In Search of NPY Y₄R Antagonists: Incorporation of Carbamoylated Arginine, Aza-Amino Acids, or d-Amino Acids into Oligopeptides Derived from the C-Terminal of the Endogenous Agonists

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Supporting Information

ABSTRACT: The cross-linked pentapeptides (2R,7R)-diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((2R,7R)-BVD-74D, (2R,7R)-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 Y₄ receptor (Y₄R) partial agonists. Here, we report on a series of analogues of (2R,7R)-1 and 2 in which Arg¹, Leu³, or Arg⁷ were replaced by the respective aza-amino acids. The replacement of Arg¹ 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°-(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 Y₄R functional activity in assays with complementary readouts: aquorin Ca²⁺ and β-arrestin 1 or β-arrestin 2 assays. In contrast to the parent compounds, which are Y₄R agonists, several ligands were able to suppress the effect elicited by the endogenous ligand pancreatic polypeptide and therefore represent a novel class of peptide Y₄R antagonists.

1. INTRODUCTION

Among the neuropeptide Y (NPY) receptors, designated Y₁R, Y₂R, Y₅R, and Y₄R, the Y₄R plays a special role because it preferentially binds pancreatic polypeptide. Compared to the Y₁R, Y₂R, and Y₅R subtypes, fewer ligands by far (e.g., see Figure 1) have been reported for the Y₄R in particular, high-affinity Y₄R antagonists are still lacking.³⁻⁶ Y₄R agonists are considered to be potential antiobesity agents.³⁻⁶ 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 Y₄R agonist,⁴ having an effect on food intake in mice.⁹ As reported previously, (2R,7R)-1 (note: in the following, positions 2 and 7 refer to the stereo centers in the 2,7-diaminobutyric acid moiety) was, by a factor of 3—10, more potent than (2S,7S)-1, depending on the type of assay.¹⁰ Even though Y₄R agonists should have higher clinical potential, antagonists are of interest as well, in particular, as pharmacological tools. The pentapeptide 3, a Y₄R agonist with an affinity in the two-digit nanomolar range (Kₓ = 50 nM), shares the same amino acid sequence with the peptide moieties of 1. The incorporation of cyclic β-amino acids such as (1R,2S)-2-aminocyclohexanone-1-carboxylic acid resulted in partial agonism.⁶

To investigate the impact of backbone modifications in Y₄R agonists on functional activity in more detail, additional analogues of (2R,7R)-1, ²,¹¹ and 3 were prepared. Here, we report on the synthesis of Y₄R 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-aza¹-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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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 (9H-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-aza1-dipeptides in yields >70%. Subsequent ester cleavage by hydrogenation gave access to the N-Fmoc-aza1-dipeptides Fmoc-aza-Leu-Arg(Pbf)-OH (17), Fmoc-aza-Orn(Boc)-Tyr(tBu)-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 aza1-peptide, the model peptide H-Ala-aza-Leu-Arg(Pbf)-NH2 (20) was synthesized under various conditions (Scheme 3). When 3-[bis-

Scheme 1. Synthesis of the Hydrazines 10 and 12

Reagents and conditions: (a) Boc2O, Na2CO3, tetrahydrofuran (THF), 0 °C, 10 min, followed by room temperature (rt), 14 h; (b) sulfur trioxide pyridine complex, DMSO, triethylamine (TEA), CH2Cl2, 0 °C, 1 h, followed by rt, 3 h; (c) Fmoc-Cl, MeCN/H2O (1:1), 0 °C, 10 min, followed by rt, 12 h; (d) 7, CH2Cl2, 14 h; (e) isobutyraldehyde, CH2Cl2, 50 h; (f) sodium cyanotrihydridoborate, CH2Cl2/MeOH (4:3), 2 M HCl (pH 2−3), rt, 2−6 h.
The dimeric aza-peptide (2R,7R)-26, an aza-Leu analogue of (2R,7R)-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-1H-pyrazole-1-carboxamidine resulting in 29 and 31, which were aza-Arg-containing analogues of 2.

Very recently, we reported that the replacement of Arg2 with the Nω-carbamoylated arginine 32 (Figure 3) in one of the pentapeptide chains of 2 led to increased Y4R 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(tBu)-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. Y4R Binding. The Kᵢ values of all of the target
compounds were determined in competition binding studies on live cells expressing Y4R (CHO-hY4R-Gqi5-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).

Among the structural modifications of pentapeptide 3, replacement of the N-terminal acetic acid with octanoic acid (compound 33) resulted in an increase in Y4R affinity by a factor of almost six. The replacement of Arg$^2$ in 3 with the carbamoylated arginine building block $^{32}$ 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 Y4R 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 ($K_i = 501$ nM).

Previous studies revealed that C-terminal amidation is crucial for Y4R binding of the endogenous ligand hPP.21 Similarly, the

**Scheme 4. Synthesis of “Homodimeric” Aza-Peptides 26–31**

| 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$_2$Cl$_2$/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$_2$O (95:5), rt, 2.5 h; (g) N,N’-di-Boc-1H-pyrazole-1-carboxamidine, DMF, DIPEA, rt, 4 h; Boc-deprotection, TFA/CH$_2$Cl$_2$/H$_2$O (10/10/1), rt, 3 h.

Figure 3. Structure of the Nω-carbamoylated arginine building block 32.20

Among the structural modifications of pentapeptide 3, replacement of the N-terminal acetic acid with octanoic acid (compound 33) resulted in an increase in Y4R 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 Y4R 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 ($K_i = 501$ nM).

Following the structural optimization of 3, a d-amine acid scan was performed with the hexapeptide 35. Whereas replacement of Arg$^2$ 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-amine acid led to a marked decrease in Y4R affinity (cf. 38–40).

Previous studies revealed that C-terminal amidation is crucial for Y4R binding of the endogenous ligand hPP.21 Similarly, the
affinity of the carboxylic acid 43 (IC\textsubscript{50} > 5000 nM) was much lower than the affinity of the amide 35. Cyclization of 43 resulted in an increase in Y4Ra affinity. However, the cyclic peptide 44 (K\textsubscript{i} = 1650 nM) was only a weak binder compared to the linear analogue 35.

### 2.3. NPY Receptor Subtype Selectivity.

K\textsubscript{i} values of all of the target compounds were also determined at the Y1, Y2, and Y5 receptors using the radioligands \([\text{3H}]\text{propionyl-pNPY}\) (Y2R and Y5R) or \([\text{3H}]\text{UR-MK136}\) (Y1R, Figure 5). Neither the linear peptides 34−43 nor the cyclic peptide 44 displayed remarkable affinity to the Y1, Y2, or Y5 receptors. Having a more than 1000-fold selectivity for the Y4R over the other NPY receptors, peptide 35 proved to be superior to (2\text{R},7\text{R})-1 and 2. The pentapeptide 33 bound to the Y4R with a K\textsubscript{i} value in the submicromolar range (K\textsubscript{i} (Y4R) = 589 nM), that is, the lipophilic N-terminal octanoyl residue is disadvantageous with respect to Y4R selectivity.

The cross-linked aza-peptides (2\text{R},7\text{R})-26, 27, and 29 displayed only low Y1R, Y2R, and Y4R affinities, whereas the affinity of 31 (K\textsubscript{i} = 53 nM) at the Y4R was markedly higher than that of the peptide analogue 2. In a Fura-2 Ca\textsuperscript{2+} assay on human erythroleukemia (HEL) cells, 31 revealed Y4R antagonism (Figure 7) with a K\textsubscript{b} value of 11.5 nM.

### 2.4. Functional Studies at the Y4R.

The target compounds were investigated for Y4R agonism and antagonism in an aequorin Ca\textsuperscript{2+} assay as well as \(\beta\)-arrestin 1 and \(\beta\)-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. Y4R Agonism.** (2\text{R},7\text{R})-1 was a partial agonist in both the Ca\textsuperscript{2+} assay and the \(\beta\)-arrestin recruitment assays (\(\alpha\) = 0.62, (Ca\textsuperscript{2+} assay), \(\alpha\) = 0.54 (\(\beta\)-arrestin 1) or 0.58 (\(\beta\)-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). Y4R 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\text{R},7\text{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 Y4R agonism in the aequorin Ca\textsuperscript{2+} assay and the \(\beta\)-arrestin recruitment assays (SI Figures 2−7). The aza-peptide 29 displayed weak partial agonism with extremely low intrinsic activity (EC\textsubscript{50} = 542 nM, \(\alpha\) = 0.07, (Ca\textsuperscript{2+} assay); EC\textsubscript{50} = 875 nM, \(\alpha\) = 0.09 (\(\beta\)-arrestin 1);
EC_{50} = 473, \alpha = 0.12 (\beta\text{-arrestin 2}). Compounds (2R,7R)-26 and 27 were also investigated for Y4R agonism in a luciferase reporter gene assay. Surprisingly, both aza-peptides displayed partial agonism in this assay (\alpha = 0.81, EC_{50} = 176 \text{nM} ((2R,7R)-26), \alpha = 0.75, EC_{50} = 220 \text{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.\textsuperscript{28} Likewise, (2R,7R)-1, a partial agonist in the Ca\textsuperscript{2+} assay and the arrestin assay, appeared as a full agonist in the luciferase assay.\textsuperscript{11}

2.6. Y_{1}R Antagonism. The incorporation of aza-amino acids into the cross-linked peptides (2R,7R)-1 and 2 had an impact on the quality of action. The dimeric aza-peptides (2R,7R)-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_{1}R antagonism, with compound 40, in which Leu\textsuperscript{4} was replaced by D-Leu, displaying the strongest antagonism in the Ca\textsuperscript{2+} assay (Table 2, SI Figure 8). Compound 37, in which Arg\textsuperscript{5} was replaced by the carbamoylated arginine 32, and the cyclic hexapeptide 44 were identified as weak Y_{1}R 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.
Table 1. Binding Data at NPY Receptor Subtypes

| compd | Y1R | Y2R | Y3R | Y5R |
|-------|-----|-----|-----|-----|
|       | Ki [nM] | Ki [nM] | Ki [nM] | Ki [nM] |
| hPP  | 440 | >5000 | 0.65 | 17 |
| (2R,7R)-1 | 440 | 830 | 0.45 | 1500 |
| 2    | 720 | 1700 | 3.5 | 280 |
| 3    | >5000 | >5000 | 337 ± 110 | >5000 |
| 4    | 24 | 920 | 660 | >5000 |
| (2R,7R)-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 |
| 32   | 589 ± 120 | >5000 | 56.9 ± 5.3 | 3440 ± 210 |
| 33   | >5000 | >5000 | 19.5 ± 2.6 | >5000 |
| 34   | 4830 ± 1400 | >5000 | 2.87 ± 0.78 | >5000 |
| 35   | 1180 ± 380 | >5000 | 3.43 ± 1.3 | >5000 |
| 36   | 1120 ± 270 | >5000 | 501 ± 200 | >5000 |
| 37   | >5000 | >5000 | 724 ± 140 | >5000 |
| 38   | >5000 | >5000 | 568 ± 76 | >5000 |
| 39   | >5000 | >5000 | 248 ± 23 | >5000 |
| 40   | >5000 | >5000 | 10.4 ± 3.3 | >5000 |
| 41   | >5000 | >5000 | 40.5 ± 15 | >5000 |
| 42   | >5000 | >5000 | >5000 | >5000 |
| 43   | >5000 | >5000 | >5000 | >5000 |
| 44   | >5000 | >5000 | 1650 ± 290 | >5000 |

Radioligand competition binding assay with [3H]propionyl-pNPY24 (Ki = 1.4 nM, c = 1 nM) using CHO-hY2-Gqi5-mtAEQ cells.25 Radioligand competition binding assay with [3H]propionyl-pNPY (Ki = 4.83 nM, c = 4 nM) using HEC-1b hY2R cells.27 K4 value reported by Berlicki et al.25 K4 value reported by Kuhn et al.11 K4 value reported by Keller et al. Presented are mean values ± SEM from at least three independent experiments (performed in triplicate). n.d.: not determined.

3. CONCLUSIONS

The affinity and potency of the Y4R partial agonist 3 was considerably increased by the replacement of Arg2 with the carbamoylated arginine 32 and the introduction of an additional arginine at the N-terminus. Several backbone modifications of the C-terminal tripeptide (i.e., 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 Y4R peptide ligands from partial agonism to antagonism. The resulting compounds displayed weaker Y4R antagonism than the reported Y4R antagonist 4. However, due to their extremely facile synthetic accessibility, linear peptides derived from 35 containing d-amino acids (e.g., 40 (Ki = 248 nM)) are promising building blocks for further structural modifications and might pave the way for the development of peptide Y4R antagonists with increased affinity.

6. 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 (Niddereau, Germany). Fmoc-Sieber-PS resin (0.61 mmol/g), Fmoc-Arg(Prf)-OH, Fmoc-Tyr(bBu)-OH, H-L-Tyr(bBu)-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, CH₂Cl₂, diethyl ether, and Triton X-100 were from Sigma-Aldrich (Deisenhofen, Germany); di-tert-butyl dicarbonate (>97%) and 3-aminoapropan-1-ol were from Alfa Aesar (Karlsruhe, Germany). MeCN for HPLC (gradient grade), Fmoc-Leu-OH, DCC, sulfur trioxide pyridine complex, hydrazine hydroxide, and methanol were from Merck (Darmstadt, Germany). Human pancreatic polypeptide and porcine NPY and coelenterazine h was obtained from Biotrend (Cologne, Germany). The synthesis of (2R,7R)-1,11,3,4,5,24,11,13,25,30,32,20,45,11,46,22 and [3H]propionyl-pNPY24 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
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(tBu)-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 2 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 block32 and the dipeptides17–19 (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 azamino 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(tBu)-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 CH2Cl2 (25 mL) and a solution of DCC (943 mg, 1.05 equiv) in CH2Cl2 (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): Rf = 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 CH2Cl2, 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 $\rightarrow$ 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 (d, 2H, J 8.5 Hz), 5.05 (s, 2H), 6.84 (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]$^+$ calc for...
A solution of methanol-ω-dihydrobenzofuran-5-yl)sulfonyl-L-arginine (10:0.6) + 1% AcOH): Rf column chromatography (eluent: CH2Cl2/MeOH (10:0.5)→CH2Cl2/MeOH (1:1) + 0.5% TFA). Removal of the volatiles under reduced pressure and by lyophlization afforded 14 as a white lyophilisate (370 mg, 88.5%). TLC (CH2Cl2/MeOH (10:0.6) + 1% AcOH): RF = 0.28. 1H NMR (400 MHz, methanol-d4): 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 7.5 Hz), 7.37 (t, 2H, J 7.5 Hz), 7.54–7.70 (m, 2H), 7.77 (d, 2H, J 7.5 Hz). HRMS (ESI): m/z [M + H]+ calcd for [C39H51N6O8S]+: 763.3484, found: 763.3494. C37H42N8O8S (782.92).

Aequorin calcium mobilization assay on CHO-hY4-Gqi5-mtAEQ cells. Antagonism (IC50 values given in italics) was determined in the presence of 3 nM hPP (EC50 (ARRB1) = 3.54 nM; EC50 (ARRB2) = 2.74 nM). Presented are mean values ± SEM from at least three independent experiments (performed in triplicate). n.a.: not applicable; n.d.: not determined.

Table 2. NPY Y4R Agonist Potencies (EC50) and Intrinsic Activities (α) or Antagonism (IC50 Values)

| compd | aequorin assayα | β-arrestin 1 assayb | β-arrestin 2 assayb |
|-------|----------------|-------------------|-------------------|
|       | EC50 or IC50 [nM] | α                | EC50 or IC50 [nM] | α       |
| hPP   | 9.7              | 3.45 ± 0.59       | 1                 |
| (2R,7R)-1 | 6.9       | 0.24 ± 0.04     | 2.08 ± 0.23       |
| 2     | 49              | n.d.             | n.d.              |
| 3     | 6390 ± 450      | 0.55             | n.d.              |
| 4     | 226             | n.a.             | 202 ± 78          |
| (2R,7R)-26 | 388        | 5430 ± 350       | 1320 ± 230        |
| 27α   | 1430 ± 290      | n.a.             | 712 ± 87          |
| 29    | 542 ± 110       | 875 ± 260        | 473 ± 65          |
| 31    | 1340 ± 530      | n.a.             | n.d.              |
| 33    | 2050 ± 84       | 2200 ± 130       | 1790 ± 1070       |
| 34    | 1460 ± 83       | 1070 ± 520       | 658 ± 120         |
| 35    | 187 ± 23        | 144 ± 28         | 124.1 ± 24        |
| 36    | 303 ± 68        | 187 ± 40         | 140 ± 45          |
| 37    | 9580 ± 2000     | 6640 ± 780       | 8050 ± 120        |
| 38    | 18 800 ± 6500   | 1310 ± 390       | 1120 ± 250        |
| 39    | 8340 ± 2900     | n.a.             | n.d.              |
| 40    | 6000 ± 2600     | n.a.             | n.d.              |
| 41    | 608 ± 140       | 2050 ± 780       | 710 ± 200         |
| 42    | 756 ± 230       | 2160 ± 600       | 624 ± 150         |
| 43    | 12 400 ± 5500   | 5390 ± 1630      | 3940 ± 660        |

“"Aequorin calcium mobilization assay on CHO-hY4-Gqi5-mtAEQ cells." Antagonism (IC50 values given in italics) was determined in the presence of 100 nM hPP (EC50 = 9.7 nM). β-Arrestin 1,2 recruitment assay on HEK293T cells stably expressing the Y4R and ARRB1 or ARRB2. Antagonism (IC50 values given in italics) was determined in the presence of 3 nM hPP (EC50 (ARRB1) = 3.54 nM; EC50 (ARRB2) = 2.74 nM). EC50 value reported by Kuhn et al. Reported as Kp = 20 nM by Keller et al. Y4R agonism on HEK293-hY4-R-CRE Luc cells (luciferase reporter gene assay): EC50 = 176 ± 50 nM, α = 0.81. Y4R agonism on HEK293-hY4-R-CRE Luc cells (luciferase reporter gene assay): EC50 = 220 ± 73 nM, α = 0.75. DOI: 10.1021/acsomega.7b00451

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H$_4^+$ calcd for [C$_{37}$H$_{47}$N$_4$O$_8$]$^+$: 675.3388, found: 675.3403. C$_{37}$H$_{46}$N$_4$O$_8$ (674.80).

4.3.4. [1-(9H-Fluoren-9-yl)-12,12-dimethyl-3,10-dioxo-2,11-dioxo-4,5,9-triazatridecan-5-yl]carbonyl-L-leucine (19).

Triphosgene (52.5 mg, 0.4 equiv) was dissolved in CH$_2$Cl$_2$ (2 mL). A solution of 16 (97.7 mg, 0.442 mmol) and DIPEA (150 μL, 2 equiv) in CH$_2$Cl$_2$ (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$_2$Cl$_2$ (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 CH$_2$Cl$_2$/MeOH (10:0.4) 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$_2$Cl$_2$/MeOH (10:0.6) + 0.1% TFA).

Figure 8. Y$_4$R agonism of (2R,7R)-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$_4$R-mtAEQ-Gqi5 cells. (C, D) Induced β-arrestin 1 recruitment in HEK293T-ARRB1-Y$_4$R cells. (E, F) Induced β-arrestin 2 recruitment in HEK293T-ARRB2-Y$_4$R cells. Data points shown for (A)–(F) are the mean ± SEM of at least three independent experiments performed in triplicate.
Removal of the volatiles afforded 19 as a sticky solid (132 mg, 52.5%). TLC (CH2Cl2/MeOH (10:0.6) + 0.5% AcOH): \( R_f = 0.55 \). \( ^1H \) NMR (400 MHz, methanol-\( d_4 \)): \( \delta \) (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\(_4\)O\(_{7}\) (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\(_2\)·C\(_2\)HF\(_3\)O\(_2\) (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 \([C_{60}H_{95}N_{11}O_{12}S]^{2+}\): 

Figure 9. Y\(_4\)R antagonism of (2\(R\),7\(R\))-26 and 27 determined in a calcium (aequorin) assay, a \( \beta \)-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\(_4\)R-mtAEQ-Gqi5 cells. (B) Inhibition of hPP (EC\(_{50}\) (Arr1) = 3.54 nM, EC\(_{50}\) (Arr2) = 2.74 nM, \( c = 3 \) nM)-induced \( \beta \)-arrestin 1,2 recruitment in HEK293T-ARRB1-Y\(_4\)R cells (\( \beta \)-arrestin 1, solid lines) and HEK293T-ARRB2-Y\(_4\)R cells (\( \beta \)-arrestin 2, dashed lines). (C) Inhibition of forskolin-stimulated (2 \( \mu \)M) luciferase activity (corresponding to 100%) in hY\(_4\)R 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 (\( \alpha = 1.0 \)). Data points shown for (A)–(C) are the mean ± SEM of at least three independent experiments performed in triplicate.

Figure 10. Y\(_4\)R functional activity of 38 and 44 determined in a calcium (aequorin) assay and a \( \beta \)-arrestin recruitment assay. (A) Inhibition of hPP (EC\(_{50}\) = 9.7 nM, \( c = 100 \) nM)-induced intracellular Ca\(_{2+}\) mobilization in CHO-hY\(_4\)R-mtAEQ-Gqi5 cells. (B) Inhibition of hPP (EC\(_{50}\) (Arr1) = 3.54 nM, EC\(_{50}\) (Arr2) = 2.74 nM, \( c = 3 \) nM)-induced \( \beta \)-arrestin 1,2 recruitment in HEK293T-ARRB1-Y\(_4\)R cells (\( \beta \)-arrestin 1, solid lines) and HEK293T-ARRB2-Y\(_4\)R cells (\( \beta \)-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).
4.3.7. Tyrt(tBu)-aza-Orn(Boc)-Leu-Arg(Pbf)-Tyrt(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, \( t_f = 14.6 \) min) afforded 23 as a white solid (36 mg, 45.2%). HRMS (ESI): \( m/z \) [M + 2H]\(^{2+}\) calcd for \([\text{C}_{60}\text{H}_{95}\text{N}_{11}\text{O}_{12}\text{S}]^{2+}\) : 596.8436, found: 596.8446. 

17.72 min, stirred at rt for 2.5 h. Water (100 mL) was added, followed by water (95:5 v/v) (2 mL) 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, \( t_f = 26.4 \) min). The eluates were subjected to lyophilization, and the residue was take up in TFA/CH\(_2\)Cl\(_2\)/H\(_2\)O (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. 4.3.11. Octanediol-bis(Tyr-Arg-Leu-aza-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (29). Compound 28 (3.7 mg, 2.05 \( \mu \)mol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (300 \( \mu \)L). A solution of \( \text{NN'-di-Boc-1H-pyrazole-1-carboximide} \) (1 mg/10 \( \mu \)L, 16 \( \mu \)L, 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, \( t_f = 26.4 \) min). The eluates were subjected to lyophilization, and the residue was take up in TFA/CH\(_2\)Cl\(_2\)/H\(_2\)O (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. 4.3.12. Octanediol-bis(Tyr-aza-Orn-Leu-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (30). Compound 23 (16.7 mg, 12.8 \( \mu \)mol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 \( \mu \)L). Compound 25 (1.88 mg, 5.1 \( \mu \)mol) 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\(_2\)Cl\(_2\) (2 \( \times \) 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, \( t_f = 16.5 \) min). Freeze-drying of the eluate gave (2R,7R)-26 as a white fluffy solid (3.45 mg, 28.3%). HRMS (ESI): \( m/z \) [M + 4H]\(^{4+}\) calcd for \([\text{C}_{78}\text{H}_{120}\text{N}_{26}\text{O}_{16}]^{4+}\): 420.2417, found: 420.2435. RP-HPLC (220 nm): 99% (\( t_f = 20.15 \) min, \( k = 6.0 \)). \( \text{C}_{78}\text{H}_{120}\text{N}_{26}\text{O}_{16}\) \( \text{C}_{8}\text{H}_{14}\text{F}_{2}\text{O}_{2} \) (1677.98 + 456.08). 

4.3.9. Octanediol-bis(Tyr-aza-Leu-Arg-Tyr-amide) Tetrakis(hydrotrifluoroacetate) (27). Compound 21 (16.7 mg, 19.2 \( \mu \)mol) was dissolved in anhydrous DMF/DIPEA (99:1 v/v) (600 \( \mu \)L). Compound 25 (2.83 mg, 7.7 \( \mu \)mol) 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\(_2\)Cl\(_2\) (2 \( \times \) 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. 

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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 \([C_{49}H_{77}N_{18}O_{11}]^{3+}\): 361.2174, found: 361.2169. RP-HPLC (220 nm): 95% (\( t_R = 14.9 \) min, \( k = 4.2 \)). C\(_{49}\)H\(_{80}\)N\(_{18}\)O\(_{10}\) C\(_{6}\)H\(_{5}\)F\(_{9}\)O\(_{6}\) (1083.11 + 456.08).

4.3.22. Ac-Ar-g-o-Tyr-{N\(^{w}\)-[N-(4-aminobutylyl)aminocarbonyl]}Arg-Leu-Arg-Tyr-amide Tetakis-(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\(_{10}\) C\(_{6}\)H\(_{5}\)F\(_{9}\)O\(_{6}\) (1083.11 + 456.08).

4.3.23. Ac-Ar-g-o-Tyr-{N\(^{w}\)-[N-(4-aminobutylyl)aminocarbonyl]}Arg-Leu-Arg-Tyr-amide Tetakis-(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): 95% (\( t_R = 14.9 \) min, \( k = 4.2 \)). C\(_{49}\)H\(_{80}\)N\(_{18}\)O\(_{10}\) C\(_{6}\)H\(_{5}\)F\(_{9}\)O\(_{6}\) (1083.11 + 456.08).

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

| receptor source             | Y1R      | Y2R      | Y4R      | Y5R      |
|-----------------------------|----------|----------|----------|----------|
| buffer                      | CHO-hY1R-G45-mtAEQ cells (6) | CHO-hY2R-G45-mtAEQ cells (26) | CHO-hY4R-G45-mtAEQ cells (26) | HEC-1B-hY1 cells (57) |
| buffer composition          | sodium-free HEPES | sodium-free HEPES | sodium-free HEPES | HEPES buffer containing 150 mM NaCl |
| radioligand                 | [3H]propionyl-pNPY | [3H]propionyl-pNPY | [3H]propionyl-pNPY | [3H]propionyl-pNPY |
| IC50                        | 0.67 nM  | 0.6 nM   | 4 nM     | 4.8 nM   |
| separation                  | suction  | filtration| suction  | suction  |
| scintillation cocktail      | Optiphase Supermix | Optiphase Supermix | Rotiscint eco plus | Optiphase Supermix |

aIsotonic HEPES buffer pH 7.4 (150 mM NaCl, 10 mM HEPES, 25 mM NaHCO3, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 5 mM KCl). bHypotonic, sodium-free HEPES buffer pH 7.4 (25 mM HEPES, 2.5 mM CaCl2, 1 mM MgCl2). Binding buffers were supplemented with BSA (1%) and bacitracin (0.1 mg/mL).

### 4.8. Aequorin Calcium Assay
The assay was performed on CHO-hY2R-G45-mtAEQ cells as previously described6 using a Genios Pro plate reader (Tecan, Salzburg, Austria). Areas under the curve were calculated using SigmalPlot 12.5 software (Systat Software Inc., Chicago, IL).

### 4.9. Luciferase Assay
The Luciferase assay was performed on HEK293-hY4-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-hY1R and HEK293T-ARRB2-hY1R cells using the Genios Pro microplate reader (Tecan, Salzburg, Austria) as previously described for HEK293T-ARRB1-hY1R and HEK293T-ARRB2-hY1R, 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 EC50 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, EC50 = 3.54 nM (ARRB1), EC50 = 2.74 nM (ARRB2)), 100 nM hPP (equinorin assay, EC50 = 9.7 nM) or 10 nM pNPY (Fura-2 Ca2+ assay, EC50 = 0.87 nM (Y1R). Kβ and Kβ (Fura-2 Ca2+ (Y1R)) values were calculated from IC50 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, (2R,7R)-26, 27, 29, 31, 37–40, and 44; aequorin Ca2+ assay with compounds (2R,7R)-26, 27, 29, 31, 37–40, and 44; saturation binding experiments with 45 at HEK293T-ARRB1-Y1R and HEK293T-ARRB2-Y1R cells; chromatograms of the HPLC purity control of all target...
1-piperazineethanesulfonic acid; HOBt, hydroxybenzotriazole; hexa-
H solid phase peptide synthesis; TEA, triethylamine; Y1,2,4,5R, oxytripyrrolidinophosphonium hexa-
NPY Y1,2,4,5 receptor

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