Bioorganic & Medicinal Chemistry 16 (2008) 9858–9866

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Modular synthesis of non-peptidic bivalent NPY $Y_1$ receptor antagonists

Stefan Weiss [a], Max Keller [b], Günther Bernhardt [b], Armin Buschauer [b], Burkhard König [a,*]

[a] Institut für Organische Chemie, Universität Regensburg, D-93040 Regensburg, Germany
[b] Institut für Pharmazie, Universität Regensburg, D-93040 Regensburg, Germany

ARTICLE INFO

*Article history:*
Received 16 May 2008
Revised 4 September 2008
Accepted 10 September 2008
Available online 13 September 2008

*Keywords:*
Neuropeptide Y
Bivalent ligands
Guanidinium compounds
Binding
Huisgen reaction
Cycloaddition

ABSTRACT

According to a 'bivalent ligand approach' to increase the affinity of the potent argininamide-type NPY $Y_1$ receptor antagonist BIBP-3226, dimeric ligands were synthesized in which two molecules of the parent compound were linked by different spacers via $N^G$-acylation at the guanidino groups. A synthetic route for the preparation of the title compounds was developed, which includes a copper(I)-catalyzed azide alkyne cycloaddition as the key step. Three bivalent analogues of BIBP-3226 were prepared showing nanomolar antagonistic activity and binding affinity to the NPY $Y_1$ receptor (calcium assay on HEL cells, radioligand binding assay on SK-N-MC cells), but these ligands were not superior to the parent compound and there was no correlation with the length or the chemical nature of the spacer. A trivalent BIBP-3226 derivate showed, surprisingly, no affinity to the NPY $Y_1$ receptor at all.



## 1. Introduction

Neuropeptide Y (NPY) is a highly conserved peptide that plays an important role as a neurotransmitter in the central and peripheral nervous system. Together with the pancreatic polypeptide (PP) and peptide YY (PYY), NPY belongs to the neuropeptide Y family. In contrast to PP and PYY, which have hormone character, NPY acts as a neurotransmitter. Five $G_{i/o}$ protein coupled NPY receptor subtypes are known.[1] In humans, the biological effects of NPY are mediated by four receptor subtypes, referred to as NPY $Y_1$, $Y_2$, $Y_4$ and $Y_5$ receptors. For instance, in the periphery NPY $Y_1$ receptor ($Y_1R$) stimulation causes an increase in blood pressure. In the central nervous system (CNS) $Y_1R$ activation elicits anxiolytic and sedative effects and is involved in the stimulation of food intake.[2] Potent and selective antagonists and agonists are required as pharmacological tools to investigate the numerous biological effects of NPY and to study the ligand receptor interactions on the molecular level.

The present study deals with a 'bivalent ligand approach'[3] to synthesize $Y_1R$ antagonists. Bivalent ligands are molecules that contain two covalently linked biologically active chemical entities; their potency can exceed the effect of the corresponding monomeric ligand at double concentration, as shown for opioid receptors by Portoghese.[4] It is a matter of speculation whether such affinity-enhancing effects may be attributed to thermodynamically more favourable binding interactions of the dimeric structures resulting from the bridging of independent recognition sites.[5–7]

The (*R*)-argininamide BIBP-3226 (**1**) is a potent NPY $Y_1R$ antagonist.[8] Previous investigations revealed that acylation at the $N^G$-position of **1** are tolerated over a wide range of structural variations including the introduction of bulky fluorophores.[9] Compared to guanidines, the $pK_a$ values of the acylguanidines are 4–5 orders of magnitude lower. Depending on the nature of the $N^G$-substituent, highly potent and selective $Y_1R$ antagonists, superior to the parent compound and capable of penetrating into the brain, were obtained. Based on the structure–activity relationships of these compounds the $N^G$-terminus of **1** has been considered an appropriate position for covalent linkage of two molecules to obtain putative bivalent $Y_1R$ ligands, for instance, of general structure **2** (Fig. 1). The synthesis of such derivatives from **1** is hampered by the high polarity and basicity of the guanidino group.[10] Therefore, $N^G$-protected or functionalized $N^G$-acylated derivatives of **1** were preferred as a building block. Here we present two versatile synthetic routes based on copper(I)-catalyzed azide–alkyne cycloaddition reactions as key steps that facilitate the preparation of bivalent $Y_1R$ antagonists derived from BIBP-3226.

## 2. Results and discussion

First, the BIBP-3226 derivative **8** with azide functionality was prepared (Scheme 1). Commercially available *S*-methylisothiourea **3** was mono-Boc-protected and coupled with 4-azidobutanoic acid (**5**) using standard peptide coupling procedures. Azide **6** and amine **7**, which is available from D-ornithine in six steps in 32% overall

* Corresponding author. Tel.: +49 941 943 4576; fax: +49 941 943 1717.
*E-mail address:* Burkhard.koenig@chemie.uni-regensburg.de (B. König).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.09.033

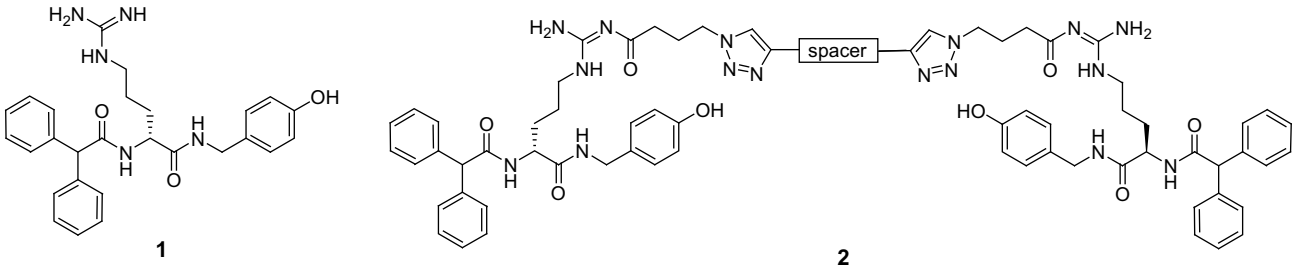
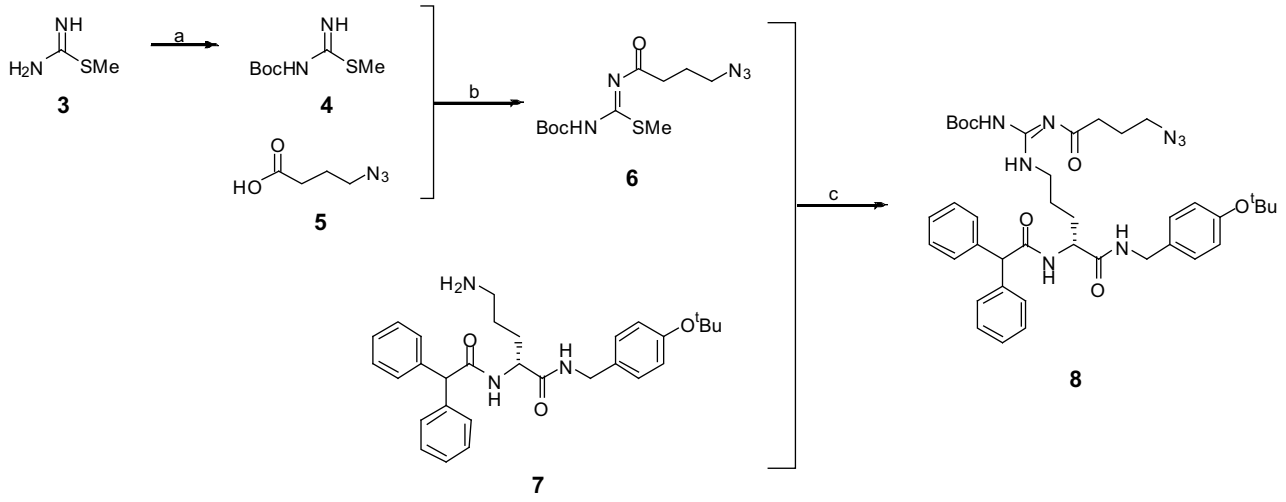



**Figure 1.** NPY $Y_1R$ antagonist BIBP-3226 (**1**) and general structure of bivalent $Y_1R$ antagonists (**2**) constructed from $N^G$-acylated **1** by 'click' reaction.

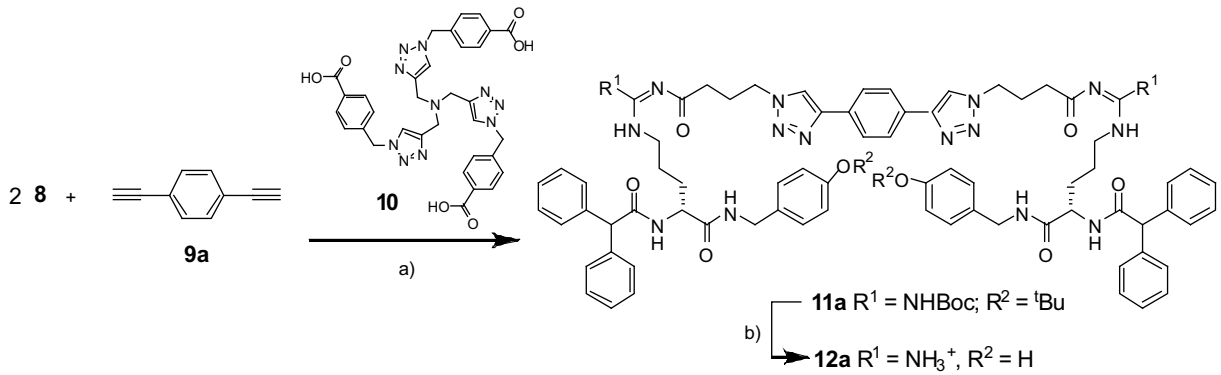



**Scheme 1.** Synthesis of azide building block **8.** (a) $Boc_2O$, NaOH, $^t$BuOH, 90%; (b) DIPEA, EDC, HOBT, dichloromethane, 89%; (c) $HgCl_2$, $NEt_3$, DMF, 76%.

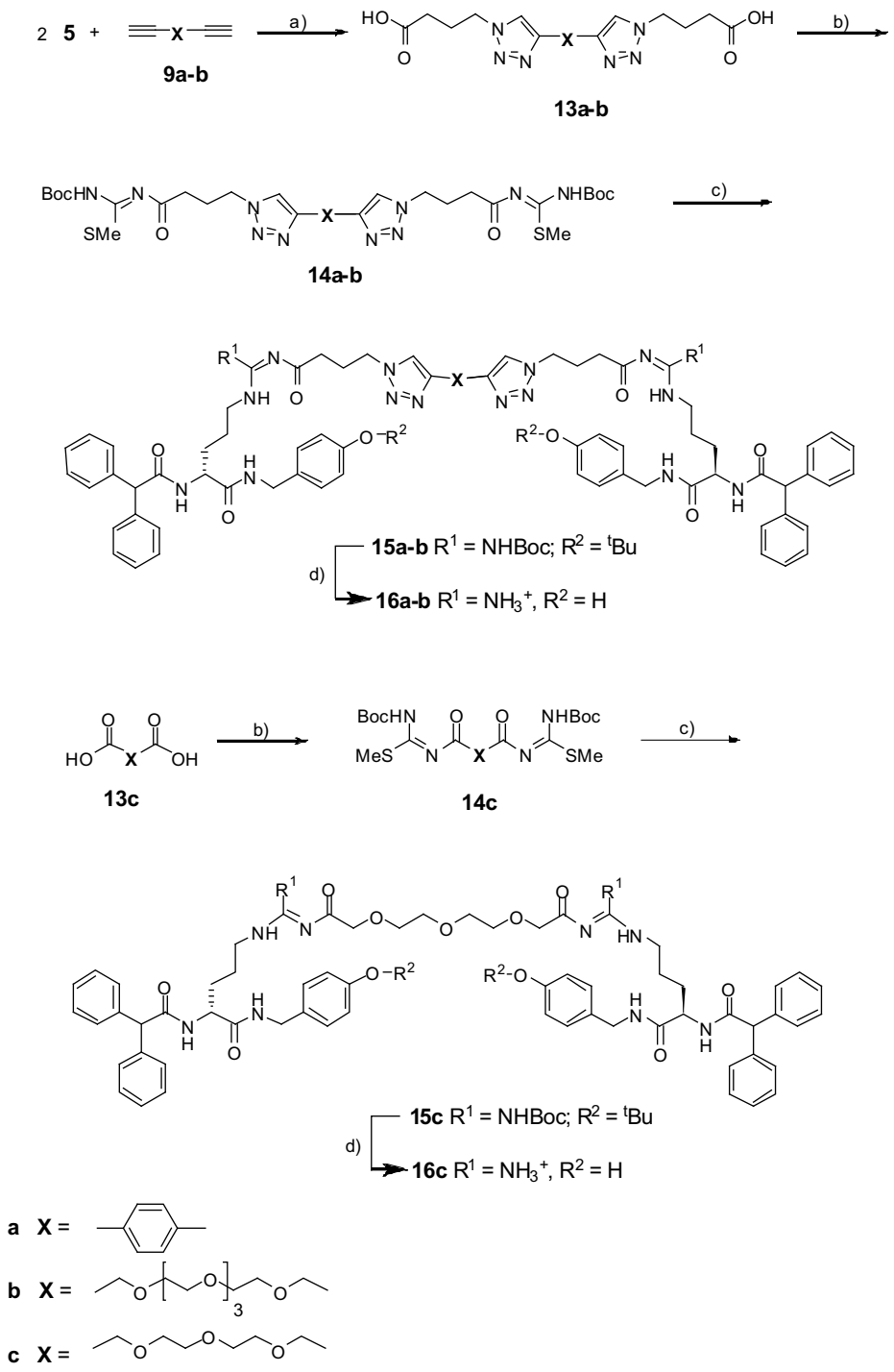
yield,[11] were smoothly converted into compound **8** by a mercury chloride mediated guanidinylation reaction.[12]

Initial attempts to react azide **8** with 1,4-diethynylbenzene (**9a**) in the presence of $CuSO_4$ and ascorbate were unsuccessful: At room temperature no conversion was detectable, whereas heating above 60 °C destroyed the starting materials. However, by addition of 20 mol% of the Cu(I)-stabilizing tris–triazole ligand **10**[13] (Scheme 2) the dual cycloaddition product **11a** was obtained in 44% yield at room temperature. Deprotection with TFA/dichloromethane 1:1 yielded the target compound **12a**.

Although the synthetic route provides access to bivalent BIBP-3226 derivatives, the introduction of the BIBP-3226 skeleton with compound **7** at an early stage of the synthesis is not very efficient. Therefore, a second route was developed introducing the $Y_1R$ antagonist moieties at the end of the synthesis. The cycloaddition of two equivalents of azide **5** with diynes **9** becomes the first step (Scheme 3). Diyne **9b** was prepared from propargyl bromide and the respective glycol according to described procedures.[14] The resulting dicarboxylic acids **13a** and **13b** were obtained in high yield and good purity using $CuSO_4$ and ascorbate to catalyze the reaction. Their conversion into bis-isothiourea derivatives **14** by the dual reaction with compound **4** proceeded in very good yield. Finally, guanidinylation of compound **7** with isothioureas **14** gave compounds **15a** and **15b** in good yields, and deprotection with DCM/TFA resulted in the target bivalent BIBP-3226 derivatives **16a** and **16b**. The obtained overall yield based on consumption of compound **7** improved significantly, when compared to the previously discussed route. Three bivalent BIBP-3226 derivatives **16a–c**,



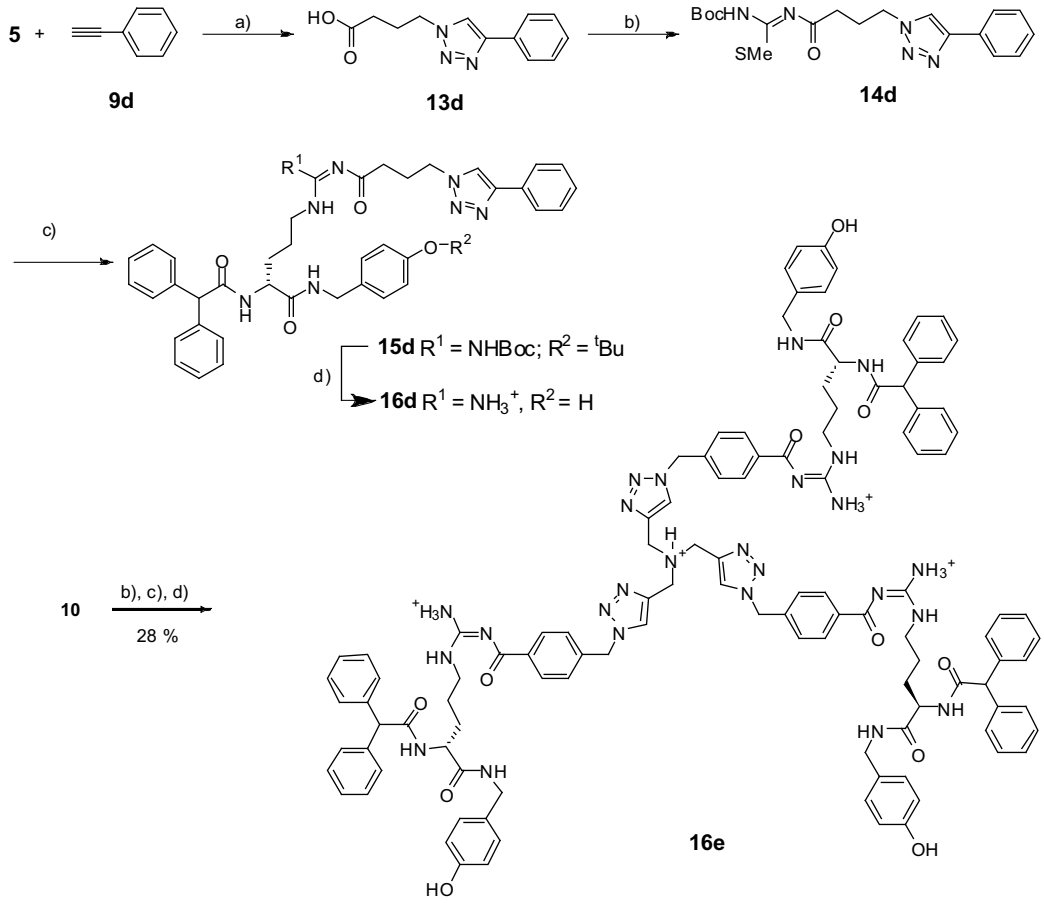

**Scheme 2.** Synthesis of the bivalent BIBP-3226 derivative **12a** via a dual 'click' reaction. (a) $CuSO_4$, **10**, sodium ascorbate, 44%; (b) TFA, DCM, quantitative yield.




**Scheme 3.** Synthesis of bivalent BIBP derivatives **16a–c** using bis-methylisothioureas **14a–c**. (a) $CuSO_4$, sodium ascorbate, $MeOH/H_2O$, 65–75%; (b) EDC, HOBt, DIPEA, DMF or DCM, **4**, 63–71%; (c) $HgCl_2$, $NEt_3$, DMF, **7**, 55–65%; (d) TFA/DCM, quantitative yield.

varying in length[15] and rigidity of the spacer, monovalent compound **16d** (for comparison) and a trivalent ligand **16e**, based on tricarboxylic acid **10**, were prepared (Scheme 4).

The compounds **16a–e** were investigated for NPY $Y_1R$ antagonism in a calcium (Fura-2) assay on human erythroleukemia (HEL) cells. In addition, the $Y_1R$ affinities were determined in a radioligand binding assay on SK-N-MC human neuroblastoma cells using $N^G$-([2,3-$^3$H]propionyl)-BIBP-3226 as radioligand (prepared and pharmacologically evaluated in our laboratory, to be reported elsewhere). $K_b$ and $K_i$ values are summarized in Table 1. The $Y_1R$ antagonistic activities of the BIBP-3226 derivatives **16a–d** in the functional assay remain in the same range as that of the parent monovalent compound **1**, whereas the $K_i$ values of **16a**, **b**, **d** are about 5- to 44-fold higher than that of **1**. As small changes of the acyl substituent may result in significantly altered binding affinities, a direct comparison of a monovalent and a bivalent ligand is only possible for compounds **16a** and **16d**. While the $K_b$ is slightly lower for the bivalent compound **16a**, the $K_i$ values of **16a** and **16d** are identical. Thus a beneficial effect of the second pharmacophore is not observed in this specific case. The comparison of the varia-




**Scheme 4.** Synthesis of mono- and trivalent BIBP-3226 derivatives **16d** and **e**. (a) $CuSO_4$, sodium ascorbate, MeOH/$H_2O$, 53%; (b) EDC, HOBt, DIPEA, DCM, **4**, 66–78%; (c) $HgCl_2$, $NEt_3$, DMF, **7**, 43–72%; (d) TFA/DCM, quantitative yield.

**Table 1**
$IC_{50}$ and $K_i$ values for NPY $Y_1R$ antagonism on HEL cells (calcium assay) and $Y_1R$ binding determined on SK-N-MC cells

| Compound | Number of BIBP moieties | Spacer length (number of atoms) | $Y_1R$ antagonism ($Ca^{2+}$-assay) $K_b$[a] [nM] | $Y_1R$ binding (SK-N-MC cells) $K_i$ [b] [nM] |
|---|---|---|---|---|
| **1**[c] | 1 | — | 1.5 ± 0.3 | 1.3 ± 0.2 |
| **16a** | 2 | 18 | 0.6 ± 0.2 | 43 ± 12 |
| **16b** | 2 | 29 | 1.8 ± 0.6 | 58 ± 12 |
| **16c** | 2 | 11 | 7.0 ± 2.5 | 6.1 ± 1.9 |
| **16d** | 1 | — | 0.9 ± 0.2 | 49 ± 6 |
| **16e** | 3 | 21 | —[d] | >50,000 |

[a] $K_b$ values for inhibition of NPY (10 nM) induced calcium mobilization in HEL cells (Fura-2 assay). Mean values ± SEM from two independent experiments.
[b] $K_i$ values determined from the displacement of $N^G$-([2,3-$^3$H]propionyl)-BIBP-3226 on SK-N-MC cells. Mean values ± SEM from two independent experiments performed in triplicate.
[c] BIBP-3226.
[d] Not determined.

tion in spacer length in **16a–c** with changes in their binding affinities does not reveal a simple correlation either. The trivalent derivative **16e** proved to be completely inactive.

## 3. Conclusion

The copper(I) catalyzed alkyne–azide cycloaddition to triazoles facilitates the preparation of bivalent argininamide-type NPY $Y_1R$ antagonists derived from BIBP-3226 (**1**). The affinity-conferring moiety can be introduced either as $N^G$-(4-azidobutanoyl)-substituted **1** (**8**), which is allowed to react with a diyne, or, more efficiently, at the end of the synthesis by guanidinylation of the corresponding ornithine derivative using bis-methylisothiurea building blocks. The new BIBP-3226 derivatives retain, despite significant variation in length and rigidity of the spacer unit, binding affinities to the NPY $Y_1$ receptor, which are in the same range as that of the parent ligand. Surprisingly, the trivalent compound **16e** is completely inactive. Although of limited scope, the small study shows that the principle of bivalent ligands, as reported for opioid receptors, does not follow a simple structure–activity relationship in the case of NPY $Y_1$ receptors, if transferable at all.

## 4. Experimental

$^1$H NMR spectra were recorded at 300 MHz on a Bruker Avance 300 spectrometer. $^{13}$C NMR spectra were recorded at 76 MHz. All chemical shifts values are reported in ppm. IR-spectra were measured with a Varian Biorad FT-IR spectrometer Excalibur FTS

3000. UV/vis spectra were recorded with a Varian Cary BIO 50 UV/vis/NIR spectrophotometer. Mass spectra: Varian CH-5(EI), Finnigan MAT 95(CI), Finnigan MAT TSQ 7000(ESI). Compounds **16a–e** were purified as TFA salts using HPLC: Phenomenex Luna C18 (RP-HPLC) column; detection: DAD at 220 nm; temperature: 25 °C; gradient: 3% AcN 97% $H_2O$[0.0059%TFA]($t$ = 0) → 98% AcN 2% $H_2O$[0.0059%TFA]($t$ = 30 min.); flow: 35.0 mL/min. Melting points were determined with a Büchi Tottoli apparatus, they are not corrected. Thin layer chromatography (TLC) was performed on alumina plates coated with silica gel (Merck silica gel 60 $F_{245}$, thickness 0.2 mm). Column chromatography (CC) was performed with Merck Geduran SI 60 silica gel as the stationary phase. PE means petrol ether with a boiling point range from 60 to 70 °C. EA is ethyl acetate.

Compounds **5**,[16] **7**[17] and **10**[13] were prepared according to literature procedures. The previously described synthesis of compound **4**[17] was improved.

### 4.1. *tert*-Butyl [imino(methylthio)methyl]carbamate (4)

*S*-Methylisothiourea hemisulphate **3** (5.0 g, 35.9 mmol, 1.0 equiv) was dissolved in 10 mL of aqueous NaOH (1.44 g, 35.9 mmol, 1.0 equiv) and $^t$BuOH (100 mL). $Boc_2O$ (6.26 g, 28.7 mmol, 0.8 equiv), dissolved in 50 mL of $^t$BuOH, was added dropwise under cooling (ice bath) to this solution over a period of 1 h. The reaction mixture was stirred overnight. Dichloromethane (100 mL) was added, the solution was washed with aqueous satd sodium carbonate solution (100 mL), the organic layer was separated, dried over magnesium sulphate and the solvent was removed under reduced pressure yielding compound **4** (4.9 g, 90%), as a white solid, mp = 77 °C.

$^1$H NMR (300 MHz, DMSO-$d_6$): 1.40 (s, 9H), 2.31 (s, 3H), 8.56 (br s, 2H). $^{13}$C NMR (300 MHz, DMSO-$d_6$): 12.7, 27.8, 77.7, 160.6, 171.4. $C_7H_{14}N_2O_2S$: MS (ES): *m/z* (%): 190(17, M+), 175(50), 134(44), 57(100).

### 4.2. *tert*-Butyl [(1*Z*)-[(4-azidobutanoyl)amino](methylthio)-methylene]carbamate (6)

4-Azidobutanoic acid (**5**, 170 mg, 1.32 mmol), HOBT·$H_2O$ (242 mg, 1.58 mmol), DIPEA (341 mg, 2.64 mmol), and EDC (303 mg, 1.58 mmol) were dissolved in cold dichloromethane (ice bath) under stirring. After 15 min, compound **4** (2.55 g, 1.32 mmol) was added, the reaction mixture was stirred overnight, diluted with water and the organic layer was washed with aqueous citric acid solution (2 × 50 mL) and aqueous satd sodium hydrogen carbonate solution (2 × 50 mL). The organic layer was dried, the solvent was removed under reduced pressure and the crude product was purified by column chromatography (EA/PE, 1:1 $R_f$ = 0.7) yielding compound **6** (354 mg, 89% yield), as a white solid, mp = 43 °C. $^1$H NMR (300 MHz, $CDCl_3$): 1.45 (s, 9H), 1.89 (m, 2H), 2.33 (s, 3H), 2.50 (m, 2H), 3.34 (m, 2H), 12.30 (d, 1H). $^{13}$C NMR (75 MHz, $CDCl_3$): 14.6, 24.1, 28.0, 36.1, 50.6, 82.5, 170.0, 171.1, 185.2. $C_{11}H_{19}N_5O_3S$: MS (CI-MS, $NH_3$): *m/z* (%) 203 (1), 274 (8), 302 (100, M+).

### 4.3. *tert*-Butyl [(1*Z*)-[(4-azidobutanoyl)amino]({(4*R*)-5-[(4-*tert*-butoxybenzyl)amino]-4-[(diphenylacetyl)amino]-5-oxopentyl}-amino)methylene]carbamate (8)

Compound **7** (120 mg, 0.40 mmol), compound **6** (194 mg, 0.40 mmol) and $HgCl_2$ (109 mg, 0.40 mmol) were dissolved separately in small amounts of DMF (1–2 mL). Solutions of compound **7** and compound **6** were combined in a small flask under nitrogen atmosphere. $NEt_3$ (404 mg, 4 mmol) was added under stirring, the $HgCl_2$ solution was added and the mixture was stirred overnight at room temperature. DMF was removed completely and the residue was dissolved in DCM. The precipitate of mercury salts was removed by filtering over celite, the organic phase was washed with sodium carbonate (50 mL), citric acid (50 mL), brine (50 mL) and dried over magnesium sulphate. DCM was removed and the crude product was purified by column chromatography (EA/PE 1:1 $R_f$ = 0.4) yielding compound **8** (223 mg, 76%), as a white solid, mp > 190 °C. $^1$H NMR (300 MHz, $CDCl_3$): 1.33 (m, 9H), 1.44 (m, 9H), 1.54–1.99 (m, 6H), 2.37–2.54 (m, 2H), 3.20–3.30 (m, 2H), 3.30–3.40 (m, 2H), 4.20–4.40 (m, 2H), 4.52–4.70 (m, 1H), 4.90–5.00 (m, 1H), 6.84–6.90 (m, 2H), 7.02–7.09 (m, 2H), 7.18–7.45 (m, 10H). $C_{40}H_{52}N_8O_6$: MS (ES): *m/z* (%) 641 (13), 665 (17), 739 (100, M+).

### 4.4. Di-*tert*-butyl (1,4-phenylenebis{1H-1,2,3-triazole-4,1-diyl-(1-oxobutane-4,1-diyl)imino[(*Z*)-({(4*R*)-5-[(4-*tert*-butoxybenzyl)amino]-4-[(diphenylacetyl)amino]-5-oxopentyl}-amino)methylylidene]})biscarbamate (11a)

Compound **8** (50.0 mg, 67.5 μmol) was dissolved in 2 mL of MeOH and a solution of compound **9a** (4.3 mg, 33.7 μmol) in MeOH (2 mL) was added. Then the solid ligand **10** (4.5 mg, 6.8 μmol) was added. A solution of sodium ascorbate (5.4 mg, 20.7 μmol) in 0.5 mL of $H_2O$ and subsequent under vigorous stirring a solution of copper(II) sulphate pentahydrate (1.7 mg, 6.8 μmol) in 0.5 mL of $H_2O$ was added to the reaction mixture. The mixture was stirred at room temperature overnight. MeOH was removed completely under reduced pressure. Water (20 mL) was added and the product was extracted with DCM (3 × 20 mL). The organic phase was dried over magnesium sulphate and the solvent was evaporated. The crude material was purified by column chromatography: (EA $R_f$ = 0.6); the product is a yellow solid (24 mg, 44%). For analytical data see compound **15a**.

### 4.5. General procedure for preparation of compounds 13a and 13b

To a solution of the appropriate diyne (**13a**: 3.69 mmol, **13b**: 5.55 mmol) in MeOH (20 mL) 4-azidobutanoic acid (**13a**: 7.75 mmol, **13b**: 11.10 mmol) was added under stirring. A solution of sodium ascorbate (**13a**: 0.37 mmol, **13b**: 0.56 mmol) in water (1 mL) and a solution of copper(II) sulphate pentahydrate (**13a**: 0.07 mmol, **13b**: 0.11 mmol) in water (1 mL) were added slowly. The reaction mixture was stirred overnight at room temperature and then refluxed for 3 h.

#### 4.5.1. 4,4′-[1,4-Phenylenebis(1H-1,2,3-triazole-4,1-diyl)]-dibutanoic acid (13a)

The yellow, insoluble product was filtered off and dissolved in 20 mL of NaOH (1 mol/L). The aqueous phase was washed with dichloromethane (2 × 20 mL) and PE (1 × 20 mL), and acidified with citric acid (pH < 2) yielding a yellow precipitate (0.99 g, 70%), mp > 190 °C. $^1$H NMR (300 MHz, DMSO-$d_6$): 2.10 (m, 4H), 2.29 (t, $J$ = 7.2, 4H), 4.45 (t, $J$ = 7.0, 4H), 7.93 (s, 4H), 8.64 (s, 2H), 12.12 (br s, 2H). $^{13}$C NMR (75 MHz, DMSO-$d_6$): 25.0, 30.4, 48.8, 121.3, 125.5, 130.1, 146.0, 173.5. $C_{18}H_{20}N_6O_4$: MS (ES): *m/z* (%) 385(100, M+). IR ($cm^{-1}$): 2559, 1685, 1430, 1291, 1215, 1045, 910, 822.

#### 4.5.2. 4,4′-[2,5,8,11,14-Pentaoxapentadecane-1,15-diylbis(1H-1,2,3-triazole-4,1-diyl)]dibutanoic acid (13b)

The solvent was removed completely under reduced pressure and the remaining yellow oil was purified by column chromatography (EA/MeOH 1:1 $R_f$ = 0.2) yielding compound **13b** (1.98 g, 67%) as a yellow oil. $^1$H NMR (300 MHz, $CD_3OD$): 2.20 (m, 4H), 2.30 (t, $J$ = 6.9, 4H), 3.60 (m, 16H), 4.50 (t, $J$ = 6.9, 4H), 4.62 (s, 4H), 7.98

(s, 2H). $^{13}$C NMR (75 MHz, $CD_3OD$): 26.9, 31.9, 50.7, 65.0, 70.7, 71.4, 125.3, 146.0, 176.7. $C_{22}H_{36}N_6O_9$: MS (ES): *m/z* (%) 529(100, M+), 551 (12). IR ($cm^{-1}$): 3458, 1720, 1558, 1219, 1084, 836.

### 4.6. General procedure for the preparation of compounds 14a–14e

The appropriate carboxylic acid **13** (1 mmol), HOBT·$H_2O$ (1.2 mmol for monovalent, 2.4 mmol for bivalent and 3.6 mmol for trivalent ligands), EDC (1.2 mmol, 2.4 mmol, 3.6 mmol) and DIPEA (2 mmol, 4 mmol, 6 mmol) were combined in a flask under nitrogen atmosphere with 10 mL of cold dichloromethane (**14d** and **14e**) or DMF (**14a, 14b** and **14c**). After 15 min compound **4** (1 mmol, 2 mmol and 3 mmol) was added and the mixture was stirred overnight at room temperature.

#### 4.6.1. Di-*tert*-butyl (1,4-phenylenebis{1H-1,2,3-triazole-4,1-diyl(1-oxobutane-4,1-diyl)imino[(*Z*)-(methylthio)-methylylidene]})biscarbamate (14a)

DMF was removed completely under reduced pressure and the crude product was purified by column chromatography (EA/PE 1:1 → EA $R_f$ = 0.6[EA]) yielding compound **14a** (460 mg, 63%) as a pale yellow solid, mp = 148 °C. $^1$H NMR (300 MHz, $CDCl_3$): 1.47 (s, 18H), 2.30 (m, 4H), 2.38 (s, 6H), 2.55 (m, 4H), 4.51 (t, *J* = 6.42, 4H), 7.84 (s, 2H), 7.89 (s, 4H), 12.30 (d, 2H). $^{13}$C NMR (75 MHz, $CDCl_3$): 14.6, 25.9, 28.0, 37.7, 49.2, 83.6, 119.8, 128.4, 130.6, 147.9, 160.4, 170.2, 184.3. $C_{32}H_{44}N_{10}O_6S_2$: MS (ES): *m/z* (%) 653 (5), 727(100, M+). IR ($cm^{-1}$): 2978, 1725, 1640, 1555, 1409, 1279, 1226, 1130, 810.

#### 4.6.2. Di-*tert*-butyl (2,5,8,11,14-pentaoxapentadecane-1,15-diylbis{1H-1,2,3-triazole-4, 1-diyl(1-oxobutane-4,1-diyl)-imino[(*Z*)-(methylthio)methylylidene]})biscarbamate (14b)

DMF was removed completely under reduced pressure; the remaining crude oil was dissolved in dichloromethane, washed with satd aqueous sodium carbonate solution (50 mL) and brine (50 mL), dried over magnesium sulphate and the solvent was evaporated. The crude product was purified by column chromatography [EA → EA/MeOH 1:1 $R_f$ = 0.8 (EA/MeOH 1:1)] yielding compound **14b** (556 mg, 64%) as a viscous oil. $^1$H NMR (300 MHz, $CDCl_3$): 1.49 (s, 18H), 2.20 (m, 4H), 2.37 (s, 6H), 2.50 (m, 4H), 3.60 (m, 16 H), 4.40 (t, *J* = 6.81, 4H), 4.66 (s, 4H), 7.59 (s, 2H), 12.30 (d, 2H). $^{13}$C NMR (75 MHz, $CDCl_3$): 14.6, 25.4, 28.0, 37.7, 49.5, 64.7, 69.7, 70.5, 84.1, 122.7, 145.3, 160.8, 170.0, 184.6. $C_{36}H_{60}N_{10}O_{11}S_2$: MS (ES): *m/z* (%) 873(100, M+), 895(15). IR ($cm^{-1}$): 2978, 1728, 1644, 1559, 1395, 1277, 1228, 1132, 809.

#### 4.6.3. *tert*-Butyl [(1*Z*,14*Z*)-19,19-dimethyl-1,15-bis(methylthio)-3,13,17-trioxo-5,8,11,18-tetraoxa-2,14,16-triazaicosa-1,14-dien-1-yl]carbamate (14c)

Compound **14c** was worked up as described for **14b**. The crude product was purified by column chromatography (PE/EA 3:1 $R_f$ = 0.1) yielding compound **14c** (402 mg, 71%) as a white solid, mp = 103 °C. $^1$H NMR (300 MHz, $CDCl_3$): 1.38 (s, 18H), 2.26 (s, 6H), 3.64 (s, 8H), 4.02 (s, 4H), 12.30 (d, 2H). $^{13}$C NMR (75 MHz, $CDCl_3$): 14.6, 27.9, 69.7, 70.5, 84.1, 160.8, 170.0, 184.6. $C_{22}H_{38}N_4O_9S_2$: MS (ES): *m/z* 567(100, M+), 589(7), 605(2). IR ($cm^{-1}$): 2978, 1720, 1650, 1553, 1425, 1365, 1272, 1228, 1144, 813.

#### 4.6.4. *tert*-Butyl((1*Z*)-(methylthio){[4-(4-phenyl-1H-1,2,3-triazol-1-yl)butanoyl]amino} methylene)carbamate (14d)

The organic layer was washed with sodium carbonate (50 mL), citric acid (50 mL) and brine (50 mL), dried over magnesium sulphate and the solvent was removed in vacuum. The crude product was purified by column chromatography (PE/EA 1:1 $R_f$ = 0.3) yielding compound **14d** (313 mg, 78%) as a white solid, mp = 140 °C. $^1$H NMR (300 MHz, $CDCl_3$): 1.50 (s, 9H), 2.30 (m, 2H), 2.37 (s, 3H), 2.50 (m, 2H), 4.49 (m, 2H), 7.29–7.44 (m, 3H), 7.81 (m, 2H), 7.83 (s, 1H), 12.30 (d, 1H). $^{13}$C NMR (75 MHz, $CDCl_3$): 14.6, 25.9, 28.0, 37.7, 49.5, 83.6, 119.8, 125.7, 128.2, 128.9, 130.6, 147.9, 160.9, 170.8, 184.7. $C_{19}H_{25}N_5O_3S$: MS (ES): *m/z* (%) 304(35), 404 (100, M+), 807(15). IR ($cm^{-1}$): 2978, 1715, 1640, 1557, 1412, 1279, 1226, 1150, 1119, 869, 815, 762, 691.

#### 4.6.5. Di-*tert*-butyl[({1-[4-({[(*Z*)-[(*tert*-butoxycarbonyl)imino](methylthio)methyl]amino}carbonyl)benzyl]-1H-1,2,3-triazol-4-yl}methyl)imino]bis{methylene-1H-1,2,3-triazole-4,1-diylmethylene-4,1-phenylenecarbonylimino[(E)-(methylthio)methylylidene]})biscarbamate (14e)

The DCM phase was washed with sodium carbonate (50 mL) and sodium hydrogensulphate (50 mL), dried over magnesium sulphate and the DCM was removed under reduced pressure. The crude product was recrystallized with MeOH yielding the product as white solid (783 mg, 66%), mp > 190 °C. $^1$H NMR (300 MHz, $CDCl_3$): 1.52 (s, 27H), 2.55 (s, 9H), 3.71 (s, 6H), 5.57 (s, 6H), 7.30 (d, *J* = 8.20, 6H), 7.73 (s, 3H), 8.20 (d, *J* = 8.20, 6H), 12.52 (s, 3H). $^{13}$C NMR (75 MHz, $CDCl_3$): 15.0, 28.0, 47.0, 53.7, 83.6, 124.1, 127.7, 130.8, 137.0, 139.3, 144.2, 150.9, 172.6, 175.1. $C_{54}H_{66}N_{16}O_9S_3$: MS (ES): *m/z* (%) 591(100), 1180(40, M+).

### 4.7. General procedure for preparation of compounds 15a–15e

The appropriate guanidinylation reagent **14** (0.2 mmol), compound **7** (0.2 mmol for monovalent, 0.4 mmol for bivalent or 0.6 mmol for trivalent) and $HgCl_2$ (0.2 mmol, 0.4 mmol, 0.6 mmol) were dissolved separately in small amounts of DMF (1–2 mL). Solutions of appropriate compound **14** and compound **7** were combined in a small flask under nitrogen atmosphere. $NEt_3$ (2 mmol, 4 mmol and 6 mmol) was added under stirring, the $HgCl_2$ solution was added and the mixture was stirred overnight at room temperature. DMF was removed completely and the residue was dissolved in DCM. The precipitate of mercury salts was removed by filtering over celite, the organic phase was washed with sodium carbonate (50 mL), citric acid (50 mL), brine (50 mL) and dried over magnesium sulphate. DCM was removed and the crude product purified by column chromatography.

#### 4.7.1. Di-*tert*-butyl (1,4-phenylenebis{1H-1,2,3-triazole-4,1-diyl(1-oxobutane-4,1-diyl)imino[(*Z*)-({(4R)-5-[(4-*tert*-butoxybenzyl)amino]-4-[(diphenylacetyl)amino]-5-oxopentyl}-amino)methylylidene]})biscarbamate (15a)

Column chromatography: (EA $R_f$ = 0.6); the product is a yellow solid (173 mg, 55%), mp > 190 °C. $^1$H NMR (300 MHz, $CDCl_3$): 1.29 (m, 18H), 1.40–1.47 (m, 18H), 1.50–1.90 (m, 8H), 2.10–2.50 (m, 8H), 3.15–3.45 (m, 4H), 4.20–4.50 (m, 8H), 4.52–4.70 (m, 2H), 4.96 (m, 2H), 6.80–6.90 (m, 4H), 7.00–7.09 (m, 4H), 7.18–7.45 (m, 24H), 7.67–7.85 (m, 6H). $C_{90}H_{110}N_{16}O_{12}$: MS (ES): *m/z* (%) 705 (23), 755 (30), 805 (100), 1509 (8), 1608 (16, M+), 1630 (6).

#### 4.7.2. *tert*-Butyl [(1*Z*)-({(4*R*)-5-[(4-*tert*-butoxybenzyl)amino]-4-[(diphenylacetyl)amino]-5-oxopentyl}amino)({4-[4-(15-{1-[4-({(1*E*)-({(4*R*)-5-[(4-*tert*-butoxybenzyl)amino]-4-[(diphenylacetyl)amino]-5-oxopentyl}amino)[(*tert*-butoxycarbonyl)amino]-methylene}amino)-4-oxobutyl]-1H-1,2,3-triazol-4-yl}-2,5,8,11,-14-pentaoxapentadec-1-yl)-1H-1,2,3-triazol-1-yl]butanoyl}-amino)methylene]carbamate (15b)

Column chromatography: (DCM/MeOH 17:3 $R_f$ = 0.4); the product is obtained as a pale yellow very viscous oil (218 mg, 64%). $^1$H NMR (300 MHz, $CD_3OD$): 1.28 (m, 18H), 1.40–1.50 (m, 18H), 1.50–2.50 (m, 16H), 3.20–3.40 (m, 4H), 3.50–3.70 (m, 16H), 4.20–4.60 (m, 14H), 5.08 (m, 2H), 6.86–6.89 (m, 4H), 7.05–7.15 (m, 4H),

7.15–7.30 (m, 20H), 7.90–7.95 (m, 2H). $C_{94}H_{126}N_{16}O_{17}$: MS (ES): *m/z* (%) 827 (20), 877 (100), 1653 (6), 1753 (18, M+).

#### 4.7.3. *tert*-Butyl{(1*E*,14*E*,20*R*)-20-{[(4-*tert*-butoxybenzyl)-amino]carbonyl}-1-({(4*R*)-5-[(4-*tert*-butoxybenzyl)amino]-4-[(diphenylacetyl)amino]-5-oxopentyl}amino)-15-[(*tert*-butoxycarbonyl)amino]-3,13,22-trioxo-23,23-diphenyl-5,8,11-trioxa-2,14,16,21-tetraazatricosa-1,14-dien-1-yl}carbamate (15c)

Column chromatography: (EA $R_f$ = 0.6); the product is a white solid (190 mg, 65%), mp > 190 °C. $^1$H NMR (300 MHz, $CDCl_3$): 1.31 (m, 18H), 1.42–1.48 (m, 18H), 1.50–1.90 (m, 8H), 3.20–3.50 (m, 4H), 3.65–3.85 (m, 8H), 4.20–4.40 (m, 8H), 4.52–4.70 (m, 2H), 4.87–4.98 (m, 2H), 6.80–6.90 (m, 4H), 7.00–7.10 (m, 4H), 7.18–7.30 (m, 20H). $C_{80}H_{104}N_{10}O_{15}$: MS (ES): *m/z* (%) 623(40), 673(30), 734(100), 1346(7), 1446(48, M+).

#### 4.7.4. *N*-(4-*tert*-Butoxybenzyl)-N5-((*Z*)-[(*tert*-butoxycarbonyl)-imino]{[4-(4-phenyl-1H-1,2,3-triazol-1-yl)butanoyl]amino}-methyl)-N2-(diphenylacetyl)-D-ornithinamide (15d)

Column chromatography: (EA $R_f$ = 0.6); the product is a white solid (122 mg, 72%), mp > 190 °C. $^1$H NMR (300 MHz, $CDCl_3$): 1.31 (m, 9H), 1.40 (m, 9H), 1.50–1.90 (m, 4H), 2.10–2.50 (m, 4H), 3.15–3.45 (m, 2H), 4.20–4.50 (m, 4H), 4.52–4.70 (m, 1H), 4.96 (m, 1H), 6.84–6.90 (m, 2H), 7.02–7.09 (m, 2H), 7.18–7.45 (m, 13H), 7.68–7.71 (m, 1H), 7.78–7.83 (m, 2H). $C_{48}H_{58}N_8O_6$: MS (ES): *m/z* (%) 843(100, M+). $C_{48}H_{58}N_8O_6$: calc. C 68.4 H 6.9 N 13.3, found: C 67.2 H 7.6 N 13.0.

#### 4.7.5. *tert*-Butyl [(*Z*)-[(4-{[4-({bis[(1-{4-[({(1*Z*)-({5-[(4-*tert*-butoxybenzyl)amino]-4-[(diphenylacetyl)amino]-5-oxopentyl}-amino)[(*tert*-butoxycarbonyl)amino]methylene}amino)carbonyl]benzyl}-1H-1,2,3-triazol-4-yl)methyl]amino}methyl)-1H-1,2,3-triazol-1-yl]methyl}benzoyl)amino]({5-[(4-*tert*-butoxybenzyl)amino]-4-[(diphenylacetyl)amino]-5-oxopentyl}imino)-methyl]carbamate (15e)

Column chromatography: (EA/MeOH 9:1 $R_f$ = 0.7); the product is a pale yellow solid (208 mg, 43%), mp > 190 °C. $C_{141}H_{165}N_{25}O_{18}$: MS (ES): *m/z* (%) 834 (20), 1250 (100), 2400 (2), 2500 (8, M+).

### 4.8. General procedure for preparation of compounds 16a–16e

The appropriate Boc- and $^t$Bu-protected ligand (**15**) (0.03–0.15 mmol) was dissolved in 5 mL of DCM/TFA 1:1 and stirred for 2 h. The solvent was removed completely under reduced pressure and the oily residue was repeatedly dissolved in DCM and the solvent evaporated to remove TFA traces. The deprotected ligands were purified by HPLC for the cell assays.

#### 4.8.1. (*E*)-{[4-(4-{4-[1-(4-{[(1*Z*)-Ammonio({(4*R*)-4-[(diphenylacetyl)amino]-5-[(4-hydroxybenzyl)amino]-5-oxopentyl}amino)methylene]amino}-4-oxobutyl)-1H-1,2,3-triazol-4-yl]phenyl}-1H-1,2,3-triazol-1-yl)butanoyl]amino}-({(4*R*)-4-[(diphenylacetyl)amino]-5-[(4-hydroxybenzyl)amino]-5-oxopentyl}imino)methanaminium bis(trifluoroacetate) (16a)

The product is obtained as a pale yellow solid (75 mg, 100%), mp > 190 °C. $^1$H NMR (300 MHz, $CD_3OD$): 1.40–1.90 (m, 8H), 2.20–2.60 (m, 8H), 3.10–3.25 (m, 4H), 4.15–4.25 (m, 4H), 4.30–4.40 (m, 2H), 4.40–4.60 (m, 4H), 5.06 (s, 2H), 6.70 (d, *J* = 8.53, 4H), 7.00 (d, *J* = 8.49, 4H), 7.20–7.35 (m, 20H), 7.85–7.90 (m, 2H), 8.35–8.40 (m, 2H). $^{13}$C NMR (75 MHz, DMSO-$d_6$): 24.3, 29.4, 33.0, 40.5, 41.7, 48.6, 52.3, 56.0, 115.0, 116.0, 121.4, 125.1, 126.6, 127.9, 129.2, 130.8, 140.4, 146.4, 152.8, 156.3, 159.1, 171.0, 174.3. $C_{72}H_{78}N_{16}O_8$: MS (ES): *m/z* (%) 649(100), 1296(6, M+). IR ($cm^{-1}$): 3285, 2934, 1638, 1514, 1445, 1359, 1194, 1128, 699. UV (MeOH): $\lambda(\varepsilon)$ 277 ($16 \times 10^3$). HPLC: $t_r$ = 13.3 min.

#### 4.8.2. (*E*)-{[4-(4-{15-[1-(4-{[(1Z)-Ammonio({(4*R*)-4-[(diphenylacetyl)amino]-5-[(4-hydroxybenzyl)amino]-5-oxopentyl}-amino)methylene]amino}-4-oxobutyl)-1H-1,2,3-triazol-4-yl]-2,5,8,11,14-pentaoxapentadec-1-yl}-1H-1,2,3-triazol-1yl) butanoyl]amino}({(4*R*)-4-[(diphenylacetyl)amino]-5-[(4-hydroxybenzyl)amino]-5-oxopentyl}imino)methanaminium bis(trifluoroacetate) (16b)

The product is obtained as a white solid (77 mg, 100%), mp > 190 °C. $^1$H NMR (300 MHz, DMSO-$d_6$): 1.30–1.75 (m, 8H), 2.00 –2.20 (m, 4H), 2.40–2.60 (t, *J* = 6.87, 4H), 3.15–3.30 (dd, J1 = 5.89, J2 = 11.55, 4H), 3.45–3.60 (m, 16H), 4.15–4.30 (m, 4H), 4.35–4.50 (m, 6H), 4.55–4.60 (br s, 4H), 5.13 (s, 2H), 6.65–6.70 (br d, *J* = 8.36, 4H), 6.95–7.05 (br d, *J* = 8.32, 4H), 7.15–7.30 (m, 20H), 7.96 (s, 2H), 8.35–8.40 (t, *J* = 5.49, 2H), 8.45–8.55 (d, *J* = 7.97, 2H), 8.50–8.90 (br s, 4H), 9.14 (br s, 2H), 11.81 (s, 2H). $^{13}$C NMR (75 MHz, DMSO-$d_6$): 24.2, 29.3, 33.0, 40.5, 41.5, 48.2, 52.2, 55.8, 63.4, 68.9, 69.6, 114.9, 123.8, 126.5, 128.1–128.9, 140.2, 143.9, 152.6, 156.2, 170.9, 174.1. $C_{76}H_{94}N_{16}O_{13}$: MS (ES): *m/z* (%) 494 (11), 721 (100), 1440 (5, M+). IR ($cm^{-1}$): 3286, 2926, 1656, 1515, 1450, 1362, 1198, 1127, 700. UV (MeOH): $\lambda(\varepsilon)$ 269($3 \times 10^3$). HPLC: $t_r$ = 12.6 min.

#### 4.8.3. ((14*Z*,20*R*)-15-Ammonio-1-({(4*R*)-4-[(diphenylacetyl)-amino]-5-[(4-hydroxybenzyl)amino]-5-oxopentyl}amino)-20-{[(4-hydroxybenzyl)amino]carbonyl}-3,13,22-trioxo-23,23-diphenyl-5,8,11-trioxa-2,14,16,21-tetraazatricos-14-en-1-ylidene)ammonium bis(trifluoroacetate) (16c)

The product is obtained as a white solid (70 mg, 100%), mp > 190 °C. $^1$H NMR (300 MHz $CD_3OD$): 1.40–1.90 (m, 8H), 3.20–3.30 (br m, 4H), 3.65–3.80 (m, 8H), 4.15–4.30 (m, 4H), 4.40–4.50 (m, 2H), 5.07 (s, 2H), 6.65–6.75 (br d, *J* = 8.37, 4H), 7.00–7.05 (br d, *J* = 8.37, 4H), 7.20–7.30 (m, 20H). $^{13}$C NMR (75 MHz, DMSO-$d_6$): 25.5, 30.3, 42.1, 43.8, 54.3, 58.7, 71.3, 71.4, 72.2, 116.4, 128.2, 129.6–130.3, 140.9, 154.7, 157.9, 173.3–174.9. $C_{62}H_{72}N_{10}O_{11}$: MS (ES): *m/z* (%) 567(100), 1133(13, M+). IR ($cm^{-1}$): 3284, 2928, 1655, 1515, 1451, 1358, 1199, 1130, 1023, 1000, 823, 700. UV (MeOH): $\lambda(\varepsilon)$ 277($2 \times 10^3$). HPLC: $t_r$ = 12.6 min.

#### 4.8.4. ({(4*R*)-4-[(Diphenylacetyl)amino]-5-[(4-hydroxybenzyl)-amino]-5-oxopentyl}amino){[4-(4-phenyl-1H-1,2,3-triazol-1-yl)butanoyl]amino}methaniminium trifluoroacetate (16d)

The product is obtained as a white solid (117 mg, 100%), mp > 190 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): 1.35–1.49 (m, 2H), 1.49–1.75 (m, 2H), 2.12–2.21 (p, J1 = 7.04, J2 = 7.14, 2H), 2.47–2.53 (m, 2H), 3.17–3.26 (dd, J1 = 6.59, J2 = 13.01, 2H), 4.09–4.20 (m, 2H), 4.30–4.36 (dd, J1 = 7.87, J2 = 14.15, 1H), 4.43–4.49 (t, *J* = 6.91, 2H), 5.12 (s, 1H), 6.65–6.69 (d, *J* = 8.49, 2H), 6.98–7.02 (d, *J* = 8.51, 2H), 7.16–7.24 (m, 2H), 7.25–7.30 (m, 8H), 7.31–7.35 (m, 1H), 7.40–7.46 (m, 2H), 7.80–7.85 (m, 2H), 8.30–8.36 (t, *J* = 5.80, 1H), 8.45–8.48 (d, *J* = 8.06, 1H), 8.56 (s, 1H), 8.60–8.90 (m, 2H), 9.12–9.20 (m, 1H), 11.85 (s, 1H). $^{13}$C NMR (101 MHz, DMSO-$d_6$): 24.3, 29.4, 33.0, 40.5, 41.7, 48.6, 52.3, 56.0, 115.0, 116.0, 121.4, 125.1, 126.6, 127.9–129.2, 130.8, 140.4, 146.4, 152.8, 156.3, 159.1, 171.0, 174.3. $C_{39}H_{42}N_8O_4$: MS (ES): *m/z* (%) 687(100, M+). IR ($cm^{-1}$): 3285, 2930, 1628, 1516, 1448, 1379, 1199, 1132, 694. UV (MeOH): $\lambda(\varepsilon)$ 235($15 \cdot 10^3$). HPLC: $t_r$ = 13.8 min.

#### 4.8.5. (Ammoniotris{methylene-1H-1,2,3-triazole-4,1-diylmethylene-4,1-phenylenecarbonylimino[({4-[(diphenylacetyl)amino]-5-[(4-hydroxybenzyl)amino]-5-oxopentyl}amino)methylylidene]}) triammonium tetrakis(trifluoroacetate) (16e)

The product is a pale yellow solid (82 mg, 100%), mp > 190 °C. $^1$H NMR (300 MHz DMSO-$d_6$): 1.40–1.70 (m, 12H), 3.30–3.40 (br m, 6H), 4.15–4.50 (m, 15H), 5.13 (s, 3H), 5.82 (s, 6H), 6.65–6.70

(br d, $J$ = 8.46, 6H), 7.00–7.05 (br d, $J$ = 8.47, 6H), 7.15–7.30 (m, 30 H), 7.45–7.55 (br d, 6H), 7.95–8.00 (br d, 6H), 8.35–8.55 (br m, 9H), 8.85–8.95 (br s, 5H), 9.26 (br s, 3H), 11.58 (br s, 2H). $^{13}$C NMR (101 MHz, DMSO-$d_6$): 24.4, 29.3, 33.2, 41.5, 45.9, 52.2, 52.3, 55.8, 114.9, 126.5, 128.1–129.0, 131.2, 140.2, 140.4, 141.5, 153.2, 156.2, 167.2, 170.9. $C_{114}H_{117}N_{25}O_{12}$: MS (ES): $m/z$ (%) 509(30), 678(100), 1015(40), 2030(8, M+). IR ($cm^{-1}$): 3284, 2930, 1642, 1515, 1451, 1358, 1170, 1132, 699. HPLC: $t_r$ = 13.5 min.

### 4.9. Radioligand binding

$Y_1$R radioligand binding studies on SK-N-MC cells were performed as described elsewhere,[18] except for [$^3$H]$N^G$-propionyl-BIBP-3226 as radioligand (specific activity 3.6 TBq/mmol, $K_d$ = 1.1 nM, $c$ = 1.5 nM) and an incubation period of 20 min. $K_i$ values were calculated using the Cheng–Prusoff equation.[19]

### 4.10. NPY $Y_1$ receptor antagonist activity in human erythroleukemia (HEL) cells

The title compounds were screened for NPY $Y_1$ receptor antagonism by measuring the inhibition of the NPY-stimulated increase in intracellular [$Ca^{2+}$].

#### 4.10.1. Cell culture

The human HEL erythroleukemia cell line was kindly provided by Dr. M.C. Michel, Department of Pharmacology and Pharmacotherapy, University of Amsterdam, The Netherlands. Cell line banking and quality control were performed according to the 'seed stock concept'.[20] Cells were maintained as suspension cultures in antibiotic-free RPMI 1640 medium (Sigma, Deisenhofen, Germany) containing 0.3 g/l L-glutamine, 2 g/l $NaHCO_3$ and 5% foetal calf serum (Biochrom AG, Berlin, Germany) using 75-cm$^2$-culture flasks (Thermo Fisher Scientific (Nunc GmbH & Co. KG), Langenselbold, Germany) in a water saturated atmosphere (95% air / 5% $CO_2$) at 37 °C. The cells were passaged weekly by 1:10 dilution with fresh culture medium. Cells were routinely monitored for, and shown to be free of, *Mycoplasma* contamination with the Venor™*GeM*-Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany).

#### 4.10.2. Preparation of the cells

The day before testing, 25 ml of a plateau-phase culture were transferred to a 175-cm$^2$ flask Thermo Fisher Scientific (Nunc GmbH & Co. KG), Langenselbold, Germany) containing 125 ml of fresh culture medium. After 24 h, the suspension (2–4 × 10$^5$ cells/ml), was centrifuged for 10 min at 200 g and room temperature. After resuspension in 10 ml of loading buffer (25 mM HEPES (Sigma, Deisenhofen, Germany), 120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose), pH 7.4, cell number was determined with a hemocytometer (Neubauer, improved), and the cells were adjusted to a density of 1.3 × 10$^6$/ml by addition of an appropriate volume of loading buffer.

#### 4.10.3. Loading of the cells with the $Ca^{2+}$ indicator Fura-2/AM

To three volumes of the prepared cell suspension, one volume of loading dispersion[22] was added, before the cells were incubated in the dark at room temperature for 30 min. The loading dispersion was freshly made by mixing 10 ml of loading buffer, containing 20 mg/ml bovine serum albumin (BSA), with 50 µl of Pluronic-F-127 (Invitrogen, Karlsruhe, Germany) (20% in DMSO), and 40 µl of Fura-2/AM (Invitrogen) (1 mM in anhydrous DMSO).

Cells were centrifuged (200$g$, 7 min), resuspended in fresh loading buffer and allowed to stand for another 30 min at room temperature in the dark. After two washing/centrifugation cycles (loading buffer, 200$g$, 7 min) and adjustment of the cell number to a value of 10$^6$/ml, cells were incubated for at least 15 min at 20 °C in the dark.

#### 4.10.4. Fluorimetric determination of intracellular $Ca^{2+}$

One-millilitre aliquots of loading buffer were filled into disposable acrylic cuvettes (Sarstedt, No. 67.755). Immediately after addition of 1 ml of the Fura-2/AM loaded cell suspension and a magnetic stirrer, the cuvette was placed into the thermostatted (25 °C) stirred cell holder of a LS 50 B Luminescence Spectrometer (Perkin Elmer, Überlingen, Germany), equipped with a fast filter accessory. Fluorescence signals were registered (instrument settings: excitation 340/380 nm, emission 510 nm, slits 10 nm, resolution 0.1, stirrer low) after addition of 10 µl of 20 µM porcine NPY (Bachem Biochemica GmbH, Heidelberg, Germany), dissolved in 10 mM HCl, supplemented with 0.1% BSA, for 300 s.

#### 4.10.5. Calculation of $Ca^{2+}$ concentrations

Calcium concentrations were calculated from dual wavelength fluorescence intensities according to the Grynkiewicz equation:[21]

$$[Ca^2+] = K_d \cdot \frac{(R - R_{min})}{(R_{max} - R)} \cdot SFB \quad (1)$$

were $K_d$ (224 nM)[22] is the dissociation constant of the Fura-2–$Ca^{2+}$-complex, $R$ is the experimental fluorescence ratio value ($F_{340}/F_{380}$), $R_{min}$ and $R_{max}$ are the fluorescence value ratios ($F_{340}/F_{380}$) under $Ca^{2+}$-free and $Ca^{2+}$-saturation conditions, respectively, and SFB is the ratio of fluorescence intensities for $Ca^{2+}$-bound/$Ca^{2+}$-free indicator, measured at 380 nm. $R_{min}$, $R_{max}$ and SFB were determined by calibration experiments, performed in every test series.

To measure $R_{max}$, 10 µl of an aqueous solution of 2% digitonin (Sigma), were pipetted into the cuvette, whereas $R_{min}$ was determined after subsequent addition of 50 µl of a 0.6 M EGTA solution (in 1 M Tris/HCl, pH 8.7).

#### 4.10.6. Determination of NPY $Y_1$ receptor antagonist activity

The cells were pre-incubated with 10 µl of the putative antagonists (dissolved either in 50% ethanol or DMSO) for 1 min, prior to the stimulation with porcine NPY at a final concentration of 10 nM. The $Ca^{2+}$-signal in every fifth cuvette was taken as the non-inhibited reference (100%), that is, the cells were only exposed to the respective solvent, before NPY stimulation.

#### 4.10.7. Calculation of $IC_{50}$- and $K_b$-values

$IC_{50}$- values were calculated from at least two antagonist concentrations, inhibiting the NPY-stimulated increase in intracellular [$Ca^{2+}$] between 20% and 80%. The mean percentual inhibition values ($P$), determined from at least two independent experiments, performed on different days, were logit transformed, and $IC_{50}$-values (logit $P$ = 0) were determined according to the equation

$$\text{logit}(P) = \log \frac{P}{100 - P} \quad (2)$$

by linear regression (SigmaPlot 8.0 software).

$K_b$-values were calculated according to the Cheng–Prusoff equation[19] with an $EC_{50}$-value for pNPY of 1.8 nM.

### Acknowledgements

The authors are grateful to Mrs. Elvira Schreiber for expert technical assistance. This work was supported by the Graduate Training Program (Graduiertenkolleg) GRK 760, 'Medicinal Chemistry:

Molecular Recognition—Ligand–Receptor Interactions', of the Deutsche Forschungsgemeinschaft.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.09.033.

## References


1. Thorsell, A.; Ehlers, C. L. Neuropeptide Y in Brain Function. In *Handbook of Neurochemistry and Molecular Neurobiology: Neuroactive Proteins and Peptides*, 3rd ed.; Springer: New York, Berlin, 2006; pp 523–543.
2. Michel, M. C.; Beck-Sickinger, A.; Cox, H.; Doods, H. N.; Herzog, H.; Larhammar, D.; Quirion, R.; Schwartz, T.; Westfall, T. *Pharmacol. Rev.* **1998**, *50*, 143.
3. (a) Messer, William S. *Curr. Pharm. Des.* **2004**, *10*, 2015; (b) Decker, M.; Lehmann, J. *Curr. Top. Med. Chem.* **2007**, *7*, 347; (c) Halazy, S. *Exp. Opin. Ther. Patents* **1999**, *9*, 431.
4. Portoghese, P. S. *J. Med. Chem.* **2001**, *44*, 2259.
5. Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4046.
6. Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531.
7. Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem. Int. Ed.* **1998**, *37*, 2754.
8. Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Beck-Sickinger, A. G.; Doods, H. N. *Eur. J. Pharm.* **1994**, *271*, R11.
9. (a) Hutzler, C.; Kracht, J.; Mayer, M.; Graichen, F.; Bauer, B.; Schreiber, E.; Bollwein, S.; Bernhardt, G.; Dove, S.; Fricker, G.; Buschauer, A. *Arch. Pharm. Med. Chem.* **2001**, *334*(17), 7; (b) Brennauer, A.; Dove, S.; Buschauer, A. Structure–Activity Relationships of Nonpeptide Neuropeptide Y Receptor Antagonists. In *Handbook of Experimental Pharmacology*; Michel, M. C., Ed.; Springer: Berlin, Heidelberg, New York, 2004; Vol. 162, pp 505–546; (c) Schneider, E.; Keller, M.; Brennauer, A.; Hoefelschweiger, B. K.; Gross, D.; Wolfbeis, O. S.; Bernhardt, G.; Buschauer, A. *Chembiochem* **2007**, *8*, 1981.
10. (a) Suhs, T.; König, B. *Chem. Eur. J.* **2006**, *12*, 8150; (b) Suhs, T.; König, B. *Mini-Rev. Org. Chem* **2006**, *3*, 315.
11. To be reported elsewhere.
12. DeMong, D. E.; Williams, R. M. *Tetrahedron Lett.* **2001**, *42*, 3529.
13. (a) Chan, T. R.; Hildegraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004**, *6*, 2853; (b) Ritter, S. C.; König, B. *Chem. Commun.* **2006**, 4694.
14. Zhang, G.; Fang, L.; Zhu, L.; Sun, D.; Wang, P. G. *Bioorg. Med. Chem.* **2006**, *14*, 426.
15. Dimerized peptidic $NPY_1$ antagonists showed slightly increased binding affinities when a spacer length between 16 and 32 atoms was used. Therefore spacer molecules in this range were selected Daniels, A.; Matthews, J. E.; Slepetis, R. J.; Jansen, M.; Viveros, O. H.; Tadepalli, A.; Harrington, W.; Heyer, D.; Landavazo, A.; Leban, J. J.; Spaltenstein, A. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9067.
16. Khoukhi, N.; Vaultier, M.; Carrié, R. *Tetrahedron* **1987**, *43*, 1811.
17. Brennauer, A. Dissertation, University Regensburg, 2006.
18. Aiglstorfer, I.; Uffrecht, A.; Geßele, K.; Moser, C.; Schuster, A.; Merz, S.; Malawska, B.; Bernhardt, G.; Dove, S.; Buschauer, A. *Regul. Pept.* **1998**, *76*, 9.
19. Cheng, Y. C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.
20. Hay, R. J. In *Animal Cell Culture: A Practical Approach*; Freshney, R. I., Ed., 2nd ed.; IRL Press: Oxford, 1992; pp 95–148.
21. Grynkiewicz, A. G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, *260*, 3440.
22. Thomas, A. P.; Delaville, F. In *Cellular Calcium: A Practical Approach*; McCormack, J. G., Cobbold, P. H., Eds.; IRL Press: Oxford, 1991; pp 1–54.