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In Search of NPY Y_4R Antagonists: Incorporation of Carbamoylated Arginine, Aza-Amino Acids, or D-Amino Acids into Oligopeptides Derived from the C-Termini of the Endogenous Agonists

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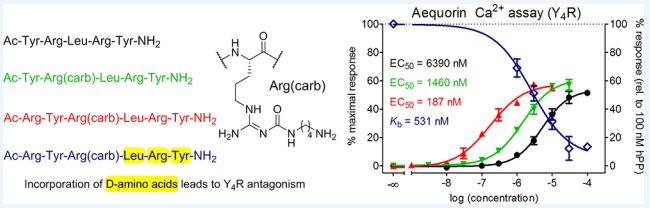
Supporting Information

ABSTRACT: The cross-linked pentapeptides (2*R*,7*R*)-diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((2*R*,7*R*)-BVD-74D, (2*R*,7*R*)-**1**) and octanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) (**2**) as well as the pentapeptide Ac-Tyr-Arg-Leu-Arg-Tyr-amide (**3**) were previously described as neuropeptide Y Y_4 receptor (Y_4R) partial agonists. Here, we report on a series of analogues of (2*R*,7*R*)-**1** and **2** in which Arg^2, Leu^3, or Arg^4 were replaced by the respective aza-amino acids. The replacement of Arg^2 in **3** with a carbamoylated arginine building block and the extension of the N-terminus by an additional arginine led to the high-affinity hexapeptide Ac-Arg-Tyr-N^{ω}-[(4-aminobutyl)aminocarbonyl]Arg-Leu-Arg-Tyr-amide (**35**), which was used as a precursor for a D-amino acid scan. The target compounds were investigated for Y_4R functional activity in assays with complementary readouts: aequorin Ca^{2+} and β-arrestin 1 or β-arrestin 2 assays. In contrast to the parent compounds, which are Y_4R agonists, several ligands were able to suppress the effect elicited by the endogenous ligand pancreatic polypeptide and therefore represent a novel class of peptide Y_4R antagonists.



1. INTRODUCTION

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

Y_4R agonists are considered to be potential antiobesity agents.[7,8] The diastereomeric mixture of the cross-linked ("dimeric") pentapeptide **1** (BVD-74D), a mimic of the C-terminus of pancreatic polypeptide, was described as a high-affinity Y_4R agonist,[4] having an effect on food intake in mice.[9] As reported previously, (2*R*,7*R*)-**1** (note: in the following, positions 2 and 7 refer to the stereo centers in the 2,7-diaminosuberic acid moiety) was, by a factor of 3−10, more potent than (2*S*,7*S*)-**1**, depending on the type of assay.[10,11] Even though Y_4R agonists should have higher clinical potential, antagonists are of interest as well, in particular, as pharmacological tools. The pentapeptide **3**,[6] a Y_4R agonist with an affinity in the two-digit nanomolar range (K_i = 50 nM), shares the same amino acid sequence with the peptide moieties of **1**. The incorporation of cyclic β-amino acids such as (1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid resulted in partial agonism.[6]

To investigate the impact of backbone modifications in Y_4R agonists on functional activity in more detail, additional analogues of (2*R*,7*R*)-**1**, **2**,[11] and **3** were prepared. Here, we report on the synthesis of Y_4R ligands in which aza-amino acids or D-amino acids were introduced. The title compounds were characterized in binding and functional cellular assays with complementary readouts.

2. RESULTS AND DISCUSSION

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

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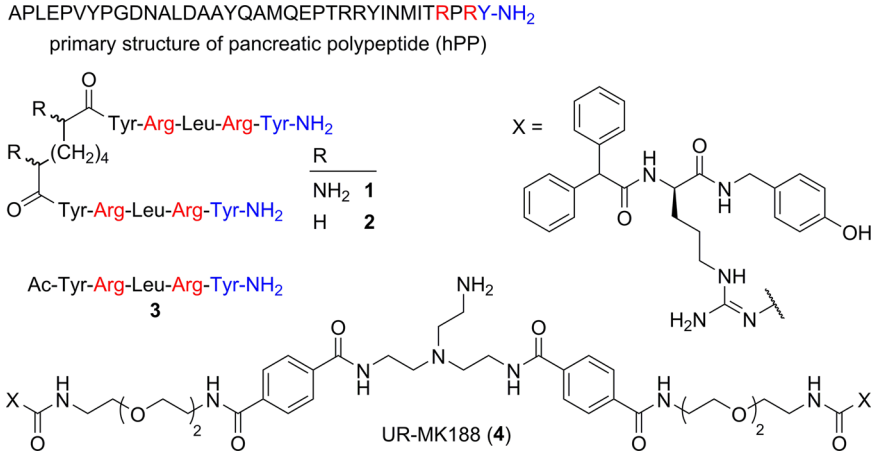


Figure 1. Amino acid sequence of human PP and structures of the described NPY Y_4R agonists **1**−**3** and the Y_1R/Y_4R antagonist UR-MK188 (**4**).

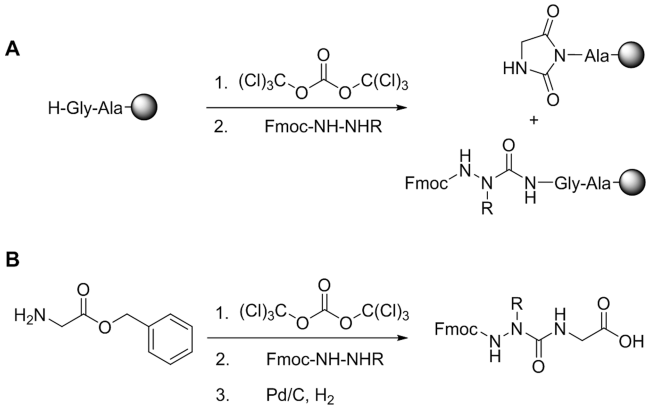


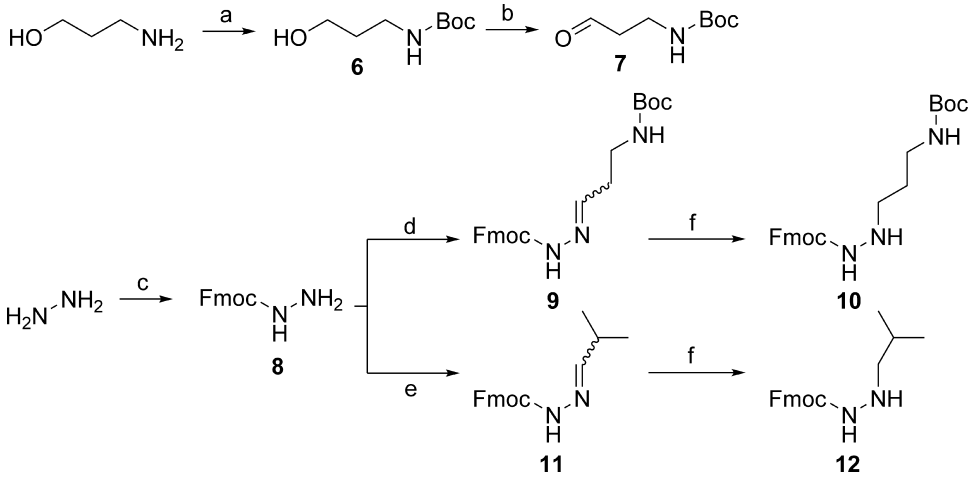
Figure 2. Synthesis of aza-peptides on a solid phase (A) or in solution (B).

Two different Fmoc-protected hydrazines were synthesized to mimic the side chains of Orn or Leu, respectively (Scheme 1). 3-Aminopropan-1-ol was Boc-protected and subsequently oxidized under Parikh−Döring conditions using dimethyl sulfoxide (DMSO) as an oxidant to form the aldehyde **7**. Condensation of (9*H*-fluoren-9-yl)methyl hydrazinecarboxylate **8** and the aldehyde **7** or isobutyric aldehyde resulted in hydrazones **9** and **11**. Subsequent reduction using sodium cyanotrihydridoborate gave access to the substituted hydrazines **10** and **12**.

The benzyl ester-protected amino acids **14**−**16** were treated with triphosgene to give the respective isocyanates in situ (Scheme 2). Without purification, the isocyanates were treated with hydrazines **10** and **12** affording the benzyl-protected *N*-Fmoc-aza[1]-dipeptides in yields >70%. Subsequent ester cleavage by hydrogenation gave access to the *N*-Fmoc-aza[1]-dipeptides Fmoc-aza-Leu-Arg(Pbf)-OH (**17**), Fmoc-aza-Orn-(Boc)-Tyr(*t*Bu)-OH (**18**), and Fmoc-aza-Orn(Boc)-Leu-OH (**19**). The dipeptides **17**−**19** were employed in solid phase synthesis under the same conditions as those for the standard Fmoc-protected amino acids, resulting in quantitative coupling efficiencies.

To optimize the coupling of a resin-bound aza[1]-peptide, the model peptide H-Ala-aza-Leu-Arg(Pbf)-NH_2 (**20**) was synthesized under various conditions (Scheme 3). When 3-[bis-

Scheme 1. Synthesis of the Hydrazines 10 and 12[a]



[a]Reagents and conditions: (a) Boc_2O, Na_2CO_3, tetrahydrofuran (THF), 0 °C, 10 min, followed by room temperature (rt), 14 h; (b) sulfur trioxide pyridine complex, DMSO, triethylamine (TEA), CH_2Cl_2, 0 °C, 1 h, followed by rt, 3 h; (c) Fmoc-Cl, MeCN/H_2O (1:1), 0 °C, 10 min, followed by rt, 12 h; (d) 7, CH_2Cl_2, 14 h; (e) isobutyraldehyde, CH_2Cl_2, 50 h; (f) sodium cyanotrihydridoborate, CH_2Cl_2/MeOH (4:3), 2 M HCl (pH 2−3), rt, 2−6 h.

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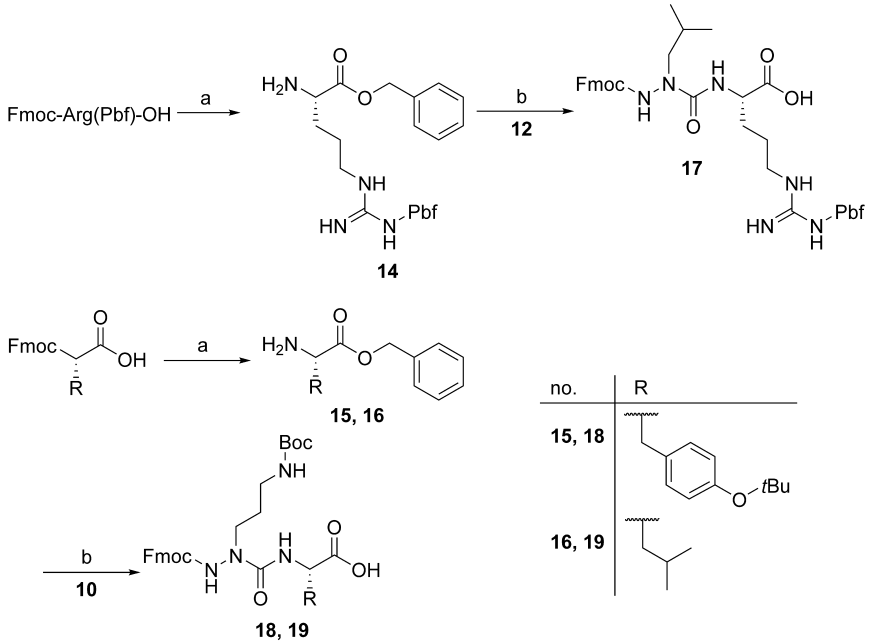
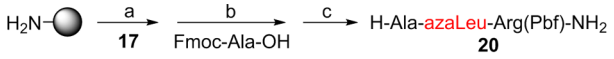
Scheme 2. Synthesis of the *N*-Fmoc-aza[1]-dipeptides 17−19[a]



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

Scheme 3. Incorporation of aza-Leu into the Model Peptide 20 via SPPS[a]



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

coupling reagent	excess of amino acid	time	temp.	solvent	yield of **20** (%)
HBTU/HOBt	4 equiv.	10 h	35 °C	DMF/NMP	5-10
Fmoc-Ala-OSu	4 equiv.	10 h	35 °C	DMF/NMP	-
HATU	4 equiv.	10 h	30 °C	DMF/NMP	50-60
triphosgene	4 equiv.	12 h	30 °C	THF	50-60
triphosgene	4 equiv.	12 h	30 °C	DCM	0-5

[a]Reagents and conditions: (a) SPPS (Fmoc strategy), **17**/HBTU/HOBt/DIPEA (3/3/3/6 equiv), solvent DMF, 35 °C, 14 h; Fmoc deprotection was carried out with 20% piperidine in DMF/*N*-methyl-2-pyrrolidone (NMP) (8:2), rt, 2 × 10 min; (b) variation of the conditions according to the table; Fmoc deprotection as under (a); (c) Fmoc deprotection, followed by CH_2Cl_2/trifluoroacetic acid (TFA) (97:3), 10 × 6 min.

(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate/hydroxybenzotriazole (HBTU/HOBt) was used as coupling reagent at 35 °C, only 5−10% of the desired products were formed. The same holds for the application of the succinimidyl ester, Fmoc-Ala-OSu. The coupling efficiency was increased to approximately 50% when the 7-aza-benzotriazole derivative HATU was used as coupling reagent. The best results (>50% coupling efficiency) were achieved by in situ activation of Fmoc-Ala-OH to the corresponding acyl chloride using triphosgene in THF. Surprisingly, when CH_2Cl_2 was used as a solvent instead of THF, only minor amounts of **20** were detected.

The dimeric aza-peptide (2*R*,7*R*)-**26**, an aza-Leu analogue of (2*R*,7*R*)-**1**, was prepared from the protected pentapeptide **21** and the diacid (*R*,*R*)-**24** (Scheme 4). For the preparation of the aza-peptides **27**−**31**, octanedioic acid disuccinimidyl ester (**25**) was used for cross-linking. Cross-linking of **21** with **25** yielded the dimeric aza-peptide **27**, an aza-Leu analogue of **2**. Dimerization of the protected peptides **22** and **23** resulted in the aza-peptides **28** and **30** containing ornithine instead of arginine. Subsequently, guanidinylation was performed with *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine resulting in **29** and **31**, which were aza-Arg-containing analogues of **2**.

Very recently, we reported that the replacement of Arg[2] with the N^{ω}-carbamoylated arginine **32** (Figure 3) in one of the pentapeptide chains of **2** led to increased Y_4R affinity.[11] Similarly, **32** was used for the preparation of peptides **33**−**42** (Figure 4), which are analogues of the pentapeptide **3**, on an Fmoc-Sieber-PS resin by manual SPPS according to the Fmoc strategy.

Finally, the applicability of **32** to the preparation of cyclic peptides via head-to-side-chain cyclization was investigated. Cyclic peptides are structurally more constrained than their linear counterparts and can mimic secondary structural elements of native peptides or proteins. Additionally, cyclic peptides are less prone to enzymatic degradation. The formation of head-to-tail cyclic peptides is often compromised by rather rigid backbones. However, the side-chain of **32** is very flexible, facilitating an approximation of the activated C-terminus and the terminal amino group. The hexapeptide **43** was prepared on an H-L-Tyr(*t*Bu)-2CT resin followed by global side-chain deprotection (Scheme 5). Cyclization was achieved at a peptide concentration of 1 mM using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as coupling reagent. Under these conditions, the formation of peptide dimers was not observed.

2.2. Competition Binding Studies at NPY Receptor Subtypes. Y_4R Binding. The K_i values of all of the target

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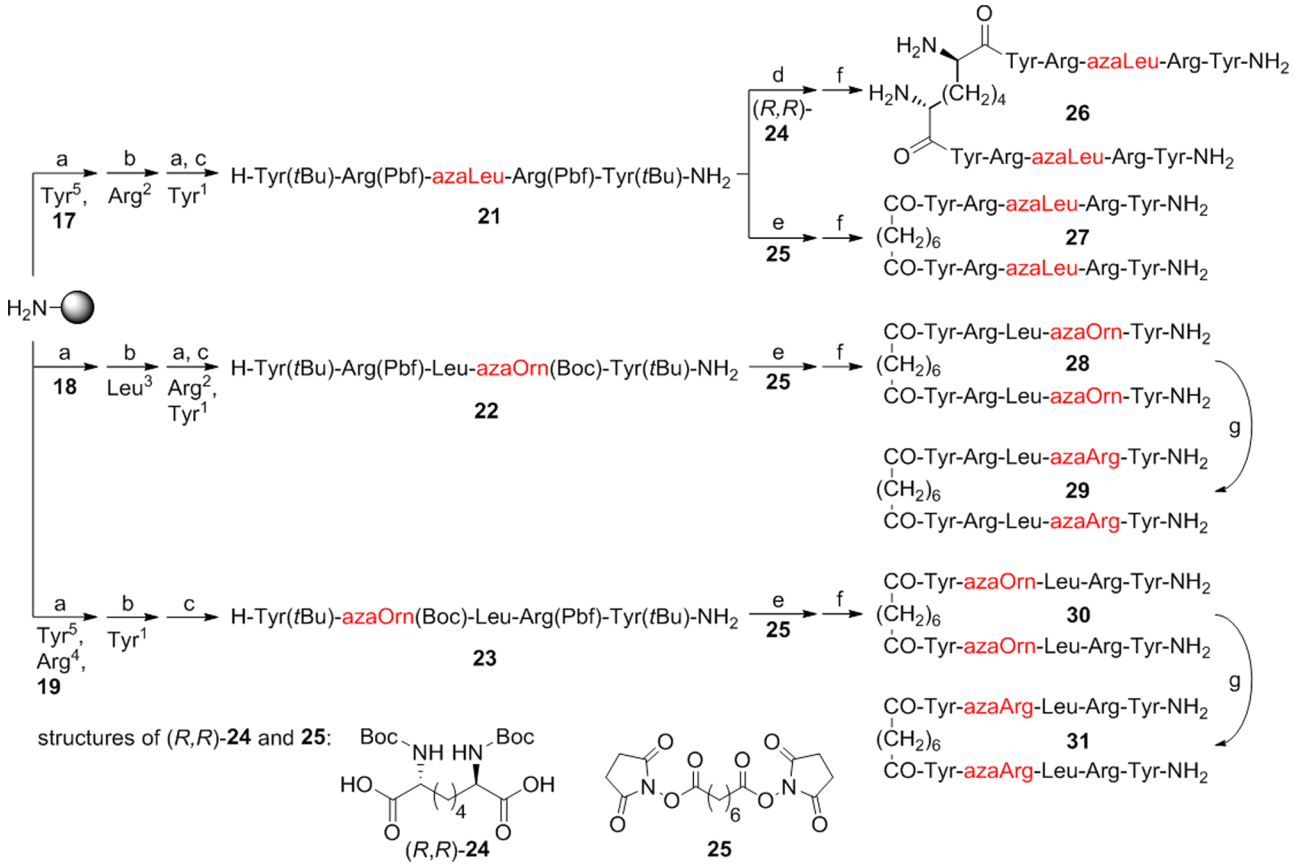
Scheme 4. Synthesis of "Homodimeric" Aza-Peptides 26−31[a]



[a]Reagents and conditions: (a) SPPS (Fmoc strategy), Fmoc-aa/HBTU/HOBt/DIPEA (5/5/5/10 equiv), solvent DMF/NMP (8:2), "double" coupling at rt, 60 min or dipeptide (**17**, **18**, or **19**)/HBTU/HOBt/DIPEA (3/3/3/6), solvent: DMF, 35 °C, 14 h; Fmoc deprotection as under (a) in Scheme 3; (b) triphosgene, DIPEA, THF, 35 °C, 14 h; Fmoc deprotection as under (a) in Scheme 3; (c) CH_2Cl_2/TFA (97:3), 10 × 6 min; (d) HBTU, HOBt, DIPEA, anhydrous DMF, 35 °C, 16 h; (e) 1 equiv of **25** in 1% DIPEA in anhydrous DMF, followed by 2.5 equiv of **21**, **22**, or **23**, 35 °C, 16 h; (f) TFA/H_2O (95:5), rt, 2.5 h; (g) *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine, DMF, DIPEA, rt, 4 h; Boc-deprotection, TFA/CH_2Cl_2/H_2O (10/10/1), rt, 3 h.

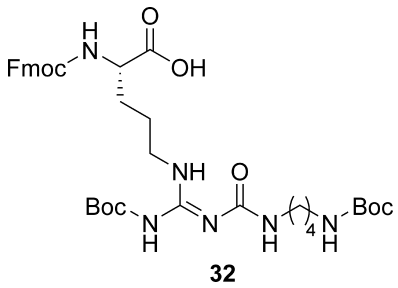


Figure 3. Structure of the N^{ω}-carbamoylated arginine building block **32**.[20]

compounds were determined in competition binding studies on live cells expressing Y_4R (CHO-hY_4R-G_{qi5}-mtAEQ cells) using the radioligand [^{3}H]UR-KK193 (**45**, Figure 5) (Table 1, Figure 6).[11] The affinities of the aza-peptides **27** (K_i = 134 nM), **29** (K_i = 52.7 nM), and **31** (K_i = 113 nM) were lower than the affinity of the respective reference compound **2** (K_i = 3.5 nM). (*R*,*R*)-2,7-Diaminosuberic acid was superior to octanedioic acid as a linker in the dimeric aza-peptides (cf. (2*R*,7*R*)-**26**: K_i = 30.8 nM), as in the case of peptide **1** compared to **2**. The aza-Orn precursors of **29** and **31**, compounds **28** (K_i = 130 nM) and **30** (K_i = 385 nM), bind to the Y_4R as well, though with lower affinities compared to their aza-Arg counterparts.

Among the structural modifications of pentapeptide **3**, replacement of the N-terminal acetic acid with octanoic acid (compound **33**) resulted in an increase in Y_4R affinity by a factor of almost six. The replacement of Arg^2 in **3** with the carbamoylated arginine **32** was even more favorable: the affinity of **34** (K_i = 19.5 nM) was 17-fold higher than the affinity of its parent compound **3**. Extension of the N-terminus by an additional arginine led to a further increase in affinity: the K_i value of compound **35** was in the single-digit nanomolar range (K_i = 2.87 nM). Up to now, comparable binding data were achieved only in the case of cross-linked or significantly longer linear peptides. The extension of the N-terminus of **3** by an additional arginine alone had almost the same impact on Y_4R affinity, that is, **36** (K_i = 3.43 nM) showed nearly the same affinity as peptide **35**. Interestingly, the replacement of the arginine adjacent to the C-terminus with the modified arginine building block **32** was not tolerated (**37**: K_i = 501 nM).

Following the structural optimization of **3**, a D-amino acid scan was performed with the hexapeptide **35**. Whereas replacement of Arg^1 or Tyr^2 with D-Arg or D-Tyr, respectively, was tolerated (cf. **41**, **42**), replacement of Leu^4, Arg^5, or Tyr^6 by the corresponding D-amino acid led to a marked decrease in Y_4R affinity (cf. **38**−**40**).

Previous studies revealed that C-terminal amidation is crucial for Y_4R binding of the endogenous ligand hPP.[21] Similarly, the

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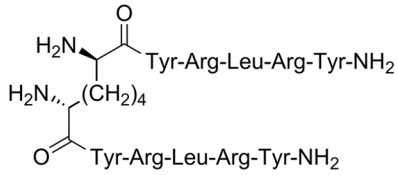
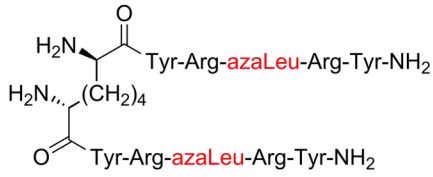
compd no.	sequence
(2*R*,7*R*)-**1**	
2	CO-Tyr-Arg-Leu-Arg-Tyr-NH_2 $(CH_2)_6$ CO-Tyr-Arg-Leu-Arg-Tyr-NH_2
26	
27	CO-Tyr-Arg-azaLeu-Arg-Tyr-NH_2 $(CH_2)_6$ CO-Tyr-Arg-azaLeu-Arg-Tyr-NH_2
28	CO-Tyr-Arg-Leu-azaOrn-Tyr-NH_2 $(CH_2)_6$ CO-Tyr-Arg-Leu-azaOrn-Tyr-NH_2
29	CO-Tyr-Arg-Leu-azaArg-Tyr-NH_2 $(CH_2)_6$ CO-Tyr-Arg-Leu-azaArg-Tyr-NH_2
30	CO-Tyr-azaOrn-Leu-Arg-Tyr-NH_2 $(CH_2)_6$ CO-Tyr-azaOrn-Leu-Arg-Tyr-NH_2
31	CO-Tyr-azaArg-Leu-Arg-Tyr-NH_2 $(CH_2)_6$ CO-Tyr-azaArg-Leu-Arg-Tyr-NH_2

compd no.	sequence
3	Ac-Tyr-Arg-Leu-Arg-Tyr-NH_2
33	Oct-Tyr-Arg-Leu-Arg-Tyr-NH_2
34	Ac-Tyr-Arg(carb)-Leu-Arg-Tyr-NH_2
35	Ac-Arg-Tyr-Arg(carb)-Leu-Arg-Tyr-NH_2
36	Ac-Arg-Tyr-Arg-Leu-Arg-Tyr-NH_2
37	Ac-Arg-Tyr-Arg-Leu-Arg(carb)-Tyr-NH_2
38	Ac-Arg-Tyr-Arg(carb)-Leu-Arg-*D*-Tyr-NH_2
39	Ac-Arg-Tyr-Arg(carb)-Leu-*D*-Arg-Tyr-NH_2
40	Ac-Arg-Tyr-Arg(carb)-*D*-Leu-Arg-Tyr-NH_2
41	Ac-Arg-*D*-Tyr-Arg(carb)-Leu-Arg-Tyr-NH_2
42	Ac-*D*-Arg-Tyr-Arg(carb)-Leu-Arg-Tyr-NH_2
43	Ac-Arg-Tyr-Arg(carb)-Leu-Arg-Tyr-OH
44	Ac-Arg-Tyr-cyclo[Arg(carb)-Leu-Arg-Tyr]

Figure 4. Primary structures of all investigated peptides.

affinity of the carboxylic acid **43** (IC_{50} > 5000 nM) was much lower than the affinity of the amide **35**. Cyclization of **43** resulted in an increase in Y_4R affinity. However, the cyclic peptide **44** (K_i = 1650 nM) was only a weak binder compared to the linear analogue **35**.

2.3. NPY Receptor Subtype Selectivity. K_i values of all of the target compounds were also determined at the Y_1, Y_2, and Y_5 receptors using the radioligands [^{3}H]propionyl-pNPY (Y_2R and Y_5R) or [^{3}H]UR-MK136 (**46**, Y_1R, Figure 5). Neither the linear peptides **34**−**43** nor the cyclic peptide **44** displayed remarkable affinity to the Y_1, Y_2, or Y_5 receptors. Having a more than 1000-fold selectivity for the Y_4R over the other NPY receptors, peptide **35** proved to be superior to (2*R*,7*R*)-**1** and **2**. Only the pentapeptide **33** bound to the Y_1R with a K_i value in the submicromolar range (K_i (Y_1R) = 589 nM), that is, the lipophilic N-terminal octanoyl residue is disadvantageous with respect to Y_4R selectivity.

The cross-linked aza-peptides (2*R*,7*R*)-**26**, **27**, and **29** displayed only low Y_1R, Y_2R, and Y_5R affinities, whereas the affinity of **31** (K_i = 53 nM) at the Y_1R was markedly higher than that of the peptide analogue **2**. In a Fura-2 Ca^{2+} assay on human erythroleukemia (HEL) cells, **31** revealed Y_1R antagonism (Figure 7) with a K_b value of 11.5 nM.

2.4. Functional Studies at the Y_4R. The target compounds were investigated for Y_4R agonism and antagonism in an aequorin Ca^{2+} assay as well as β-arrestin 1 and β-arrestin 2 recruitment assays on genetically engineered CHO or HEK293T cells, respectively (for data see Table 2, Figures 8−10, SI Figures 1−8).

2.5. Y_4R Agonism. (2*R*,7*R*)-**1** was a partial agonist in both the Ca^{2+} assay and the β-arrestin recruitment assays (α = 0.62, (Ca^{2+} assay), α = 0.54 (β-arrestin 1) or 0.58 (β-arrestin 2), Figure 8A,C,E). The pentapeptides **33** and **34**, and the hexapeptides **35** and **36**, were partial agonists achieving between 44 and 78% of the maximal response of hPP in both the calcium and the arrestin assay (Figure 8). Y_4R agonism was retained, though at a lower level, when D-amino acids were introduced into the N-terminal part of **35** (cf. **41**, **42**; Figure 8). In contrast, the aza-peptides (2*R*,7*R*)-**26**, **27**, and **31**, the carbamoyl-Arg modified peptides **37**−**40**, including those bearing D-amino acids in the C-terminus (**38**−**40**), as well as the cyclic hexapeptide **44** were devoid of Y_4R agonism in the aequorin Ca^{2+} assay and the β-arrestin recruitment assays (SI Figures 2−7). The aza-peptide **29** displayed weak partial agonism with extremely low intrinsic activity (EC_{50} = 542 nM, α = 0.07, (Ca^{2+} assay); EC_{50} = 875 nM, α = 0.09 (β-arrestin 1);

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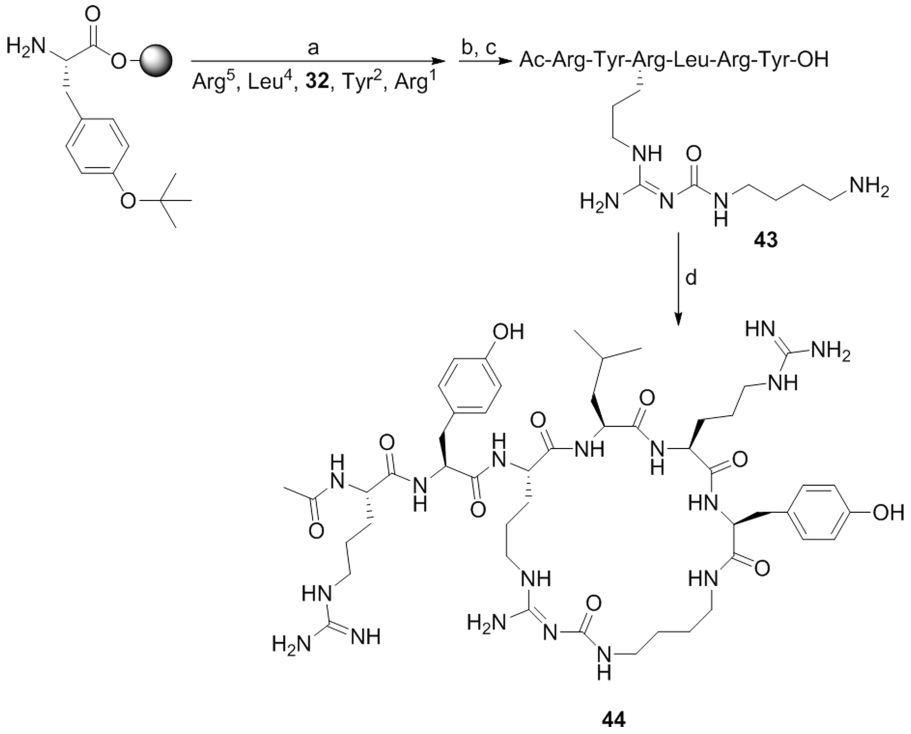
Scheme 5. Synthesis of the Head-to-Side-Chain Cyclic Peptide 43[a]



[a]Reagents and conditions: (a) SPPS (Fmoc strategy), Fmoc-aa/HBTU/HOBt/DIPEA (5/5/5/10 equiv), solvent DMF/NMP (8:2), double coupling at rt, 60 min or **32**/HBTU/HOBt/DIPEA (3/3/3/6), solvent: DMF, 35 °C, 14 h; Fmoc deprotection as under (a) in Scheme 3; (b) CH_2Cl_2/TFA (97:3), 10 × 6 min; (c) TFA/H_2O (95:5), rt, 2.5 h; (d) PyBOP, HOBt, DIPEA, DMF, rt, 24 h.

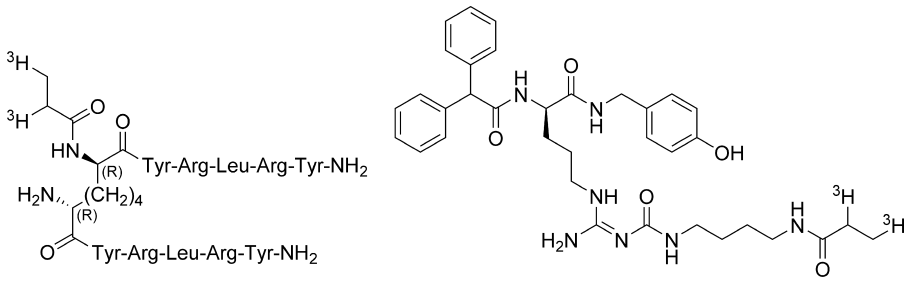


Figure 5. Structures of the Y_4R radioligand [^{3}H]UR-KK193 (**45**) and the Y_1R radioligand [^{3}H]UR-MK136 (**46**).

EC_{50} = 473, α = 0.12 (β-arrestin 2)). Compounds (2*R*,7*R*)-**26** and **27** were also investigated for Y_4R agonism in a luciferase reporter gene assay. Surprisingly, both aza-peptides displayed partial agonism in this assay (α = 0.81, EC_{50} = 176 nM ((2*R*,7*R*)-**26**), α = 0.75, EC_{50} = 220 nM (**27**)). Functional assays with distal readouts such as the luciferase reporter gene assay can reflect pronounced signal amplifications. It should be taken into account that the incubation period in the luciferase reporter gene assays was 4.5 h compared to 60 min for the arrestin assays and a few seconds in the case of the aequorin assay. Therefore, compounds displaying negligible agonistic activity in assays with proximal readouts may show much higher efficacies in reporter gene assays, as demonstrated, for example, for histamine H_4 receptor ligands.[28] Likewise, (2*R*,7*R*)-**1**, a partial agonist in the Ca^{2+} assay and the arrestin assay, appeared as a full agonist in the luciferase assay.[11]

2.6. Y_4R Antagonism. The incorporation of aza-amino acids into the cross-linked peptides (2*R*,7*R*)-**1** and **2** had an impact on the quality of action. The dimeric aza-peptides (2*R*,7*R*)-**26**, **27**, **29**, and **31** were able to suppress the response elicited by the endogenous ligand hPP (Figure 9, SI Figure 8). The introduction of D-amino acids into the C-terminal tripeptide of **35** led to Y_4R antagonism, with compound **40**, in which Leu^4 was replaced by D-Leu, displaying the strongest antagonism in the Ca^{2+} assay (Table 2, SI Figure 8). Compound **37**, in which Arg^5 was replaced by the carbamoylated arginine **32**, and the cyclic hexapeptide **44** were identified as weak Y_4R antagonists (Figure 10, SI Figure 1).

In summary, modification of the C-terminal part of the monomeric and cross-linked peptide partial agonists by the introduction of aza-amino acids or D-amino acids as well as rigidization of the backbone by head-to-side-chain cyclization

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Table 1. Binding Data at NPY Receptor Subtypes

	Y_1R	Y_2R	Y_4R	Y_5R
compd	K_i [nM][a]	K_i [nM][b]	K_i [nM][c]	K_i [nM][d]
hPP	440[e]	>5000[e]	0.65[f]	17[e]
(2*R*,7*R*)-**1**	440[f]	830[f]	0.45[f]	1500[f]
2	720[f]	1700[f]	3.5[f]	280[f]
3	>5000	>5000	337 ± 110	>5000
4	24[g]	920[g]	660[f]	>5000[g]
(2*R*,7*R*)-**26**	1840 ± 380	>5000	30.8 ± 7.5	>5000
27	574 ± 49	>5000	134 ± 20	2700 ± 490
28	n.d.	n.d.	130 ± 39	n.d.
29	3730 ± 350	>5000	52.7 ± 7.1	3560 ± 440
30	n.d.	n.d.	385 ± 127	n.d.
31	53 ± 12	3270 ± 870	113 ± 14	2100 ± 240
33	589 ± 120	>5000	56.9 ± 5.3	3440 ± 210
34	>5000	>5000	19.5 ± 2.6	>5000
35	4830 ± 1400	>5000	2.87 ± 0.78	>5000
36	1180 ± 380	>5000	3.43 ± 1.3	>5000
37	1120 ± 270	>5000	501 ± 200	>5000
38	>5000	>5000	724 ± 140	>5000
39	>5000	>5000	568 ± 76	>5000
40	>5000	>5000	248 ± 23	>5000
41	>5000	>5000	10.4 ± 3.3	>5000
42	>5000	>5000	40.5± 15	>5000
43	>5000	>5000	>5000	>5000
44	>5000	>5000	1650 ± 290	>5000

[a]Radioligand competition binding assay with **46**[22] (K_d = 6.2 nM, c = 4 nM) using MCF-7-hY_1 cells.[23] [b]Radioligand competition binding assay with [^{3}H]propionyl-pNPY[24] (K_d = 1.4 nM, c = 1 nM) using CHO-hY_2-G_{qi5}-mtAEQ cells.[25] [c]Radioligand competition binding assay with **45**[11] (K_d = 0.67 nM, c = 0.6 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells.[26] [d]Radioligand competition binding assay with [^{3}H]propionyl-pNPY (K_d = 4.83 nM, c = 4 nM) using HEC-1b hY_5R cells.[27] [e]K_i value reported by Berlicki et al.[6] [f]K_i value reported by Kuhn et al.[11] [g]K_i value reported by Keller et al.[5] Presented are mean values ± SEM from at least three independent experiments (performed in triplicate). n.d.: not determined.

changed the quality of action to antagonism in the aequorin and β-arrestin assays. Unfortunately, these modifications went along with a decrease in Y_4R affinity. The EC_{50} values of the reported Y_4R partial agonists (**33**−**36**, **41**, **42**) were substantially higher than the K_i values. This effect was previously discussed and can be attributed to the presence or absence of sodium ions in the buffers.[11,29]

3. CONCLUSIONS

The affinity and potency of the Y_4R partial agonist **3** was considerably increased by the replacement of Arg^2 with the carbamoylated arginine **32** and the introduction of an additional arginine at the N-terminus. Several backbone modifications of the C-terminal tripeptide (e.g., the introduction of aza-amino acids or D-amino acids) of **35** as well as head-to-side-chain cyclization changed the quality of action of the linear or dimeric Y_4R peptide ligands from partial agonism to antagonism. The resulting compounds displayed weaker Y_4R antagonism than the reported Y_4R antagonist **4**. However, due to their extremely facile synthetic accessibility, linear peptides derived from **35** containing D-amino acids (e.g., **40** (K_i = 248 nM)) are promising building blocks for further structural modifications and might pave the way for the development of peptide Y_4R antagonists with increased affinity.

4. EXPERIMENTAL SECTION

4.1. Chemistry: General Conditions. Chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise indicated. DMF for peptide synthesis, NMP for peptide synthesis, 4-DMAP, sodium cyanotrihydroborate, and HOBt hydrate were from Acros Organics/Fisher Scientific (Nidderau, Germany). Fmoc-Sieber-PS resin (0.61 mmol/g), Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(*t*Bu)-OH, H-L-Tyr(*t*Bu)-2CT resin (0.68 mmol/g), PyBOP, and HBTU were from Iris Biotech (Marktredwitz, Germany). MeCN for HPLC (gradient grade), Fmoc-Leu-OH, DCC, sulfur trioxide pyridine complex, hydrazine hydroxide, and methanol were from Merck (Darmstadt, Germany). Trifluoroacetic acid, TEA, triphosgene, Fmoc chloride, isobutyraldehyde, DEA, CH_2Cl_2, diethyl ether, and Triton X-100 were from Sigma-Aldrich (Deisenhofen, Germany); di-*tert*-butyl dicarbonate (>97%) and 3-aminopropan-1-ol were from Alfa Aesar (Karlsruhe, Germany). DIPEA (99%) was from ABCR (Karlsruhe, Germany), and Fmoc-Ala-OSu was from Advanced Chemtech (Louisville, KY). Bovine serum albumin (BSA) and bacitracin were from Serva (Heidelberg, Germany) and coelenterazin h was obtained from Biotrend (Cologne, Germany). Human pancreatic polypeptide and porcine NPY were from Synpeptide (Shanghai, China). The synthesis of (2*R*,7*R*)-**1**,[11] **3**,[6] **4**,[5] **24**,[11] **25**,[30] **32**,[20] **45**,[11] **46**,[22] and [^{3}H]propionyl-pNPY[24] was previously described. Millipore water was used throughout for the preparation of buffers and HPLC eluents. Stock solutions of test compounds were prepared in Millipore water containing 0.1% TFA. Polypropylene reaction vessels (1.5 or 2 mL) with a screw cap (Süd-Laborbedarf, Gauting, Germany) were used for small scale reactions (e.g., the preparation of **44**) and for the storage of stock solutions. Thin-layer and column chromatography, NMR spectroscopy, mass spectrometry, preparative and analytical

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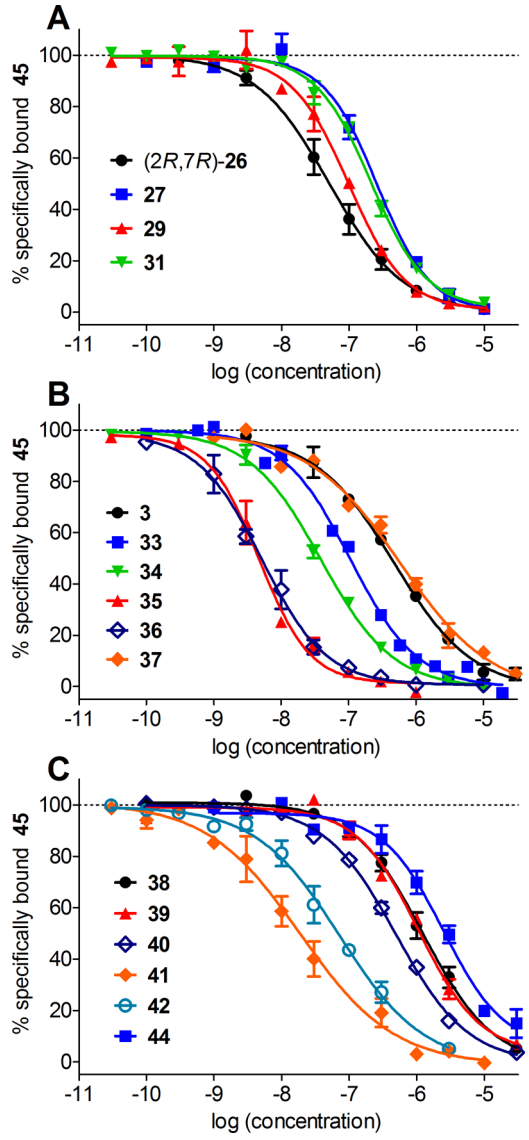

Figure 6. Competition binding performed with the radioligand **45** at CHO-hY$_4$R-G$_{qi5}$-mtAEQ cells. (A–C) Displacement of **45** (K_d = 0.67 nM, c = 0.6 nM). Data represent mean values ± SEM of at least three independent experiments, each performed in triplicate.

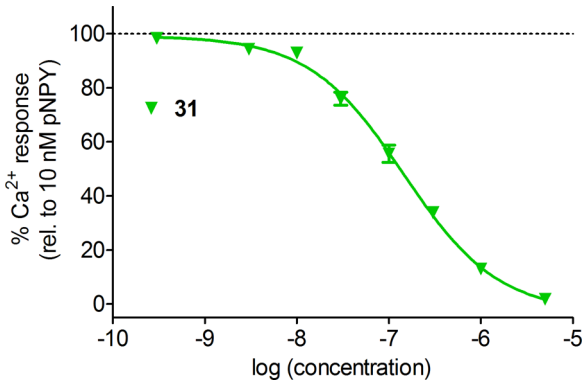

Figure 7. Y$_1$R antagonism of **31**. Inhibition of pNPY (c = 10 nM, EC$_{50}$ = 0.87 nM)-induced intracellular Ca^{2+} mobilization in HEL cells by **31** (K_b = 11.5 ± 1.1 nM), determined in a Fura-2 assay. Mean ± SEM from three independent experiments.

HPLC, as well as freeze-drying were performed as described previously.[11]

4.2. Compound Characterization. Compounds were characterized as described previously.[11] Purities determined by reversed-phase high-performance liquid chromatography (RP-HPLC) were >95%.

4.3. Chemistry: Experimental Protocols and Analytical Data. General Procedure for Solid Phase Peptide Synthesis. Peptides were synthesized by manual SPPS using the Fmoc strategy on an Fmoc-Sieber-PS resin or an H-L-Tyr(*t*Bu)-2CT resin (**43**) as described with minor modifications. Five milliliters Discardit II syringes (Becton Dickinson, Heidelberg, Germany) were equipped with 35 μm polyethylene frits (Roland Vetter Laborbedarf, Ammerbuch, Germany) and used as reaction vessels. For the coupling of standard D- or L-amino acid to the N-terminus of an amino acid or the amino group of the Fmoc-Sieber-PS resin, DMF/NMP (8:2) was used as solvent. Fmoc amino acids (5-fold excess) were preactivated with HBTU/HOBt/DIPEA (5/5/10 equiv) for 5 min and added to the resin. Double coupling at rt was performed for all standard amino acids for 45 min. The coupling of the arginine building block **32** and the dipeptides **17**–**19** (3-fold excess, preactivated with HBTU/HOBt/DIPEA (3/3/6 equiv)) was performed at 35 °C for 14 h ("single coupling") using anhydrous DMF as solvent. For the coupling of Fmoc amino acids to a resin-bound aza-amino acid, triphosgene was used as the coupling reagent. Triphosgene (1.75 equiv) was dissolved in anhydrous THF and cooled to 0 °C. A solution of Fmoc amino acid (5 equiv) and DIPEA (15 equiv) in anhydrous THF was added dropwise causing the formation of a white precipitate. After addition, stirring was continued for 5 min. The suspension was centrifuged and the supernatant was added to the resin. Coupling was performed at 35 °C for 12 h.

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

*4.3.1. Benzyl (S)-2-Amino-3-[4-(tert-butoxy)phenyl]-propanoate (**15**).* Fmoc-Tyr(*t*Bu)-OH (2 g, 4.35 mmol, 1 equiv), 4-DMAP (53.2 mg, 0.1 equiv), and benzyl alcohol (1 mL, 2.2 equiv) were dissolved in CH_2Cl_2 (25 mL) and a solution of DCC (943 mg, 1.05 equiv) in CH_2Cl_2 (6 mL) was added dropwise under ice-cooling. The mixture was stirred at rt for 14 h. After completion of the reaction (monitored by thin-layer chromatography (TLC) (light petroleum/EtOAc 2:1): R_f = 0.76), the mixture was filtered, and the filtrate was washed with 0.1 M HCl solution (30 mL) and brine (20 mL). The volume of the organic layer was adjusted to 70 mL with CH_2Cl_2, and diethylamine (12 mL) was added for removal of the Fmoc group. After all of the starting material had been consumed, the volatiles were removed on a rotary evaporator, and the residue was taken up in light petroleum/EtOAc (1:1, 10 mL) and subjected to column chromatography (eluent: light petroleum/EtOAc 1:1 → EtOAc/MeOH 4:1). The desired compound was obtained as a yellowish highly viscous oil (1.05 g, 73.7%). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 1.26 (s, 9H), 1.80 (br s, 2H), 2.71–2.89 (m, 2H), 3.60 (t, 2H, J 6.9 Hz), 5.05 (s, 2H), 6.84 (d, 2H, J 8.5 Hz), 7.06 (d, 2H, J 8.4 Hz), 7.25–7.39 (m, 5H). ^{13}C NMR (100.6 MHz, DMSO-d_6): δ (ppm) 29.0 (3 carb), 56.4, 66.0, 78.0, 123.8 (2 carb), 128.40 (2 carb), 128.43, 128.8 (2 carb), 130.2 (2 carb), 132.9, 136.5, 153.9, 175.4. HRMS (ESI): m/z [M + H]$^+$ calcd for

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Table 2. NPY Y_4R Agonist Potencies (EC_{50}) and Intrinsic Activities (α) or Antagonism (IC_{50} Values)

	aequorin assay[a]		β-arrestin 1 assay[b]		β-arrestin 2 assay[b]	
compd	EC_{50} or IC_{50} [nM]	α	EC_{50} or IC_{50} [nM]	α	EC_{50} or IC_{50} [nM]	α
hPP	9.7[c]	1	3.54 ± 0.59	1	2.74 ± 0.23	1
(2*R*,7*R*)-**1**	6.9[c]	0.62	40.4 ± 11	0.54	31.8 ± 12	0.58
2	49[c]	0.73	n.d.	n.d.	n.d.	n.d.
3	6390 ± 450	0.55	n.d.	n.d.	n.d.	n.d.
4	*226*[d]	n.a.	*187 ± 72*	n.a.	*202 ± 78*	n.a.
(2*R*,7*R*)-**26**[e]	*388 ± 51*	n.a.	*5430 ± 350*	n.a.	*1320 ± 230*	n.a.
27[f]	*1430 ± 290*	n.a.	*1090 ± 350*	n.a.	*712 ± 87*	n.a.
29	542 ± 110	0.07	875 ± 260	0.09	473 ±65	0.12
	572 ± 59	n.a.	n.d.	n.d.	n.d.	n.d.
31	*1340 ± 530*	n.a.	n.d.	n.d.	n.d.	n.d.
33	2050 ± 84	0.44	2200 ± 130	0.56	1790 ± 1070	0.61
34	1460 ± 83	0.61	1070 ± 520	0.62	568 ± 120	0.60
35	187 ± 23	0.58	144 ± 28	0.48	124.1 ± 24	0.55
36	303 ± 68	0.78	187 ± 40	0.51	140 ± 45	0.54
37	*9580 ± 2000*	n.a.	*6640 ± 780*	n.a.	*8050 ± 120*	n.a.
38	*18 800 ± 6500*	n.a.	*1310 ± 390*	n.a.	*1120 ± 250*	n.a.
39	*8340 ± 2900*	n.a.	n.d.	n.d.	n.d.	n.d.
40	*6000 ± 2600*	n.a.	n.d.	n.d.	n.d.	n.d.
41	608 ± 140	0.61	2050 ± 780	0.46	710 ± 200	0.49
42	756 ± 230	0.63	2160 ± 600	0.45	624 ± 150	0.56
44	*12 400 ± 5500*	n.a.	*5390 ± 1630*	n.a.	*3940 ± 660*	n.a.

[a]Aequorin calcium mobilization assay on CHO-hY_4-G_{qi5}-mtAEQ cells.[26] Antagonism (IC_{50} values given in italics) was determined in the presence of 100 nM hPP (EC_{50} = 9.7 nM). [b]β-Arrestin 1,2 recruitment assay on HEK293T cells stably expressing the Y_4R and ARRB1 or ARRB2. Antagonism (IC_{50} values given in italics) was determined in the presence of 3 nM hPP (EC_{50} (ARRB1) = 3.54 nM; EC_{50} (ARRB2) = 2.74 nM). [c]EC_{50} value reported by Kuhn et al.[11] [d]Reported as K_b = 20 nM by Keller et al.[5] [e]Y_4R agonism on HEK293-hY_4R-CRE Luc cells (luciferase reporter gene assay): EC_{50} = 176 ± 50 nM, α = 0.81. [f]Y_4R agonism on HEK293-hY_4R-CRE Luc cells (luciferase reporter gene assay): EC_{50} = 220 ± 73 nM, α = 0.75. Presented are mean values ± SEM from at least three independent experiments (performed in triplicate). n.a.: not applicable; n.d.: not determined.

$[C_{20}H_{26}NO_3]^+$: 328.1907, found: 328.1915. $C_{20}H_{25}NO_3$ (327.42).

*4.3.2. N^2-(2-{[(9H-Fluoren-9-yl)methoxy]carbonyl}-1-isobutylhydrazine-1-carbonyl)-N^{ω}-[(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl]-L-arginine (**17**).* Triphosgene (152 mg, 0.4 equiv) was dissolved in CH_2Cl_2 (5 mL). A solution of **14** (145 mg, 0.442 mmol) and DIPEA (450 μL, 2 equiv) in CH_2Cl_2 (5 mL) was added dropwise under ice-cooling. The mixture was allowed to warm to ambient temperature under stirring for 30 min. A solution of **12** (200 mg, 1.1 equiv) in CH_2Cl_2 (2 mL) was added and stirring was continued for 90 min. The mixture was diluted with EtOAc (60 mL) and washed with water. The volatiles were removed under reduced pressure, the residue was taken up in EtOAc/hexane (2:1, 10 mL) and subjected to column chromatography (eluent: EtOAc/hexane (2:1)). The protected intermediate was dissolved in MeOH (10 mL), and Pd/C-catalyst (50 mg) was added. The mixture was stirred vigorously under hydrogen. After completion of the hydrogenation, the solids were removed by filtration through celite, the volume was reduced on a rotary evaporator. The desired compound was purified via column chromatography (eluent: CH_2Cl_2/MeOH (10:1) → CH_2Cl_2/MeOH (1:1) + 0.5% TFA). Removal of the volatiles under reduced pressure and by lyophilization afforded **14** as a white lyophilisate (370 mg, 88.5%). TLC (CH_2Cl_2/MeOH (10:0.6) + 1% AcOH): R_f = 0.28. ^{1}H NMR (400 MHz, methanol-d_4): δ (ppm) 0.85 (s, 6H), 1.41 (s, 6H), 1.47−1.69 (m, 3H), 1.69−1.79 (m, 1H), 1.79−1.92 (m, 1H), 2.06 (s, 3H), 2.49 (s, 3H), 2.56 (s, 3H), 2.95 (s, 2H), 3.15 (s, 2H), 4.12−4.30 (m, 2H), 4.52 (s, 2H), 7.28 (t, 2H, *J* 7.3 Hz), 7.37 (t, 2H, *J* 7.3 Hz), 7.54−7.70 (m, 2H), 7.77 (d, 2H, *J* 7.5 Hz). HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $[C_{39}H_{51}N_6O_8S]^+$: 763.3484, found: 763.3494. $C_{39}H_{50}N_6O_8S$ (762.92).

*4.3.3. (S)-5-({[(9H-Fluoren-9-yl)methoxy]carbonyl}amino)-2-[4-(tert-butoxy)benzyl]-12,12-dimethyl-4,10-dioxo-11-oxa-3,5,9-triazatridecanoic Acid (**18**).* Triphosgene (52.5 mg, 0.4 equiv) was dissolved in CH_2Cl_2 (2 mL). A solution of **15** (145 mg, 0.442 mmol) and DIPEA (150 μL, 2 equiv) in CH_2Cl_2 (2 mL) was added dropwise under ice-cooling. The mixture was allowed to warm to ambient temperature under stirring for 30 min. A solution of **10** (200 mg, 1.1 equiv) in CH_2Cl_2 (2 mL) was added, and stirring was continued for 90 min. The mixture was diluted with EtOAc (40 mL) and washed with water. The volatiles were removed under reduced pressure, the residue was taken up in EtOAc/hexane (1:1, 10 mL) and subjected to column chromatography (eluent: EtOAc/hexane (1:1), R_f = 0.57). The protected intermediate was dissolved in MeOH (10 mL), and Pd/C-catalyst (30 mg) was added. The mixture was stirred vigorously under hydrogen. After completion of the hydrogenation, the solids were removed by filtration through celite, the volume was reduced on a rotary evaporator. The desired compound was purified via column chromatography (eluent: CH_2Cl_2/MeOH (10:0.5) → CH_2Cl_2/MeOH (10:0.6) + 1% AcOH). Removal of the volatiles under reduced pressure and by lyophilization afforded **18** as a white lyophilisate (221 mg, 74.1%). TLC (CH_2Cl_2/MeOH (10:0.6) + 0.5% AcOH): R_f = 0.35. ^{1}H NMR (400 MHz, methanol-d_4): δ (ppm) 1.20 (s, 9H), 1.42 (s, 9H), 1.51−1.66 (m, 2H), 2.81−3.20 (m, 5H), 3.69 (br s, 1H), 4.21 (s, 1H), 4.35−4.60 (m, 3H), 6.79 (s, 2H), 7.04 (s, 2H), 7.24−7.35 (m, 2H), 7.39 (t, 2H, *J* 7.2 Hz), 7.54−7.73 (m, 2H), 7.80 (d, 2H, *J* 7.5 Hz). HRMS (ESI): *m*/*z* [M +

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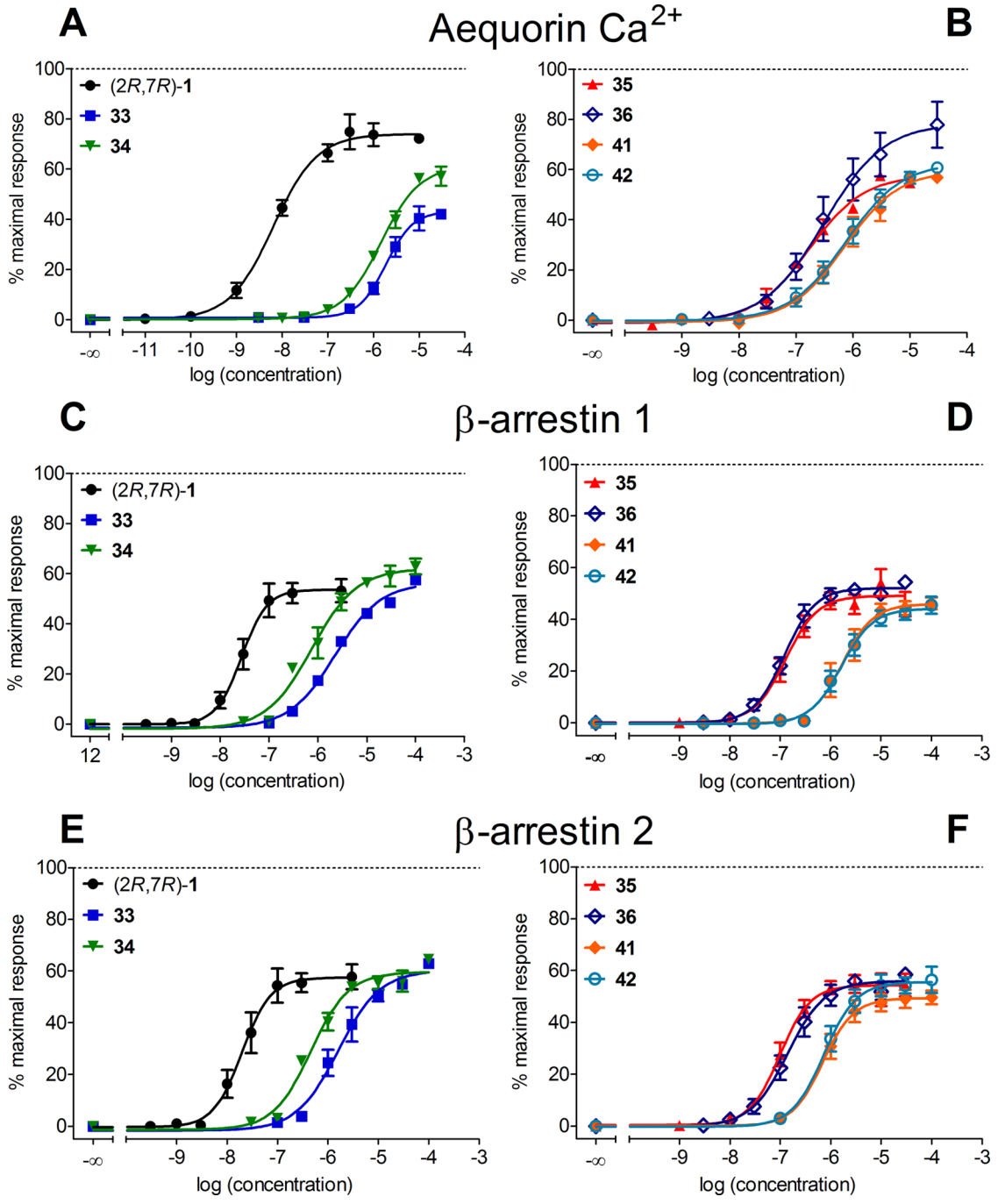

Figure 8. Y_4R agonism of (2*R*,7*R*)-**1**, **33**−**36**, **41**, and **42** determined in a calcium (aequorin) assay, a *β*-arrestin recruitment assay, and a luciferase reporter gene assay. (A, B) Induced intracellular Ca^{2+} mobilization in CHO-hY_4R-mtAEQ-G_{qi5} cells. (C, D) Induced *β*-arrestin 1 recruitment in HEK293T-ARRB1-Y_4R cells. (E, F) Induced *β*-arrestin 2 recruitment in HEK293T-ARRB2-Y_4R cells. Data points shown for (A)−(F) are the mean ± SEM of at least three independent experiments performed in triplicate.

H]$^+$ calcd for $[C_{37}H_{47}N_4O_8]^+$: 675.3388, found: 675.3403. $C_{37}H_{46}N_4O_8$ (674.80).

*4.3.4. {[1-(9H-Fluoren-9-yl)-12,12-dimethyl-3,10-dioxo-2,11-dioxa-4,5,9-triazatridecan-5-yl]carbonyl}-L-leucine (**19**).* Triphosgene (52.5 mg, 0.4 equiv) was dissolved in CH_2Cl_2 (2 mL). A solution of **16** (97.7 mg, 0.442 mmol) and DIPEA (150 *μ*L, 2 equiv) in CH_2Cl_2 (2 mL) was added dropwise under ice-cooling. The mixture was allowed to warm to ambient temperature under stirring for 30 min. A solution of **10** (200 mg, 1.1 equiv) in CH_2Cl_2 (2 mL) was added and stirring was continued for 90 min. The mixture was diluted with EtOAc (40 mL) and washed with water. The volatiles were removed under reduced pressure and the residue was taken up in EtOAc/hexane (1:1, 10 mL) and subjected to column chromatography (eluent: EtOAc/hexane (1:1), R_f = 0.71). The protected intermediate was dissolved in MeOH (10 mL), and Pd/C-catalyst (30 mg) was added. The mixture was stirred vigorously under hydrogen. After completion of the hydrogenation, the solids were removed by filtration through celite, and the volume was reduced on a rotary evaporator. The desired compound was purified via column chromatography (eluent: CH_2Cl_2/MeOH (10:0.4) → CH_2Cl_2/MeOH (10:0.6) + 0.1% TFA).

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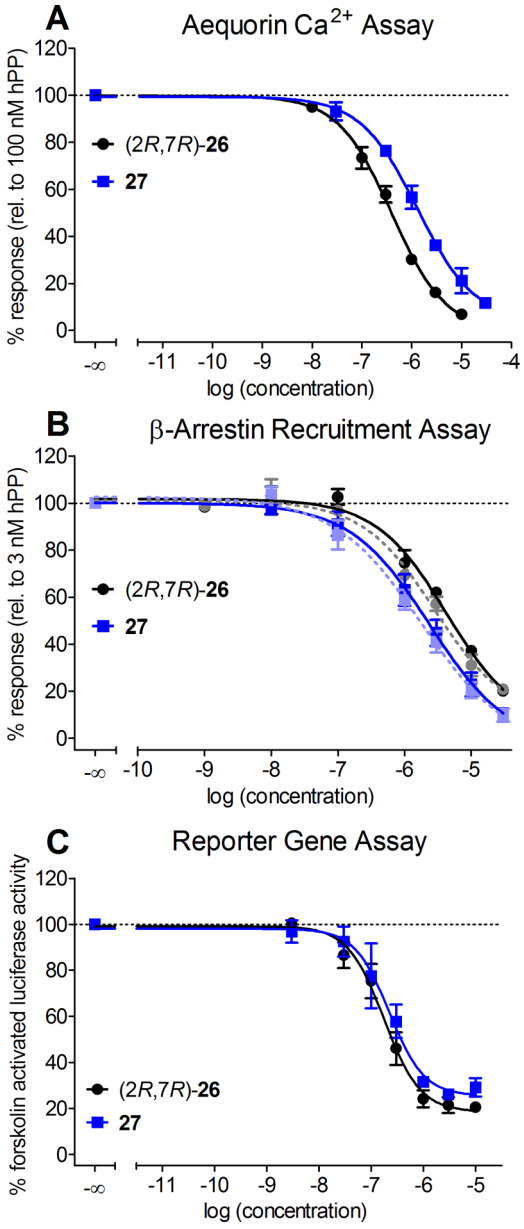

Figure 9. Y_4R antagonism of (2*R*,7*R*)-**26** and **27** determined in a calcium (aequorin) assay, a *β*-arrestin recruitment assay, and a luciferase reporter gene assay. (A) Inhibition of hPP (EC_{50} = 9.7 nM, *c* = 100 nM)-induced Ca^{2+} mobilization in CHO-hY_4R-mtAEQ-G_{qi5} cells. (B) Inhibition of hPP (EC_{50} (Arr1) = 3.54 nM, EC_{50} (Arr2) = 2.74 nM, *c* = 3 nM)-induced *β*-arrestin 1,2 recruitment in HEK293T-ARRB1-Y_4R cells (*β*-arrestin 1, solid lines) and HEK293T-ARRB2-Y_4R cells (*β*-arrestin 2, dashed lines). (C) Inhibition of forskolin-stimulated (2 *μ*M) luciferase activity (corresponding to 100%) in hY_4R expressing HEK293 cells with the maximum inhibitory effect of the endogenous ligand hPP, which was set to 0% luciferase activity and corresponds to full agonism (*α* = 1.0). Data points shown for (A)–(C) are the mean ± SEM of at least three independent experiments performed in triplicate.

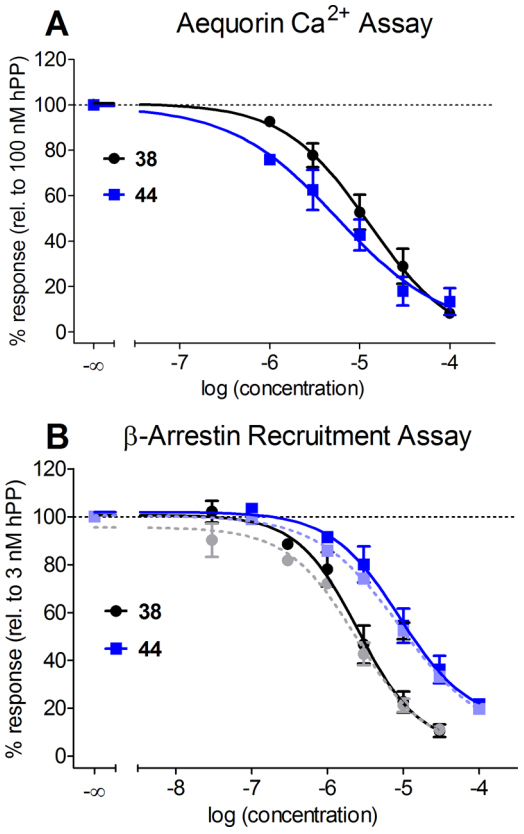

Figure 10. Y_4R functional activity of **38** and **44** determined in a calcium (aequorin) assay and a *β*-arrestin recruitment assay. (A) Inhibition of hPP (EC_{50} = 9.7 nM, *c* = 100 nM)-induced intracellular Ca^{2+} mobilization in CHO-hY_4R-mtAEQ-G_{qi5} cells. (B) Inhibition of hPP (EC_{50} (Arr1) = 3.54 nM, EC_{50} (Arr2) = 2.74 nM, *c* = 3 nM)-induced *β*-arrestin 1,2 recruitment in HEK293T-ARRB1-Y_4R cells (*β*-arrestin 1, solid lines) and HEK293T-ARRB2-Y_4R cells (*β*-arrestin 2, dashed lines). Data points shown for (A) and (B) are the mean ± SEM of at least three independent experiments performed in triplicate. Note: Inhibition of hPP-induced response appears incomplete, however, concentrations of antagonists higher than those given in the curves could not be applied due to a shortage of the test compounds. There was no intrinsic activity of both compounds in the respective assays when performed in the agonist mode (SI Figures 3, 5, and 7).

Removal of the volatiles afforded **19** as a sticky solid (132 mg, 52.5%). TLC (CH_2Cl_2/MeOH (10:0.6) + 0.5% AcOH): R_f = 0.55. ^{1}H NMR (400 MHz, methanol-d_4): *δ* (ppm) 0.90 (d, 6H, *J* 4.4 Hz), 1.42 (s, 9H), 1.52–1.77 (m, 5H), 3.03 (s, 2H), 3.35–3.95 (m, 2H), 4.23 (t, 1H, *J* 6.3 Hz), 4.29 (t, 1H, *J* 7.0 Hz), 4.52 (s, 2H), 7.31 (t, 2H, *J* 7.3 Hz), 7.39 (t, 2H, *J* 7.4 Hz), 7.59–7.71 (m, 2H), 7.79 (d, 2H, *J* 7.5 Hz). HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $[C_{30}H_{41}N_4O_7]^+$: 569.2970, found: 569.2969. $C_{30}H_{40}N_4O_7$ (568.67).

*4.3.5. Tyr(tBu)-Arg(Pbf)-aza-Leu-Arg(Pbf)-Tyr(tBu)-amide Hydrotrifluoroacetate (**21**).* Compound **21** was synthesized according to the general procedure (100 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0–18 min MeCN/0.1% aq TFA 42:58–78:22, t_R = 14.9 min) afforded **21** as a white solid (39 mg, 42.6%). HRMS (ESI): *m*/*z* $[M + 2H]^{2+}$ calcd for $[C_{69}H_{105}N_{13}O_{13}S_2]^{2+}$: 693.8693, found: 693.8708. $C_{69}H_{103}N_{13}O_{13}S_2{\cdot}C_2HF_3O_2$ (1386.06 + 114.02).

*4.3.6. Tyr(tBu)-Arg(Pbf)-Leu-aza-Orn(Boc)-Tyr(tBu)-amide Hydrotrifluoroacetate (**22**).* Compound **22** was synthesized according to the general procedure (100 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0–20 min MeCN/0.1% aq TFA 42:58–78:22, t_R = 14.2 min) gave **22** as a white solid (42 mg, 52.7%). HRMS (ESI): *m*/*z* $[M + 2H]^{2+}$ calcd for $[C_{60}H_{95}N_{11}O_{12}S]^{2+}$:

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596.8436, found: 596.8445. $C_{60}H_{93}N_{11}O_{12}S{\cdot}C_2HF_3O_2$ (1192.53 + 114.02).

*4.3.7. Tyr(tBu)-aza-Orn(Boc)-Leu-Arg(Pbf)-Tyr(tBu)-amide Hydrotrifluoroacetate (**23**).* Compound **23** was synthesized according to the general procedure (100 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−20 min MeCN/0.1% aq TFA 42:58−78:22, t_R = 14.6 min) afforded **23** as a white solid (36 mg, 45.2%). HRMS (ESI): m/z $[M + 2H]^{2+}$ calcd for $[C_{60}H_{95}N_{11}O_{12}S]^{2+}$: 596.8436, found: 596.8446. $C_{60}H_{93}N_{11}O_{12}S{\cdot}C_2HF_3O_2$ (1192.53 + 114.02).

*4.3.8. (2R,7R)-Diaminooctanedioyl-bis(Tyr-Arg-aza-Leu-Arg-Tyr-amide) Hexakis(hydrotrifluoroacetate) ((2R,7R)-**26**).* Compound **24** (2.08 mg, 5.1 μmol), HBTU (4.1 mg, 2.1 equiv), and HOBt (1.57 mg, 2 equiv) were dissolved in anhydrous DMF (400 μL). DIPEA (10 μL, 5 equiv) was added. Stirring was continued at rt for 5 min, followed by addition of a solution of **21** (19.3 mg, 12.8 μmol) in DMF. The mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic phases were concentrated, and the residue was dried in vacuo. TFA/water (95:5, 2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilization. The product was purified by preparative HPLC (gradient: 0−20 min: MeCN/0.1% aq TFA 3:97−42:58, t_R = 15.6 min). Freeze-drying of the eluate gave (2*R*,7*R*)-**26** as a white fluffy solid (3.45 mg, 28.3%). HRMS (ESI): m/z $[M + 4H]^{4+}$ calcd for $[C_{78}H_{126}N_{28}O_{16}]^{4+}$: 427.7471, found: 427.7485. RP-HPLC (220 nm): 99% (t_R = 17.72 min, k = 5.2). $C_{78}H_{122}N_{28}O_{16}{\cdot}C_{12}H_6F_{18}O_{12}$ (1708.01 + 684.12).

*4.3.9. Octanedioyl-bis(Tyr-Arg-aza-Leu-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (**27**).* Compound **21** (28.8 mg, 19.2 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Compound **25** (2.83 mg, 7.7 μmol) was added, and the mixture was stirred at 35 °C for 16 h. Water (10 mL) was added, and the protected intermediate was extracted with CH_2Cl_2 (2 × 10 mL). The combined extracts were evaporated, and the residue was dried in vacuo. TFA/water (95:5 v/v) (2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilization. The product was purified by preparative HPLC (gradient: 0−20 min: MeCN/0.1% aq TFA 3:97−42:58, t_R = 16.5 min). Lyophilization of the eluate afforded **27** as a white fluffy solid (6.3 mg, 38.3%). HRMS (ESI): m/z $[M + 4H]^{4+}$ calcd for $C_{78}H_{124}N_{26}O_{16}$: 420.2417, found: 420.2433. RP-HPLC (220 nm): 95% (t_R = 19.13 min, k = 5.7). $C_{78}H_{120}N_{26}O_{16}{\cdot}C_8H_4F_{12}O_8$ (1677.98 + 456.08).

*4.3.10. Octanedioyl-bis(Tyr-Arg-Leu-aza-Orn-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (**28**).* Compound **22** (16.7 mg, 12.8 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Compound **25** (1.9 mg, 5.1 μmol) was added, and the mixture was stirred at 35 °C for 16 h. After addition of water (10 mL), the protected intermediate was extracted with CH_2Cl_2 (2 × 10 mL). The combined extracts were evaporated, and the residue was dried in vacuo. TFA/water (95:5 v/v) (2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added, followed by lyophilization. The product was purified by preparative HPLC (gradient: 0−20 min: MeCN/0.1% aq TFA 3:97−42:58, t_R = 16.1 min). Lyophilization of the eluate afforded **28** as a white fluffy solid (4.2 mg, 40.2%). HRMS (ESI): m/z $[M + 4H]^{4+}$ calcd for $C_{76}H_{120}N_{22}O_{16}$: 399.2308, found: 399.2327. RP-HPLC (220 nm): 99% (t_R = 19.55 min, k = 5.8). $C_{76}H_{116}N_{22}O_{16}{\cdot}C_8H_4F_{12}O_8$ (1593.90 + 456.08).

*4.3.11. Octanedioyl-bis(Tyr-Arg-Leu-aza-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (**29**).* Compound **28** (3.7 mg, 2.05 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (300 μL). A solution of *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine (1 mg/10 μL, 16 μL, 2.5 equiv) was added, and the mixture was stirred at rt for 4 h. After addition of water (5 mL), the protected intermediate was purified by preparative HPLC (gradient: 0−25 min: MeCN/0.1% aq TFA 3:97−52:48, t_R = 26.4 min). The eluates were subjected to lyophilization, and the residue was taken up in TFA/CH_2Cl_2/H_2O (5:5:0.5, 3 mL). The resulting mixture was stirred for 3 h. The volatiles were removed on a rotary evaporator and by lyophilization. **29** was obtained as a white fluffy solid (1.62 mg, 37.0%). HRMS (ESI): m/z $[M + 4H]^{4+}$ calcd for $C_{78}H_{124}N_{26}O_{16}$: 420.2417, found: 420.2434. RP-HPLC (220 nm): >99% (t_R = 20.15 min, k = 6.0). $C_{78}H_{120}N_{26}O_{16}{\cdot}C_8H_4F_{12}O_8$ (1677.98 + 456.08).

*4.3.12. Octanedioyl-bis(Tyr-aza-Orn-Leu-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (**30**).* Compound **23** (16.7 mg, 12.8 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 μL). Compound **25** (1.88 mg, 5.1 μmol) was added, and the mixture was stirred at 35 °C for 16 h. After addition of water (10 mL), the protected intermediate was extracted with CH_2Cl_2 (2 × 10 mL). The combined extracts were evaporated, and the residue was dried in vacuo. TFA/water (95:5 v/v) (2 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (100 mL) was added followed by lyophilization. The product was purified by preparative HPLC (gradient: 0−20 min: MeCN/0.1% aq TFA 6:94−37:63, t_R = 17.4 min). Lyophilization of the eluate afforded **30** as a white fluffy solid (3.9 mg, 37.3%). HRMS (ESI): m/z $[M + 4H]^{4+}$ calcd for $C_{76}H_{120}N_{22}O_{16}$: 399.2308, found: 399.2317. RP-HPLC (220 nm): 98% (t_R = 20.13 min, k = 6.0). $C_{76}H_{116}N_{22}O_{16}{\cdot}C_8H_4F_{12}O_8$ (1593.90 + 456.08).

*4.3.13. Octanedioyl-bis(Tyr-aza-Arg-Leu-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (**31**).* Compound **30** (3.0 mg, 1.66 μmol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (300 μL). A solution of *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine (1 mg/10 μL, 13 μL, 2.5 equiv) was added, and the mixture was stirred at rt for 4 h. After addition of water (5 mL), the protected intermediate was purified by preparative HPLC (gradient: 0−25 min: MeCN/0.1% aq TFA 3:97−52:48, t_R = 26.1 min). The solvents were removed by lyophilization, the residue was taken up in TFA/CH_2Cl_2/H_2O (5:5:0.5, 3 mL), and the resulting mixture was stirred for 3 h. The volatiles were removed on a rotary evaporator and by lyophilization. The title compound was obtained as a white fluffy solid (1.50 mg, 42.3%). HRMS (ESI): m/z $[M + 4H]^{4+}$ calcd for $C_{78}H_{124}N_{26}O_{16}$: 420.2417, found: 420.2437. RP-HPLC (220 nm): 96% (t_R = 20.86 min, k = 6.3). $C_{78}H_{120}N_{26}O_{16}{\cdot}C_8H_4F_{12}O_8$ (1677.98 + 456.08).

*4.3.14. Octanoyl-Tyr-Arg-Leu-Arg-Tyr-amide Bis(hydrotrifluoroacetate) (**33**).* The pentapeptide **33** was synthesized according to the general procedure (82 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 22:78−51:49, t_R = 13.2 min) afforded **33** as a white solid (33.4 mg, 59.5%). HRMS (ESI): m/z $[M + 2H]^{2+}$ calcd for $[C_{44}H_{72}N_{12}O_8]^{2+}$: 448.2793, found: 448.2800. RP-HPLC (220 nm): 99% (t_R = 28.04 min, k = 8.8). $C_{44}H_{70}N_{12}O_8{\cdot}C_4H_2F_6O_4$ (895.13 + 228.04).

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*4.3.15. Ac-Tyr-N$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Leu-Arg-Tyr-amide Tris(hydrotrifluoroacetate) (**34**).* The pentapeptide **34** was synthesized according to the general procedure (18.8 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 14.4 min) gave **34** as a white solid (7.7 mg, 53.0%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for $[C_{43}H_{71}N_{14}O_9]^{3+}$: 309.1837, found: 309.1850. RP-HPLC (220 nm): 97% (t_R = 16.96 min, k = 4.9). $C_{43}H_{68}N_{14}O_9{\cdot}C_6H_3F_9O_6$ (925.12 + 342.06).

*4.3.16. Ac-Arg-Tyr-N$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Leu-Arg-Tyr-amide Tetrakis(hydrotrifluoroacetate) (**35**).* The hexapeptide **35** was synthesized according to the general procedure (18.8 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 13.6 min) yielded **35** as a white solid (9.3 mg, 52.7%). HRMS (ESI): m/z [M + 4H]$^{4+}$ calcd for $[C_{49}H_{84}N_{18}O_{10}]^{4+}$: 271.1649, found: 217.1657. RP-HPLC (220 nm): 97% (t_R = 15.22 min, k = 4.3). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

*4.3.17. Ac-Arg-Tyr-Arg-Leu-Arg-Tyr-amide Tris(hydrotrifluoroacetate) (**36**).* The hexapeptide **36** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 13.7 min) afforded **36** as a white solid (18.3 mg, 65.5%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for $[C_{44}H_{70}N_{16}O_9]^{3+}$: 323.1910, found: 323.1927. RP-HPLC (220 nm): 99% (t_R = 15.98 min, k = 4.6). $C_{44}H_{70}N_{16}O_9{\cdot}C_6H_3F_9O_6$ (967.15 + 342.06).

*4.3.18. Ac-Arg-Tyr-Arg-Leu-N$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Tyr-amide Tetrakis(hydrotrifluoroacetate) (**37**).* The hexapeptide **37** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 13.6 min) gave **37** as a white solid (7.2 mg, 21.9%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$: 361.2174, found: 361.2187. RP-HPLC (220 nm): 99% (t_R = 15.17 min, k = 4.3). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

*4.3.19. Ac-Arg-Tyr-N$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Leu-Arg-D-Tyr-amide Tetrakis(hydrotrifluoroacetate) (**38**).* The hexapeptide **38** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 13.6 min) afforded **38** as a white solid (5.7 mg, 17.4%). HRMS (ESI): m/z [M + 4H]$^{4+}$ calcd for $[C_{49}H_{84}N_{18}O_{10}]^{4+}$: 271.1649, found: 217.1666. RP-HPLC (220 nm): 98% (t_R = 15.25 min, k = 4.3). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

*4.3.20. Ac-Arg-Tyr-N$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-Leu-D-Arg-Tyr-amide Tetrakis(hydrotrifluoroacetate) (**39**).* The hexapeptide **39** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 13.3 min) yielded **39** as a white solid (6.0 mg, 18.3%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$: 361.2174, found: 361.2190. RP-HPLC (220 nm): 95% (t_R = 14.77 min, k = 4.1). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

*4.3.21. Ac-Arg-Tyr-N$^{\omega}$-[(4-aminobutyl)aminocarbonyl]Arg-D-Leu-Arg-Tyr-amide Tetrakis(hydrotrifluoroacetate) (**40**).* The hexapeptide **40** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 13.4 min) afforded **40** as a white solid (6.6 mg, 20.1%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$: 361.2174, found: 361.2189. RP-HPLC (220 nm): 96% (t_R = 14.33 min, k = 4.0). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

*4.3.22. Ac-Arg-D-Tyr-{N$^{\omega}$-[N-(4-aminobutyl)-aminocarbonyl]}Arg-Leu-Arg-Tyr-amide Tetrakis(hydrotrifluoroacetate) (**41**).* The hexapeptide **41** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 13.5 min) gave **41** as a white solid (6.7 mg, 20.5%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$: 361.2174, found: 361.2190. RP-HPLC (220 nm): 95% (t_R = 15.1 min, k = 4.3). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

*4.3.23. Ac-D-Arg-Tyr-{N$^{\omega}$-[N-(4-aminobutyl)-aminocarbonyl]}Arg-Leu-Arg-Tyr-amide Tetrakis(hydrotrifluoroacetate) (**42**).* The hexapeptide **42** was synthesized according to the general procedure (35 mg Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 13.5 min) afforded **42** as a white solid (7.0 mg, 21.4%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for $[C_{49}H_{83}N_{18}O_{10}]^{3+}$: 361.2174, found: 361.2190. RP-HPLC (220 nm): 99% (t_R = 14.94 min, k = 4.2). $C_{49}H_{80}N_{18}O_{10}{\cdot}C_8H_4F_{12}O_8$ (1081.31 + 456.08).

*4.3.24. Ac-Arg-Tyr-{N$^{\omega}$-[N-(4-aminobutyl)-aminocarbonyl]}Arg-Leu-Arg-Tyr-OH Tetrakis(hydrotrifluoroacetate) (**43**).* The hexapeptide **43** was synthesized according to the general procedure (44.1 mg, H-L-Tyr(*t*Bu)-2CT resin (loading: 0.68 mmol/g)). Purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 13:87−42:58, t_R = 10.7 min) yielded **43** as a white solid (15.2 mg, 32.9%). HRMS (ESI): m/z [M + 4H]$^{4+}$ calcd for $[C_{49}H_{83}N_{17}O_{11}]^{4+}$: 271.4109, found: 271.4124. RP-HPLC (220 nm): 95% (t_R = 16.28 min, k = 4.7). $C_{49}H_{79}N_{17}O_{11}{\cdot}C_8H_4F_{12}O_8$ (1082.28 + 456.08).

*4.3.25. (S)-2-Acetamido-N-((S)-1-{[(12S,15S,18S,21S)-2-amino-15-(3-guanidinopropyl)-12-(4-hydroxybenzyl)-18-isobutyl-4,11,14,17,20-pentaoxo-1,3,5,10,13,16,19-heptaazacyclotetracos-2-en-21-yl]amino}-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)-5-guanidinopentanamide Tris(hydrotrifluoroacetate) (**44**).* Compound **43** (9.0 mg, 5.85 μmol), HOBt (1.79 mg, 2 equiv), and DIPEA (0.9 μL, 8 equiv) were dissolved in anhydrous DMF (5850 μL) to give a 1 mM solution. A solution of PyBOP (3.65 mg, 1.2 equiv) was added dropwise. After stirring at rt for 24 h, 0.1% aq TFA (20 mL) was added, and purification by preparative HPLC (gradient: 0−18 min MeCN/0.1% aq TFA 3:97−42:58, t_R = 15.9 min) afforded **44** as a white solid (3.9 mg, 47.4%). HRMS (ESI): m/z [M + 3H]$^{3+}$ calcd for $[C_{49}H_{80}N_{17}O_{10}]^{3+}$: 355.5419, found: 355.5434. RP-HPLC (220 nm): 95% (t_R = 18.4 min, k = 5.4). $C_{49}H_{77}N_{17}O_{10}{\cdot}C_6H_3F_9O_6$ (1064.27 + 342.06).

4.4. Cells. HEC-1B (HTB-113) human endometrial cancer cells were from the American Type Culture Collection (Rockville, MD). MCF-7-Y$_1$ cells were previously established in our laboratory.[23] HEL cells were kindly provided by Dr. M. C. Michel (Universitätsklinikum Essen, Germany), and CHO-K1 (ACC-110) cells and HEK293T (ACC 635) cells were from

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Table 3. Conditions of the Radioligand Competitions Binding Assays at the NPY Receptor Subtypes

	Y_1R	Y_2R	Y_4R	Y_5R
receptor source	MCF-7-Y_1 cells[23]	CHO-hY_2R-G_{qi5}-mtAEQ cells[25]	CHO-hY_4R-G_{qi5}-mtAEQ cells[26]	HEC-1B-hY_5 cells[27]
buffer	*N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) buffer containing 150 mM NaCl[a]	sodium-free HEPES buffer[b]	sodium-free HEPES buffer[b]	HEPES buffer containing 150 mM NaCl[a]
radioligand	compd **46**	[^{3}H]propionyl-pNPY	compd **45**[11]	[^{3}H]propionyl-pNPY
K_d	6.2 nM	1.4 nM	0.67 nM	4.8 nM
concentration	4 nM	1.0 nM	0.6 nM	4 nM
incubation period	90 min	90 min	90 min	120 min
separation	suction	suction	filtration	suction
scintillation cocktail	Optiphase Supermix	Optiphase Supermix	Rotiscint eco plus	Optiphase Supermix

[a]Isotonic HEPES buffer pH 7.4 (150 mM NaCl, 10 mM HEPES, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM KCl). [b]Hypotonic, sodium-free HEPES buffer pH 7.4 (25 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$). Binding buffers were supplemented with BSA (1%) and bacitracin (0.1 mg/mL).

Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

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

4.4.1. HEK293T-ARRB1-hY_4R and HEK293T-ARRB2-hY_4R Cells. The β-arrestin recruitment was quantified by a luciferase complementation assay based on the emerald luciferase (ELuc) from the brazilian click-beetle *Pyrearinus termitilluminans*. The fusion construct of the human hY_4R (the hY_4R cDNA was from cDNA Resource Center, Bloomsburg, PA) and the C-terminal luciferase fragment (hY_4R-ELucC) was generated using the previously described construct SSTR2-ELucC[31,32] by replacing the cDNA of SSTR2 with the cDNA of the hY_4R. HEK293T cells were stably transfected with the pcDNA3.1/myc-HIS (B) vector encoding the β-arrestin isoform 1 or 2, N-terminally fused with the N-terminus of the luciferase (ELucN-ARRB1 or ELucN-ARRB2, respectively)[31] and the pcDNA4/V5-HIS (B) vector encoding hY_4R-ELucC using G418 (600 μg/mL) and zeocin (40 μg/mL) as selection antibiotics, as previously described for the H_1R.[33] To determine the hY_4R-ELucC expression, saturation binding studies were performed with both HEK293T-ARRB1-hY_4R cells and HEK293T-ARRB2-hY_4R cells using the radioligand **45** (cf. SI Figures S6 and S7).

4.5. Cell Culture. Cells were maintained in 25 or 75 cm^2 flasks (Sarstedt, Nümbrecht, Germany) in a humidified atmosphere (95% air, 5% CO_2) at 37 °C. MCF-7-Y_1 cells,[22] HEL cells,[34] CHO-hY_2-G_{qi5}-mtAEQ cells,[26] CHO-hY_4-G_{qi5}-mtAEQ cells,[26] and HEK293T-hY_4R-CRE Luc cells[11] were cultured as described previously. HEK293T-ARRB1-hY_4R and HEK293T-ARRB2-hY_4R cells were maintained in Dulbecco's modified Eagle's medium containing 10% FCS, 600 μg/mL G418, and 40 μg/mL zeocin.

4.6. Radioligand Binding Assays. Radioligand binding assays at all NPY receptor subtypes were performed at 22 ± 1 °C according to the experimental protocols described in detail previously.[11] Radioactivity (dpm) was measured with a MicroBeta2 plate counter (PerkinElmer, Rodgau, Germany). Cells, buffers, radioligands, and the respective K_d values and concentrations, as well as the assay conditions are summarized in Table 3.

4.7. Fura-2 Calcium Assay. The assay was performed with HEL cells as previously described.[34,35]

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

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

4.10. β-Arrestin Recruitment Assay. The recruitment of β-arrestin was measured via the split-luciferase complementation technique.[32] Agonist potencies were determined on HEK293T-ARRB1-Y_4R and HEK293T-ARRB2-Y_4R cells using the GENios Pro microplate reader (Tecan, Salzburg, Austria) as previously described for HEK293T-ARRB1-H_1R and HEK293T-ARRB2-H_2R, respectively.[33] For the determination of antagonism, the cells were preincubated in the presence of the antagonist for 15 min. Ten microliters of an hPP solution (30 nM, final concentration 3 nM) was added and incubation was continued at 25 °C for 60 min. The plates were further processed as in the case of the agonist mode.

4.11. Data Analysis. Concentration response curves from functional assays and displacement curves from radioligand competition binding were analyzed by four-parameter sigmoidal fits (GraphPad Prism 5.0, San Diego, CA). Agonist potencies are given as EC_{50} values, intrinsic activities are expressed as α values with respect to the effect of 1 μM hPP (maximal response α = 1.0). Antagonistic activities were determined in the presence of 3 nM hPP (β-arrestin 1/2 assay, EC_{50} = 3.54 nM (ARRB1), EC_{50} = 2.74 nM (ARRB2)), 100 nM hPP (aequorin assay, EC_{50} = 9.7 nM) or 10 nM pNPY (Fura-2 Ca^{2+} assay, EC_{50} = 0.87 nM (Y_1R)). K_i and K_b (Fura-2 Ca^{2+} (Y_1R)) values were calculated from IC_{50} values using the Cheng–Prusoff equation.[36]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00451.

Synthesis of compounds **6**–**12**, **14**, and **16**; β-arrestin recruitment assay with compounds **4**, (2*R*,7*R*)-**26**, **27**, **29**, **31**, **37**–**40**, and **44**; aequorin Ca^{2+} assay with compounds (2*R*,7*R*)-**26**, **27**, **29**, **31**, **37**–**40**, and **44**; saturation binding experiments with **45** at HEK293T-ARRB1-Y_4R and HEK293T-ARRB2-Y_4R cells; chromatograms of the HPLC purity control of all target

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compounds (SI Figures 11−28); ^{1}H NMR and ^{13}C NMR spectra (SI Figures 29−42) (PDF)
Molecular formula strings and summary of pharmacological data (CSV)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

br s, broad singlet; CHO, Chinese hamster ovary; DEA, diethylamine; DIPEA, *N*,*N*-diisopropylethylamine; EtOAc, ethyl acetate; FCS, fetal calf serum; H_2, hydrogen gas; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HBTU, 3-[bis(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate; HEC, human endometrial cancer; HEL, human erythroleukemia cell line; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, hydroxybenzotriazole; hPP, human pancreatic polypeptide; K_b, inhibition constant; MCF-7, Michigan cancer foundation; MeCN, acetonitrile; MeOH, methanol; Orn, ornithine; OSu, succinimidyl ester; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl; pNPY, porcine neuropeptide Y; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; PYY, peptide YY; RLU, relative light unit; RP, reversed phase; SPPS, solid phase peptide synthesis; TEA, triethylamine; $Y_{1,2,4,5}R$, NPY $Y_{1,2,4,5}$ receptor

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