15-trioxo-19,22-dioxa-2,8,10,16-tetraazatetracos-9-en-24-yl)-2,6-dimethyl-4-(3-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)prop-1-enyl)pyridinium hydrotrifluoroacetate trifluoroacetate (62).** Prepared from **37** and Py-6; gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–75:25 ($t_R$ = 38.4 min); 15% (1.51 mg); RP-HPLC (gradient 1): 97% (210 nm, $t_R$ = 21.3 min, $k$ = 6.9); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): $m/z$ 528.5 $[M^+ + H]^{2+}$, 1055.6 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{63}H_{75}N_8O_7^+$: 1055.5753, found: 1055.5732; $C_{63}H_{75}N_8O_7^+ \times C_4HF_6O_4^-$ (1283.3).

**4.2.30.10. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,16-trioxo-20,23-dioxa-2,8,10,17-tetraazapentacos-9-en-25-yl)-2,6-dimethyl-4-(3-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)prop-1-enyl)pyridinium hydrotrifluoroacetate trifluoroacetate (63).** Prepared from **38** and Py-6; gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–75:25

($t_R$ = 38.6 min); 16% (1.68 mg); $^1$H NMR (COSY, 600 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.49–1.56 (m, 1H), 1.56–1.62 (m, 1H), 1.63–1.73 (m, 5H), 1.81–1.87 (m, 1H), 1.98 (s, 6H), 2.21 (m, 2H), 2.47 (t, 2H, $^3J$ = 6.4 Hz), 2.76 (s, 6H), 3.20–3.28 (m, 2H), 3.31 (m, 2H), 3.42 (s, 3H), 3.44 (t, 2H, $^3J$ = 5.8 Hz), 3.54 (m, 2H), 3.58 (m, 2H), 3.91 (t, 2H, $^3J$ = 5.1 Hz), 4.24 (m, 2H), 4.42 (m, 1H), 4.56 (t, 2H, $^3J$ = 5.2 Hz), 5.06 (s, 1H), 5.82 (d, 1H, $^3J$ = 12.5 Hz), 6.22 (d, 1H, $^3J$ = 14.3 Hz), 6.70 (m, 2H), 7.05 (m, 2H), 7.21–7.30 (m, 10H), 7.31–7.36 (m, 2H), 7.47 (s, 2H), 7.52 (m, 1H), 7.88 (m, 2H), 8.12 (d, 1H, $^3J$ = 8.5 Hz), 8.18 (dd, 1H, $^3J$ = 14.3, 12.5 Hz); RP-HPLC (gradient 1): 96% (210 nm, $t_R$ = 21.5 min, $k$ = 7.0); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): $m/z$ 535.5 $[M^+{+}H]^{2+}$, 1069.7 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{64}H_{77}N_8O_7^+$: 1069.5915, found: 1069.5905; $C_{64}H_{77}N_8O_7^+ \times C_4HF_6O_4^-$ (1297.3).

#### 4.2.30.11. 1-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,18-trioxo-22,25-dioxa-2,8,10,19-tetraazaheptacos-9-en-27-yl)-2,6-dimethyl-4-(3-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)prop-1-enyl)pyridinium hydrotrifluoroacetate trifluoroacetate (64).

Prepared from **40** and Py-6; gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 15:85–78:22 ($t_R$ = 40.5 min); 13% (1.43 mg); RP-HPLC (gradient 1): 89% (210 nm, $t_R$ = 21.7 min, $k$ = 7.0); MS (ES, DCM/MeOH + 10 mM $NH_4OAc$): $m/z$ 549.5 $[M^+{+}H]^{2+}$, 1097.7 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{66}H_{81}N_8O_7^+$: 1097.6223, found: 1097.6230; $C_{66}H_{81}N_8O_7^+ \times C_4HF_6O_4^-$ (1325.4).

#### 4.2.31. 3-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,15,20-tetraoxo-2,8,10,16,19-pentaazatricos-9-en-23-yl)-2-((1E,3E)-4-(4-(dimethylamino)phenyl)buta-1,3-dienyl)benzo[d]thiazol-3-ium hydrotrifluoroacetate trifluoroacetate (65)

Carbonyldiimidazole (1.1 mg, 6.6 μmol) was added to a solution of **31** (6.5 mg, 7.6 μmol) in anhydrous DMF (500 μL) and the mixture was stirred for 40 min at rt. UR-DE99 (**51**) (2 mg, 5.1 μmol) and $NEt_3$ (1.8 mg, 2.5 μL, 17.8 μmol) were added and stirring was continued at rt for 6.5 h. The reaction was stopped by addition of 10% aq TFA (corresponding to 10 equiv of TFA) and the product was purified with preparative HPLC (gradient: 0–60 min: MeCN/0.1% aq TFA 20:80–75:25, $t_R$ = 35.6 min). 23% (1.43 mg); RP-HPLC (gradient 1): 94% (210 nm, $t_R$ = 16.9 min, $k$ = 5.3); MS (ES, acetonitrile/TFA): $m/z$ 502.9 $[M^+{+}H]^{2+}$, 1004.6 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{57}H_{66}N_9O_6S^+$: 1004.4851, found: 1004.4847; $C_{57}H_{66}N_9O_6S^+ \times C_4HF_6O_4^-$ (1232.3).

#### 4.2.32. 3-((R)-9-Amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,18-trioxo-2,8,10,12,17-pentaazahenicos-9-en-21-yl)-2-((1E,3E)-4-(4-(dimethylamino)phenyl)buta-1,3-dienyl)benzo[d]thiazol-3-ium hydrotrifluoroacetate trifluoroacetate (66)

Compound **41** (5.6 mg, 6.9 μmol) and UR-DE99 succinimidyl ester (**52**) (2.6 mg, 4.3 μmol) were dissolved in anhydrous DMF (150 μL). $NEt_3$ (1.7 mg, 2.4 μL, 17.2 μmol) was added and the mixture was stirred for 3 h at rt. The reaction was stopped by addition of 10% aq TFA (corresponding to 4 equiv of TFA) and the product was purified with preparative HPLC (gradient: 0–50 min: MeCN/0.1% aq TFA 20:80–75:25, $t_R$ = 34.9 min). 24% (1.23 mg); RP-HPLC (gradient 2): 95% (220 nm, $t_R$ = 21.7 min, $k$ = 7.0); MS (ES, acetonitrile/TFA): $m/z$ 481.5 $[M^+{+}H]^{2+}$, 962 $M^+$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{55}H_{64}N_9O_5S^+$: 962.4751, found: 962.4741; $C_{55}H_{64}N_9O_5S^+ \times C_4HF_6O_4^-$ (1190.2).

#### 4.2.33. 1-((R)-9-Amino-20-((R)-9-amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,16-trioxo-2,8,10,17-tetraazanonadec-9-en-19-yl)-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,16-trioxo-2,8,10,17,20-pentaazadocos-9-en-22-yl)-4-((E)-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-ij]quinolin-9-yl)ethenyl)-2,6-dimethylpyridinium tris(hydrotrifluoroacetate) trifluoroacetate (70)

Prepared from **67** and Py-1 (cf. general procedure **4.2.30**); gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–75:25 ($t_R$ = 34.0 min); 26% (1.32 mg); $^1$H NMR (COSY, 600 MHz, MeOH-$d_4$): $\delta$ (ppm) 1.54 (m, 2H), 1.60 (m, 2H), 1.66 (m, 8H), 1.71 (m, 2H), 1.84 (m, 2H), 1.94 (p, 4H, $^3J$ = 6.0 Hz), 2.24 (t, 4H, $^3J$ = 6.5 Hz), 2.47 (t, 4H, $^3J$ = 6.2 Hz), 2.73 (m, 8H), 2.79 (s, 6H), 2.99 (t, 2H, $^3J$ = 6.9 Hz), 3.20–3.27 (m, 8H), 3.29 (m, 4H), 4.23 (m, 4H), 4.42 (m, 4H), 5.06 (s, 2H), 6.69 (m, 4H), 6.81 (d, 1H, $^3J$ = 15.8 Hz), 7.04 (m, 4H), 7.11 (s, 2H), 7.21–7.30 (m, 20H), 7.61–7.65 (m, 3H); RP-HPLC (gradient 1): 98% (210 nm, $t_R$ = 18.0 min, $k$ = 5.7); MS (ES, TFA/acetonitrile): $m/z$ 801.1 $[M^+{+}H]^{2+}$, 543.5 $[M^+{+}2H]^{3+}$; HRMS: (ESI): $m/z$ $[M^+{+}H]^{2+}$ calcd for $C_{93}H_{115}N_{15}O_{10}^+$: 801.4491, found: 801.4489; $C_{93}H_{114}N_{15}O_{10}^+ \times C_8H_3F_{12}O_8^-$ (2057.1).

#### 4.2.34. 1-((R)-9-Amino-22-((R)-9-amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,18-trioxo-2,8,10,19-tetraazahenicos-9-en-21-yl)-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,18-trioxo-2,8,10,19,22-pentaazatetracos-9-en-24-yl)-4-((E)-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-ij]quinolin-9-yl)ethenyl)-2,6-dimethylpyridinium tris(hydrotrifluoroacetate) trifluoroacetate (71)

Prepared from **68** and Py-1 (cf. general procedure **4.2.30**); gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–80:20 ($t_R$ = 33.3 min); 28% (1.54 mg); RP-HPLC (gradient 1): 90% (210 nm, $t_R$ = 18.8 min, $k$ = 6.0); MS (ES, TFA/acetonitrile): $m/z$ 553.2 $[M^+{+}2H]^{3+}$, 829.2 $[M^+{+}H]^{2+}$; HRMS: (ESI): $m/z$ $[M^+{+}H]^{2+}$ calcd for $C_{97}H_{123}N_{15}O_{10}^+$: 829.4806, found: 829.4804; $C_{97}H_{122}N_{15}O_{10}^+ \times C_8H_3F_{12}O_8^-$ (2113.2).

#### 4.2.35. 1-((R)-9-Amino-24-((R)-9-amino-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,15,20-tetraoxo-2,8,10,16,21-pentaazatricos-9-en-23-yl)-4-(2,2-diphenylacetamido)-1-(4-hydroxyphenyl)-3,11,15,20-tetraoxo-2,8,10,16,21,24-hexaazahexacos-9-en-26-yl)-4-((E)-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-ij]quinolin-9-yl)ethenyl)-2,6-dimethylpyridinium tris(hydrotrifluoroacetate) trifluoroacetate (72)

Prepared from **69** and Py-1 (cf. general procedure **4.2.30**); gradient for preparative HPLC: 0–50 min: MeCN/0.1% aq TFA 20:80–75:25 ($t_R$ = 31.9 min); 28% (1.53 mg); RP-HPLC (gradient 1): 93% (210 nm, $t_R$ = 16.7 min, $k$ = 5.2); MS (ES, TFA/acetonitrile): $m/z$ 872.2 $[M^+{+}H]^{2+}$, 581.9 $[M^+{+}2H]^{3+}$; HRMS: (FAB): $m/z$ $M^+$ calcd for $C_{99}H_{124}N_{17}O_{12}^+$: 1743.9648, found: 1743.9646; $C_{99}H_{124}N_{17}O_{12}^+ \times C_8H_3F_{12}O_8^-$ (2199.2).

### 4.3. Fluorescence spectroscopy and determination of quantum yields

Determination of quantum yields was performed with a Cary Eclipse spectrofluorimeter and Cary 100 UV/VIS photometer (Varian Inc., Mulgrave, Victoria, Australia). The photomultiplier voltage of the Cary Eclipse spectrofluorimeter was set to 400 V throughout. Recording of excitation spectra was performed with an excitation slit of 5 nm and an emission slit of 10 nm. Emission spectra depicted in Figure 3 were recorded with an excitation slit of 10 nm and an emission slit of 5 nm.

The concentrations of the fluorescent ligands were determined by UV–vis spectroscopy; absorption spectra were recorded within a concentration range of 2–6 μM. For fluorescence spectroscopy solutions with absorbances between 0.1 and 0.2 at the respective excitation wavelength were used. The excitation wavelength was chosen as close to the absorption maximum as possible, but it was strictly avoided to excite the fluorescent compounds in a flank of the excitation spectrum. With a quantum yield of 54% in ethanol,[50] cresyl violet perchlorate (Acros Organics, Geel, Belgium) was used as a red fluorescent standard. Spectra were recorded in glass cuvettes (Hellma, 100-OS, 10 × 10 mm) or in acryl cuvettes (10 × 10 mm, Ref. 67.755, Sarstedt, Nümbrecht, Germany).

Solutions of the fluorescent ligands in PBS, PBS containing 1% BSA or ethanol were freshly prepared from 1 or 2 mM stock solutions of the compounds in DMSO and immediately protected from light. Fluorescence spectra were recorded at three different slit adjustments (excitation/emission): 5/5 nm, 10/5 nm and 10/10 nm. Spectra of the cresyl violet standard were recorded in ethanol. For the determination of reference spectra, the pure solvents with the same DMSO content were used. The solutions were always maintained in the dark.

The emission spectra were recorded within 15–20 min at a temperature of 22 °C using the 'medium scan rate'. The filter settings were 'auto' for the excitation and 'open' for the emission filter. The emission starting point was set 10 nm above the excitation wavelength for the slit adjustments 5/5 nm and 10/5 nm as well as 15 nm above the excitation wavelength for the slit combination 10/10 nm. From every emission spectrum the corresponding reference spectrum was subtracted, yielding the net spectra, which were multiplied with the corresponding lamp correction spectra. The resulting corrected net spectra were integrated up to 850 nm.

The absorbance at the excitation wavelength was determined by recording absorption spectra immediately after the recording of the emission spectra (within 30 min after preparation of test solutions). Baselines were stored using reference solutions and subtracted from the raw spectra. The quantum yield was calculated according to the following equation:

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

where $A_s$ is the absorbance and $F_s$ the integral of the corrected emission spectrum of the cresyl violet standard solution. $A_x$ and $F_x$ stand for the absorbance and the integral of the corrected emission spectrum of the fluorescent ligand. The refractive indices of the solvents for the fluorescent ligands and the cresyl violet standard are denoted $n_x$ and $n_s$, respectively. $\Phi_{F(S)}$ is the reported quantum yield of cresyl violet, in this case 54%.

### 4.4. Investigation of the chemical stability

The stability of the fluorescent $Y_1R$ antagonists **53**, **54**, **57**, **66** and **70** regarding the formation of BIBP 3226 (**1**) was investigated at neutral pH in phosphate buffered saline (1:10 diluted with water). Incubation was started by addition of 10 μL of a 1 mM solution of the compounds in DMSO to 190 μL of buffer to give a final concentration of 50 μM. After 20 and 90 min a 80 μL aliquot was taken and diluted with a mixture of acetonitrile, $H_2O$ and 1% aq TFA (5:1:4, 80 μL). 100 μL of the resulting solution (pH = 2–3) was analysed by 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 acetonitrile (A) and 0.05% aq TFA (B) were used as mobile phase (gradient: 0–28 min: A/B 25:75–70:30, 28–31 min: 70:30–95:5, 31–40 min: 95:5). The flow rate was set to 0.80 mL/min, the column temperature to 30 °C and the detection wavelength to 220 nm. A five-point calibration curve (0.3, 1.5, 4, 10 and 25 μM) of **1** was acquired for quantification of the decomposition product **1**.

### 4.5. Receptor binding and functional assays

#### 4.5.1. Cell culture

HEL and SK-N-MC cells were cultured as described elsewhere.[51,52] HEL cells were subcultured by 1:6-dilution with fresh culture medium 24 h prior to the Fura $Ca^{2+}$-assay. MCF-7-$Y_1$[a] cells were maintained in EMEM (Sigma), supplemented with 5% FCS (Biochrom AG, Berlin, Germany). The CHO cells, transfected with the human $Y_2R$, $G\alpha_{qi5}$ and aequorin, were cultured as previously described.[2] CHO cells, transfected with the human $Y_4R$, $G\alpha_{qi5}$ and aequorin, were cultured under the same conditions. HEC-1B cells, transfected with the human $Y_5R$, were cultured as previously described.[53]

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

#### 4.5.2. Fura-2 calcium assay and radioligand competition binding assay

The Fura assay was performed with HEL cells as previously described using a Perkin–Elmer LS50 B spectrofluorimeter (Perkin Elmer, Überlingen, Germany).[35] Radioligand competition experiments with [$^3$H]-UR-MK114 were performed as described elsewhere.[6]

#### 4.5.3. Flow cytometry

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

#### 4.5.4. $Y_1R$ binding of 66 and displacement by BIBP 3226

MCF-7-$Y_1$ cells were seeded in a 175-cm$^2$ culture flask 5–6 days prior to the experiment. 17β-Estradiol (1 nM) was added three days prior to the experiment using a 1 μM solution in ethanol. Cells were treated with trypsin, suspended in culture medium and centrifuged. The cell pellet was re-suspended in buffer (HEPES 10 mM, NaCl 150 mM, KCl 5 mM, $CaCl_2{\cdot}2H_2O$ 2.5 mM, $KH_2PO_4$ 1.2 mM, $Mg_2SO_4{\cdot}7H_2O$ 1.2 mM and $NaHCO_3$ 25 mM), cells were centrifuged and re-suspended in buffer to a density of $0.5 \times 10^6$–$1 \times 10^6$ cells/mL. Compound **66** was applied at final concentrations between 1 and 50 nM for saturation binding experiments. Non-specific binding was determined in the presence of BIBP 3226 (200-fold excess). For displacement of the fluorescent ligand **66** (10 nM) from the $Y_1R$, BIBP 3226 (**1**) was used as competitor (final concentrations: 0.03–3000 nM). The final DMSO concentration was 1%.

After 15 min of incubation in darkness at room temperature the samples were measured by flow cytometry using an excitation wavelength of 635 nm (red diode laser) and recording the fluorescence signals in channel FL-4 (661 ± 18 nm). Measurements were stopped when 20,000 gated events had been counted (highest flow rate). The geometrical mean values of fluorescence intensities obtained from the competition assay were converted to percentage inhibition values according to the procedure described in detail previously.[2,54]

#### 4.5.5. Determination of binding affinities of 54 and 66 to the $Y_2R$, $Y_4R$ and $Y_5R$

To evaluate the $Y_2R$, $Y_4R$ and $Y_5R$ affinity of fluorescent ligands **54** and **66** their abilities to replace fluorescently labelled peptides from these receptors were studied applying concentrations of 1 and 10 μM for **54** as well as 0.3 and 1 μM for **66**. $IC_{50}$ and $K_i$ values could not be determined due to limited amounts of the fluorescent title compounds and very high concentrations required to displace the labelled peptides from NPY receptors other than $Y_1R$. As the optical properties of fluorescent ligand **66** interfere with those of the fluorescently labelled peptides, the data were corrected by subtraction of the fluorescence signal of **66** (acquired in the absence of the fluorescent peptides). This procedure turned out to be reliable at concentrations up to 1 μM.

The binding assays on CHO-h$Y_2$, HEC-1B-h$Y_5$ and CHO-h$Y_4$ cells using Cy5-pNPY ($K_d$ 5.2 nM ($Y_2R$), $K_i$ 4.4 nM ($Y_5R$)), Dy-635-pNPY ($K_i$ 5.5 nM, $Y_2R$) and Cy5-[K$^4$]-hPP ($K_d$ 5.6 nM, $Y_4R$) as labelled

**Table 5**
Settings for the detection of the fluorescent ligands **53**, **54**, **57**, **60**, **66**, **70** and **72** by confocal microscopy (Zeiss Axiovert 200 M)

| Compounds | Excitation (laser transmission) | Filter | Pinhole (μm) |
|---|---|---|---|
| **53**, **54**, **60** | 488 nm (10%) | LP 560 | 170 |
| **57**, **72** | 488 nm (5.1%) | LP 505 | 71 |
| **66** | 633 nm (10%) | LP 650 | 164 |
| **70** | 488 nm (5.1%) | LP 560 | 240 |

ligands were essentially performed as previously described[2,5,38,39] with minor modifications. For $Y_4R$ binding studies, instead of S0586-[K$^4$]-hPP,[5] Cy5-[K$^4$]-hPP was used at concentrations of 5 nM and 3 nM in case of **54** and **66**, respectively. For $Y_2R$ binding studies with **54**, Dy-635-pNPY (10 nM) was used instead of Cy5-pNPY. The cell density in loading buffer was in the range of $0.5 \times 10^6$ to $10^6$ cells/mL. Two independent determinations were performed in duplicate or triplicate.

#### 4.5.6. Confocal microscopy

Two days prior to the experiment MCF-7-$Y_1$ cells (between 175th and 217th passage) were trypsinized and seeded in ibiTreat μ-slide 8-well cover glasses (Ibidi, Planegg, Germany) (for **53**, **54**, **60**, **66**) or in Nunc LabTek™ II 8-chambered coverglasses (Nunc, Wiesbaden, Germany) (for **57**, **70**, **72**) in EMEM containing 1 nM 17β-estradiol and 5% FCS. On the day of the experiment confluency of the cells was 50–80%. The culture medium was removed and the cells were washed once with Leibowitz L15 culture medium (**53**, **54**, **60**, **66**: 200 μL, **57**, **70**, **72**: 400 μL). In case of compounds **53**, **54**, **60** and **66** the cells were covered with L15 medium (100 μL) and L15 medium (100 μL) containing the fluorescent ligand (twofold concentrated) for total binding. Non-specific binding was determined by analogy, but blank L15 medium (100 μL) was replaced by L15 medium (100 μL) containing BIBP 3226 (twofold concentrated). In case of compounds **57**, **70** and **72** cells were covered with L15 medium (240 μL) and L15 medium (80 μL) containing the fluorescent probe (fivefold concentrated) for total binding. For non-specific binding L15 medium (80 μL) with the competing agent BIBP 3226 (fivefold concentrated) and L15 medium (80 μL) with the fluorescent probe (fivefold concentrated) was added. Images of total and non-specific binding were acquired with a Zeiss Axiovert 200 M microscope equipped with the LSM 510 laser scanner after an incubation period of 5–8 min (**53**, **54**, **57**, **60**), 12–15 min (**66**) or 21–23 min (**70**, **72**) at room temperature. Table 5 shows the settings for the detection of the investigated fluorescent ligands.

### 4.6. Data processing

Data from competition binding experiments were analysed by four parameter sigmoidal fits (SigmaPlot 9.0, Systat Software) and resulting $IC_{50}$ values were converted to $K_i$ values according to the Cheng–Prusoff equation[55] using the respective $K_d$ value of the radioligand or fluorescence ligand. Three data points (between 20% and 80% inhibition, from the Fura-2 $Ca^{2+}$ assay) 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 using an $EC_{50}$ value of 1.8 nM for pNPY (mean value from 4 independently determined concentration-effect curves on HEL cells). Data from saturation binding experiments were evaluated by one-site saturation fits (SigmaPlot 9.0, Systat Software) to obtain $K_d$ values. For Schild analysis $\log_{10}(r-1)$ was plotted against $\log_{10}$[antagonist] with $r = 10^{\Delta pEC50}$. Raw data from flow cytometric experiments were processed with the aid of the WinMDI 2.7 software. The confocal images were processed with the Carl Zeiss LSM image browser 4.2TM. Specific angles of rotation were calculated according to $[\alpha] = \alpha/(c \cdot l)$, for which $\alpha$ = angle of rotation, $c$ = concentration [g cm$^{-3}$] and $l$ = length of the cuvette [dm]. The retention (capacity) factor was calculated according to $k = (t_R - t_0)/t_0$.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## Supplementary data

Supplementary data (NMR spectra of compounds **12**, **13**, **17**, **19**, **27**, **29**, **31–34**, **36–38**, **40**, **41**, **49–52**, **54**, **57**, **58**, **60**, **63** and **70** as well as chromatograms (analytical HPLC analysis) of building block **12**, parent compound **13** and the fluorescent ligands (compounds **53–66** and **70–72**)) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.045.

## References and notes


1. Böhme, I.; Beck-Sickinger, A. G. *Cell Commun. Signal.* **2009**, *7*, 16.
2. Schneider, E.; Mayer, M.; Ziemek, R.; Li, L.; Hutzler, C.; Bernhardt, G.; Buschauer, A. *Chembiochem* **2006**, *7*, 1400.
3. Dumont, Y.; Gaudreau, P.; Mazzuferi, M.; Langlois, D.; Chabot, J. G.; Fournier, A.; Simonato, M.; Quirion, R. *Br. J. Pharmacol.* **2005**, *146*, 1069.
4. Ingenhoven, N.; Beck-Sickinger, A. G. *J. Recept. Signal Transduct. Res.* **1997**, *17*, 407.
5. Schneider, E.; Keller, M.; Brennauer, A.; Hoefelschweiger, B. K.; Gross, D.; Wolfbeis, O. S.; Bernhardt, G.; Buschauer, A. *Chembiochem* **2007**, *8*, 1981.
6. Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. *J. Med. Chem.* **2008**, *51*, 8168.
7. Igel, P.; Schnell, D.; Bernhardt, G.; Seifert, R.; Buschauer, A. *ChemMedChem* **2009**, *4*, 225.
8. Keller, M.; Teng, S.; Bernhardt, G.; Buschauer, A. *ChemMedChem* **2009**, *4*, 1733.
9. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B. *Bioorg. Med. Chem.* **2008**, *16*, 9858.
10. 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.
11. Reubi, J. C.; Gugger, M.; Waser, B.; Schaer, J. C. *Cancer Res.* **2001**, *61*, 4636.
12. Magni, P.; Motta, M. *Ann. Oncol.* **2001**, *12*, S27.
13. Körner, M.; Waser, B.; Reubi, J. C. *Clin. Cancer Res.* **2004**, *10*, 8426.
14. Körner, M.; Waser, B.; Reubi, J. C. *Lab. Invest.* **2004**, *84*, 71.
15. Körner, M.; Waser, B.; Reubi, J. C. *Int. J. Cancer* **2005**, *115*, 734.
16. Körner, M.; Reubi, J. C. *J. Neuropathol. Exp. Neurol.* **2008**, *67*, 741.
17. Körner, M.; Reubi, J. C. *Peptides* **2007**, *28*, 419.
18. Khan, I. U.; Zwanziger, D.; Bohme, I.; Javed, M.; Naseer, H.; Hyder, S. W.; Beck-Sickinger, A. G. *Angew. Chem., Int. Ed.* **2010**, *49*, 1155.
19. Guerin, B.; Dumulon-Perreault, V.; Tremblay, M. C.; Ait-Mohand, S.; Fournier, P.; Dubuc, C.; Authier, S.; Benard, F. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 950.
20. 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.
21. Wieland, H. A.; Engel, W.; Eberlein, W.; Rudolf, K.; Doods, H. N. *Br. J. Pharmacol.* **1998**, *125*, 549.
22. Wetzl, B. K.; Yarmoluk, S. M.; Craig, D. B.; Wolfbeis, O. S. *Angew. Chem., Int. Ed.* **2004**, *43*, 5400.
23. Craig, D. B.; Wetzl, B. K.; Duerkop, A.; Wolfbeis, O. S. *Electrophoresis* **2005**, *26*, 2208.
24. Buffa, R.; Zahradnik, P.; Foltinova, P. *Heterocycl. Commun.* **2001**, *7*, 331.
25. Engel, W.; Eberlein, W.; Rudolf, K.; Doods, H.; Wieland, H.-A.; Willim, K.-D.; Entzeroth, M.; Wienen, W. German Patent DE 19544687, 1997; *Chem. Abstr.* **1997**, *127*, 81788.
26. Callahan, J. F.; Ashton-Shue, D.; Bryan, H. G.; Bryan, W. M.; Heckman, G. D.; Kinter, L. B.; McDonald, J. E.; Moore, M. L.; Schmidt, D. B., et al *J. Med. Chem.* **1989**, *32*, 391.
27. Duschinsky, R.; Dolan, L. A. *J. Am. Chem. Soc.* **1946**, *68*, 2350.
28. Marx, J.; Marx-Moll, L. *Chem. Ber.* **1954**, *87*, 1499.

29. Kirmse, W.; Buschhoff, M. *Chem. Ber.* **1967**, *100*, 1491.
30. Eschalier, A.; Dureng, G.; Duchene-Marullaz, P.; Berecoechea, J.; Anatol, J. *Eur. J. Med. Chem.* **1983**, *18*, 139.
31. Fegley, G.; Bell, S. C.; Costenza, S.; Duke, J.; Reddy, E. P.; Reddy, M. V. R. WO Patent 2008033475, 2008; *Chem. Abstr. 148*, 379331.
32. Brennauer, A.; Keller, M.; Freund, M.; Bernhardt, G.; Buschauer, A. *Tetrahedron Lett.* **2007**, *48*, 6996.
33. Majer, P.; Randad, R. S. *J. Org. Chem.* **1994**, *59*, 1937.
34. Caro, B.; Le Guen-Robin, F.; Salmain, M.; Jaouen, G. *Tetrahedron* **1999**, *56*, 257.
35. Müller, M.; Knieps, S.; Gessele, K.; Dove, S.; Bernhardt, G.; Buschauer, A. *Arch. Pharm. (Weinheim)* **1997**, *330*, 333.
36. Brennauer, A.; Dove, S.; Buschauer, A. In *Handbook of Experimental Pharmacology*, Michel, M. C., Ed.; Springer: Heidelberg, 2004; Vol. 162, p 506.
37. Vanderheyden, P. M.; Van Liefde, I.; de Backer, J. P.; Vauquelin, G. *J. Recept. Signal Transduct.* **1998**, *18*, 363.
38. Ziemek, R.; Brennauer, A.; Schneider, E.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. *Eur. J. Pharmacol.* **2006**, *551*, 10.
39. Ziemek, R.; Schneider, E.; Kraus, A.; Cabrele, C.; Beck-Sickinger, A. G.; Bernhardt, G.; Buschauer, A. *J. Recept. Signal Transduct.* **2007**, *27*, 217.
40. Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; König, B. *Bioorg. Med. Chem.* **2010**, *18*, 6292.
41. Reubi, J. C.; Waser, B. *Eur. J. Nucl. Med. Mol. Imaging* **2003**, *30*, 781.
42. Amlal, H.; Faroqui, S.; Balasubramaniam, A.; Sheriff, S. *Cancer Res.* **2006**, *66*, 3706.
43. Kenakin, T. P. *A Pharmacology Primer: Theory, Applications, and Methods*; Academic Press, 2007.
44. Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Rudolf, K.; Eberlein, W.; Engel, W.; Doods, H. N. *J. Pharmacol. Exp. Ther.* **1995**, *275*, 143.
45. Kraus, A.; Ghorai, P.; Birnkammer, T.; Schnell, D.; Elz, S.; Seifert, R.; Dove, S.; Bernhardt, G.; Buschauer, A. *ChemMedChem* **2008**.
46. Bauer, J.; Martin, R. O.; Rodriguez, S. U.S. Patent 2005-113443, 2005; *Chem. Abstr. 143*, 267243.
47. Henichart, J. P.; Houssin, R.; Bernier, J. L.; Catteau, J. P. *J. Chem. Soc., Chem. Commun.* **1982**, 1295.
48. Donati, D.; Morelli, C.; Porcheddu, A.; Taddei, M. *J. Org. Chem.* **2004**, *69*, 9316.
49. GB Patent 945536, 1964; *Chem. Abstr.* **1964**, *60*, 60474.
50. Magde, D.; Brannon, J. H.; Cremers, T. L.; Olmsted, J., III *J. Phys. Chem.* **1979**, *83*, 696.
51. Aiglstorfer, I.; Uffrecht, A.; Gessele, K.; Moser, C.; Schuster, A.; Merz, S.; Malawska, B.; Bernhardt, G.; Dove, S.; Buschauer, A. *Regul. Pept.* **1998**, *75–76*, 9.
52. Biedler, J. L.; Helson, L.; Spengler, B. A. *Cancer Res.* **1973**, *33*, 2643.
53. Moser, C.; Bernhardt, G.; Michel, J.; Schwarz, H.; Buschauer, A. *Can. J. Physiol. Pharmacol.* **2000**, *78*, 134.
54. Burchiel, S. W.; Edwards, B. S.; Kuckuck, F. W.; Lauer, F. T.; Prossnitz, E. R.; Ransom, J. T.; Sklar, L. A. *Methods* **2000**, *21*, 221.
55. Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.