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Mimicking of Arginine by Functionalized N^{ω}-Carbamoylated Arginine As a New Broadly Applicable Approach to Labeled Bioactive Peptides: High Affinity Angiotensin, Neuropeptide Y, Neuropeptide FF, and Neurotensin Receptor Ligands As Examples

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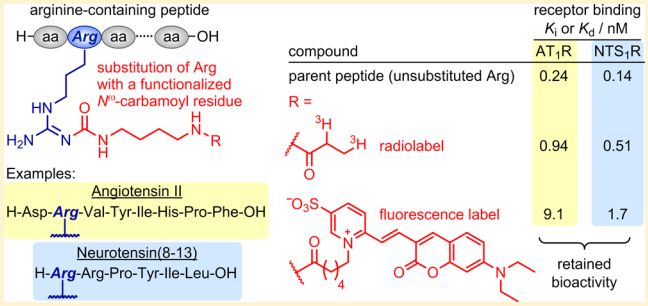
Supporting Information

ABSTRACT: Derivatization of biologically active peptides by conjugation with fluorophores or radionuclide-bearing moieties is an effective and commonly used approach to prepare molecular tools and diagnostic agents. Whereas lysine, cysteine, and *N*-terminal amino acids have been mostly used for peptide conjugation, we describe a new, widely applicable approach to peptide conjugation based on the nonclassical bioisosteric replacement of the guanidine group in arginine by a functionalized carbamoylguanidine moiety. Four arginine-containing peptide receptor ligands (angiotensin II, neurotensin(8−13), an analogue of the C-terminal pentapeptide of neuropeptide Y, and a neuropeptide FF analogue) were subject of this proof-of-concept study. The N^{ω}-carbamoylated arginines, bearing spacers with a terminal amino group, were incorporated into the peptides by standard Fmoc solid phase peptide synthesis. The synthesized chemically stable peptide derivatives showed high receptor affinities with K_i values in the low nanomolar range, even when bulky fluorophores had been attached. Two new tritiated tracers for angiotensin and neurotensin receptors are described.



1. INTRODUCTION

Conjugation of biologically active peptides, e.g., to fluorophores, radionuclide-bearing moieties, or surfaces, is widely applied to prepare pharmacological tools, diagnostic agents, or drug delivery systems.[1−16] Proteinogenic amino acids, allowing site-specific and efficient conjugation of peptides, are lysine (ε-amino group), cysteine (sulfhydryl group), and uncapped *N*-terminal amino acids (α-amino group). Among them, lysine is most commonly used due to the high reactivity of the basic ($pK_a \approx 10$) ε-amino group in its nonionized form allowing various reactions, i.e., acylation, alkylation, arylation, and amidination.[17] However, retained affinity of the modified peptide to the respective biological target cannot be taken for granted.

Numerous biologically active endogenous or truncated peptides such as angiotensin II (AngII), bombesin, bradykinin, gonadoliberin, vasopressin, neuropeptide FF (NPFF), pancreatic polypeptide,[18,19] neurotensin(8−13),[20] and kisspeptin-13,[21] are devoid of lysine and cysteine residues but contain arginine. Moreover, arginine-containing chemically modified peptides such as selective or metabolically stable neurotensin NTS_2 receptor ligands,[22−24] NPFF analogues (e.g., [D-Tyr[1],NMePhe[3]]-NPFF and EYF (Figure 1B)),[25,26] C-terminal neuropeptide Y (NPY) analogues,[27−29] integrin binding RGD peptides,[30−32] cyclic pentapeptides addressing the $CXCR_4$ chemokine receptor,[33,34] opioid receptor ligands,[35] and peptidic melanocortin receptor ligands[36−38] were described. Generally, the strongly basic guanidine groups (pK_a 12−13) in arginine-containing peptides are considered to undergo key interactions with acidic amino acids of the respective receptor. In combination with the low chemical reactivity of the positively charged moiety,[17] this may be the reason why the development

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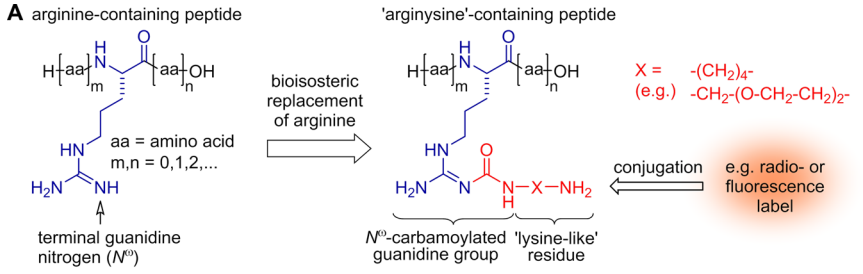

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peptide	aa sequence / structure	target (receptor)
Angiotensin II (AngII)	H-Asp-**Arg**-Val-Tyr-Ile-His-Pro-Phe-OH	AT_1, AT_2
Neurotensin(8-13) (NT(8-13))	H-**Arg**-**Arg**-Pro-Tyr-Ile-Leu-OH	NTS_1, NTS_2
1, **2**[a]	Tyr-**Arg**-Leu-**Arg**-Tyr-NH_2, $(CH_2)_4$, Tyr-**Arg**-Leu-**Arg**-Tyr-NH_2; **1** R = H; **2** R = NH_2	NPY Y_4
EYF	H-Glu-Tyr-Trp-Ser-Leu-Ala-Ala-Pro-Gln-**Arg**-Phe-NH_2 (NPFF = H-Phe-Leu-Phe-Gln-Pro-Gln-**Arg**-Phe-NH_2)	$NPFF_2$

Figure 1. Concept of the present study applied to selected arginine-containing peptides. (A) Bioisosteric replacement (in a nonclassical sense[51]) of the guanidine group in arginine by a carbamoylguanidine moiety bearing an amino-functionalized residue enables a versatile conjugation of arginine-containing peptides. (B) Arginine-containing peptidic receptor ligands, which were subject of the proof-of-concept study. [a]Balasubramaniam et al.[28]

of labeling strategies based on the derivatization of the guanidine group in arginine has been neglected. Previously, we demonstrated that acylation of guanidine groups in nonpeptide ligands of G-protein-coupled receptors (GPCRs), e.g., histamine and NPY receptors (see Supporting Information, Figure S1),[39−43] results in retained affinities regardless of basicity reduced by 4−5 orders of magnitude. Acylation of the guanidine group of arginine residues in bioactive peptides was recently reported for leuprolide, a gonadotropin-releasing hormone receptor agonist, and microcystin, a cyanobacterial toxin, to introduce, e.g., a fluorescence label or a biotin tag.[44−46] However, the acylguanidine moiety was shown to be unstable in aqueous solution at pH > 4.[42,47−49] By contrast, carbamoylation instead of acylation of the guanidine group resulted in chemically stable histamine and NPY receptor ligands with retained affinity.[42,48,49]

This prompted us to explore if the guanidine-carbamoylguanidine bioisosteric approach is applicable to arginine replacement in biologically active peptides by incorporating amino-functionalized N^{ω}-carbamoylated arginine ("arginysine") enabling versatile conjugation and labeling by analogy to methodologies applied to lysine (Figure 1A). In the present study, we report on a proof-of-concept covering the peptide ligands of subtypes of four GPCR families: AngII, neurotensin, NPY, and NPFF receptors (in the following encoded by color shading in yellow, blue, green, and gray, respectively). The selected parent ligands, comprising one, two, or four arginine residues in different positions, were AngII, NT(8−13), the dimeric peptide **1**, a derivative of the reported NPY Y_4 receptor agonist **2**,[28] and EYF,[26,50] an analogue of NPFF (Figure 1B). Appropriately modified arginine building blocks were synthesized and used in standard solid phase peptide synthesis (SPPS) (Figure 2). The prepared peptide derivatives, including new radio- and fluorescence labeled molecular tools, were characterized in binding and functional assays at the receptors of interest.

2. RESULTS

N^{ω}-Carbamoylated Arginine Building Blocks for SPPS.

N^{α}-Fmoc protected, N^{ω}-carbamoylated arginine derivatives (**10**, **11**, **16**), bearing Boc-protected terminal amino groups, were prepared to be incorporated into peptides using standard Fmoc strategy SPPS (Scheme 1, Figure 2A). Whereas compounds **10** and **16** contain an aliphatic tetramethylene spacer in the N^{ω}-substituent, compound **11** comprises a dioxaoctamethylene spacer. Unlike **10** and **11**, which were Boc-protected at the guanidine group, the respective position in **16** was unprotected (Scheme 1, Figure 2A). The convenient synthesis of arginine building blocks **10**, **11**, and **16**, which were obtained in 99% chemical purity, is shown in Scheme 1: Fmoc-Orn(Boc)-OH (**3**) was esterified with benzylic alcohol to give the ornithine derivative **4**,[52] which was treated with TFA to obtain amine **5**.[53] Guanidinylation of **5** using various *S*-methyl-isothiourea derivatives (**6**,[42] **7**,[54] or **14**) afforded the intermediates **8**, **9**, and **15**, which were converted to the arginine building blocks **10**, **11**, and **16** by hydrogenolytic cleavage of the benzyl ester (Scheme 1).

To investigate the suitability of **10**, **11**, and **16** (Scheme 1, Figure 2A) for SPPS, the model peptide H-Tyr-(N^{ω}-carbamoyl)-Arg-Leu-NH_2 (**17**) was synthesized on a Rink amide resin using the arginine building blocks **11** and **16** (Figure 2B). The coupling of **11** was performed under various conditions. When coupling was performed at 22 °C, the incorporation of **11** was incomplete (giving the side product H-Tyr-Leu-NH_2), irrespective of both coupling reagents (HBTU, PyBOP) and repeated coupling procedure. Peptide **17** was obtained in high yield when anhydrous DMF was used and the temperature increased to 35 °C. Unlike **11**, building block **16** was inappropriate for SPPS due to the formation of lactam **18** by an intramolecular ring closure upon activation of the carboxylic group with HBTU, showing that protection of the guanidine group as in **10** and **11** is indispensible (Figure 2C).

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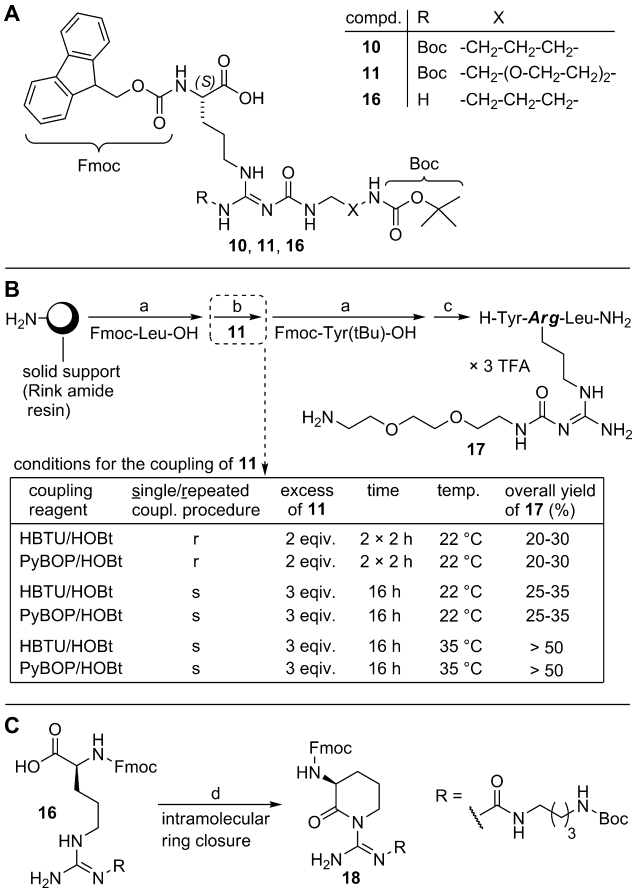

coupling reagent	single/repeated coupl. procedure	excess of **11**	time	temp.	overall yield of **17** (%)
HBTU/HOBt	r	2 eqiv.	2 × 2 h	22 °C	20-30
PyBOP/HOBt	r	2 eqiv.	2 × 2 h	22 °C	20-30
HBTU/HOBt	s	3 eqiv.	16 h	22 °C	25-35
PyBOP/HOBt	s	3 eqiv.	16 h	22 °C	25-35
HBTU/HOBt	s	3 eqiv.	16 h	35 °C	> 50
PyBOP/HOBt	s	3 eqiv.	16 h	35 °C	> 50

Figure 2. N^{ω}-Carbamoylated arginine building blocks for SPPS. (A) Structure of the synthesized arginine building blocks **10**, **11**, and **16**. (B) Incorporation of **11** into a model peptide via SPPS. Reagents and conditions: (a) Fmoc-strategy SPPS using 5 equiv of Fmoc-aa, HBTU/HOBt (4.9/5 equiv), and DIPEA (10 equiv); solvent, DMF/NMP (8:2 v/v), rt, 2 × 60 min (coupling procedure repeated once); Fmoc-deprotection, 20% piperidine in DMF/NMP (8:2 v/v). (b) Varying conditions (see inserted table); solvent, (DMF/NMP 8:2 v/v) using either DMF for peptide synthesis packed under nitrogen (coupling at 22 °C) or anhydrous DMF over molecular sieve (coupling at 35 °C), Fmoc-deprotection as under (a); (c) trifluoroacetic acid (TFA)/H_2O (95:5 v/v), rt, 2 h. (C) Formation of lactam **18** from arginine derivative **16**. Reagents and conditions: (d) HBTU/HOBt (1/1 equiv), DIPEA (2 equiv), DMF/NMP (8:2 v/v), rt, >90% conversion after 5 min.

Incorporation of N^{ω}-Carbamoylated Arginines into Peptides. The parent peptides in the present study were the octapeptide AngII with arginine in position 2, the hexapeptide NT(8−13) containing two arginines in positions 8 and 9, the dimeric pentapeptide **1** comprising four arginines, and the undecapeptide EYF, a derivative of NPFF, with arginine in position 10 close to the C-terminus (Figure 1B). Instead of the previously reported Y_4R agonist **2**, an analogue of the C-terminal pentapeptide of NPY containing two chiral centers in the linker moiety,[28] we synthesized analogue **1** containing an unsubstituted suberyl linker (Figure 1B, Supporting Information, Figure S2).

Incorporation of the arginine building blocks **10** and **11** into the parent peptides using Fmoc strategy SPPS yielded the peptide analogues **19**, **20**, **25**, **26**, **31**, **33**, and **35** bearing amino-functionalized carbamoyl residues at the guanidine group of arginine (Figure 3). Remarkably, the synthesis of the EYF derivative **35**, which required the subsequent coupling of nine amino acids after the incorporation of **10**, was obtained in 34% overall yield. In NT(8−13), either Arg^8 or Arg^9 was replaced by a N^{ω}-substituted arginine (analogues **25**, **26**, and **31**), and in peptide **1**, arginine in position 2 was replaced (**33**). The amino-functionalized peptides **19**, **20**, **25**, **26**, **31**, **33**, and **35** were propionylated to obtain the propionamide derivatives **21**, **22**, **27**, **28**, **32**, **34**, and **36** (Figure 3, Supporting Information, Figure S2). To explore if bulkier moieties than propionyl are tolerated with respect to receptor binding, a chromene or a cyanine dye was attached to give the fluorescently labeled AngII and NT(8−13) derivatives **23**, **24**, **29**, and **30** (Figure 3, for fluorescence spectra and quantum yields, see Supporting Information, Figures S19 and S20, Table S1). The fluorescence properties of the fluorophores were comparable to those of the labeled peptides, as demonstrated for compounds **24** and **30** (Supporting Information, Figure S20, Table S1). For the synthesis of the peptide derivatives (**1**, **19**−**36**), see Supporting Information (Figure S2) and Experimental Section.

Stability of the Peptides in Buffer and under Assay Conditions. The chemical stability of the synthesized N^{ω}-carbamoylated peptide derivatives was assessed for the AngII derivatives **19**−**21**, **23**, and **24**, the NT(8−13) analogues **25**, **27**, **29**, and **30**, and the EYF derivatives **35** and **36** in phosphate buffer (pH 7.0, 21 °C) over periods of 24 or 48 h. All peptides proved to be stable (**21**, **27**, Figure 4A; **19**, **20**, **23**−**25**, **29**, **30**, **35**, and **36**, Supporting Information, Figures S3−S11). The "carba" analogue of the NT(8−13) derivative **25**, containing an acylguanidine instead of the carbamoylguanidine moiety (compound **44**, Supporting Information, Figure S16) was cleaved, giving the parent compound NT(8−13) devoid of the N^{ω}-acyl substituent (Supporting Information, Figures S17 and S18). This is consistent with the reported instability of previously reported non-peptide NPY Y_1 and histamine H_2 receptor ligands containing an acylguanidine moiety.[42,47−49] Additionally, the stabilities of AngII and derivatives **21** and **22** as well as of NT(8−13) and analogues **27** and **28** were investigated under assay conditions in the presence of CHO-hAT_1 and HT-29 cells, respectively, over a period of up to 6 h. Under these conditions, a small additional peak, amounting to <5.5% of the respective initial peak area of the intact peptide, was detected by HPLC at 220 nm for both the parent and the modified peptides (Figure 4B; Supporting Information, Figures S12−S15). This demonstrates that degradation of the peptides during saturation and competition binding experiments (incubation period of 2 h) is negligible.

Functional Studies with Analogues of AngII and NT(8−13). The potencies of the AngII analogues **19**−**22** (AT_1R) and the NT(8−13) derivatives **25** and **27** (NTS_1R) were investigated in comparison with the respective parent peptide in whole cell functional assays by measuring intracellular calcium (Ca^{2+}) mobilization (Figure 5). In an aequorin Ca^{2+} assay performed with CHO-hAT_1-AEQ-$G_{\alpha 16}$ cells, the AngII analogues **19**−**22** were full agonists exhibiting potencies (pEC_{50}) comparable to that of AngII (Figure 5A). Likewise, in a fura-2 Ca^{2+} assay performed with $hNTS_1$ receptor expressing HT-29 colon carcinoma cells, the NT(8−13) analogues **25** and **27** proved to be equipotent and equi-effective compared to NT(8−13) (Figure 5B).

Preparation of the Tritiated Peptides [^{3}H]21 and [^{3}H]27. The tritiated AngII derivative [^{3}H]**21** and the tritiated NT(8−13) analogue [^{3}H]**27** were prepared by treating an excess of the precursor peptides **19** and **25**, respectively, with commercially available succinimidyl [^{3}H]propionate ([^{3}H]**37**) (Figure 6A). [^{3}H]**21** and [^{3}H]**27** were obtained in high radiochemical yields and radiochemical purities (Figure 6B) and

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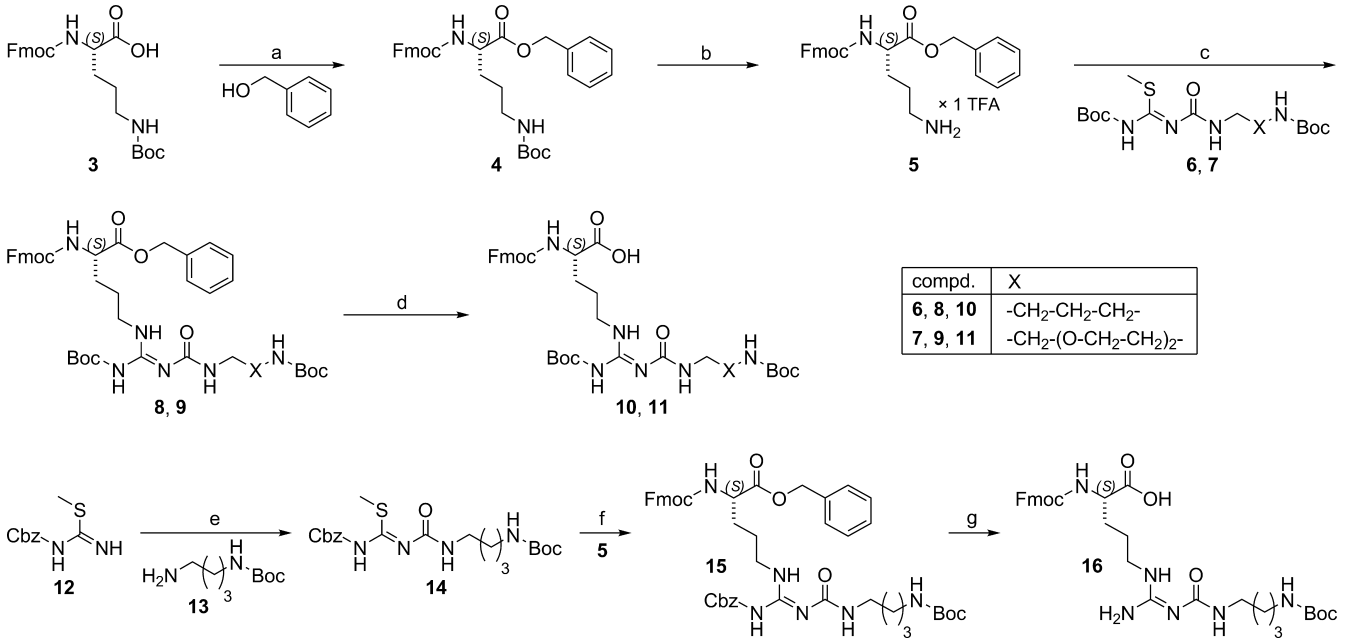
Scheme 1. Synthesis of the N^{ω}-Carbamoylated Arginine Building Blocks 10, 11, and 16[a]



[a]Reagents and conditions: (a) DCC, DMAP, CH_2Cl_2, 0 °C to rt, 20 h, 94%; (b) TFA/CH_2Cl_2 (1:5 v/v), rt, 3 h, 98%; (c) $HgCl_2$, DIPEA, rt, 1.5 h, 63% (**8**), 76% (**9**); (d) 10% Pd/C, H_2, 2-propanol, rt, 3−4.5 h, 76% (**10**), 81% (**11**); (e) triphosgene, DIPEA, CH_2Cl_2, 34%; (f) $HgCl_2$, DIPEA, rt, 3 h, 67%; (g) 10% Pd/C, H_2, MeOH, rt, 60 min, 46%.

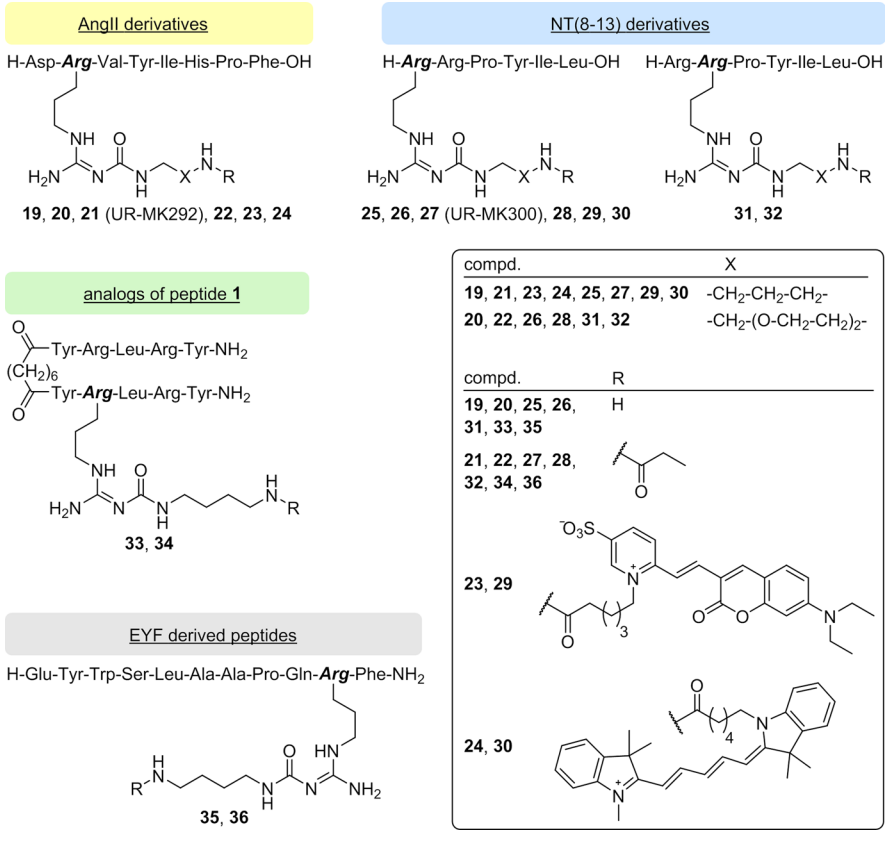


Figure 3. Structures of the synthesized analogues of AngII, NT(8−13), **1**, and EYF, containing N^{ω}-substituted arginines (peptides **19**−**36**).

proved to be stable upon storage at −20 °C (Supporting Information, Figure S21).

Receptor Binding Studies with the Radiolabeled Peptides [^{3}H]21 and [^{3}H]27. Saturation binding studies with the AngII analogue [^{3}H]**21** at live CHO cells expressing the human AT_1R revealed a dissociation constant (K_d) of 0.94 nM (Figure 7A, Table 1A). The number of specific binding sites per cell amounted to approximately 20,000 and was stable over at

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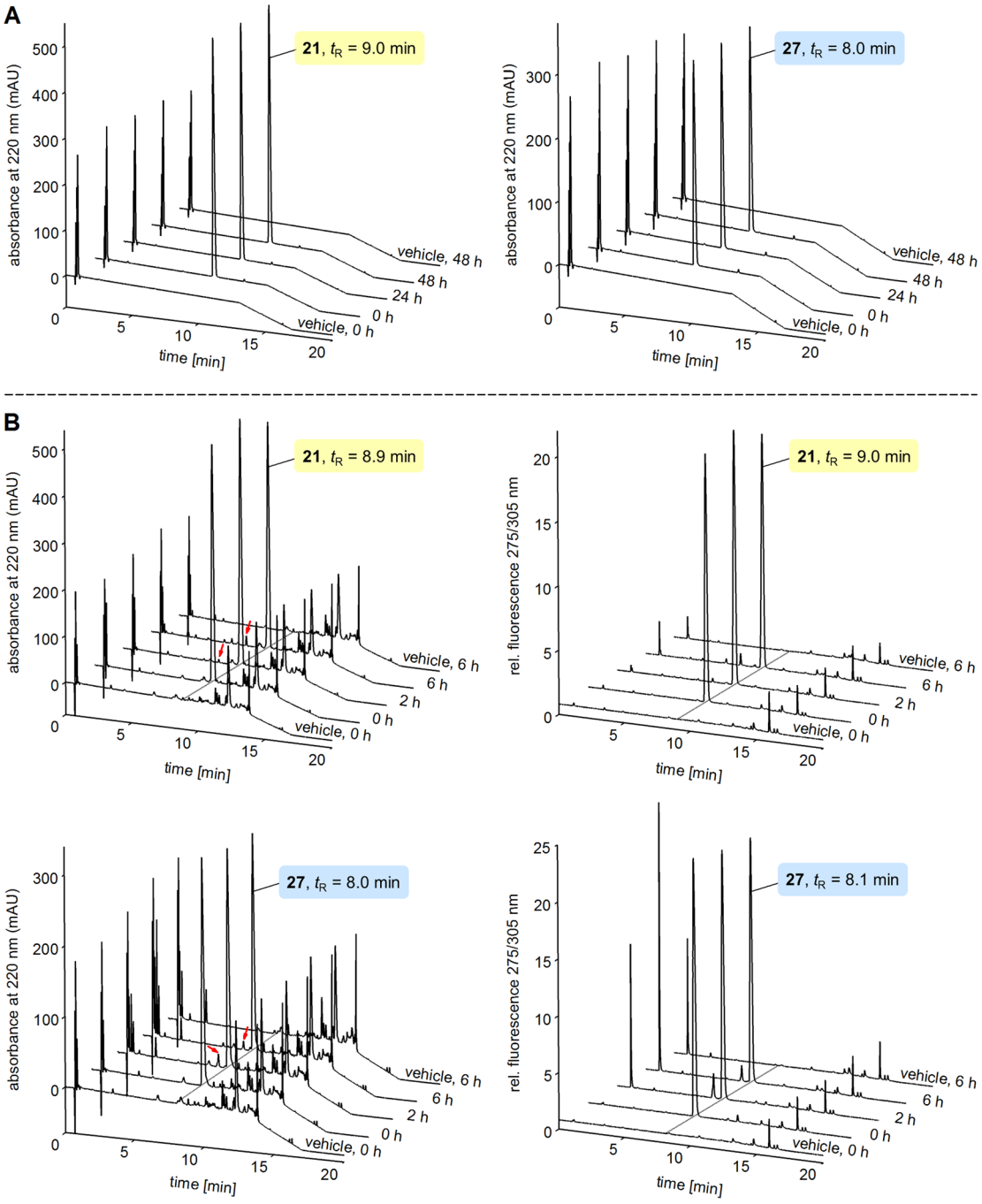

Figure 4. Chemical stability of the AngII derivative **21** and the NT(8−13) derivative **27** investigated in PBS pH 7.0 at 21 °C (A) and under assay conditions in the presence of CHO-hAT$_1$ and HT-29 cells, respectively (B). Shown are the chromatograms of the reversed-phase HPLC analysis after incubation for 48 h (A) or 6 h (B). A small additional peak (marked with red arrow; t_R 7.3 min in case of **21** and 7.4 min in case of **27**), corresponding to <2% and <4% of the respective initial peak areas of **21** and **27**, respectively, was detected (220 nm) after 2 and 6 h of incubation.

least 45 passages. The K_d value of [^{3}H]**21** determined at rat mesangial cells natively expressing the AT$_1$ receptor subtype, amounted to 1.5 ± 0.12 nM (mean ± SEM from five independent experiments) with a B_{max} corresponding to approximately 25,000 sites per cell (a representative saturation isotherm is shown in the Supporting Information, Figure S25A).

Saturation binding studies with the tritiated NT(8−13) analogue [^{3}H]**27** were performed at human HT-29 colon carcinoma cells natively expressing the NTS$_1$ receptor subtype (Figure 7A) and at CHO cells stably transfected with the human NTS$_1$ receptor gene (Supporting Information, Figure S26), revealing high NTS$_1$R affinity with K_d values of 0.51 and 0.58 nM, respectively (Table 1B). The numbers of receptors per cell were approximately 45,000 and 300,000, respectively. To investigate if the NTS$_2$ receptor subtype is expressed by HT-29 cells, too, saturation binding of the nonselective radioligand [^{3}H]**27** (K_i of the "cold" form (**27**) at the NTS$_2$R: 2.5 nM, Table 1B) was performed in the presence of an excess of the highly selective NTS$_2$R ligand **45**[23] (structure, see Supporting Information, Figure S29). The B_{max} was the same in the presence and in the absence of **45** (Figure 7A), suggesting, in contrast to a report in the literature,[55] that the NTS$_2$ receptor is not expressed by

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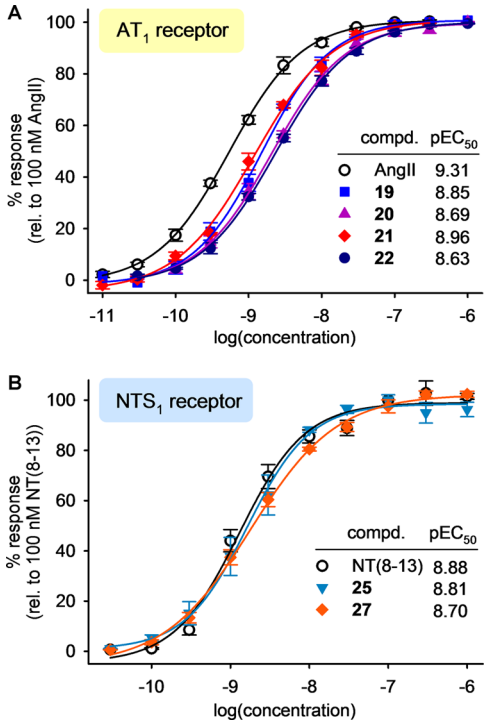

Figure 5. Agonistic effect of the AngII derivatives **19**–**22** and the NT(8–13) derivatives **25** and **27** on the mobilization of intracellular Ca^{2+}. (A) Concentration–response curves (CRCs) of AngII and **19**–**22** from an aequorin assay on CHO-hAT_1-AEQ-$G_{\alpha 16}$ cells. Mean values ± SEM from ≥2 independent experiments (performed in triplicate). (B) CRCs of NT(8–13), **25**, and **27** from a fura-2 assay on HT-29 colon carcinoma cells. Mean values ± SEM from ≥3 independent experiments (performed in singlet).

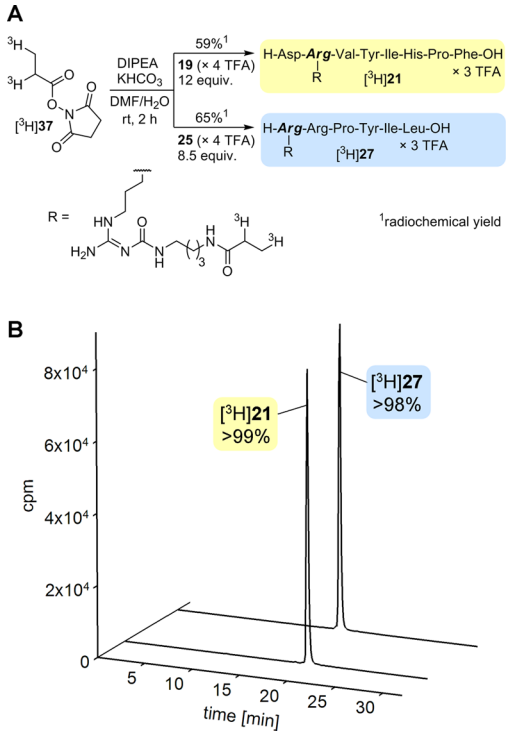

Figure 6. Preparation and HPLC analysis of the tritiated peptides [^{3}H]**21** and [^{3}H]**27**. (A) [^{3}H]propionylation of the precursor peptides **19** and **25**, yielding the radiolabeled peptides [^{3}H]**21** and [^{3}H]**27**. (B) Radiochemical purity of [^{3}H]**21** and [^{3}H]**27** determined by RP-HPLC analysis using radiometric detection (column: Luna C18(2), 3 μm, 150 mm × 4.6 mm; gradient: 0–20 min, acetonitrile + 0.04% TFA/0.05% aq TFA 14:86–30:70; 20–25 min, 30:70–95:5; 25–32 min, 95:5) (see Experimental Section). Retention times: 19.4 and 17.4 min, respectively.

HT-29 cells. This is supported by Western blot analysis as shown in Figure 8. Unspecific binding of both radioligands, [^{3}H]**21** and [^{3}H]**27**, was very low, that is, <10% of total binding at concentrations corresponding to the K_d values (Figure 7A, Supporting Information, Figures S25A and S26).

Receptor association and dissociation kinetics of [^{3}H]**21** and [^{3}H]**27** were determined at CHO-hAT_1-AEQ-$G_{\alpha 16}$ and HT-29 cells, respectively (results shown in the Supporting Information, Figures S23, S24, and Tables S2, S3). Specific binding of [^{3}H]**21** and [^{3}H]**27** was reversible, although mild acid treatment (acid stripping), which is considered as the method of choice to investigate internalization of peptidic membrane receptor agonists,[56–58] revealed a high degree of internalization of the radioligands (Supporting Information, Figures S27 and S28). This phenomenon can be explained by receptor recycling and ligand externalization as previously described for the AT_1R and [^{125}I]AngII.[59] The suitability of [^{3}H]**27** for the labeling of neurotensin receptors in tissues was demonstrated by autoradiography with sections of a HT-29 tumor xenograft (Figure 7C).

Competition Binding Studies at AT_1, NTS_1, NTS_2, Y_4, and $NPFF_2$ Receptors. K_i values of the new peptide analogues **19**–**36** were determined by competition binding at intact cells (AT_1R, NTS_1R, Y_4R), cell membranes ($NPFF_2R$), or cell homogenates (NTS_2R) using the radioligands [^{3}H]**21** (AT_1R), [^{3}H]**27** (NTS_1R), [^{3}H]NT(8–13)[60] (NTS_2R), and [^{3}H]EYF[50] ($NPFF_2R$) or the fluorescently labeled peptide S0586-[K^4]hPP[61] (Y_4R). All AngII analogues (**19**–**24**) and all NT(8–13) derivatives (**25**–**32**) exhibited high AT_1R and NTS_1R affinities, respectively, with K_i values in the picomolar or one-digit nanomolar range (Table 1A,B). Competition binding curves of the peptides **19**, **21**, **23**, **25**, **27**, **29**, and **32** are shown in Figure 7B. The K_i values of the AngII derivative **21** and NT(8–13) analogue **27** were in excellent agreement with the K_d values of their tritiated analogues [^{3}H]**21** and [^{3}H]**27**, respectively (Table 1A,B). Binding data of reported AT_1 and NTS_1 receptor ligands (AT_1: AngII, candesartan, and losartan; NTS_1: NT(8–13) and **46**[62] (SR142948A, structure see Supporting Information, Figure S29), determined with [^{3}H]**21** or [^{3}H]**27**, were in good accordance with reported data (see Table 1A,B). In addition to NTS_1 receptor binding, affinities of the NT(8–13) analogues **25** and **27** were determined at the NTS_2 receptor subtype. K_i values were comparable to that of the parent peptide NT(8–13) (Table 1B). The Y_4R ligand **1** and its N^{ω}-carbamoylated derivatives **33** and **34** showed comparable affinities with K_i values in the range of 1.5–7.4 nM (Table 1C). The N^{ω}-carbamoylated EYF analogues **35** and **36** exhibited K_i values of 27 and 41 nM, respectively (Table 1D).

3. DISCUSSION

The concept of bioisosteric replacement of the guanidine group in arginine by a carbamoylguanidine moiety was applied to obtain amino-functionalized N^{ω}-carbamoylated arginine (Figure 1A), enabling radio and fluorescence labeling of peptides, as demonstrated for AngII, NT(8–13), peptide **1**, and EYF, ligands of AT_1, NTS_1, NTS_2, NPY Y_4, and $NPFF_2$ receptors:

AngII Derivatives. The octapeptide AngII contains one arginine, which is essential for AT_1 receptor binding of AngII, as

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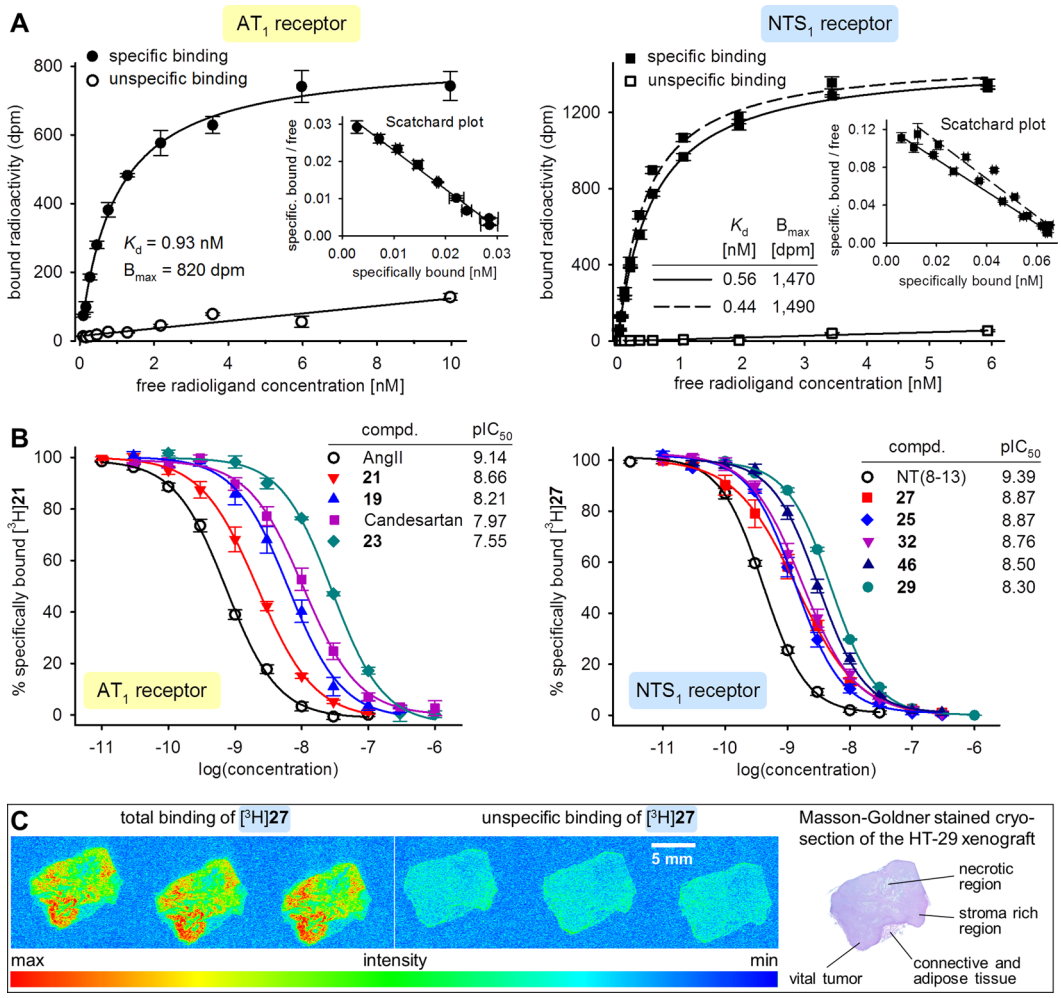

Figure 7. Binding and displacement of the radioligands [^{3}H]**21** and [^{3}H]**27** at the AT$_1$R or the NTS$_1$R. (A) Saturation binding with [^{3}H]**21** at CHO-hAT$_1$-AEQ-G$_{\alpha 16}$ cells and with [^{3}H]**27** at HT-29 cells: saturation isotherm, unspecific binding, and Scatchard transformation. Error bars of "specific binding" and error bars in the Scatchard plot represent propagated errors calculated according to the Gaussian law of errors. Data of "unspecific binding" represent the mean ± SEM (n = 3). Dashed line: experiment performed in the presence of the selective NTS$_2$R ligand **45**[23] (structure, see Supporting Information, Figure S29) (200-fold excess compared to [^{3}H]**27**). (B) Displacement of [^{3}H]**21** (c = 2 nM) by AngII, **19**, **21**, **23**, and candesartan (structure see Supporting Information, Figure S29) at the hAT$_1$ receptor, and displacement of [^{3}H]**27** (c = 1 nM) by NT(8−13), **25**, **27**, **29**, **32**, and **46** (structure, see Supporting Information, Figure S29) at the NTS$_1$R. Mean values ± SEM from ≥3 independent experiments (performed in triplicate). (C) Autoradiography of a HT-29 colon carcinoma xenograft (first in vivo passage) using the tritiated NTS$_1$/NTS$_2$ receptor ligand [^{3}H]**27**. For histological micrographs at higher magnification, see Supporting Information, Figure S30.

becomes obvious from the AT$_1$R affinities of [DesAsp^1]AngII (AngIII) and [DesAsp^1,desArg^2]AngII (AngIV): whereas AngIII exhibits almost the same AT$_1$R affinity as AngII, AngIV, devoid of arginine, is by ca. 3 orders of magnitude less potent than AngII.[66] The replacement of arginine in AngII by a carbamoylated arginine bearing an amino group (**19**, **20**), a propionamide moiety (**21**, **22**), or bulky fluorophores (**23**, **24**) was well tolerated, resulting in derivatives with high AT$_1$R affinities (K_i of **19**−**24**: 0.71 to 13 nM) and high agonistic potencies (EC$_{50}$ of **19**−**22**: 1.1 to 2.4 nM). The radiolabeled AngII analogue [^{3}H]**21**, characterized by saturation binding studies and kinetic binding experiments, represents a valuable high affinity molecular tool (K_d 0.94 nM (human AT$_1$R), 1.5 nM (rat AT$_1$R)), which can be economically prepared from the precursor peptide **19** and commercially available succinimidyl [^{3}H]propionate.

NT(8−13) Analogues. In NT(8−13), both Arg^8 and Arg^9 contribute to receptor binding as demonstrated by alanine replacement.[23,67] This is in accordance with X-ray data of the rat NTS$_1$R in complex with NT(8−13), proving the interaction of both arginines with acidic amino acids of the receptor protein (Supporting Information, Figure S31).[68,69] Remarkably, in the present study, replacement of either arginine in NT(8−13) by a N^{ω}-carbamoylated arginine afforded derivatives with high NTS$_1$R affinities, irrespective of a conjugation to space filling fluorophores (K_i of **25**−**32** (HT-29 cells): 0.26 to 4.1 nM). The NT(8−13) derivative [^{3}H]**27**, conveniently prepared from precursor peptide **25**, represents a high affinity radioligand (K_d (NTS$_1$R, HT-29 cells) 0.51 nM) to study the expression of neurotensin receptors in cells and tissues and to determine binding constants by competition binding. The high affinity fluorescently labeled NT(8−13) derivatives **29** and **30** (K_i (NTS$_1$R, HT-29 cells) 1.7 and 4.1 nM, respectively) can be used, e.g., in flow cytometric binding studies or to investigate receptor trafficking.

Analogues of the NPY Y$_4$ Receptor Ligand 1. In case of the homodimeric peptidic ligand **1**, one of the four arginine residues was replaced. The carbamoylated analogues of **1**, the amine **33**, and the propionamide **34** had Y$_4$R affinities

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Table 1. Receptor Binding Data (K_i) of the Parent Peptides AngII, NT(8−13), 1, EYF, and Their Analogues Containing a N^{ω}-Substituted Arginine (19−36), and K_d Values of the Radioligands [^{3}H]21 and [^{3}H]27 As Well As AT_1 and NTS_1 Receptor Agonist Potencies (EC_{50}) of Selected Peptides

(A)

	hAT$_1$ receptor		
	EC_{50} [nM][a]	K_i [nM][b]	K_d [nM]
AngII	0.52 ± 0.024	0.24 ± 0.019 (lit. K_i 0.16 nM[63])	
19	1.4 ± 0.18	2.1 ± 0.33	
20	2.1 ± 0.31	2.8 ± 0.13	
21	1.1 ± 0.21	0.71 ± 0.053	
[^{3}H]**21**			0.94 ± 0.021[c]
22	2.4 ± 0.18	3.1 ± 0.14	
23	nd	9.0 ± 0.040	
24	nd	13 ± 0.57	
Candesartan		3.5 ± 0.48 (lit. IC_{50} 0.69 nM[57])	
Losartan		12 ± 0.50 (lit. K_i 17 nM[64])	

(B)

	hNTS$_1$ receptor				hNTS$_2$R receptor
	EC_{50} [nM][d]	K_i [nM][e]	K_i [nM][f]	K_d [nM]	K_i [nM][g]
NT(8−13)	1.4 ± 0.25	0.14 ± 0.010 (lit. K_i 0.24 nM[23])			1.2 ± 0.17
25	1.7 ± 0.51	0.47 ± 0.049	0.69 ± 0.052		0.74 ± 0.040
26	nd	0.26 ± 0.010			
27	2.0 ± 0.23	0.47 ± 0.085	0.63 ± 0.030		2.5 ± 0.15
[^{3}H]**27**				0.51 ± 0.040[h] 0.58 ± 0.069[i]	nd
28	nd	0.41 ± 0.047			nd
29	nd	1.7 ± 0.031			nd
30	nd	4.1 ± 0.17	7.9 ± 1.7		
31	nd	0.36 ± 0.077			nd
32	nd	0.59 ± 0.092	1.7 ± 0.32		nd
46		1.1 ± 0.096 (lit. K_i 1.0 nM[65])			nd

(C)

	hY$_4$ receptor
	K_i [nM][j]
hPP	0.50 ± 0.068
1	7.4 ± 2.4
33	1.5 ± 0.14
34	3.6 ± 0.28

(D)

	hNPFF$_2$ receptor
	K_i [nM][k]
hNPFF	1.2 ± 0.42
EYF	0.53 ± 0.18
[^{3}H]EYF[50]	(lit. K_d 0.54 nM[50])
35	27 ± 7.5
36	41 ± 1.5

[a]Half-maximally effective concentration determined in an aequorin Ca^{2+} assay on CHO-hAT$_1$-AEQ-G$_{\alpha 16}$ cells. Mean ± SEM ($n \geq 2$). [b]Dissociation constant determined by competition binding with [^{3}H]**21** (c = 2 nM) at CHO-hAT$_1$-AEQ-G$_{\alpha 16}$ cells. Mean ± SEM ($n \geq 3$). [c]Dissociation constant determined by saturation binding at CHO-hAT$_1$-AEQ-G$_{\alpha 16}$ cells. Mean ± SEM (n = 4). [d]Determined in a fura-2 Ca^{2+} assay at human HT-29 colon carcinoma cells. Mean ± SEM ($n \geq 3$). [e]Determined by competition binding with [^{3}H]**27** (c = 1 nM) at HT-29 cells. Mean ± SEM ($n \geq 3$). [f]Determined by competition binding with [^{3}H]**27** (c = 1 nM) at CHO-hNTS$_1$ cells. Mean ± SEM ($n \geq 5$). [g]Determined by competition binding with [^{3}H]NT(8−13) (c = 0.5 nM) at HEK-hNTS$_2$ cell homogenates. Mean ± SEM ($n \geq 2$). [h]Determined by saturation binding at HT-29 cells. Mean ± SEM (n = 4). [i]Determined by saturation binding at CHO-hNTS$_1$ cells. Mean ± SEM (n = 4). [j]Determined by competition binding with the fluorescent Y$_4$R ligand S0586-[K^4]hPP (c = 10 nM) at CHO-hY$_4$-G$_{qi5}$-mtAEQ cells. Mean ± SEM ($n \geq 2$). [k]Determined by competition binding with [^{3}H]EYF (c = 0.35 nM) at CHO-hNPFF$_2$ cell membranes. Mean ± SEM (n = 2). n represents the number of independent experiments

comparable to that of the parent peptide **1** (K_i (Y$_4$R) 7.4 nM (**1**), 1.5 nM (**33**), and 3.6 nM (**34**)). Peptide **34** represents a potential radioligand, which can be prepared by [^{3}H]propionylation from the precursor peptide **33** by analogy with the protocols for the synthesis of [^{3}H]**21** and [^{3}H]**27**.

EYF Derivatives. The undecapeptide EYF, containing one arginine in position 10, close to the C-terminus, is an analogue of the RFamide peptide NPFF with a preference for the NPFF$_2$ receptor.[26,50] In these peptides, the arginine residue is of utmost relevance, as becomes obvious from the replacement by alanine, resulting in a more than 10,000-fold decrease in receptor affinity.[70] N^{ω}-carbamoylation of the arginine in EYF (K_i (NPFF$_2$R) 0.53 nM) resulted in a by far less pronounced decrease in affinity (K_i (NPFF$_2$R) 27 nM (**35**), 41 nM (**36**)). However, unlike the retained or even improved binding data of the modified angiotensin, neurotensin, and NPY receptor ligands, the reduced NPFF$_2$R binding affinities of **35** and **36** compared to the parent peptide recall that the success of the presented approach, as for any labeling strategy, depends on the peculiarities of the individual ligand−receptor interactions. Such differences between structure−activity relationships may be exploited by the arginine-carbamoylarginine replacement with respect to increasing receptor subtype selectivity and specificity, as recently demonstrated for the discrimination of nonpeptide argininamide-type antagonists between NPY Y$_1$ and NPFF receptors.[71]

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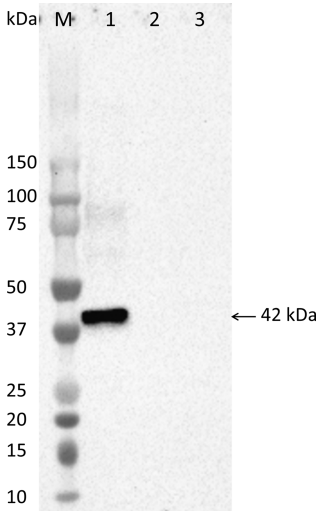

Figure 8. Immunoblot analysis of homogenates (12 μg protein) of HEK-hNTS$_2$ (1), CHO-hNTS$_1$ (2), and HT-29 colon carcinoma (3) cells using a polyclonal NTS$_2$R specific antibody. An immunopositive band was only detected in case of the homogenate of the NTS$_2$ receptor expressing control cells (1). M = molecular weight standard.

The incorporation of amino-functionalized N^{ω}-carbamoylated arginines into peptides containing arginine either close to the N- or the C-terminus (Figures 1B and 3) was conveniently achieved by standard Fmoc SPPS using appropriately protected amino-functionalized arginine building blocks (**10** and **11**). In contrast to both nonpeptidic[42,47−49] and peptidic (Supporting Information, Figures S17 and S18) receptor ligands containing an acylguanidine moiety, the N^{ω}-carbamoylated peptides presented in this study proved to be stable in buffer and in the presence of cells (Figure 4 and Supporting Information, Figures S3−S15). Therefore, misinterpretation of binding and functional assays due to the release of the bioactive parent compounds can be precluded.

We demonstrated that the synthesized (precursor) peptides, bearing a primary amine functionality at the modified arginine side chain, can be conjugated and labeled in a versatile manner by analogy with the procedures established for lysine. Notably, whereas acylation of the ε-amino group in lysine is accompanied by loss of basicity, the strong basicity of the guanidine group in arginine is reduced but not abolished by N^{ω}-carbamoylation (pK_a value of the carbamoylguanidine moiety: 7−8).[72] This guarantees a sufficient portion of the carbamoylguanidine group to be positively charged at physiological pH, a prerequisite for retained affinity, as arginine residues of biologically active peptides typically interact with acidic amino acids of the receptor.

The high receptor affinities of the fluorescently labeled analogues of AngII (**23**, **24**) and NT(8−13) (**29**, **30**) revealed that even the attachment of bulky moieties to arginine is tolerated. This can be explained by the fact that acidic residues of peptide-binding receptors, preferentially interacting with arginine residues of the peptide, are mainly located in regions of the receptor facing the extracellular space. This assumption is supported by the crystal structures of the rat NTS$_1$[68,69] and the human AT$_1$ receptor[73] (Supporting Information, Figures S31 and S32) by a homology model of the NPY Y$_4$ receptor (Supporting Information, Figure S33) as well as reported models of the AngII AT$_2$, apelin, bradykinin B$_1$ and B$_2$, bombesin BB$_1$ and BB$_2$, melanocortin MC$_3$, neurotensin NTS$_2$, NPFF$_1$, NPFF$_2$, NPY Y$_1$ and Y$_2$, and the vasopressin V$_1$ and V$_2$ receptors (see *GPCRDB*[74]).

4. CONCLUSION

The presented concept harbors a high potential for the development of peptide-based pharmacological tools and is of interest for the development of radiopharmaceuticals, as numerous peptide-binding receptors have been suggested as biomarkers and targets for cancer diagnosis and treatment, respectively.[4,7,9,13,15,75−77]

5. EXPERIMENTAL SECTION

Materials. If not otherwise stated, solvents, reagents, and buffer components were of analytical grade purchased from commercial suppliers. Technical grade solvents (ethyl acetate, light petroleum (40−60 °C), and dichloromethane) were distilled before use. Acetonitrile and MeOH for HPLC (gradient grade) were obtained from Merck (Darmstadt, Germany). 2-Propanol (HPLC grade) for hydrogenations was obtained from Carl Roth (Karlsruhe, Germany). *N*,*N*-Diisopropylethylamine (99%) was from ABCR (Karlsruhe, Germany). Anhydrous DMF over molecular sieve (>99.8%), trifluoroacetic acid (ReagentPlus, 99%), phenol, triisopropylsilane (99%), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (>99%), Triton X-100, amine **13**, the fluorescent dye Fluorescence Red Mega 480 succinimidyl ester (**38**), and suberic acid monomethyl ester (**42**) were purchased from Sigma-Aldrich (Deisenhofen, Germany). HBTU, PyBOP, and Fmoc-Orn(Boc)-OH (**3**) were from Iris Biotech (Marktredwitz, Germany). The succinimidyl ester **39** (S2197) of the fluorescent dye S0223 was purchased from FEW Chemicals (Wolfen, Germany). HOBt hydrate, NMP (for peptide synthesis, nitrogen flushed), and DMF (for peptide synthesis, packed under nitrogen, code D/3848/PB17) were obtained from Acros Organics/Fisher Scientific (Nidderau, Germany). Bovine serum albumin (BSA) was purchased from Serva (Heidelberg, Germany). Fura-2 AM and Pluronic F-127 were obtained from Calbiochem/Merck Biosciences (Beeston, UK). AngII was obtained from Bachem (Bubendorf, Switzerland). Coelenterazin h was obtained from Biotrend (Cologne, Germany). Human pancreatic polypeptide (hPP),[78] compounds **6**,[42] **7**,[54] and *S*-methylisothiourea hydroiodide[43] were prepared according to previously reported procedures. The syntheses of NT(8−13),[60] S0586-[K^4]hPP,[61] and compound **41**[41] were described previously. EYF was prepared by SPPS according to the general procedure (see below). Millipore water was used throughout for the preparation of buffers and HPLC eluents. The 1.5 or 2 mL polypropylene reaction vessels with screw cap from Süd-Laborbedarf (Gauting, Germany) (in the following referred to as "reaction vessel with screw cap") were used for the preparation and storage of stock solutions, for small scale reactions (e.g., the synthesis of radiolabeled and fluorescently labeled peptides) and for the investigation of chemical stabilities. The 1.5 or 2 mL polypropylene reaction vessels from Sarstedt (Nümbrecht, Germany) (in the following referred to as "reaction vessel") were used to dilute compounds, e.g., for assays and HPLC analyses.

General Experimental Conditions. Thin layer chromatography was performed on Merck silica gel 60 F$_{254}$ TLC aluminum plates. If not otherwise stated Silica Gel 60 (40−63 μm, Merck) was used for column chromatography. In the case of the purification of acid sensitive compounds (Boc protecting group (except for **10**, **11** and **16**)), the stationary phase was conditioned with the respective solvent containing 0.2% triethylamine. Melting points were determined with a Büchi 510 apparatus (Büchi, Essen, Germany) and are uncorrected. Specific optical rotations at 589 nm (Na-D line) were measured on a polarimeter P8000-T equipped with an electronic Peltier thermostat PT31 (A. KRÜSS Optronic, Hamburg, Germany) using a microcuvette (layer thickness, 100 mm; volume, 1 mL; thermostat-controlled at 20 °C). IR spectra were measured with a NICOLET 380 FT-IR spectrophotometer (Thermo Electron Corporation). NMR spectra were recorded on a Bruker Avance 300 instrument (7.05 T, ^{1}H 300 MHz; ^{13}C 75 MHz), a Bruker Avance 400 instrument (9.40 T, ^{1}H 400 MHz; ^{13}C 100 MHz) or

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a Bruker Avance 600 instrument with cryogenic probe (14.1 T, ^{1}H 600 MHz; ^{13}C 150 MHz) (Bruker, Karlsruhe, Germany). Elemental analysis was performed with a Vario MICRO Cube elemental analyzer (Elementar Analysensysteme, Hanau, Germany). Low-resolution mass spectrometry (MS) was performed on a Finnigan ThermoQuest TSQ 7000 instrument (Thermo Finnigan, San Jose, CA) equipped with an electrospray ionization (ESI) source. High-resolution mass spectrometry (HRMS) analysis was performed on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) using an ESI source. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Gemini-NX C18, 5 μm, 250 mm × 21 mm (Phenomenex, Aschaffenburg, Germany), a Kinetex-XB C18, 5 μm, 250 mm × 21 mm (Phenomenex), or a YMC-Actus Triart C8, 5 μm, 250 mm × 20 mm (YMC Europe, Dinslaken, Germany) served as RP-columns at flow rates of 22 (Gemini-NX) or 20 mL/min (Kinetex-XB, YMC-Actus Triart). Mixtures of 0.1% aqueous TFA and acetonitrile (or MeOH) were used as mobile phase, and a detection wavelength of 220 nm was used throughout. The solvent from collected fractions was removed by lyophilization using a Christ alpha 2−4 LD lyophilization apparatus equipped with a vacuubrand RZ 6 rotary vane vacuum pump. Analytical HPLC analysis of compounds **10**, **11**, **16**, **19**−**32**, **35**, **36**, **44**, and EYF (concentrations between 70 and 120 μM) was performed with a system from Agilent Technologies composed of a 1290 Infinity binary pump equipped with a degasser, a 1290 Infinity autosampler, a 1290 Infinity thermostated column compartment, a 1260 Infinity diode array detector, and a 1260 Infinity fluorescence detector. A Kinetex-XB C18, 2.6 μm, 100 mm × 3 mm (Phenomenex), served as stationary phase at a flow rate of 0.6 mL/min. Mixtures of acetonitrile (A) and 0.04% aq TFA (B) were used as mobile phase. The following linear gradients were applied. Compounds **10**, **11**, and **16**: 0−20 min, A/B 20:80−95:5; 20−25 min, 95:5. Compounds **19**−**22**, **25**−**28**, **31**, **32**, **35**, **36**, and **44**: 0−12 min, A/B 10:90−30:70; 12−16 min, 30:70−95:5; 16−20 min, 95:5. Compounds **23**, **24**, **29**, and **30**: 0−15 min, A/B 15:85−42:58; 15−18 min, 42:58−95:5; 18−23 min, 95:5. The oven temperature was 25 °C, and the injection volume was 20 μL. Detection was performed at 220 nm throughout and additionally at 500 nm (**23**, **29**) or 630 nm (**24**, **30**). Analytical HPLC analysis of compounds **1**, **33**, and **34** (concentration: 100 μM) was performed on a system from Merck-Hitachi composed of a L-6200-A pump, an AS-2000A autosampler, a L-4000A UV detector, and a D-6000 interface. A Kinetex-XB C18, 5 μm, 250 mm × 4.6 mm (Phenomenex) served as stationary phase at a flow rate of 0.8 mL/min. Mixtures of acetonitrile (A) and 0.1% aq TFA (B) were used as mobile phase. The following linear gradient was applied: 0−25 min, A/B 10:90−40:60; 25−35 min, 60:40−95:5; 35−45 min, 95:5. Detection was performed at 220 nm, the oven temperature was 30 °C, and the injection volume was 50 μL.

Compound Characterization. New synthetic organic compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy, HRMS, optical rotation (if applicable), and melting point (if applicable). In addition, the key compounds **10**, **11**, and **16** were characterized by 2D-NMR spectroscopy (^{1}H-COSY, HSQC, HMBC), RP-HPLC analysis, IR spectroscopy, and elemental (combustion) analysis. New peptides (fully deprotected), obtained after SPPS and cleavage from the solid support, were characterized by ^{1}H and ^{13}C NMR, 2D-NMR (^{1}H-COSY, HSQC, HMBC, TOCSY), HRMS, and RP-HPLC analysis. Propionylated derivatives of these peptides were characterized by ^{1}H NMR, HRMS, and RP-HPLC analysis. Fluorescently labeled derivatives were characterized by HRMS and RP-HPLC analysis.

The purity of final compounds, determined by RP-HPLC (220 nm), was ≥96% throughout. Elemental analysis data (**10**, **11**, and **16**) fulfilled the requirement of less than ±0.4% deviation.

General Procedure for SPPS. Peptides were synthesized by manual SPPS applying the Fmoc strategy. First, 5 mL BD Discardit II syringes (Becton Dickinson, Heidelberg, Germany) equipped with a 35 μm polyethylene frit (Roland Vetter Laborbedarf OHG, Ammerbuch, Germany) served as reaction vessels. Protected standard L-amino acids (Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, and Fmoc-Val-OH) and the solid supports Fmoc-Rink amide AM polystyrene (PS) resin, H-Phe-2-ClTrt PS resin, and H-Leu-2-ClTrt PS resin were purchased from Merck Chemicals (Schwalbach am Taunus, Germany). The Sieber PS resin was obtained from Iris Biotech. DMF/NMP (8:2 v/v) was used as solvent throughout (ca. 2.2 mL per 1 mmol Fmoc-aa). Standard amino acids (5-fold excess) were preactivated with HBTU/HOBt/DIPEA (4.9/5/10 equiv) for 5 min and added to the resin. The vessel was shaken at rt for 60 min. The coupling procedure was repeated in case of standard amino acids. The coupling of the arginine building blocks **10** and **11** (3-fold excess, preactivated with HBTU/HOBt/DIPEA (2.95/3/6 equiv) for 5 min) was performed at 35 °C for 16 h ("single" coupling) using anhydrous DMF. After completed coupling of a Fmoc-aa the resin was washed with solvent (4×) followed by Fmoc deprotection with 20% piperidine in DMF/NMP (8:2 v/v) (rt, 2 × 10 min; after incorporation of **10** or **11**, 2 × 5 min) and thorough washing of the resin with solvent (6×). After the last coupling and Fmoc deprotection step, the resin was washed with solvent (4×) and CH_2Cl_2 (6×) (in case of 2-ClTrt resins CH_2Cl_2 was treated with K_2CO_3). Peptides synthesized at the Rink aminde AM resin (**17**, **35**) were cleaved off with TFA/water (95:5 v/v) at rt for 2 h (**17**) or with TFA/phenolum liquefactum/water/triisopropylsilane (95:2.4:1.6:1 v/v/v/v) at rt for 2.5 h (**35**). The resin was separated by filtration and washed three times with MeCN/water (40:60 v/v) (2 mL each). All filtrates were collected and combined in a round-bottom flask containing water (120 mL), and the mixture was lyophilized to obtain the crude peptide, which was purified by preparative HPLC. Peptides synthesized at the 2-ClTrt resin (**19**, **20**, **25**, **26**, **31**) were cleaved off with CH_2Cl_2/HFIP (3:1 v/v) (rt, 2 × 20 min). The resin was separated by filtration and washed once with CH_2Cl_2/HFIP (3:1) (1 mL). The filtrates were combined, the volatiles were evaporated and the residue taken up in CH_2Cl_2 (20 mL), followed by evaporation of the solvent. TFA/water (95:5 v/v) (1−2 mL) was added, and the mixture was incubated at rt for 2 h for peptides without Arg(Pbf) or 3.5 h for peptides containing Arg(Pbf). Water (120 mL) was added followed by lyophilization and purification of the crude peptide by preparative HPLC. Peptides synthesized at the Sieber resin (**33**, **40**, **43**) were cleaved off with CH_2Cl_2/TFA (97:3 v/v) (rt, 10 × 6 min). The resin was removed by filtration. All filtrates were collected and combined in a round-bottom flask containing water (10 times the volume of the filtrate). The organic solvent was evaporated, and the aqueous phase was lyophilized followed by side chain deprotection (**33**) or purification by preparative HPLC (**40** and **43**).

Chemistry: Synthesis Protocols and Analytical Data of Compounds 1, 10, 11, 16, and 19−36. *Octanedioyl-bis(tyrosyl-arginyl-leucyl-arginyl-tyrosinamide) Tetrakis(hydrotrifluoroacetate) (1).* Compound **40** (20 mg, 13.3 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Compound **41** (1.96 mg, 5.3 μmol) was added, and the mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate extracted with CH_2Cl_2 (2 × 10 mL). The combined extracts were evaporated and the residue dried in vacuo. TFA/water (95:5 v/v) (2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added, followed by lyophilization. The product was purified by preparative HPLC (column, Kinetex-XB C18 250 mm × 21 mm; gradient, 0−20 min; MeCN/0.1% aq TFA 14:86−52:48 (flow rate: 15 instead 20 mL/min); t_R = 11 min). Lyophilization of the eluate afforded **1** as a white fluffy solid (5.7 mg, 51%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.83 (d, 6H, *J* = 6.5 Hz), 0.87 (d, 6H, *J* = 6.5 Hz), 1.03 (br s, 4H), 1.30 (br s, 4H), 1.37−1.56 (m, 16H), 1.56−1.73 (m, 6H), 1.95−2.05 (m, 4H), 2.57−2.65 (m, 2H), 2.68−2.75 (m, 2H), 2.81−2.89 (m, 4H), 3.02−3.13 (m, 8H), 4.17−4.24 (m, 2H), 4.24−4.36 (m, 6H), 4.39−4.46 (m, 2H), 6.59−6.65 (m, 8H), ca. 6.9 (br s, 8H), 6.95−6.99 (m, 4H), 6.99−7.03 (m, 4H), 7.05 (s, 2H), ca. 7.3 (br s, 8H), 7.36 (s, 2H), 7.49−7.57 (m, 4H), 7.74 (d, 2H, *J* = 7.9 Hz), 7.85−7.94 (m, 4H), 8.03 (d, 2H, *J* = 7.7 Hz), 8.17 (d, 2H, *J* = 7.8 Hz), 9.16 (s, 2H), 9.17 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 21.4 (2 carb), 23.1 (2 carb), 24.1 (2 carb), 24.9 (2 carb), 25.0 (2 carb), 25.1 (2 carb), 28.4 (2 carb), 28.9 (2 carb), 29.0 (2 carb), 35.2 (2 carb), 36.6 (2 carb), 36.8 (2 carb), 40.4 (4 carb), 40.7 (2 carb), 50.9 (2 carb), 52.1 (2 carb), 52.2 (2 carb), 53.9 (2 carb), 54.1 (2 carb), 114.8 (4 carb), 114.9 (4 carb), 127.5 (2 quat carb), 127.9 (2 quat carb), 129.9 (4 carb), 130.0 (4 carb),

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155.7 (2 quat carb), 155.8 (2 quat carb), 155.6 (2 quat carb), 155.7 (2 quat carb), 170.8 (2 quat carb), 171.1 (2 quat carb), 171.8 (2 quat carb), 172.0 (2 quat carb), 172.3 (2 quat carb), 172.7 (2 quat carb). HRMS (ESI): m/z $[M + 4H]^{4+}$ calcd for $[C_{80}H_{126}N_{24}O_{16}]^{4+}$ 419.7440, found 419.7452. RP-HPLC (220 nm): 98% (t_R = 20.2 min, k = 6.0). $C_{80}H_{122}N_{24}O_{16}\cdot C_8H_4F_{12}O_8$ (1676.01 + 456.09).

*N^{ω}-tert-Butoxycarbonyl-$N^{\omega'}$-[N-(4-tert-butoxycarbonylaminobutyl)-aminocarbonyl]-N^{α}-fluoren-9-ylmethoxycarbonyl-L-arginine Monohydrate (**10**).* In a two-necked 100 mL round-bottom flask, under an atmosphere of argon, a 10% Pd/C catalyst (300 mg) was added to a solution of **8** (2.05 g, 2.56 mmol) and acetic acid (8 μL) in 2-propanol (30 mL), and a slow stream of hydrogen was passed through a glass tube into the vigorously stirred suspension for 4.5 h (additional catalyst was added after 2.5 h (150 mg) and 3.5 h (100 mg)). The main part of the catalyst was removed by filtration through a pad of cotton wool, followed by a filtration using a 25 mm syringe filter (Nylon, 0.45 μm, PP housing). Water (300 mL) was added to the filtrate, and the mixture was lyophilized. The product was purified by column chromatography (Merck silica gel Geduran 60 (0.063−0.200 mm), eluent, MeCN/EtOH 40:1 to 10:1; upon appearance of the product in the eluate, the eluent was changed from MeCN/EtOH 10:1 to MeCN/EtOH/AcOH 1000:125:1). After completion of elution, the eluate was immediately diluted with water (600 mL) and lyophilized. Product **10** was obtained as a white powder (1.42 g, 75%) and stored at −20 °C. TLC (MeCN/EtOH/AcOH 100:10:1 v/v/v): R_f = 0.6. $[\alpha]_D^{20}$ (0.037 M in MeCN/H_2O 7:3 v/v). ^{1}H NMR (600 MHz, DMSO-d_6) (two major rotamers with a ratio of approximately 1:1 were evident in the ^{1}H and ^{13}C NMR spectra): δ 1.30−1.39 (m, 13H), 1.40 (s, 4.5H), 1.44 (s, 4.5H), 1.46−1.64 (m, 3H), 1.68−1.77 (m, 1H), 2.83−2.92 (m, 2H), 2.92−2.98 (m, 1H), 2.98−3.05 (m, 1H), 3.20−3.30 (m, 2H), 3.86 (br s, 1H), 4.19−4.27 (m, 2H), 4.27−4.34 (m, 1H), 6.75 (t, 0.5H, J = 5.5 Hz), 6.78 (t, 0.5H, J = 5.3 Hz), 6.96 (t, 0.5H, J = 5.6 Hz), 7.24 (br s, 1H), 7.29−7.34 (m, 2H), 7.37−7.43 (m, 2H), 7.67−7.73 (m 2H), 7.83−7.91 (m, 2.5H), 8.14 (br s, 0.5H), 9.16 (t, 0.5H, J = 5.1 Hz), 11.06 (s, 0.5H), 12.52 (s, 0.5H), 12.8 (br s, 0.5H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 25.5, 26.3, 26.86, 26.91, 27.0, 27.7, 28.1, 28.3, 29.0, 38.8, 39.0, 39.6, 39.8, 46.7, 54.3, 65.4, 77.28, 77.33, 81.9, 120.1, 125.2, 127.0, 127.6, 140.7, 143.8, 143.9, 152.4, 153.0, 154.6, 155.6, 155.8, 156.4, 162.6, 164.3, 174.3. IR (KBr): 3340, 3065, 2975, 2935, 2870, 1715, 1635, 1605, 1515, 1450, 1415, 1365, 1250, 1150 cm^{-1}. HRMS (ESI): m/z $[M + H]^+$ calcd for $[C_{36}H_{51}N_6O_9]^+$ 711.3712, found 711.3729. Anal. Calcd for $C_{36}H_{50}N_6O_9\cdot H_2O$: C 59.33, H 7.19, N 11.53. Found: C 59.14, H 6.83, N 11.70. RP-HPLC (220 nm): 99% (t_R = 10.0 min, k = 12.2). $C_{36}H_{50}N_6O_9\cdot H_2O$ (710.82 + 18.02).

*N^{ω}-tert-Butoxycarbonyl-$N^{\omega'}$-[N-(8-tert-butoxycarbonylamino-3,6-dioxaoctyl)aminocarbonyl]-N^{α}-fluoren-9-ylmethoxycarbonyl-L-arginine Monohydrate (**11**).* Compound **11** was prepared from **9** (2.32 g, 2.69 mmol) using the procedure for the preparation of **10**. Added portions of catalyst: 400 mg (at first), 200 mg (after 1 h), and 100 mg (after 2 h). The conversion was complete after 3 h. Column chromatography was performed as described for **10**. Lyophilization of the eluate afforded the product as a white solid, which contained about 40 mol % of acetic acid. For removal of the acetic acid, lyophilization was repeated three times after uptake in MeCN/water 40:60 (250 mL). Compound **11** was obtained as a white powder (1.72 g, 81%). TLC (MeCN/EtOH/AcOH 100:10:1 v/v/v): R_f = 0.5. $[\alpha]_D^{20}$ 3.1 (0.021 M in MeCN/H_2O 7:3 v/v). ^{1}H NMR (600 MHz, DMSO-d_6) (two major rotamers with a ratio of approximately 1:1 were evident in the ^{1}H and ^{13}C NMR spectra): δ 1.36 (s, 9H), 1.40 (s, 4.5H), 1.45 (s, 4.5H), 1.48−1.66 (m, 3H), 1.68−1.77 (m, 1H), 3.00−3.09 (m, 2H), 3.10−3.16 (m, 1H), 3.17−3.20 (m, 1H), 3.21−3.30 (m, 2H), 3.33−3.53 (m, 8H), 3.90−3.98 (m, 1H), 4.19−4.25 (m, 2H), 4.25−4.35 (m, 1H), 6.74 (t, 1H, J = 5.2 Hz), 6.87 (t, 0.5H, J = 5.6 Hz), 7.29−7.35 (m, 2H), 7.37−7.43 (m, 2H), 7.54 (br s, 1H), 7.69−7.75 (m 2H), 7.86−7.90 (m, 2H), 7.92 (t, 0.5H, J = 5.2 Hz), 8.28 (br s, 0.5H), 9.13 (t, 0.5H, J = 5.0 Hz), 11.06 (s, 0.5H), 12.43 (s, 0.5H), 12.6 (br s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 25.5, 25.6, 27.7, 28.1, 28.2, 28.4, 39.1, 39.6 (two carbon nuclei), 46.7, 53.8, 65.6, 68.9, 69.17, 69.20, 69.46, 69.52, 77.4, 77.6, 82.0, 120.1, 125.3, 127.1, 127.6, 140.71, 140.72, 143.8, 143.9, 152.4, 153.2, 154.7, 155.6, 156.0, 156.3, 162.5, 164.4, 174.0. IR (KBr): 3340, 3065, 2975, 2935, 2870, 1715, 1635, 1605, 1515, 1450, 1415, 1365, 1250, 1150 cm^{-1}. HRMS (ESI): m/z $[M + H]^+$ calcd for $[C_{38}H_{55}N_6O_{11}]^+$ 771.3923, found 771.3933. Anal. Calcd for $C_{38}H_{54}N_6O_{11}\cdot H_2O$: C 57.85, H 7.15, N 10.65. Found: C 58.14, H 6.94, N 10.59. RP-HPLC (220 nm): 99% (t_R = 9.8 min, k = 11.9). $C_{38}H_{54}N_6O_{11}\cdot H_2O$ (770.87 + 18.02).

*N^{ω}-[N-(4-tert-Butoxycarbonylaminobutyl)aminocarbonyl]-N^{α}-fluoren-9-ylmethoxycarbonyl-L-arginine Dihydrate (**16**).* In a two-necked round-bottom flask, under an atmosphere of argon, a 10% Pd/C catalyst (160 mg) was added to a solution of **15** (1.45 g, 1.74 mmol) in MeOH, and a slow stream of hydrogen was passed through a glass tube into the vigorously stirred suspension for 60 min. The catalyst was removed by filtration, and the solvent was evaporated. The product was purified by column chromatography (eluent, CH_2Cl_2/MeOH 20:1 to 3:1 (column packed in CH_2Cl_2/MeOH/AcOH 200:10:1)). The eluate was concentrated under reduced pressure, water (200 mL) was added, and the mixture was lyophilized. Relyophilization after uptake in MeCN/water 60:40 (100 mL) and evaporation of the major amount of MeCN at 30 °C afforded the product as a white solid (0.515 g, 46%). TLC (CH_2Cl_2/MeOH 5:1 v/v): R_f = ca. 0.4. $[\alpha]_D^{20}$ 3.4 (0.032 M in EtOH). ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.29−1.41 (m, 13H), 1.46−1.57 (m, 2H), 1.57−1.65 (m, 1H), 1.65−1.89 (m, 1H), 2.81−2.93 (m, 2H), 3.03 (br s, 2H), 3.19 (br s, 2H), 3.70−3.89 (m, 1H), 4.17−4.33 (m, 3H), 6.76 (t, 1H, J = 5.2 Hz), 7.07 (br s, 1H), 7.24−7.34 (m, 2H), 7.36−7.58 (m, 3H), 7.64−7.75 (m, 2H), 7.86−7.91 (m, 2H), 8.39 (br s, 2H), 9.45 (br s, 1H), 13.6 (br s, 1H). ^{13}C NMR (150 MHz, MeOH-d_4): δ 25.7, 27.9, 28.2, 28.8, 30.9, 40.4, 41.0, 41.8, 48.5, 57.2, 67.6, 79.8, 120.9, 126.23, 126.26, 128.1, 128.8, 142.57, 142.61, 145.2, 145.4, 155.9, 156.1, 158.3, 158.5, 180.3. IR (KBr): 3350, 2975, 2940, 1690, 1610, 1540, 1450, 1400, 1250, 1170 cm^{-1}. HRMS (ESI): m/z $[M + H]^+$ calcd for $[C_{31}H_{43}N_6O_7]^+$ 611.3188, found 611.3198. Anal. Calcd for $C_{31}H_{42}N_6O_7\cdot H_4O_2$: C 57.57, H 7.17, N 12.99. Found: C 57.78, H 6.87, N 12.82. RP-HPLC (220 nm): 99% (t_R = 7.2 min, k = 8.5). $C_{31}H_{42}N_6O_7\cdot H_4O_2$ (610.70 + 36.03).

*H-Asp-{N^{ω}-[N-(4-aminobutyl)aminocarbonyl]}Arg-Val-Tyr-Ile-His-Pro-Phe-OH Tetrakis(hydrotrifluoroacetate) (**19**).* The peptide was synthesized according to the general procedure using a H-Phe-2-ClTrt resin (loading: 0.73 mmol/g). Purification by preparative HPLC was performed with a YMC-Actus Triart C8 250 mm × 20 mm (gradient, 0−30 min; MeCN/0.1% aq TFA 10:90−35:65; t_R = 22 min), and the product fraction still containing byproduct was rechromatographed on a Gemini-NX C18 250 mm × 21 mm (gradient, 0−30 min; MeOH/0.1% aq TFA 17:83−57:43, t_R = 25 min). Lyophilization of the eluate afforded peptide **19** as a white fluffy solid (47.9 mg, 45%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.71−0.81 (m, 12H), 1.00−1.09 (m, 1H), 1.31−1.39 (m, 1H), 1.44−1.59 (m, 7H), 1.60−1.68 (m, 2H), 1.75−1.88 (m, 3H), 1.89−1.97 (m, 1H), 1.99−2.06 (m, 1H), 2.60−2.68 (m, 2H), 2.75−2.84 (m, 4H), 2.89−2.96 (m, 2H), 2.99−3.07 (m, 2H), 3.08−3.14 (m, 2H), 3.18−3.27 (m, 2H), 3.45−3.50 (m, 1H), 3.61−3.67 (m, 1H), 4.09−4.16 (m, 2H), 4.18 (dd, 1H, J = 8.6, 6.6 Hz), 4.34−4.45 (m, 3H), 4.47−4.53 (m, 1H), 4.74−4.80 (m, 1H), 6.58−6.63 (m, 2H), 6.99−7.03 (m, 2H), 7.17−7.22 (m, 1H), 7.23−7.29 (m, 4H), 7.37 (s, 1H), 7.61 (br s, 1H), 7.80 (br s, 4H), 7.85 (d, 1H, J = 8.5 Hz), 8.03 (d, 1H, J = 8.0 Hz), 8.19 (br s, 3H), 8.28 (d, 1H, J = 7.7 Hz), 8.31 (d, 1H, J = 7.5 Hz), 8.46 (br s, 2H), 8.60 (d, 1H, J = 7.8 Hz), 8.93 (s, 1H), 9.04 (br s, 1H), 9.21 (br s, 1H), 10.7 (br s, 1H), 12.9 (br s, 2H), 14.3 (br s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 10.8, 15.2, 17.7, 19.2, 24.2, 24.3, 24.4, 24.6, 26.0, 26.3, 29.0, 29.2, 30.9, 35.5, 36.5, 36.57, 36.62, 38.5, 38.6, 40.5, 46.9, 48.8, 49.6, 52.5, 53.6, 53.9, 56.7, 57.2, 59.3, 114.8, 117.1 (q, J = 299 Hz) (TFA), 117.3, 126.4, 127.7, 128.2, 128.8, 129.3, 130.0, 133.7, 137.3, 153.8, 153.9, 155.7, 158.5 (q, J = 32 Hz) (TFA), 167.8, 168.1, 170.5, 170.6, 170.8, 170.9, 171.1, 171.8, 172.6. HRMS (ESI): m/z $[M + 2H]^{2+}$ calcd for $[C_{55}H_{83}N_{15}O_{13}]^{2+}$ 580.8142, found 580.8146. RP-HPLC (220 nm): 99% (t_R = 7.3 min, k = 8.6). $C_{55}H_{81}N_{15}O_{13}\cdot C_8H_4F_{12}O_8$ (1160.3 + 456.09).

*H-Asp-{N^{ω}-[N-(8-amino-3,6-dioxaoctyl)aminocarbonyl]}Arg-Val-Tyr-Ile-His-Pro-Phe-OH Tetrakis(hydrotrifluoroacetate) (**20**).* The peptide was synthesized according to the general procedure using a H-Phe-2-ClTrt resin (loading: 0.73 mmol/g). Purification by preparative HPLC was performed with a YMC-Actus Triart C8 250 mm × 20 mm (gradient, 0−24 min; MeCN/0.1% aq TFA 10:90−30:70;

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t_R = 23 min), and the product fraction still containing byproduct was rechromatographed on a Gemini-NX C18 250 mm × 21 mm (gradient, 0−30 min; MeOH/0.1% aq TFA 17:83−57:43; t_R = 25 min). Lyophilization of the eluate afforded peptide **20** as a white fluffy solid (66.4 mg, 26%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.71−0.81 (m, 12H), 1.00−1.09 (m, 1H), 1.31−1.39 (m, 1H), 1.44−1.59 (m, 3H), 1.60−1.68 (m, 2H), 1.75−1.88 (m, 3H), 1.89−1.97 (m, 1H), 1.99−2.07 (m, 1H), 2.60−2.68 (m, 2H), 2.76−2.83 (m, 2H), 2.89−3.00 (m, 4H), 3.00−3.08 (m, 2H), 3.18−3.30 (m, 4H), 3.43−3.51 (m, 3H), 3.53−3.61 (m, 6H), 3.61−3.67 (m, 1H), 4.10−4.16 (m, 2H), 4.18 (dd, 1H, J = 8.6, 6.6 Hz), 4.34−4.40 (m, 2H), 4.40−4.45 (m, 1H), 4.47−4.53 (m, 1H), 4.74−4.80 (m, 1H), 6.58−6.63 (m, 2H), 6.98−7.03 (m, 2H), 7.17−7.22 (m, 1H), 7.23−7.28 (m, 4H), 7.37 (s, 1H), 7.48 (br s, 1H), 7.74−7.96 (m, 5H), 8.03 (d, 1H, J = 8.1 Hz), 8.19 (br s, 3H), 8.28 (d, 1H, J = 7.7 Hz), 8.31 (d, 1H, J = 7.5 Hz), 8.47 (br s, 2H), 8.60 (d, 1H, J = 7.7 Hz), 8.95 (s, 1H), 9.06 (br s, 1H), 9.21 (br s, 1H), 10.7 (br s, 1H), 12.8 (br s, 2H), 14.2 (br s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 10.8, 15.2, 17.7, 19.2, 24.2, 24.3, 24.6, 26.3, 29.0, 29.2, 30.9, 35.5, 36.5, 36.57, 36.61, 38.5, 39.1, 40.5, 46.9, 48.8, 49.6, 52.5, 53.6, 53.9, 56.7, 57.2, 59.3, 66.7, 68.7, 69.4, 69.6, 114.8, 117.0 (q, J = 299 Hz) (TFA), 117.3, 126.4, 127.7, 128.2, 128.8, 129.3, 130.0, 133.7, 137.3, 153.7, 153.9, 155.7, 158.5 (q, J = 32 Hz) (TFA), 167.8, 168.1, 170.5, 170.6, 170.8, 170.9, 171.1, 171.8, 172.6. HRMS (ESI): m/z [M + 2H]$^{2+}$ calcd for [$C_{57}H_{87}N_{15}O_{15}$]$^{2+}$ 610.8248, found 610.8254. RP-HPLC (220 nm): 99% (t_R = 7.4 min, k = 8.7). $C_{57}H_{85}N_{15}O_{15}$·$C_8H_4F_{12}O_8$ (1220.4 + 456.09).

*H-Asp-{N$^{\omega}$-[N-(4-propanoylaminobutyl)aminocarbonyl]}Arg-Val-Tyr-Ile-His-Pro-Phe-OH Tris(hydrotrifluoroacetate) (**21**).* The reaction was performed in a 2 mL reaction vessel with screw cap equipped with a magnetic microstirrer. DIPEA (7.6 mg, 58.8 μmol) and K_2CO_3 (8.4 mg, 83.9 μmol) were added to a solution of peptide **19** (11.3 mg, 6.94 μmol) in DMF/water (1:1 v/v) (400 μL), followed by the addition of succinimidyl propionate (**37**) (0.72 mg, 4.2 μmol) dissolved in DMF (40 μL) (added as 10 μL portions with a time lag of 5 min). Then 60 min after continued stirring, 10% aq TFA (100 μL) was added. The product was purified by preparative HPLC (column, Gemini-NX C18 250 mm × 21 mm; gradient, 0−35 min; MeCN/0.1% aq TFA 10:90−37:63; t_R = 23 min). Lyophilization of the eluate afforded **21** as a white fluffy solid (5.0 mg, 77%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.72−0.82 (m, 12H), 0.98 (t, 3H, J = 7.6 Hz), 1.01−1.09 (m, 1H), 1.31−1.44 (m, 5H), 1.44−1.57 (m, 3H), 1.64 (br s, 2H), 1.75−1.88 (m, 3H), 1.90−1.97 (m, 1H), 2.00−2.08 (m, 3H), 2.61−2.67 (m, 2H), 2.75−2.83 (m, 2H), 2.89−2.96 (m, 2H), 2.99−3.06 (m, 4H), 3.06−3.12 (m, 2H), 3.22 (br s, 2H), 3.43−3.49 (m, 1H), 3.61−3.68 (m, 1H), 4.08−4.17 (m, 2H), 4.19 (dd, 1H, J = 8.7, 6.6 Hz), 4.34−4.45 (m, 3H), 4.47−4.53 (m, 1H), 4.74−4.80 (m, 1H), 6.58−6.63 (m, 2H), 6.99−7.04 (m, 2H), 7.18−7.22 (m, 1H), 7.23−7.28 (m, 4H), 7.37 (s, 1H), 7.51 (t, 1H, J = 4.9 Hz), 7.74 (t, 1H, J = 5.5 Hz), 7.81 (d, 1H, J = 9.1 Hz), 7.84 (d, 1H, J = 8.3 Hz), 8.04 (d, 1H, J = 8.1 Hz), 8.16 (br s, 3H), 8.28−8.34 (m, 2H), 8.39 (br s, 2H), 8.58 (d, 1H, J = 7.8 Hz), 8.94 (br s, 1H), 8.98 (br s, 1H), 9.16 (br s, 1H), 10.0 (br s, 1H), 12.7 (br s, 1H), 13.0 (br s, 1H), 14.1 (br s, 2H). HRMS (ESI): m/z [M + 2H]$^{2+}$ calcd for [$C_{58}H_{87}N_{15}O_{14}$]$^{2+}$ 608.8273, found 608.8281. RP-HPLC (220 nm): 99% (t_R = 9.1 min, k = 11.0). $C_{58}H_{85}N_{15}O_{14}$·$C_6H_3F_9O_6$ (1216.4 + 342.07).

*H-Asp-{N$^{\omega}$-[N-(8-propanoylamino-3,6-dioxaoctyl)aminocarbonyl]}-Arg-Val-Tyr-Ile-His-Pro-Phe-OH Tris(hydrotrifluoroacetate) (**22**).* Compound **22** was prepared from **20** (16.85 mg, 10.05 μmol) and **37** (0.9 mg, 5.26 μmol) following the procedure for the preparation of **21**. DIPEA: 15.6 mg, 121 μmol. K_2CO_3: 10 mg, 99 μmol. The product was purified by preparative HPLC (column, Gemini-NX C18 250 mm × 21 mm; gradient, 0−35 min; MeCN/0.1% aq TFA 10:90−34:66; t_R = 27 min). Lyophilization of the eluate afforded **22** as a white fluffy solid (7.4 mg, 87%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.72−0.82 (m, 12H), 0.97 (t, 3H, J = 7.6 Hz), 1.01−1.10 (m, 1H), 1.31−1.39 (m, 1H), 1.43−1.58 (m, 3H), 1.60−1.68 (m, 2H), 1.76−1.88 (m, 3H), 1.90−1.97 (m, 1H), 2.00−2.09 (m, 3H), 2.61−2.67 (m, 2H), 2.75−2.83 (m, 2H), 2.88−2.96 (m, 2H), 2.99−3.08 (m, 2H), 3.16−3.29 (m, 6H), 3.39 (t, 2H, J = 6.1 Hz), 3.43−3.49 (m, 3H), 3.49−3.54 (m, 4H), 3.62−3.67 (m, 1H), 4.11 (d, 1H, J = 6.9 Hz), 4.15 (t, 1H, J = 8.0 Hz), 4.18 (dd, 1H, J = 8.7, 6.7 Hz), 4.34−4.41 (m, 2H), 4.41−4.45 (m, 1H), 4.47−4.53 (m, 1H), 4.74−4.80 (m, 1H), 6.57−6.63 (m, 2H), 6.98−7.04 (m, 2H), 7.17−7.22 (m, 1H), 7.23−7.28 (m, 4H), 7.37 (s, 1H), 7.51 (t, 1H, J = 4.9 Hz), 7.75−7.87 (m, 3H), 8.04 (d, 1H, J = 8.1 Hz), 8.19 (br s, 3H), 8.30 (d, 1H, J = 7.8 Hz), 8.32 (d, 1H, J = 7.7 Hz), 8.43 (br s, 2H), 8.58 (d, 1H, J = 7.9 Hz), 8.94 (br s, 1H), 9.00 (br s, 1H), 9.16 (br s, 1H), 10.1 (br s, 1H), 12.8 (br s, 1H), 13.0 (br s, 1H), 14.1 (br s, 2H). HRMS (ESI): m/z [M + 2H]$^{2+}$ calcd for [$C_{60}H_{91}N_{15}O_{16}$]$^{2+}$ 638.8379, found 638.8375. $C_{60}H_{89}N_{15}O_{16}$. RP-HPLC (220 nm): 99% (t_R = 8.9 min, k = 10.7). $C_6H_3F_9O_6$ (1276.4 + 342.07).

*H-Asp-{N$^{\omega}$-[N-(4-{N-[6-(6-{2-[7-(diethylamino)-2-oxo-2H-chromen-3-yl]vinyl}-3-sulfonato-pyridin-1-ium-1-yl)hexanoyl]amino}butyl)aminocarbonyl]}Arg-Val-Tyr-Ile-His-Pro-Phe-OH Tris(hydrotrifluoroacetate) (**23**).* A solution of Fluorescence Red Mega 480 succinimidyl ester (**38**) (1 mg, 1.63 μmol) in anhydrous DMF (15 μL) was added to a solution of **19** (6.4 mg, 3.96 μmol), DIPEA (7 mg, 54 μmol), and K_2CO_3 (4 mg, 40 μmol) in DMF/water (4:6 v/v) (100 μL) in a 1.5 mL reaction vessel with screw cap, and the vessel was shaken at rt in the dark for 30 min. Then 10% aq TFA (70 μL) was added, and the product was purified by preparative HPLC (column, Kinetex 250 mm × 21 mm; gradient, 0−25 min; MeCN/0.1% aq TFA 10:90−40:60; t_R = 24.5 min). Lyophilization of the eluate yielded **23** as a dark-red solid (0.48 mg, 15%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for [$C_{81}H_{112}N_{17}O_{19}S$]$^{3+}$ 553.2685, found 553.2692. RP-HPLC (220 and 500 nm): 98% (t_R = 10.4 min, k = 12.7). $C_{81}H_{109}N_{17}O_{19}S$·$C_6H_3F_9O_6$ (1656.90 + 342.07).

*H-Asp-[N$^{\omega}$-(N-{4-[N-(6-{3,3-dimethyl-2-[5-(1,3,3-trimethyl-3H-indol-1-ium-2-yl)penta-2,4-dien-1-ylidene]indolin-1-yl}hexanoyl)-amino]butyl}aminocarbonyl)]Arg-Val-Tyr-Ile-His-Pro-Phe-OH Trifluoroacetate Tris(hydrotrifluoroacetate) (**24**).* Compound **24** was prepared from **19** (6.4 mg, 3.96 μmol) and **39** (1.7 mg, 2.57 μmol) following the procedure for the preparation of **23**. DIPEA: 7 mg, 54 μmol. K_2CO_3: 4 mg, 40 μmol. The product was purified by preparative HPLC (column, Kinetex 250 mm × 21 mm; gradient, 0−22 min; MeCN/0.1% aq TFA 10:90−37:63; 22−40 min, 37:63−45:55; t_R = 33 min). Lyophilization of the eluate afforded **24** as a dark-blue solid (1.3 mg, 25%). HRMS (ESI): m/z [M + 2H]$^{3+}$ calcd for [$C_{87}H_{120}N_{17}O_{14}$]$^{3+}$ 542.3061, found 542.3066. RP-HPLC (220 and 630 nm): 98% (t_R = 13.9 min, k = 17.3). $C_{87}H_{118}N_{17}O_{14}$·$C_8H_3F_{12}O_8$ (1625.02 + 455.09).

*H-{N$^{\omega}$-[N-(4-aminobutyl)aminocarbonyl]}Arg-Arg-Pro-Tyr-Ile-Leu-OH Tetrakis(hydrotrifluoroacetate) (**25**).* The peptide was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading: 0.81 mmol/g). Purification by preparative HPLC was performed with a Gemini-NX C18 250 mm × 21 mm (gradient, 0−35 min; MeCN/0.1% aq TFA 8:92−41:59; t_R = 19 min). Lyophilization of the eluate afforded **25** as a white fluffy solid (72.6 mg, 59%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.80 (t, 3H, J = 7.4 Hz), 0.82−0.86 (m, 6H), 0.90 (d, 3H, J = 6.6 Hz), 1.00−1.10 (m, 1H), 1.36−1.44 (m, 1H), 1.44−1.65 (m, 12H), 1.65−1.76 (m, 4H), 1.78−1.87 (m, 3H), 1.94−2.04 (m, 1H), 2.68 (dd, 1H, J = 14.0, 8.2 Hz), 2.75−2.83 (m, 2H), 2.83−2.90 (m, 1H), 3.03−3.15 (m, 4H), 3.26 (br s, 2H), 3.53−3.63 (m, 2H), 3.80−3.86 (m, 1H), 4.18−4.24 (m, 2H), 4.32−4.37 (m, 1H), 4.39−4.44 (m, 1H), 4.46−4.53 (m, 1H), 6.58−6.63 (m, 2H), 6.96−7.01 (m, 2H), ca. 7.0 (br s, 2H), ca. 7.4 (br s, 2H), 7.59 (br s, 1H), 7.72−7.84 (m, 5H), 7.88 (d, 1H, J = 7.9 Hz), 8.14−8.25 (m, 4H), 8.49 (br s, 2H), 8.68 (d, 1H, J = 7.4 Hz), 9.09 (br s, 1H), 9.21 (br s, 1H), 10.7 (br s, 1H), 12.5 (br s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 10.9, 15.1, 21.2, 22.9, 23.5, 24.1, 24.2, 24.3, 24.4, 24.6, 26.0, 28.2, 28.3, 29.1, 36.4, 37.1, 38.5, 38.6, 39.8, 40.2, 40.5, 46.8, 50.1, 50.4, 51.6, 54.1, 56.4, 59.2, 114.8, 116.9 (q, J = 298 Hz) (TFA), 127.6, 130.1, 153.9 (2 quat carb), 155.8, 156.9, 158.7 (q, J = 32 Hz) (TFA), 168.3, 169.2, 170.7, 170.9, 171.2, 173.8. HRMS (ESI): m/z [M + 2H]$^{2+}$ calcd for [$C_{43}H_{76}N_{14}O_9$]$^{2+}$ 466.2954, found 466.2956. RP-HPLC (220 nm): 96% (t_R = 5.7 min, k = 6.5). $C_{43}H_{74}N_{14}O_9$·$C_8H_4F_{12}O_8$ (931.14 + 456.09).

*H-{N$^{\omega}$-[N-(8-amino-3,6-dioxaoctyl)aminocarbonyl]}Arg-Arg-Pro-Tyr-Ile-Leu-OH Tetrakis(hydrotrifluoroacetate) (**26**).* The peptide was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading: 0.81 mmol/g). Purification by preparative HPLC was performed with a Gemini-NX C18 250 mm × 21 mm (gradient, 0−35 min; MeCN/0.1% aq TFA 8:92−41:59; t_R = 19 min). Lyophilization of the eluate afforded **26** as a white fluffy solid (76.5 mg, 59%). ^{1}H NMR

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(600 MHz, DMSO-d_6): δ 0.80 (t, 3H, J = 7.5 Hz), 0.82−0.86 (m, 6H), 0.90 (d, 3H, J = 6.6 Hz), 1.00−1.10 (m, 1H), 1.36−1.46 (m, 1H), 1.46−1.66 (m, 8H), 1.66−1.76 (m, 4H), 1.77−1.86 (m, 3H), 1.94−2.04 (m, 1H), 2.68 (dd, 1H, J = 14.0, 8.2 Hz), 2.81−2.93 (m, 1H), 2.94−3.00 (m, 2H), 3.03−3.15 (m, 2H), 3.20−3.31 (m, 4H), 3.47 (t, 2H, J = 5.6 Hz), 3.53−3.62 (m, 8H), 3.80−3.86 (m, 1H), 4.18−4.25 (m, 2H), 4.32−4.37 (m, 1H), 4.39−4.44 (m, 1H), 4.46−4.53 (m, 1H), 6.58−6.63 (m, 2H), 6.96−7.01 (m, 2H), ca. 7.0 (br s, 2H), ca. 7.4 (br s, 2H), 7.46 (br s, 1H), 7.72−7.78 (m, 2H), 7.78−7.96 (m, 4H), 8.13−8.27 (m, 4H), 8.51 (br s, 2H), 8.68 (d, 1H, J = 7.4 Hz), 9.11 (br s, 1H), 9.21 (br s, 1H), 10.7 (br s, 1H), 12.5 (br s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 10.9, 15.1, 21.2, 22.8, 23.5, 24.1, 24.2, 24.3, 24.6, 28.2, 28.3, 29.1, 36.4, 37.1, 38.5, 39.1, 39.8, 40.2, 40.5, 46.8, 50.1, 50.4, 51.6, 54.1, 56.4, 59.2, 66.7, 68.7, 69.4, 69.6, 114.8, 116.9 (q, J = 298 Hz) (TFA), 127.6, 130.1, 153.7, 153.9, 155.8, 156.9, 158.7 (q, J = 32 Hz) (TFA), 168.3, 169.2, 170.7, 170.9, 171.2, 173.8. HRMS (ESI): m/z [M + 2H]$^{2+}$ calcd for [$C_{45}H_{80}N_{14}O_{11}$]$^{2+}$ 496.3060, found 496.3065. RP-HPLC (220 nm): 98% (t_R = 5.9 min, k = 6.8). $C_{45}H_{78}N_{14}O_{11}$·$C_8H_4F_{12}O_8$ (991.19 + 456.09).

*H-{N$^{\omega}$-[N-(4-propanoylaminobutyl)aminocarbonyl]}Arg-Arg-Pro-Tyr-Ile-Leu-OH Tris(hydrotrifluoroacetate) (**27**).* Compound **27** was prepared from **25** (14.2 mg, 10.22 μmol) and **37** (1.06 mg, 6.2 μmol) following the procedure for the preparation of **21**. DIPEA: 10.6 mg, 82 μmol. K_2CO_3: 10 mg, 99 μmol. The product was purified by preparative HPLC (column, Gemini-NX C18 250 mm × 21 mm; gradient, 0−35 min; MeCN/0.1% aq TFA 10:90−37:63; t_R = 23 min). Lyophilization of the eluate afforded **27** as a white fluffy solid (7.1 mg, 86%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.80 (t, 3H, J = 7.4 Hz), 0.82−0.86 (m, 6H), 0.90 (d, 3H, J = 6.6 Hz), 0.98 (t, 3H, J = 7.6 Hz), 1.01−1.09 (m, 1H), 1.34−1.46 (m, 5H), 1.46−1.65 (m, 8H), 1.65−1.75 (m, 4H), 1.78−1.87 (m, 3H), 1.96−2.02 (m, 1H), 2.05 (q, 2H, J = 7.6 Hz), 2.67 (dd, 1H, J = 14.1, 8.2 Hz), 2.83−2.90 (m, 1H), 3.00−3.05 (m, 2H), 3.05−3.14 (m, 4H), 3.25 (br s, 2H), 3.53−3.63 (m, 2H), 3.78−3.84 (m, 1H), 4.18−4.25 (m, 2H), 4.32−4.38 (m, 1H), 4.39−4.45 (m, 1H), 4.47−4.54 (m, 1H), 6.58−6.63 (m, 2H), ca. 6.9 (br s, 2H), 6.96−7.02 (m, 2H), ca. 7.3 (br s, 2H), 7.50 (t, 1H, J = 5.1 Hz), 7.58 (t, 1H, J = 5.4 Hz), 7.71−7.78 (m, 2H), 7.90 (d, 1H, J = 7.9 Hz), 8.07−8.18 (m, 3H), 8.20 (d, 1H, J = 7.8 Hz), 8.42 (br s, 2H), 8.67 (d, 1H, J = 7.5 Hz), 9.06 (br s, 1H), 9.17 (br s, 1H), 10.1 (br s, 1H), 12.5 (br s, 1H). HRMS (ESI): m/z [M + 2H]$^{2+}$ calcd for [$C_{46}H_{80}N_{14}O_{10}$]$^{2+}$ 494.3085, found 494.3092. RP-HPLC (220 nm): 99% (t_R = 8.2 min, k = 9.8). $C_{46}H_{78}N_{14}O_{10}$·$C_6H_3F_9O_6$ (987.20 + 342.07).

*H-{N$^{\omega}$-[N-(8-propanoylamino-3,6-dioxaoctyl)aminocarbonyl]}-Arg-Arg-Pro-Tyr-Ile-Leu-OH Tris(hydrotrifluoroacetate) (**28**).* Compound **28** was prepared from **26** (12.9 mg, 8.91 μmol) and **37** (1.1 mg, 6.43 μmol) using the procedure for the preparation of **21**, but DMF/water (4:6 v/v) (200 μL) was used as solvent. DIPEA: 9.0 mg, 70 μmol. K_2CO_3: 8.6 mg, 85 μmol. The product was purified by preparative HPLC (column, Gemini-NX C18 250 mm × 21 mm; gradient, 0−35 min; MeCN/0.1% aq TFA 8:92−43:57; t_R = 21 min). Lyophilization of the eluate afforded **28** as a white fluffy solid (7.7 mg, 86%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.80 (t, 3H, J = 7.4 Hz), 0.82−0.86 (m, 6H), 0.90 (d, 3H, J = 6.6 Hz), 0.97 (t, 3H, J = 7.6 Hz), 1.00−1.09 (m, 1H), 1.37−1.46 (m, 1H), 1.46−1.65 (m, 8H), 1.65−1.76 (m, 4H), 1.78−1.88 (m, 3H), 1.96−2.04 (m, 1H), 2.06 (q, 2H, J = 7.6 Hz), 2.67 (dd, 1H, J = 14.1, 8.2 Hz), 2.83−2.90 (m, 1H), 3.04−3.15 (m, 2H), 3.16−3.21 (m, 2H), 3.21−3.31 (m, 4H), 3.39 (t, 2H, J = 6.1 Hz), 3.46 (t, 2H, J = 5.4 Hz), 3.49−3.54 (m, 4H), 3.54−3.63 (m, 2H), 3.78−3.84 (m, 1H), 4.19−4.25 (m, 2H), 4.32−4.37 (m, 1H), 4.39−4.44 (m, 1H), 4.47−4.54 (m, 1H), 6.58−6.63 (m, 2H), ca. 6.9 (br s, 2H), 6.97−7.03 (m, 2H), ca. 7.3 (br s, 2H), 7.49 (t, 1H, J = 5.4 Hz), 7.57 (t, 1H, J = 5.4 Hz), 7.75 (d, 1H, J = 8.9 Hz), 7.79 (t, 1H, J = 5.4 Hz), 7.90 (d, 1H, J = 7.9 Hz), 8.08−8.18 (m, 3H), 8.20 (d, 1H, J = 7.7 Hz), 8.44 (br s, 2H), 8.67 (d, 1H, J = 7.4 Hz), 9.07 (br s, 1H), 9.17 (br s, 1H), 10.1 (br s, 1H), 12.5 (br s, 1H). HRMS (ESI): m/z [M + 2H]$^{2+}$ calcd for [$C_{48}H_{84}N_{14}O_{12}$]$^{2+}$ 524.3191, found 524.3196. RP-HPLC (220 nm): 99% (t_R = 8.2 min, k = 9.8). $C_{48}H_{82}N_{14}O_{12}$·$C_6H_3F_9O_6$ (1047.3 + 342.07).

*H-{N$^{\omega}$-[N-(4-{N-[6-(6-{2-[7-(diethylamino)-2-oxo-2H-chromen-3-yl]vinyl}-3-sulfonato-pyridin-1-ium-1-yl)hexanoyl]amino}butyl)-aminocarbonyl]}Arg-Arg-Pro-Tyr-Ile-Leu-OH Tris-(hydrotrifluoroacetate) (**29**).* The compound was prepared from **25** (6.7 mg, 4.8 μmol) and **38** (1 mg, 1.63 μmol) following the procedure for the preparation of **23**. DIPEA: 7 mg, 54 μmol. K_2CO_3: 4 mg, 40 μmol. The product was purified by preparative HPLC (conditions as for **23**; t_R = 25 min). Lyophilization of the eluate afforded **29** as a dark-red solid (0.38 mg, 13%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for [$C_{69}H_{105}N_{16}O_{15}S$]$^{3+}$ 476.5883, found 476.5891. RP-HPLC (220 and 500 nm): 99% (t_R = 10.3 min, k = 12.6). $C_{69}H_{102}N_{16}O_{15}S$·$C_6H_3F_9O_6$ (1427.71 + 342.07).

*H-[N$^{\omega}$-(N-{4-[N-(6-{3,3-dimethyl-2-[5-(1,3,3-trimethyl-3H-indol-1-ium-2-yl)penta-2,4-dien-1-ylidene]indolin-1-yl}hexanoyl)amino]-butyl}aminocarbonyl)]Arg-Arg-Pro-Tyr-Ile-Leu-OH Trifluoroacetate Tris(hydrotrifluoroacetate) (**30**).* Compound **30** was prepared from **25** (8.4 mg, 6.06 μmol) and **39** (1.7 mg, 2.57 μmol) following the procedure for the preparation of **23**. DIPEA: 7 mg, 54 μmol. K_2CO_3: 4 mg, 40 μmol. The product was purified by preparative HPLC (column, Kinetex 250 mm × 21 mm; gradient, 0−23 min; MeCN/0.1% aq TFA 10:90−38:62; 23−40 min, 38:62−46:54; t_R = 33 min). Lyophilization of the eluate afforded **30** as a dark-blue solid (1.4 mg, 28%). HRMS (ESI): m/z [M+2H]$^{3+}$ calcd for [$C_{75}H_{113}N_{16}O_{10}$]$^{3+}$ 465.9603, found 465.9611. RP-HPLC (220 and 630 nm): 98% (t_R = 13.9 min, k = 17.3). $C_{75}H_{111}N_{16}O_{10}$·$C_8H_3F_{12}O_8$ (1396.78 + 455.09).

*H-Arg-{N$^{\omega}$-[N-(8-amino-3,6-dioxaoctyl)aminocarbonyl]}Arg-Pro-Tyr-Ile-Leu-OH Tetrakis(hydrotrifluoroacetate) (**31**).* The peptide was synthesized according to the general procedure using a H-Leu-2-ClTrt resin (loading: 0.81 mmol/g). Purification by preparative HPLC was performed with a Gemini-NX C18 250 mm × 21 mm (gradient, 0−35 min; MeCN/0.1% aq TFA 8:92−41:59; t_R = 19 min). Lyophilization of the eluate afforded **31** as a white fluffy solid (48.2 mg, 37%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.79 (t, 3H, J = 7.4 Hz), 0.82−0.86 (m, 6H), 0.90 (d, 3H, J = 6.6 Hz), 0.99−1.09 (m, 1H), 1.36−1.45 (m, 1H), 1.45−1.48 (m, 5H), 1.48−1.77 (m, 7H), 1.77−1.87 (m, 3H), 1.94−2.04 (m, 1H), 2.68 (dd, 1H, J = 14.0, 8.2 Hz), 2.81−2.92 (m, 1H), 2.94−3.00 (m, 2H), 3.06−3.14 (m, 2H), 3.17−3.31 (m, 4H), 3.46 (t, 2H, J = 5.6 Hz), 3.53−3.62 (m, 8H), 3.78−3.85 (m, 1H), 4.18−4.25 (m, 2H), 4.31−4.37 (m, 1H), 4.39−4.45 (m, 1H), 4.48−4.55 (m, 1H), 6.58−6.63 (m, 2H), 6.96−7.01 (m, 2H), ca. 7.1 (br s, 2H), ca. 7.4 (br s, 2H), 7.46 (br s, 1H), 7.75 (d, 1H, J = 8.9 Hz), 7.80 (t, 1H, J = 5.8 Hz), 7.81−7.98 (m, 4H), 8.12−8.26 (m, 4H), 8.52 (br s, 2H), 8.69 (d, 1H, J = 7.4 Hz), 9.08 (br s, 1H), 9.21 (br s, 1H), 10.7 (br s, 1H), 12.5 (br s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 10.9, 15.1, 21.2, 22.8, 24.1 (3 carb), 24.2, 24.3, 28.2, 28.4, 29.1, 36.4, 37.1, 38.6, 39.1, 39.8, 40.1, 40.6, 46.8, 50.1, 50.4, 51.7, 54.1, 56.3, 59.2, 66.7, 68.7, 69.4, 69.6, 114.8, 116.8 (q, J = 297 Hz) (TFA), 127.6, 130.1, 153.7, 153.9, 155.8, 156.8, 158.7 (q, J = 32 Hz) (TFA), 168.3, 169.2, 170.7, 170.9, 171.2, 173.8. HRMS (ESI): m/z [M + 2H]$^{2+}$ calcd for [$C_{45}H_{80}N_{14}O_{11}$]$^{2+}$ 496.3060, found 496.3063. RP-HPLC (220 nm): 99% (t_R = 6.3 min, k = 7.3). $C_{45}H_{78}N_{14}O_{11}$·$C_8H_4F_{12}O_8$ (991.19 + 456.09).

*H-Arg-{N$^{\omega}$-[N-(8-propanoylamino-3,6-dioxaoctyl)-aminocarbonyl]}Arg-Pro-Tyr-Ile-Leu-OH Tris(hydrotrifluoroacetate) (**32**).* Compound **32** was prepared from **31** (12.5 mg, 8.64 μmol) and **37** (1.05 mg, 6.13 μmol) using the procedure for the preparation of **21**, but DMF/water (4:6 v/v) (200 μL) was used as solvent. DIPEA: 9.0 mg, 70 μmol. K_2CO_3: 8.6 mg, 85 μmol. The product was purified by preparative HPLC (column, Gemini-NX C18 250 mm × 21 mm; gradient, 0−35 min; MeCN/0.1% aq TFA 8:92−43:57; t_R = 21 min). Lyophilization of the eluate afforded **32** as a white fluffy solid (7.6 mg, 89%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.80 (t, 3H, J = 7.4 Hz), 0.82−0.86 (m, 6H), 0.90 (d, 3H, J = 6.6 Hz), 0.97 (t, 3H, J = 7.6 Hz), 1.01−1.09 (m, 1H), 1.36−1.44 (m, 1H), 1.44−1.58 (m, 5H), 1.58−1.77 (m, 7H), 1.78−1.88 (m, 3H), 1.96−2.04 (m, 1H), 2.06 (q, 2H, J = 7.6 Hz), 2.67 (dd, 1H, J = 14.0, 8.1 Hz), 2.83−2.90 (m, 1H), 3.06−3.13 (m, 2H), 3.16−3.20 (m, 2H), 3.21−3.30 (m, 4H), 3.39 (t, 2H, J = 6.1 Hz), 3.46 (t, 2H, J = 5.3 Hz), 3.49−3.54 (m, 4H), 3.54−3.62 (m, 2H), 3.77−3.83 (m, 1H), 4.19−4.25 (m, 2H), 4.32−4.37 (m, 1H), 4.40−4.46 (m, 1H), 4.49−4.54 (m, 1H), 6.58−6.63 (m, 2H), 6.96−7.02 (m, 2H), ca. 7.0 (br s, 2H), ca. 7.3 (br s, 2H), 7.51 (br s, 1H), 7.58 (t, 1H, J = 5.9 Hz), 7.75 (d, 1H, J = 9.0 Hz), 7.79 (t, 1H, J = 5.1 Hz), 7.89 (d, 1H, J = 7.5 Hz), 8.07−8.16 (m, 3H), 8.19 (d, 1H, J = 7.7 Hz), 8.44 (br s, 2H), 8.67 (d, 1H, J = 7.5 Hz), 9.02 (br s, 1H), 9.17 (br s, 1H), 9.96 (br s, 1H), 12.5 (br s, 1H). HRMS (ESI): m/z [M + 2H]$^{2+}$ calcd for [$C_{48}H_{84}N_{14}O_{12}$]$^{2+}$ 524.3191,

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found 524.3199. RP-HPLC (220 nm): 99% (t_R = 8.3 min, k = 9.9). $C_{48}H_{82}N_{14}O_{12}\cdot C_6H_3F_9O_6$ (1047.3 + 342.07).

*At Position Arg^2 N$^{\omega}$-[N-(4-Aminobutyl)aminocarbonyl]-monosubstituted Octanedioyl Bis(tyrosyl-arginyl-leucyl-arginyl-tyrosinamide) Pentakis(hydrotrifluoroacetate) (**33**).* Compound **33** was synthesized on a Sieber resin (loading: 0.61 mmol/g). Fmoc-amino acids and arginine building block **10** were coupled according to the general procedure in the order Fmoc-Tyr(*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, **10**, and Fmoc-Tyr(*t*Bu)-OH followed by the coupling of building block **43** (50 mg, 32 μmol) using HBTU/HOBt/DIPEA (1/1/2 equiv to **43**) and anhydrous DMF (800 μL) as solvent (35 °C, 16 h). The peptide was cleaved from the resin according to the general procedure. The residue obtained after lyophilization was dissolved in TFA/water (95:5 v/v) (2 mL), and the mixture was stirred at rt for 2.5 h and poured into ice-cold diethyl ether (15 mL), causing the precipitation of the crude product, which was separated from the organic solvent by centrifugation and decantation. Purification by preparative HPLC (column, Kinetex-XB C18 250 mm × 21 mm; gradient, 0−25 min; MeCN/0.1% aq TFA 13:87−43:57 (flow rate: 15 instead 20 mL/min); t_R = 16 min) afforded **33** as a white fluffy solid (22 mg, 41%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.83 (d, 6H, J = 6.5 Hz), 0.87 (d, 6H, J = 6.5 Hz), 1.03 (br s, 4H), 1.30 (br s, 4H), 1.36−1.57 (m, 20H), 1.57−1.75 (m, 6H), 1.94−2.06 (m, 4H), 2.57−2.65 (m, 2H), 2.68−2.75 (m, 2H), 2.75−2.82 (m, 2H), 2.82−2.90 (m, 4H), 3.02−3.14 (m, 8H), 3.24 (br s, 2H), 4.17−4.23 (m, 2H), 4.24−4.36 (m, 6H), 4.39−4.46 (m, 2H), 6.59−6.65 (m, 8H), ca. 6.95 (br s, 6H), 6.95−6.99 (m, 4H), 6.99−7.03 (m, 4H), 7.05 (s, 2H), ca. 7.3 (br s, 6H), 7.33−7.39 (m, 2H), 7.52−7.63 (m, 4H), 7.67−7.79 (m, 4H), 7.84−7.95 (m, 4H), 7.99−8.08 (m, 2H), 8.17 (d, 1H, J = 7.8 Hz), 8.20 (d, 1H, J = 7.8 Hz), 8.45 (br s, 2H), 9.00 (s, 1H), 9.17 (br s, 4H), 10.38 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 21.4 (2 carb), 23.1, 23.1, 24.1 (2 carb), 24.4, 24.9 (3 carb), 25.0, 25.1 (2 carb), 26.0, 28.4 (2 carb), 28.9, 29.0 (3 carb), 35.2 (2 carb), 36.6 (2 carb), 36.8 (2 carb), 38.5, 38.6, 40.4 (3 carb), 40.5, 40.7 (2 carb), 51.0 (2 carb), 50.1 (2 carb), 52.26, 52.29, 53.89, 53.92, 54.1, 54.2, 114.8 (4 carb), 114.9 (4 carb), 115.6 (TFA), 117.6 (TFA), 127.5 (2 quat carb), 127.89, 127.91, 130.02 (4 carb), 130.04 (4 carb), 153.7, 153.8, 155.75 (2 quat carb), 155.82 (2 quat carb), 156.71 (2 quat carb), 156.73, 158.5 (q, J = 33 Hz) (TFA), 170.81, 170.84, 171.08, 171.13, 171.8, 171.9, 172.0 (2 quat carb), 172.31, 172.34, 172.7 (2 quat carb). HRMS (ESI): m/z $[M + 4H]^{4+}$ calcd for $[C_{85}H_{136}N_{26}O_{17}]^{4+}$ 448.2639, found 448.2652. RP-HPLC (220 nm): 96%, (t_R = 19.3 min, k = 5.7). $C_{85}H_{132}N_{26}O_{17}\cdot C_{10}H_5F_{15}O_{10}$ (1790.16 + 570.11).

*At Position Arg^2 N$^{\omega}$-[N-(4-Propanoylaminobutyl)aminocarbonyl]-monosubstituted Octanedioyl Bis(tyrosyl-arginyl-leucyl-arginyl-tyrosinamide) Tetrakis(hydrotrifluoroacetate) (**34**).* Compound **33** (10 mg, 4.24 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Succinimidyl propionate (**37**) (0.8 mg, 4.7 μmol) was added, and the mixture was stirred at rt for 2 h. Then 0.2% aq TFA (9 mL) was added, and the product was purified by preparative HPLC (column, Kinetex-XB C18 250 mm × 21 mm; gradient, 0−25 min; MeCN/0.1% aq TFA 12:88−43:57 (flow rate: 15 instead 20 mL/min); t_R = 18 min). Lyophilization of the eluate afforded **34** as a white fluffy solid (5.7 mg, 58%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.83 (d, 6H, J = 6.5 Hz), 0.87 (d, 6H, J = 6.6 Hz), 0.98 (t, 3H, 7.7 Hz), 1.03 (br s, 4H), 1.30 (br s, 4H), 1.34−1.57 (m, 20H), 1.57−1.75 (m, 6H), 1.94−2.07 (m, 6H), 2.57−2.64 (m, 2H), 2.69−2.75 (m, 2H), 2.82−2.88 (m, 4H), 2.99−3.13 (m, 10H), 3.23 (br s, 2H), 4.17−4.24 (m, 2H), 4.24−4.36 (m, 6H), 4.40−4.46 (m, 2H), 6.59−6.65 (m, 8H), ca. 6.85 (br s, 6H), 6.95−6.99 (m, 4H), 6.99−7.03 (m, 4H), 7.05 (s, 2H), ca. 7.3 (br s, 6H), 7.37 (s, 2H), 7.46−7.53 (m, 4H), 7.69−7.77 (m, 3H), 7.84−7.93 (m, 4H), 8.03 (m, 2H), 8.18 (m, 2H), 8.40 (br s, 2H), 8.96 (s, 1H), 9.16 (s, 4H), 9.87 (s, 1H). HRMS (ESI): m/z $[M + 4H]^{4+}$ calcd for $[C_{88}H_{140}N_{26}O_{18}]^{4+}$ 462.2704, found 462.2717. RP-HPLC (220 nm): 96%, (t_R = 21.1 min, k = 6.3). $C_{88}H_{136}N_{26}O_{18}\cdot C_8H_4F_{12}O_8$ (1846.22 + 456.09).

*H-Glu-Tyr-Trp-Ser-Leu-Ala-Ala-Pro-Gln-{N$^{\omega}$-[N-(4-aminobutyl)-aminocarbonyl]}Arg-Phe-NH$_2$ Tris(hydrotrifluoroacetate) (**35**).* The peptide was synthesized according to the general procedure using a Rink amide resin (loading: 0.73 mmol/g). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 mm × 21 mm (gradient, 0−30 min; MeCN/0.1% aq TFA 15:85−38:62; t_R = 18.5 min). Lyophilization of the eluate afforded **35** as a white fluffy solid (58 mg, 34%). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.84 (d, 3H, J = 6.5 Hz), 0.87 (d, 3H, J = 6.6 Hz), 1.14−1.22 (m, 6H), 1.34−1.57 (m, 9H), 1.57−1.67 (m, 2H), 1.70−1.90 (m, 5H), 1.90−1.96 (m, 2H), 2.00−2.07 (m, 1H), 2.09−2.17 (m, 2H), 2.32−2.38 (m, 2H), 2.60−2.67 (m, 1H), 2.70−2.85 (m, 3H), 2.92−3.06 (m, 3H), 3.08−3.15 (m, 3H), 3.15−3.22 (m, 2H), 3.49−3.56 (m, 2H), 3.56−3.65 (m, 2H), 3.72−3.79 (m, 1H), 4.09−4.14 (m, 1H), 4.14−4.19 (m, 1H), 4.22−4.27 (m, 1H), 4.27−4.37 (m, 3H), 4.37−4.43 (m, 1H), 4.45−4.53 (m, 2H), 4.59−4.65 (m, 1H), 6.61−6.66 (m, 2H), 6.84 (s, 1H), 6.93−6.98 (m, 1H), 7.02−7.09 (m, 3H), 7.13 (s, 1H), 7.15−7.19 (m, 2H), 7.19−7.26 (m, 4H), 7.28 (s, 1H), 7.30−7.35 (m, 2H), 7.58 (d, 1H, J = 7.9 Hz), 7.61 (br s, 1H), 7.75 (br s, 3H), 7.81−7.87 (m, 2H), 7.95 (d, 1H, J = 7.3 Hz), 7.98 (d, 1H, J = 8.2 Hz), 8.01 (d, 1H, J = 7.0 Hz), 8.02−8.09 (m, 4H), 8.19 (d, 1H, J = 7.0 Hz), 8.37 (d, 1H, J = 7.7 Hz), 8.44 (br s, 2H), 8.48 (d, 1H, J = 7.9 Hz), 8.97 (br s, 1H), 9.21 (br s, 1H), 10.4 (br s, 1H), 10.7 (s, 1H), 12.3 (br s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 16.7, 17.9, 21.4, 23.2, 24.1, 24.3, 24.4, 24.5, 26.0, 26.6, 27.2, 27.6, 28.7, 28.9, 29.0, 31.4, 36.5, 37.4, 38.5, 38.6, 40.4, 40.5, 46.3, 46.7, 47.9, 51.0, 51.4, 52.4, 52.8, 53.4, 53.8, 54.6, 54.8, 59.7, 61.7, 109.9, 111.2, 114.9, 115.6 (TFA), 117.6 (TFA), 118.2, 118.4, 120.8, 123.5, 126.2, 127.4, 127.6, 128.0, 129.1, 130.1, 136.0, 137.8, 153.7, 153.9, 155.9, 158.4 (q, J = 33 Hz) (TFA), 168.2, 170.0, 170.9 (2 quat carb), 171.0, 171.4, 171.6, 171.7, 171.9, 172.8, 173.6, 174.1. HRMS (ESI): m/z $[M + 2H]^{2+}$ calcd for $[C_{70}H_{103}N_{19}O_{17}]^{2+}$ 740.8884, found 740.8890. RP-HPLC (220 nm): 99% (t_R = 8.9 min, k = 10.7). $C_{70}H_{101}N_{19}O_{17}\cdot C_6H_3F_9O_6$ (1480.7 + 342.07).

*H-Glu-Tyr-Trp-Ser-Leu-Ala-Ala-Pro-Gln-{N$^{\omega}$-[N-(4-propanoylaminobutyl)aminocarbonyl]}Arg-Phe-NH$_2$ Tris(hydrotrifluoroacetate) (**36**).* Compound **36** was prepared from **35** (13.8 mg, 7.57 μmol) and **37** (0.91 mg, 5.32 μmol) using the procedure for the preparation of **21**, but DMF (160 μL) was used as solvent and K_2CO_3 was omitted. DIPEA: 12 mg, 92.9 μmol. The product was purified by preparative HPLC (column, Kinetex-XB C18 250 mm × 21 mm; gradient, 0−30 min; MeCN/0.1% aq TFA 15:85−38:62; t_R = 21.5 min). Lyophilization of the eluate afforded **36** as a white fluffy solid (7.8 mg, 83%). ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) 0.84 (d, 3H, J = 6.5 Hz), 0.87 (d, 3H, J = 6.6 Hz), 0.98 (t, 3H, J = 7.6 Hz), 1.15−1.21 (m, 6H), 1.35−1.46 (m, 7H), 1.46−1.54 (m, 2H), 1.56−1.67 (m, 2H), 1.70−1.90 (m, 5H), 1.90−1.96 (m, 2H), 2.00−2.08 (m, 3H), 2.09−2.16 (m, 2H), 2.32−2.38 (m, 2H), 2.61−2.67 (m, 1H), 2.78−2.84 (m, 1H), 2.92−3.06 (m, 5H), 3.06−3.15 (m, 3H), 3.15−3.21 (m, 2H), 3.50−3.55 (m, 2H), 3.56−3.63 (m, 2H), 3.72−3.77 (m, 1H), 4.09−4.15 (m, 1H), 4.15−4.21 (m, 1H), 4.23−4.27 (m, 1H), 4.27−4.36 (m, 3H), 4.38−4.44 (m, 1H), 4.45−4.53 (m, 2H), 4.60−4.65 (m, 1H), 5.04 (br s, 1H), 6.61−6.66 (m, 2H), 6.84 (s, 1H), 6.94−6.98 (m, 1H), 7.02−7.09 (m, 3H), 7.13 (s, 1H), 7.14−7.19 (m, 2H), 7.19−7.26 (m, 4H), 7.27 (s, 1H), 7.31 (d, 1H, J = 8.1 Hz), 7.33 (s, 1H), 7.49 (br s, 1H), 7.59 (d, 1H, J = 7.8 Hz), 7.73 (t, 1H, J = 5.2 Hz), 7.81−7.87 (m, 2H), 7.95 (d, 1H, J = 7.3 Hz), 7.97 (d, 1H, J = 8.5 Hz), 7.98−8.07 (m, 5H), 8.16 (d, 1H, J = 7.1 Hz), 8.36 (d, 1H, J = 7.6 Hz), 8.38 (br s, 2H), 8.46 (d, 1H, J = 7.7 Hz), 8.86 (br s, 1H), 9.18 (br s, 1H), 9.58 (br s, 1H), 10.7 (br s, 1H), 12.3 (br s, 1H). HRMS (ESI): m/z $[M + 2H]^{2+}$ calcd for $[C_{73}H_{107}N_{19}O_{18}]^{2+}$ 768.9015, found 768.9024. RP-HPLC (220 nm): 99% (t_R = 10.8 min, k = 13.2). $C_{73}H_{105}N_{19}O_{18}\cdot C_4H_2F_6O_4$ (1536.7 + 228.05).

*Preparation of [^{3}H]**21** and [^{3}H]**27**.* The radiolabeled peptides [^{3}H]**21** and [^{3}H]**27** were prepared by [^{3}H]propionylation of the precursor peptides **19** and **25**, respectively. A solution of succinimidyl [^{3}H]-propionate (specific activity: 80 Ci/mmol, purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) via Hartman Analytics (Braunschweig, Germany)) (3.2 mCi, 6.8 μg, 40 nmol, and 4.8 mCi, 10.3 μg, 60 nmol, respectively) in EtOAc (0.4 and 0.6 mL, respectively) was transferred from the delivered ampule into a 1.5 mL reaction vessel with screw cap, and the solvent was removed in a vacuum concentrator (about 30 min at ca. 30 °C). Immediately, a solution of the precursor peptide (**19**, 0.8 mg, 495 nmol; **25**, 0.7 mg, 505 nmol) and DIPEA (0.41 mg, 0.55 μL, 3.2 μmol) in DMF/250 mM bicarbonate buffer pH 9.4 (1:1 v/v) (60 μL) was added and the vessel was shaken at rt for 3 h. The mixture was acidified by the addition of 2% aq TFA (80 μL) followed by the addition of acetonitrile/water (10:90 v/v) (80 μL). [^{3}H]**21** and [^{3}H]**27** were purified using a HPLC system from Waters

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(Eschborn, Germany) consisting of two pumps 510, a pump control module, a 486 UV/vis detector, and a Flow-one beta series A-500 radiodetector (Packard, Meriden, CT). A YMC-Triart C18, 5 μm, 250 mm × 6.0 mm (YMC Europe) served as stationary phase at a flow rate of 1.4 mL/min. Mixtures of acetonitrile supplemented with 0.04% TFA (A) and 0.05% aq TFA (B) were used as mobile phase. The following linear gradient was applied: 0−20 min, A/B 10:90−37:63; 20−22 min, 37:63−95:5; 22−32 min, 95:5. For each radiolabeled peptide two HPLC runs (UV detection: 225 nm; no radiometric detection) were performed. The products (t_R = 19.9 and 19.2 min, respectively) were collected in 2 mL reaction vessels with screw cap by combining the two eluates of each product in one vessel. The volumes of the combined product fractions were reduced by evaporation to 300 and 600 μL, respectively, and ethanol containing 200 μM TFA ([^{3}H]**21**, 200 μL; [^{3}H]**27**, 400 μL) was added. The solutions were transferred into 3 mL borosilicate glass vials with conical bottom (Wheaton NextGen 3 mL V-vials). The 2 mL reaction vessels were rinsed twice with EtOH/water (40:60 v/v), and the washings were transferred to the 3 mL glass vials to reach volumes of the tentative stocks of 1270 μL ([^{3}H]**21**) or 2120 μL ([^{3}H]**27**)). For the quantification of the radioligands, a four-point calibration was performed with **21** and **27** (0.3, 0.75, 1.5, and 2.5 μM; injection volume, 100 μL; UV detection, 225 nm) using the above-mentioned HPLC system from Waters and a Luna C18(2), 3 μm, 150 mm × 4.6 mm (Phenomenex) as stationary phase at a flow rate of 0.8 mL/min. Mixtures of acetonitrile supplemented with 0.04% TFA (A) and 0.05% aq TFA (B) were used as mobile phase. The following linear gradient was applied: 0−20 min, A/B 14:86−30:70; 20−25 min, 30:70−95:5; 25−32 min, 95:5 (retention times: 18.9 and 17.3 min, respectively). Aliquots ([^{3}H]**21**, 4 μL; [^{3}H]**27**, 4.43 μL) of the tentative stock solutions were added to acetonitrile/0.05% aq TFA (14:86 v/v) (126 and 125.6 μL, respectively), 100 μL of the resulting solutions were analyzed by HPLC, and five times 3 μL were counted in 3 mL of scintillator (Rotiszint eco plus) with a LS 6500 liquid scintillation counter (Beckmann-Coulter, Munich, Germany). These analyses were performed twice. The molarities of the tentative stock solutions were calculated from the mean of the peak areas and the linear calibration curve obtained from the peak areas of the standards. To determine the radiochemical purity and to proof the identity, a 0.5 μM solution of the radiolabeled peptide (100 μL), spiked with the "cold" analogue **21** or **27** (20 μM), was analyzed by RP-HPLC using the column, flow rate, gradient, and UV detection as for the quantification and additionally radiometric detection (flow rate of the liquid scintillator (acetonitrile/Rotiszint eco plus (Carl Roth) 15:85 v/v): 4.0 mL/min). The radiochemical purities amounted to 99% and 98%, respectively (see Figure 6 and Figure S21A (Supporting Information)). This analysis was repeated after 12 months of storage at −20 °C (see Supporting Information, Figure S21B). Calculated specific activity: 1.74 TBq/mmol (47.01 Ci/mmol). The activity concentration was adjusted to 27.75 MBq/mL (0.75 mCi/mL) by addition of EtOH/water (40:60 v/v) yielding a molarity of 15.9 μM (final volumes: [^{3}H]**21**, 2.528 mL; [^{3}H]**27**, 4.156 mL). Storage conditions: −20 °C. Chemical yields (tris(hydrotrifluoroacetate) of [^{3}H]**21** and [^{3}H]**27**): 62.6 μg, 40.2 nmol, and 87.8 μg, 66.1 nmol, respectively. Radiochemical yields: [^{3}H]**21**, 1.896 mCi (70.16 MBq), 59%; [^{3}H]**27**, 3.117 mCi (115.3 MBq), 65%.

Investigation of the Chemical Stability. The chemical stability of peptides **19**−**21**, **23**−**25**, **27**, **29**, **30**, **35**, and **36** was investigated in PBS at 21 °C. The incubation of compounds **19**−**21**, **25**, **27**, **35**, and **36** was started by addition of a 5 mM solution in EtOH/20 mM HCl (40:60 v/v) (8.8 μL) to 2 mM autoclaved PBS pH 7.0 (211.2 μL) to give a final concentration of 200 μM. After 0, 4, 24, and 48 h an aliquot (40 μL) was taken and diluted with acetonitrile/1% aq TFA (2:8 v/v) (40 μL). An aliquot (20 μL) of the resulting solution (pH < 3) was analyzed by RP-HPLC (analytical HPLC system and conditions see general experimental conditions).

The incubation of compounds **23**, **24**, **29** and **30** was started by addition of a 1 mM solution in DMSO (15.6 μL) (**23**, **24**) or DMSO/water (20:80 v/v) (19.5 μL) (**29**, **30**) to 2 mM PBS (114.4 and 110.5 μL, respectively) to give final concentrations of 120 and 150 μM, respectively. After 0, 3, 6, and 24 h an aliquot (30 μL) was taken and diluted with acetonitrile/1% aq TFA (2:8 v/v) (30 μL). An aliquot (20 μL) of the resulting solution was analyzed by RP-HPLC (HPLC conditions see General Experimental Conditions).

The stability of the NT(8−13) derivative **44** was investigated as described above for **19**−**21**, **25**, **27**, **35** and **36** in PBS pH 7.0 and, additionally, in a 10 mM autoclaved phosphate buffer pH 8.0 (results shown in Supporting Information, Figure S18). The 5 mM stock solution of **44** was prepared in MeCN/0.05% aq TFA (1:1 v/v).

Peptide Stability under Assay Conditions. The stability under assay conditions was investigated for AngII and derivatives **21** and **22** in the presence of CHO-hAT$_1$ cells, and for NT(8−13) and derivatives **27** and **28** in the presence of HT-29 cells at 22 °C using the same binding buffer (Dulbecco's PBS pH 7.4 supplemented with 1% BSA and 100 μg/mL bacitracin) as for radioligand binding studies performed with [^{3}H]**21** and [^{3}H]**27** (see below). One day prior to the experiment, cells were seeded into Primaria 24-well plates (Corning Life Sciences, Oneonta, NY). On the day of the experiment, cell confluency was 90−100%. The culture medium was removed by suction, the cells were washed with PBS (500 μL), and binding buffer (250 μL) containing AngII, NT(8−13), **21**, **22**, **27**, or **28** (50 μM) was added. The plates were gently shaken during incubation at 22 °C for 30 s (designated 0 h), 2 or 6 h. After incubation, 200 μL were taken from the wells, transferred into 1.5 mL reaction vessels and cooled on ice for 5−10 min. Acetonitrile (cooled to −18 °C) (470 μL) was added carefully. To precipitate protein, the reaction vessels were allowed to warm up to rt under vortexing for 5 min. The vessels were centrifuged at 16,000 g at 10 °C for 10 min and 640 μL of the supernatants were transferred into 1.5 mL reaction vessels containing 15 μL of 10% aq TFA. The solvent was removed in a vacuum concentrator (Speed-Vac Plus SC110A Savant, Thermo Electron Corporation) at 40 °C and the dry residue was redissolved in 90 μL of acetonitrile/0.05% aq TFA (10:90 v/v) under vortexing (2 min) and sonication (8 min). The solutions were filtered with 0.2-μm syringe filters (4 mm, regenerated cellulose, Phenomenex), and 20 μL of the filtrates were analyzed by RP-HPLC (HPLC conditions see General Experimental Conditions; additionally to the UV detection at 220 nm fluorescence detection was performed at ex./em. 275/305 nm). The experiments were performed in triplicate. The recoveries of the investigated compounds (mean values ranging from 76 to 86%) were fairly constant.

Fluorescence Spectroscopy and Determination of Quantum Yields. Fluorescence quantum yields of the fluorescently labeled peptides **23**, **24**, **29**, **30** and reference compound **47** were determined in PBS (pH 7.4) and PBS containing 1% BSA with a Cary Eclipse spectrofluorimeter and a Cary 100 UV/vis photometer (Varian Inc., Mulgrave, Victoria, Australia) according to a previously described procedure using acryl cuvettes (10 mm × 10 mm, Sarstedt, ref. 67.755) and cresyl violet perchlorate as quantum yield standard.[42] The following concentrations, at which the absorbances at the excitation wavelengths were between 0.09 and 0.15, were used: **23**, **29**: 3 μM in PBS and 2.5 μM in PBS + 1% BSA; **24**, **30**, **47**: 1.6 μM in PBS and 1.3 μM in PBS + 1% BSA. Emission spectra were recorded at the slit adjustments (ex./em.) 10/5 nm and 10/10 nm, and the quantum yields obtained for these slit combinations were averaged.

Cell Culture. All cell lines were maintained in 25 or 75 cm^2 culture flasks (Sarstedt) in a water saturated atmosphere (95% water, 5% CO_2) at 37 °C. CHO-hAT$_1$-G$_{\alpha 16}$-mtAEQ cells[79,80] were cultured in Ham's F12 medium (Sigma) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), G418 (Biochrom) (1000 μg/mL) and zeocin (InvivoGen, San Diego, CA) (250 μg/mL). Rat mesangial cells (kindly provided by Prof. Dr. A. Kurtz, Department of Physiology, University of Regensburg)[81] were maintained in antibiotic-free RPMI-1640 medium (Sigma) containing 10% fetal calf serum (FCS) and bovine insulin (Sigma insulin I-5500) (660 U/L). HT-29 colon carcinoma cells (ATCC no. HTB-38) were cultured in antibiotic-free McCoy's 5A medium supplemented with 5% FCS. CHO-hNTS$_1$ cells[23] were maintained in DMEM/HAM's F12 medium (1:1) supplemented with 10% FCS and hygromycin B (250 μg/mL). CHO-hY$_4$-G$_{qi5}$-mtAEQ cells were grown as previously reported.[6]

Immunoblotting. HT-29, HEK-hNTS$_2$ and CHO-hNTS$_1$ cells were grown in 177 cm^2 cell culture dishes (150 × 20 mm, Sarstedt) to 80% confluency. Cells were rinsed twice with ice-cold PBS and scraped from the dishes in a 50 mM Tris buffer pH 7.4 supplemented with 1 mM

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EDTA and protease inhibitors (SIGMAFAST Protease Inhibitor cocktail tablets, Sigma), in the following referred to as "Tris buffer" (20 mL per cell culture dish). After centrifugation (400 g, 5 min, at 4 °C) the cells were resuspended in Tris buffer (20 mL per culture dish) and homogenized using a Ultra-Turrax (IKA Werk, Staufen, Germany) (20,000 rpm, 5 × 5 s) under cooling in an ice bath followed by centrifugation (50,000*g*, 15 min, at 4 °C). The pellets were resuspended in Tris buffer (0.5 mL per culture dish) and treated with a Potter-Elvehjem homogenizer. The protein concentration was determined by the Bradford method (BioRad, Munich, Germany) and adjusted to 1600 μg/mL for each cell homogenate. Fifteen μL of cell homogenate were diluted with 15 μL of sample buffer pH 8 (water (27 mL), 1 M Tris in water (1.5 mL), glycine (6 mL), urea (28.8 g), SDS (1.5 g), dithiothreitol (1.8 g), bromophenol blue (6 mg)), and the mixtures were incubated under shaking at 30 °C for 1 h. Samples (15 μL) were loaded onto SDS gradient gels (12 wells, Novex 8−16% Tris-Glycine Gel, Fisher Scientific). SDS-PAGE was performed at 130 V, (ca. 1.5 h, buffer: Tris (3 g), glycine (14.4 g), SDS (1 g) *ad* 1 L of water, pH 8.3). A ready-to-use stained protein standard (Precision Plus Protein Dual Color Standard (BioRad) (6 μL) served as molecular weight marker. All samples were run in duplicate. The separated proteins were transferred to a nitrocellulose membrane (pore size 0.2 μm, Peqlab Biotechnologie, Erlangen, Germany) at 200 mA for 45 min (blotting buffer: Tris (0.3 g), glycine (1.4 g) *ad* 100 mL of water/MeOH (8:2 v/v)) followed by blocking with 5% skimmed milk powder in Tris-buffered saline (20 mM Tris and 150 mM NaCl in water, pH 7.6) containing 1% Tween-20 (in the following referred to as "TBST") at rt for 1.5 h. The membrane was cut in half. One half of the membrane was incubated in TBST + 5% milk powder (7 mL), containing the primary antibody (Anti-Neurotensin Receptor 2 Antibody (AB15134), Merck Chemicals, Darmstadt, Germany) at a concentration of 1.0 μg/mL, under rotation at 4 °C overnight, the second half was treated in the same manner, but the primary antibody was omitted. The membrane was washed with TBST (5 × 5 min, rt) followed by incubation in TBST + 5% milk powder, containing the secondary antibody (donkey antirabbit IgG-HRP (sc-2313), Santa Cruz Biotechnology, Dallas, TX) at a concnetration of 0.04 μg/mL, under shaking at rt in the dark for 1 h, washing with TBST (6 × 5 min, rt), and incubation with the substrate (Clarity Western ECl Substrate, BioRad) at rt for 5 min. Bioluminescence was detected with a ChemiDoc MP Imaging System (BioRad). No bands were detectable in the absence of primary antibody (data not shown).

Fura-2 Calcium Assay (HT-29 Cells). The fura-2 Ca^{2+}-assay with HT-29 colon carcinoma cells was performed as described for human erythroleukemia cells (Ca^{2+}-response elicited by NPY) using a Perkin−Elmer LS50 B spectrofluorimeter (PerkinElmer, Rodgau, Germany).[82] HT-29 cells (80−95% confluency) were trypsinized, detached from the culture flask and treated according to the described protocol. The intracellular Ca^{2+}-mobilizations induced by NT(8−13), **25** and **27** were normalized to the effect elicited by 100 nM NT(8−13).

Functional Bioluminescence Assay (CHO-AT_1-$G_{\alpha16}$-mtAEQ cells). The aequorin assay was performed as previously described for CHO-hY_4-G_{qi5}-mtAEQ cells[6] using CHO-$G_{\alpha16}$-mtAEQ cells,[79] that were kindly donated by Dr. M. Detheux (Euroscreen s.a., Gosselies, Belgium) and were stably transfected with the human AT_1R (designated as CHO-AT_1-$G_{\alpha16}$-mtAEQ cells).[80] Measurements were carried out with a GENios Pro plate reader (Tecan, Salzburg, Austria). Areas under the curve were calculated using SigmaPlot 11.0 software (Systat Software Inc.). Net fractional luminescence data were obtained by subtraction of the fractional luminescence of the blanks (that is agonist omitted, determined at least every 9 wells) from fractional luminescence data acquired in the presence of agonist. Net data were normalized to the effect elicited by 100 nM AngII (determined in at least two triplicates per 96-well plate).

Radioligand Binding Experiments with [^{3}H]21 (AT_1R) and [^{3}H]27 (NTS_1R). All radioligand binding experiments were performed at 22 ± 2 °C. Saturation and competition binding experiments with [^{3}H]**21** at intact CHO-AT_1-$G_{\alpha16}$-mtAEQ and intact rat mesangium cells were performed in Primaria 24-well plates (Corning Life Sciences) as previously described for the NPY Y Y_1 receptor radioligand [^{3}H]UR-MK136[48] at intact SK-N-MC cells with the following modifications: Dulbecco's PBS with Ca^{2+} and Mg^{2+} (1.8 mM $CaCl_2$, 2.68 mM KCl, 1.47 mM KH_2PO_4, 3.98 mM $MgSO_4$, 136.9 mM NaCl and 8.06 mM Na_2HPO_4) supplemented with 1% BSA and 100 μg/mL of the protease inhibitor bacitracin (Serva) was used as binding buffer. PBS was used for the washing steps (prior to the incubation at rt and after incubation ice-cold). Unspecific binding was determined in the presence of AngII (500-fold excess to [^{3}H]**21**). The incubation period was 2 h. The confluency of the cells was at least 90% on the day of the experiment.

Saturation and competition binding experiments with [^{3}H]**27** at intact HT-29 colon carcinoma and CHO-NTS_1 cells were performed in tissue culture treated white 96-well plates with clear bottom (Corning Incorporated Life Sciences, Tewksbury, MA; Corning cat. no. 3610) using the binding buffer as aforementioned for [^{3}H]**21**. Cells were seeded one or 2 days prior to the experiment. On the day of the experiment confluency of the cells was at least 90% (HT-29 cells) or approximately 10% (CHO-NTS_1 cells). The culture medium was removed by suction, the cells were washed with PBS (200 μL) and covered with binding buffer (160 μL). For total binding, binding buffer (20 μL) and the same volume of binding buffer containing the radioligand 10-fold concentrated were added. For the determination of unspecific binding (in the presence of NT(8−13) in 500-fold excess to [^{3}H]**27**) and displacement of [^{3}H]**27** (competition binding experiments), binding buffer (20 μL) containing the competitor 10-fold concentrated and binding buffer (20 μL) containing the radioligand 10-fold concentrated were added. The plates were gently shaken during incubation for 2 h. After incubation, the liquid was removed by suction, the cells were washed twice with ice-cold PBS (200 μL; washing period <3 min) followed by the addition of lysis solution (urea (8 M), acetic acid (3 M) and Triton-X-100 (1%) in water) (25 μL). The plates were shaken for 30 min, liquid scintillator (Optiphase Supermix (PerkinElmer)) (200 μL) was added, and the plates were sealed with a transparent sealing tape (permanent seal for microplates, PerkinElmer, prod. no. 1450−461). The plates were turned up-side down several times in order to achieve complete mixing of scintillator and lysis solution. The plates were kept in the dark for at least 1 h prior to the measurement of radioactivity (dpm) with a MicroBeta2 plate counter (PerkinElmer).

If fluorescently labeled ligands were investigated competition binding experiments were performed in the dark.

Association and dissociation experiments with [^{3}H]**21** (intact CHO-hAT_1-$G_{\alpha16}$-mtAEQ cells) and [^{3}H]**27** (intact HT-29 cells) were performed as described for the saturation and competition binding experiments (see above) using the same equipment, buffers, and volumes. Unspecific binding was determined in the presence of AngII and NT(8−13), respectively (each in 500-fold excess). For association experiments the radioligand concentration was 3 and 1.2 nM, respectively. The incubation of the cells with the radioligand was stopped after different periods of time (between 1 and 120 min). In case of dissociation experiments cells were preincubated with 8 nM [^{3}H]**21** and 2 nM [^{3}H]**27**, respectively (each for 90 min). The radioligand solution was removed by suction, the cells were covered with binding buffer (250 and 200 μL, respectively) containing AngII (160 nM) and NT(8−13) (100 nM), respectively, and the plates were gently shaken. After different periods of time the cells were washed with ice-cold PBS followed by cell lysis and further processing as described above.

Saturation Binding and Acid Wash Experiments with [^{3}H]21 and [^{3}H]27. To estimate cell surface-bound and internalized radioligand, saturation binding experiments were performed with [^{3}H]**21** and [^{3}H]**27** at CHO-hAT_1 and HT-29 cells, respectively, as described above with the following modification: Binding experiments with [^{3}H]**27** were carried out in Primaria 24-well plates (Corning Life Sciences) as in case of [^{3}H]**21** (see above). After washing twice with ice-cold PBS (500 μL) at the end of the 2-h incubation period, the cells were washed twice with ice-cold glycine buffer pH 3.0 (50 mM glycine and 125 mM NaCl in water) for 5 min and the radioactivity of the collected and combined washings (representing the fraction of cell surface-bound radioligand) was counted in 10 mL of liquid scintillator (Rotiszint eco plus) with a LS 6500 liquid scintillation counter (Beckman-Coulter).

Lysis solution (200 μL) was added to the cells, the plates were gently shaken for 30 min and the solution was transferred into 20 mL

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scintillation vials filled with 10 mL of scintillator. The wells were washed with 200 μL of lysis solution, the washings were added to the scintillation vials and the radioactivity, representing the fraction of intracellular radioligand, was counted. Unspecific binding data were determined in the presence of AngII and NT(8−13), respectively, in 500-fold excess to the radiolabeled peptide. For each radioligand two independent experiments were performed in triplicate giving comparable results.

Radioligand Competition Binding Assay with [^{3}H]NT(8−13) (NTS_2R). Radioligand competition binding experiments with [^{3}H]NT(8−13) were performed at HEK-hNTS_2 cell homogenates as previously reported.[23]

Radioligand Competition Binding Assay with [^{3}H]EYF ($NPFF_2R$). Radioligand competition binding studies with [^{3}H]EYF were performed at CHO-h$NPFF_2$ cell membranes as previously reported.[50] The K_d value of [^{3}H]EYF[50] was determined in the same system by saturation binding and amounted to 6.9 nM. Determined binding data of the reference peptides NPFF and EYF proved to be in good agreement with reported data (see Table 1D).

Flow Cytometric Competition Binding Assay (Y_4R). The flow cytometric Y_4R competition binding assay on CHO-hY_4-G_{qi5}-mtAEQ cells using S0586-[K^4]hPP as fluorescent ligand (c = 10 nM) was performed as previously described on a FACSCalibur flow cytometer (Becton Dickinson), equipped with an argon laser (488 nm) and a red diode laser (635 nm) (settings: FSC: E-1, SSC: 280 V, Fl-4:700−750 V).[83] The cell density in loading buffer was 10^6 cells/mL. The samples were incubated in 1.5 mL reaction vessels (ref. 72.690.001, Sarstedt) in the dark at rt for 90 min. Unspecific binding was determined by addition of hPP at a concentration of 1 μM. Data acquisition was stopped after counting of 10,000−15,000 gated events. The dissociation constant (K_d value) of S0586-[K^4]hPP, was determined in two independent saturation experiments applying concentrations between 0.5 and 40 nM (nonspecific binding determined in the presence of hPP 100-fold concentrated) and amounted to 7.5 ± 2.1 nM, being in good agreement with the reported K_d of 10.2 nM.[61]

Autoradiography. NMRI (nu/nu) mice were bred in the animal facility of the University of Regensburg. Animal experiments were approved by the local veterinary authorities (district government) according to the European guidelines and national regulations of the German animal protection act (notification from 12th of May, 2011, "Propagation of tumor cells in nude mice", to the Regierung der Oberpfalz, Department 621, veterinary services).

Autoradiography at HT-29 tumor cryosections using [^{3}H]**27** as radioligand were essentially performed as previously described.[40] Subcutaneous HT-29 tumors were established in male NMRI (nu/nu) mice by subcutaneous injection of a cell suspension in FCS-free culture medium (100 μL containing about three million cells) or by subcutaneous transplantation of tumor pieces. Tumors were excised, immediately embedded in Tissue-Tek and frozen on dry ice, and were stored at −78 °C. Cryosections (12 μm) were obtained at −15 °C with a 2800 Frigocut E freezing microtome (Reichert-Jung/Leica, Germany). Adjacent sections were mounted on microscopic slides (Superfrost Plus, 25 × 75 × 1 mm (Gerhard Menzel (via Thermo Scientific), Braunschweig, Germany) and put in a chamber of 100% humidity. After 1−2 min tumor sections were either carefully covered with binding buffer (D-PBS containing 0.2% BSA and 100 μg/mL bacitracin, filtered with a 0.2-μm syringe filter) (800−1000 μL) or immersed in an alcoholic formaldehyde fixative (37% (w/w) formaldehyde in water (40 mL), 95% (v/v) ethanol (360 mL) and calcium acetate (0.2 g)) for 30 s. The incubation with radioligand was started less than 15 min after preparation of the cryosections by careful removal of the binding buffer and immediate recovering of the sections with binding buffer containing [^{3}H]**27** (6 nM) (total binding) or binding buffer containing [^{3}H]**27** (6 nM) and NT(8−13) (1.8 μM) (nonspecific binding). The sections were incubated in a humidity chamber at 22 °C for a period of 60 min followed by washing in ice-cold PBS and drying over phosphorus pentoxide according to the described protocol. The incubation period on the tritium sensitive phosphor screen (medium size (252 mm × 125 mm); PerkinElmer) was 16 days. For luminescence detection a phosphorimager (Cyclone Storage Phosphor System, Packard) was used.

Masson-Goldner staining (Jerusalem's modification) of the fixed sections was performed as previously described with the following modifications:[40] phosphoric acid-Orange G was 1:3 diluted in water and the immersion period in this solution was 7 s. The incubation period in 0.2% light green was 5 min.

Homology Model of the Human Y_4 Receptor. The homology model of the human Y_4R was generated with the modeling suite SYBYL-X 1.3 (Tripos Inc., St. Louis, MO) using the crystal structure of the rat NTS_1R (PDB ID: 3ZEV) as template.[69] Model generation was essentially performed as described.[84] Loops (ICL1-ICL3 and ECL1-ECL3) were inserted nontruncated using the Loop-Search module within SYBYL. The N-terminal sequence of 27 amino acids, not resolved in the template, is not present. The model was provided with Amber7 FF99 charges and energy-minimized with the Amber7 FF99 force field[85] up to a gradient of 0.01 kcal mol^{-1} Å^{-1} using a dielectric constant of 4.

Data Processing. Specific optical rotation was calculated according to $[\alpha] = \alpha/(c \cdot l)$, for which α = measured angle of rotation, c = concentration [g · cm^{-3}], and l = length of the cuvette Dm]. Retention (capacity) factors were calculated from retention times (t_R) according to $k = (t_R - t_0)/t_0$ (t_0 = dead time).

Relative data from functional cellular assays (aequorin assay: % fractional luminescence, fura-2 assay: % Ca^{2+} response) were plotted against log(concentration agonist) and analyzed by a four parameter logistic equation (SigmaPlot 11.0, Systat Software Inc., Chicago, IL) to obtain pEC_{50} values. Specific binding data (in dpm) from saturation binding experiments with the radioligands [^{3}H]**21** and [^{3}H]**27** were plotted against the free radioligand concentration and analyzed by an equation describing hyperbolic binding (ligand binding - one site saturation fit, SigmaPlot 11.0) to obtain the dissociation (binding) constant K_d and the maximum number of binding sites B_{max}. The free radioligand concentration (nM) was calculated by subtracting the amount of specifically bound radioligand (nM) (calculated from the specifically bound radioligand in dpm, the specific activity and the volume per well) from the total radioligand concentration. Unspecific binding data from saturation binding experiments were fitted by linear regression. The number of binding sites per cell was calculated from the B_{max}, the specific activity (Bq mol^{-1}) of the radioligand, the Avogadro constant (mol^{-1}) and the number of cells per well. Specific binding data from association experiments with [^{3}H]**21** and [^{3}H]**27** were analyzed by a two-parameter equation describing an exponential rise to a maximum (SigmaPlot 11.0) to obtain the observed association rate constant k_{obs} and the maximum of specifically bound radioligand (B_{eq}), which was used to calculate specifically bound radioligand (B) in %. Data from dissociation experiments (% specifically bound radioligand (B) plotted over time) with [^{3}H]**21** were analyzed by a four-parameter equation describing a biphasic exponential decline (SigmaPlot 11.0) to obtain the dissociation rate constants $k_{off}(1)$ and $k_{off}(2)$. Data from dissociation experiments (% B plotted over time) with [^{3}H]**27** were analyzed by a two-parameter equation describing a monophasic exponential decline (SigmaPlot 11.0) to obtain k_{off}. The association rate constant (k_{on}) of [^{3}H]**27** was calculated from k_{obs}, k_{off} and the radioligand concentration ([RL]) according to the correlation: $k_{on} = (k_{obs} - k_{off})/[RL]$. Specific binding data from radioligand or fluorescent ligand competition binding experiments were plotted as % (100% = bound radioligand in the absence of competitor) over log(concentration competitor) and analyzed by a four parameter logistic equation (SigmaPlot 11.0). Resulting pIC_{50} values were transformed to IC_{50} values, which were converted to the dissociation (binding) constant K_i according to the Cheng−Prusoff equation[86] using the K_d value of the respective radioligand or fluorescent ligand ([^{3}H]**21**: K_d 0.94 nM (CHO-hAT_1 cells), [^{3}H]**27**: K_d 0.51 nM (HT-29 cells), [^{3}H]EYF: K_d 6.9 nM (CHO-h$NPFF_2$ cell membranes), S0586-[K^4]hPP: K_d 7.5 nM).

Propagated errors were calculated according to the Gaussian law of errors.

Figures of the rNTS_1 and hAT_1 receptor crystal structures and of the homology model of the NPY Y_4 receptor (Supporting Information, Figures S31−S33) were prepared with PyMOL Molecular Graphics System, version 1.6 (Schrödinger LLC, Portland, OR).

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01495.

Examples of histamine H_2 and NPY Y_1 receptor ligands containing an acylguanidine moiety; scheme showing the synthesis of peptides **1** and **19**−**36**; chemical stability of the peptides **19**, **20**, **23**−**25**, **29**, **30**, **35**, and **36**; stability of AngII, NT(8−13), **22**, and **28** under assay conditions; control experiments using a N^{ω}-acyl-Arg containing peptide: chemical instability of the NT(8−13) derivative **44**; excitation spectra, emission spectra, and fluorescence quantum yields of **23**, **24**, **29**, **30**, and **47**; RP-HPLC analysis of [^{3}H]**21** and [^{3}H]**27**: identity, purity, and long-term stability; Coomassie staining of the gel used for the immunoblot shown in Figure 8; AT_1 receptor association and dissociation kinetics of [^{3}H]**21**; NTS_1 receptor association and dissociation kinetics of [^{3}H]**27**; saturation and competition binding with the AngII derivative [^{3}H]**21** at rat mesangial cells; saturation binding with the NT(8−13) derivative [^{3}H]**27** at CHO-hNTS_1 cells; saturation binding and acid wash experiments with [^{3}H]**21** and [^{3}H]**27**; structures of the NTS_2R ligand **45**, the NTS_1R antagonist **46**, and the AT_1R antagonists candesartan and losartan; histology of the HT-29 xenograft shown in Figure 7C; structures of the rat NTS_1, the human AT_1 and the modeled human NPY Y_4 receptor; synthesis protocols and analytical data of compounds **4**, **5**, **8**, **9**, **12**, **14**, **15**, **17**, **40**, **43**, and EYF; ^{1}H NMR and ^{13}C NMR spectra of compounds **1**, **10**, **11**, **16**, **17**, **19**, **20**, **25**, **26**, **31**, **33**, and **35**; ^{1}H NMR spectra of compounds **21**, **22**, **27**, **28**, **32**, **34**, and **36**; RP-HPLC chromatograms of compounds **1**, **10**, **11**, **16**, and **19**−**36** (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AngII, angiotensin II; aq, aqueous; AT_1R, angiotensin receptor type 1; br s, broad singlet; CH_2Cl_2, dichloromethane; 2-ClTrt, 2-chlorotrityl; DIPEA, diisopropylethylamine; dpm, disintegrations per minute; EtOAc, ethyl acetate; HBTU, *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HOBt, 1*H*-benzotriazol-1-ol; hPP, human pancreatic polypeptide; *k*, retention (or capacity) factor (HPLC); K_d, dissociation (or binding) constant obtained from a saturation binding experiment; K_i, dissociation (or binding) constant obtained from a competition binding experiment; MeCN, acetonitrile; NT(8−13), neurotensin(8−13); NTS_1R, neurotensin receptor type 1; NTS_2R, neurotensin receptor type 2; NPFF, neuropeptide FF; $NPFF_2R$, NPFF receptor type 2; NPY, neuropeptide Y; PBS, phosphate buffered saline; PS, polystyrene; PyBOP, 1*H*-benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; RGD, arginine-glycine-aspartate sequence; RP-HPLC, reversed-phase HPLC; SEM, standard error of the mean; SPPS, solid phase peptide synthesis; Y_4R, NPY Y_4 receptor

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