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Article

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High Affinity Agonists of the Neuropeptide Y (NPY) Y_4 Receptor Derived from the C-terminal Pentapeptide of Human Pancreatic Polypeptide (hPP): Synthesis, Stereochemical Discrimination and Radiolabeling

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ABSTRACT

The diastereomeric mixture of *D*/*L*-2,7-diaminooctanedioyl-bis(YRLRY-NH_2) (BVD-74D, **2**) was described in the literature as a high affinity Y_4 receptor agonist. Here we report on the synthesis and pharmacological characterization of the pure diastereomers (2*R,*7*R*)- and (2*S,*7*S*)-**2** and a series of homo- and heterodimeric analogues in which octanedioic acid was used as an achiral linker. To investigate the role of the Arg residues, one or two arginines were replaced by Ala. Moreover, N^{ω}-(6-aminohexylaminocarbonyl)Arg was introduced as an arginine replacement (**17**). (2*R,*7*R*)-**2** was superior to (2*S,*7*S*)-**2** in binding and functional cellular assays and equipotent with **17**. [^{3}H]propionylation of one amino group in the linker of (2*R,*7*R*)-**2** or at the primary amino group in **17** resulted in high affinity Y_4R radioligands ([^{3}H]-(2*R,*7*R*)-**10**, [^{3}H]**18**) with subnanomolar K_d values.

KEYWORDS

Bioisosteric replacement, carbamoylguanidine, cross-metathesis, diastereomers, neuropeptide Y, NPY Y_4 receptor agonist, pancreatic polypeptide, radioligand

1. INTRODUCTION

Neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) consist of 36 amino acids and share considerable sequence similarities (Figure 1).[1-3] NPY, PYY and PP activate NPY receptors, in humans comprising four functional subtypes of G-protein coupled receptors (Y_1R, Y_2R, Y_4R, Y_5R), which are involved in the regulation of numerous central and peripheral biological processes.[4] The Y_4R shares only low sequence identity with the other subtypes (e.g. 42% sequence identity of hY_4R with hY_1R[5]) and is an exception as it prefers PP over NPY and PYY.[5] The Y_4R is considered to play a role, for example, in feeding behavior and the regulation of energy metabolism.[6,7] Recently, Y_4R agonists were proposed as putative anti-obesity agents.[8,9] Therefore, there is a need for high affinity Y_4R ligands, agonists as well as antagonists, to explore the potential of the Y_4R as a biological target.

Previous work from different groups suggests that connecting two pharmacophoric moieties favors Y_4R binding, in particular, in case of peptidic agonists such as **1** and **2** (Figure 2), which are derived from the C-terminal pentapeptide of hNPY or hPP, respectively. Compound **3**[10] (UR-MK188) is an example of a Y_4R antagonist, composed of two molecules of an argininamide-type Y_1R antagonist ((*R*)-4-guanidino-1-({[(4-hydroxyphenyl)methyl]amino}carbonyl)butyl-α-phenylbenzeneacetamide, BIBP3226),[11] which are connected via the guanidine groups. Generally, the arginine residues in positions 33 and 35 of the endogenous peptides are important for NPY receptor binding.[12] Similarly, the arginine moieties are considered crucial structural features of the Y_4R ligands **1-3**. The "dimeric peptide" **1** (GW1229, also known as GR23118 or 1229U91)[13] is both, a high-affinity antagonist at the Y_1R and an agonist at the Y_4R.[14] The pentapeptide sequence Tyr-Arg-Leu-Arg-Tyr-NH_2 in **1** is very similar to the C-terminal sequence of hPP (…Thr^{32}-Arg^{33}-Pro^{34}-Arg^{35}-Tyr^{36}-NH_2). *D/L*-2,7-diaminooctanedioyl-

bis(YRLRY-NH_2) (BVD-74D, **2**),[15] comprising the same pentapeptide moieties as **1**, but a different linker, is more selective for the Y_4R. Data reported for **2** refer to the diastereomeric mixture of (2*R*,7*R*)-**2** and (2*S*,7*S*)-**2**,[15] not to a single stereoisomer (note: in the following, positions 2 and 7 refer to the stereo centers in the 2,7-diaminosuberic acid moiety).

NPY YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2
PYY YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2
PP APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH_2

Figure 1. Amino acid sequences of human NPY, PYY and PP

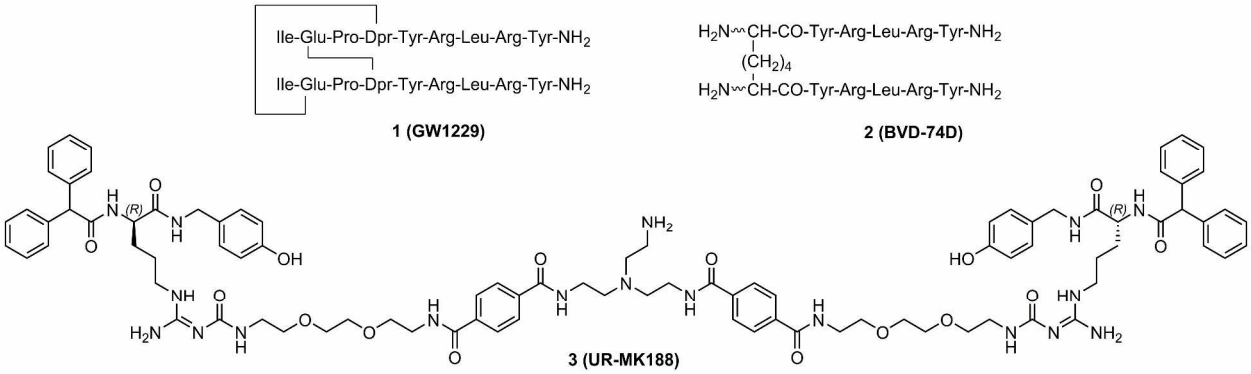


Figure 2. Structures of the described NPY Y_1R antagonist/Y_4R agonist **1**, the Y_4R agonist **2**, and the Y_1R/Y_4R antagonist **3**. Dpr: *L*-2,3-diaminopropionic acid; **2** was previously described as a mixture of two diastereomers due to (2*R*,7*R*)- and (2*S*,7*S*)-configuration of the 2,7-diaminosuberic acid linker.

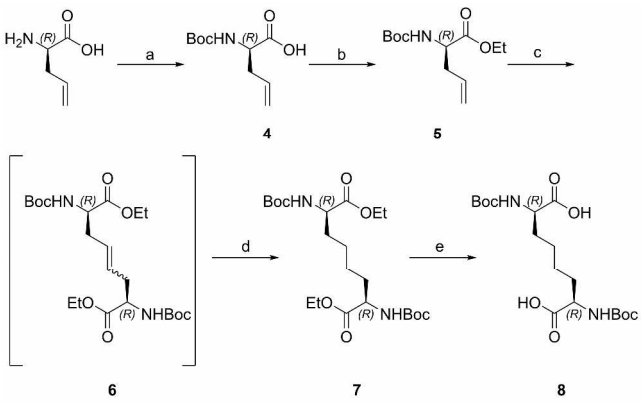
Here we report on the synthesis and pharmacological characterization of the pure stereoisomers (2*R*,7*R*)-**2** and (2*S*,7*S*)-**2**. Very recently, a pilot study from our laboratory suggested that the amino groups in the linker of **2** are dispensable.[16] Therefore, we synthesized a set of analogues using octanedioic acid instead of 2,7-diaminosuberic acid as linker. To investigate the contribution of the individual arginines to the Y_4R affinity of the dimers, we prepared compounds in which one or two Arg moieties in the pentapeptide building block were

replaced by Ala. Furthermore, the suitability of the 'dimeric' pentapeptides as precursors for the preparation of radiolabeled compounds was explored, the target compounds were characterized in functional assays, and two tritiated derivatives were synthesized and used to perform Y_4R binding studies.

2. RESULTS AND DISCUSSION

Chemistry. The enantiopure 2,7-diaminooctanedioic acids were synthesized from (*S*)- and (*R*)-2-allylglycine via the *N*-Boc protected esters (*S*)-**5** and (*R*)-**5** (Scheme 1) by analogy with procedures from the literature.[17,18] The enantiomers of **5** were submitted to a cross-metathesis reaction, employing Grubbs' II catalyst to build up the core structure **6**. After exchanging the solvent (DCM) with ethanol, compound **6** was not isolated, but hydrogenated at 20 bar in the presence of the same catalyst,[19,20] in contrast to reported procedures requiring workup of **6** and Pd/C catalyzed hydrogenation.[21] Besides the convenience of this protocol, overall yields were improved, affording (2*R*,7*R*)-**7** and (2*S*,7*S*)-**7** in 95% and 74% yield, respectively, over two steps. Hydrolysis of the ethyl ester quantitatively provided the diacids (2*R*,7*R*)-**8** and (2*S*,7*S*)-**8**.

Scheme 1. Enantioselective synthesis of the diacids (2*R,*7*R*)-**8** and (2*S,*7*S*)-**8**[a]

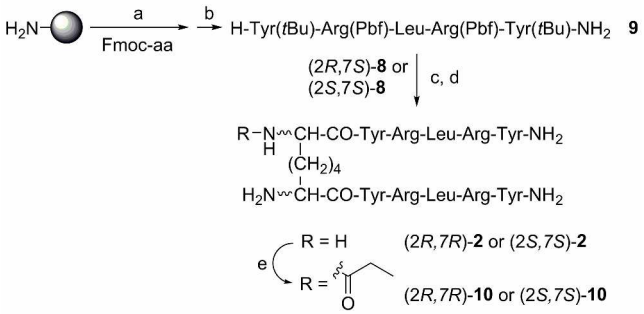


[a](2*S,*7*S*)-**8** was prepared according to the same protocol using (*S*)-2-allylglycine as starting material. Reagents and conditions: (a) Boc_2O, 1 M NaOH/dioxane (2:1), 0 °C to rt, 18 h; (b) DCC, 4-DMAP, EtOH, DCM, 0 °C to rt, 12 h; (c) 0.1 equiv Grubbs' II catalyst, anhydrous DCM, N_2 atmosphere, reflux, 12 h; compd. **6** was not isolated, but subjected to hydrogenation in the next step; (d) 0.1 equiv Grubbs' II catalyst (from reaction step (c)), ethanol, H_2 (20 bar), rt, 15 h (95% for (*R,R*)-**7**, 74% for (*S,S*)-**7** over two steps, (c) and (d)); (e) 1 M NaOH, EtOH, rt, 21 h (quant.).

Peptide synthesis was carried out manually on a Sieber-amide resin applying an Fmoc protocol and 'double' coupling procedures with HBTU/HOBt/DIPEA (Scheme 2). After cleavage from the resin, two equivalents of the protected pentapeptide **9**[16] were cross-linked with the *in situ* activated (*R*,*R*)- and (*S*,*S*)-configured enantiomers of 2,7-diaminosuberic acid (**8**) to obtain the corresponding peptides (2*R,*7*R*)-**2** and (2*S,*7*S*)-**2** after deprotection. Subsequent acylation (monitored by HPLC) of an excess of precursor with succinimidyl propionate gave the propionamides (2*R,*7*R*)-**10** (for preparation of [^{3}H]-labeled form see below) and (2*S,*7*S*)-**10**.

With respect to labeling of compounds devoid of amino groups in the linker (Scheme 3), we introduced the recently reported arginine derivative **11** (Figure 3), in which the guanidine group is replaced by a nonclassical bioisostere, a functionalized carbamoylguanidine moiety.[16]

Scheme 2. Synthesis of (2*R,*7*R*)-**2** and (2*S,*7*S*)-**2** and the propionylated analogues (2*R,*7*R*)-**10** and (2*S,*7*S*)-**10**[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; Fmoc deprotection was carried out with 20% piperidine in DMF/NMP (8:2), rt, 2 × 10 min; (b) DCM/TFA (97:3), 10 × 6 min; (c) HBTU, HOBt, DIPEA, anhydrous DMF, 35 °C, 16 h; (d) TFA/H_2O (95:5), rt, 2.5 h; (e) succinimidyl propionate, 3% DIPEA in anhydrous DMF, rt, 2 h.

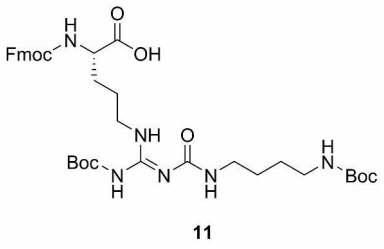
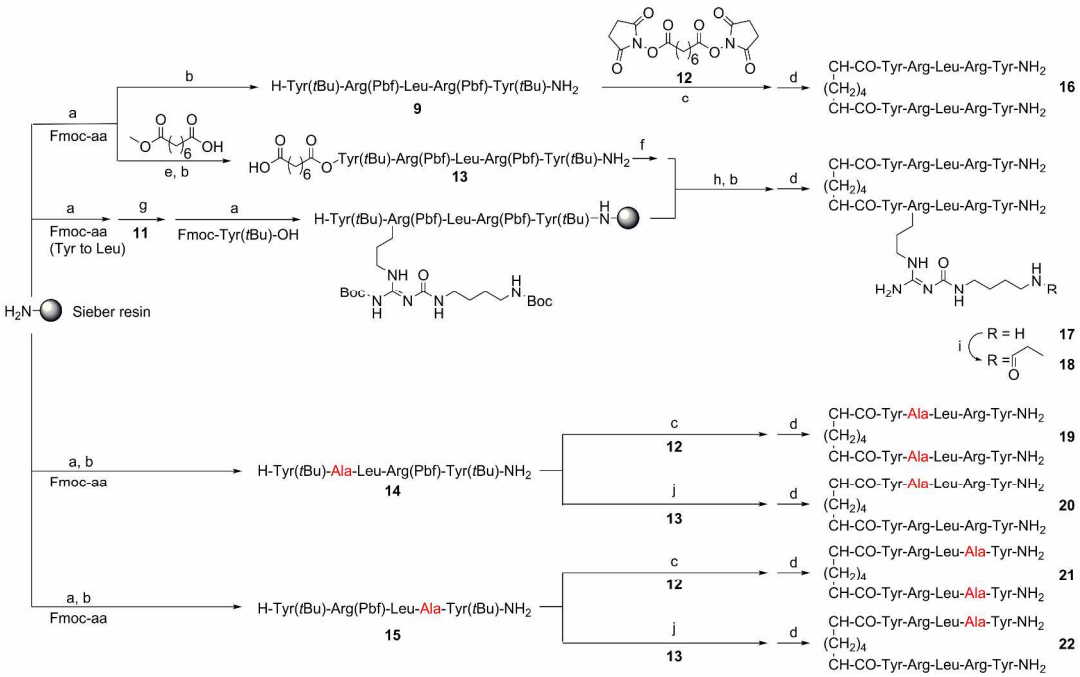


Figure 3. Structure of the N^{ω}-carbamoylated arginine building block **11**.[16]

For the preparation of the homodimeric ligands (**16**, **19**, **21**) shown in Scheme 3, octanedioic acid, activated as di-succinimidyl ester (**12**[22]), was used for cross-linking (Scheme 3). By contrast, as a building block for the synthesis of heterodimeric ligands (**17**,[16] **18**,[16] **20**, **22**,), compound **13**[16] was prepared by coupling suberic acid monomethyl ester to the resin-bound peptide sequence Tyr(*t*Bu)-Arg(Pbf)-Leu-Arg(Pbf)-Tyr(*t*Bu). The side-chain protected peptides **14** and **15** were treated with **12** to give **16**,[16] **19** and **21**), whereas *in situ* activated **13** was coupled to the side-chain protected peptides **14** and **15** for the preparation of the heterodimeric ligands **20** and **22**, respectively. By analogy with the synthesis of the potential radioligands (2*S,*7*S*)-**10** and (2*R,*7*R*)-**10**, the precursor **17**[16] was treated with succinimidyl propionate to give **18** as described very recently.[16]

Scheme 3. Synthesis of homo- and heterodimeric Y_4R ligands[a]



[a]Reagents and conditions: (a) cf. legend to Scheme 2; (b) DCM/TFA (97:3), 10 × 6 min; (c) 1 equiv of **12** in 1% DIPEA in anhydrous DMF, followed by 2.5 equiv of **9**, **14** or **15**, 35 °C, 16 h; (d) TFA/H_2O (95:5), rt, 2.5 h; (e) suberic acid monomethyl ester/HBTU/HOBt/DIPEA (4/4/4/8 equiv), solvent: DMF/NMP (8:2), single coupling at 30 °C, 14 h; (f) 0.5 M NaOH/MeOH (1:5), reflux, 3 h; (g) **11**/HBTU/HOBt/DIPEA (3/3/3/6 equiv), solvent: DMF/NMP (8:2), single coupling at 35 °C, 16 h; (h) **13**/HBTU//HOBt/DIPEA (1/1/1/2 equiv), solvent: DMF/NMP (8:2), single coupling at 35 °C, 16 h; (i) succinimidyl propionate, DMF, DIPEA, rt, 2 h; (j) **13**/HBTU/HOBt/DIPEA (1/1/1/3.5 equiv), solvent: DMF, rt, 5 min, subsequent addition of 1.1 equiv of **14** or **15**, rt, 17 h.

Synthesis of the Radiolabeled Ligands [^{3}H](2*R*,7*R*)-10 and [^{3}H]18. Based on the results obtained in the functional assays (see below), (2*R*,7*R*)-**10** and **18** were selected for the synthesis of the corresponding tritiated versions. An 8-fold excess of the precursor peptides (2*R*,7*R*)-**2** and **17**[16] was treated with commercially available succinimidyl [^{3}H]propionate (Figure 4A). The radioligands were obtained in high radiochemical purities (Figure 4B and Figure 4D). After storage of the radioligands in a mixture of EtOH/H_2O (1:1) at -20 °C over 9 months, approximately 10% were decomposed (Figure 4C and Figure 4E). No decomposition was

observed after an incubation period of 48 h, when the 'cold' analogues, (2*R*,7*R*)-**10** and **18**, were investigated for stability (Figure 5A and Figure 5B) in a HEPES buffer (buffer I, pH 7.4).

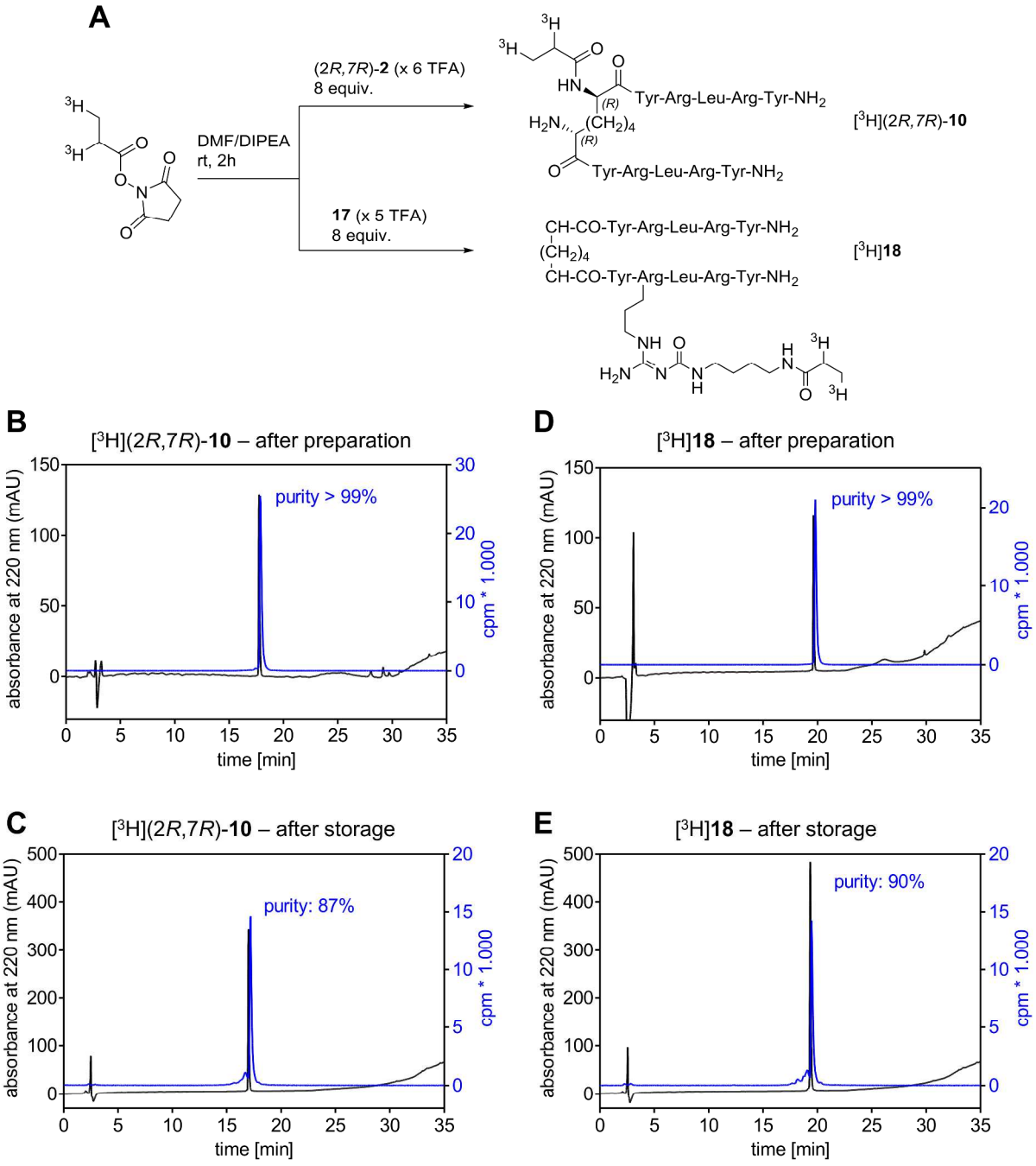


Figure 4. Preparation, purity, identity control and long-term stability of the radiolabeled peptides [^{3}H](2*R*,7*R*)-**10** and [^{3}H]**18**. (**A**) Synthesis of the radioligands [^{3}H](2*R*,7*R*)-**10** and [^{3}H]**18** by

propionylation of the precursors (2*R*,7*R*)-**2** and **17**. Radiochemical yield: 34% ([^{3}H](2*R*,7*R*)-**10**), 30% ([^{3}H]**18**). (**B**) HPLC analysis of [^{3}H](2*R*,7*R*)-**10** (ca. 0.35 μM) spiked with 'cold' (2*R*,7*R*)-**10** (5 μM), analyzed 3 days after synthesis. Radiochemical purity > 99%. (**C**) HPLC analysis of [^{3}H](2*R*,7*R*)-**10** (ca. 0.25 μM) spiked with 'cold' (*R*,*R*)-**10** (13 μM), analyzed after 9 months of storage at -20 °C in EtOH/H_2O (1:1). Radiochemical purity: 87%. (**D**) HPLC analysis of [^{3}H]**18** (ca. 0.35 μM) spiked with 'cold' **18** (5 μM), analyzed 10 days after synthesis. Radiochemical purity > 99%. (**E**) HPLC analysis of [^{3}H]**18** (ca. 0.25 μM) spiked with 'cold' **18** (13 μM), analyzed after 9 months of storage at -20 °C in EtOH/H_2O (1:1). Radiochemical purity: 90%. The minor differences in t_R result from serial detection of the UV and radiometric signals.

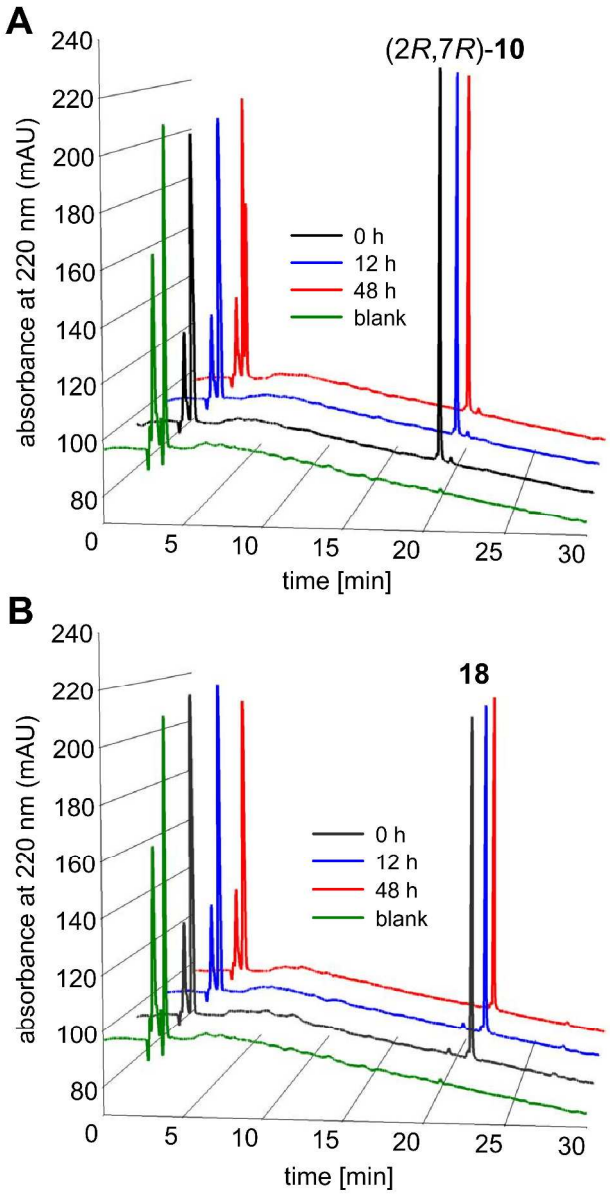


Figure 5. HPLC analysis of 'cold' (2*R*,7*R*)-**10** (**A**) and **18** (**B**) after incubation in buffer I (HEPES buffer, sodium-free) (pH 7.4) for up to 48 h. Both compounds showed no decomposition. Peaks between 0 and 5 min correspond to buffer components.

Functional Studies at the hY$_4$R. The target compounds were investigated for Y$_4$R agonism in an aequorin Ca^{2+} assay[23] and a luciferase gene reporter assay on genetically engineered CHO and HEK293 cells, respectively (data cf. Table 1 and Figure 6).

Table 1. NPY Y_4R agonist potencies (EC_{50}) and intrinsic activities (α) of 'dimeric' pentapeptides and reference compounds PP and **1**.

	Aequorin assay [a]		**Luciferase assay** [b]	
Compd.	EC_{50} [nM]	α	EC_{50} [nM]	α
PP	9.7 ± 0.4	1	0.73 ± 0.2	1
1	8.5[c]	0.62	5.5 ± 0.8	1.01
(2*R*,7*R*)-**2**	6.9 ± 0.6	0.75	1.6 ± 0.6	1.03
(2*S*,7*S*)-**2**	18 ± 1.6	0.78	19 ± 4.8	1.07
(2*R*,7*R*)-**10**	14 ± 2.0	0.76	4.0 ± 0.9	1.00
(2*S*,7*S*)-**10**	59 ± 5.6	0.70	27 ± 4.6	0.98
16	49 ± 13	0.73	86 ± 16	0.93
17	6.4 ± 1.3	0.80	1.7 ± 0.2	1.06
18	13 ± 1.1	0.67	7.1 ± 1.9	1.05
19	> 3000	-	> 3000	-
20	290 ± 33	0.85	160 ± 29	0.98
21	> 3000	-	> 3000	-
22	310 ± 38	0.85	150 ± 18	0.99

[a]Aequorin calcium mobilization assay on CHO-hY_4-G_{qi5}-mtAEQ cells.[23] [b]CRE-luciferase reporter gene assay on HEK293 cells stably co-expressing the hY_4R and the CRE-controlled luciferase gene reporter. Y_4R agonist potency was determined from the inhibition of forskolin (2 μM) stimulated luciferase activity. The maximal response (intrinsic activity, α) is referred to the effect of PP set to α = 1.0. [c]EC_{50} value reported by Ziemek et al.[23]

The configuration of the stereo centers in the 2,7-diaminosuberic acid linker in **2** had an impact on Y_4R potency. In the luciferase assay (2*R*,7*R*)-**2** (EC_{50} = 1.6 nM) was 12 times more potent than (2*S*,7*S*)-**2** (EC_{50} = 19 nM). In the aequorin assay the difference in potency was less

pronounced (EC_{50} values: 6.9 nM ((2*R*,7*R*)-**2**) vs. 18 nM ((2*S*,7*S*)-**2**)). The same tendency became obvious for the propionylated analogues (2*R*,7*R*)-**10** and (2*S*,7*S*)-**10** in both assays. Generally, propionylation of an amino group of the linker did only slightly (by a factor of two) affect Y_4R potency in both assays.

Except for **17** and **18**, compounds devoid of an amino group in the linker were significantly less potent Y_4R agonists. Interestingly, replacement of one of the 'inner arginines' in **16** by the N^G-carbamoylated arginine **11** considerably increased Y_4R agonist potency (**17**, EC_{50}: 6.4 nM and 1.7 nM in the aequorin assay and the luciferase assay, respectively), that is, **17** was equipotent with (2*R*,7*R*)-**2**. As observed for the stereoisomers of **2** and **10**, propionylation of **17**, resulting in the derivative **18**, led only to a minor decrease in Y_4R potency.

The homodimeric compounds **19** and **21**, in which two arginines were replaced by alanine, proved to be inactive at the Y_4R, suggesting that the presence of at least one unaltered pentapeptide sequence Tyr-Arg-Leu-Arg-Tyr-NH_2 is indispensable. Compounds **20** and **22**, in which only one Arg was replaced by Ala, were less potent than **16** by a factor of 5 to 6 in the aequorin assay and by a factor of 2 in the luciferase assay.

Except for **19** and **21**, all investigated compounds were partial agonists in the aequorin assay (intrinsic activities between 62% and 85% referred to hPP), but full agonists in the luciferase assay. Moreover, the potencies obtained in the luciferase assay were on average slightly higher than those obtained in the aequorin assay, presumably due to more pronounced signal amplification in the gene reporter assay.[24]

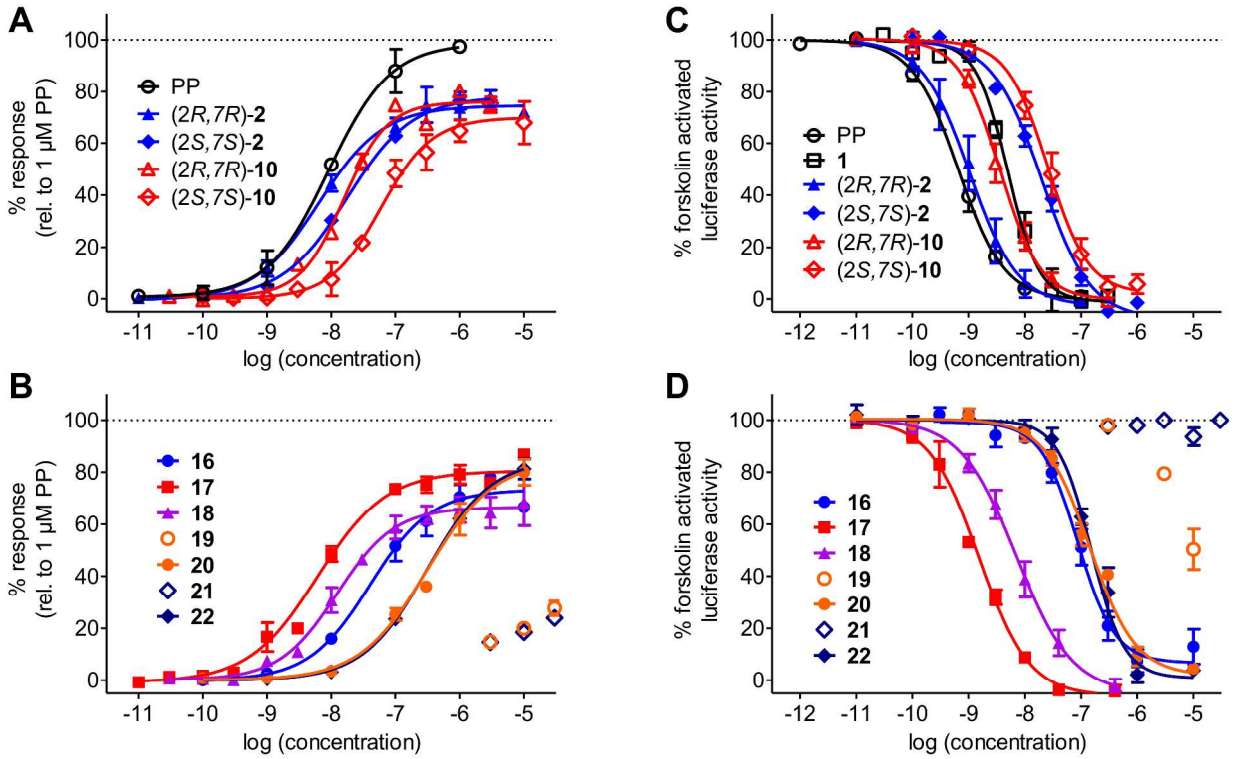


Figure 6. Y_4R agonism of PP, **1**, **2**, **10** and **16-22** determined in a calcium (aequorin) assay and a luciferase reporter gene assay. (**A**) and (**B**) Concentration-response curves (CRCs) from an aequorin assay on CHO-hY_4R-mtAEQ-G_{qi5} cells. Mean values ± SEM were from at least 3 independent experiments (performed in triplicate). (**C**) and (**D**) Inhibition of forskolin-stimulated (2 µM) luciferase activity (corresponding to 100%) in hY_4R expressing HEK293 cells by PP, **1**, **2**, **10** and **16-22**, with the maximum inhibitory effect of the endogenous ligand PP which is set to 0% luciferase activity and corresponds to full agonism (α = 1.0). Data points shown are the mean ± SEM of at least three independent experiments performed in triplicate.

Characterization of [^{3}H](2*R*,7*R*)-10 and [^{3}H]18. Saturation binding experiments with the tritiated radioligands (Figure 7A and Figure 7D), performed on intact CHO-hY_4-G_{qi5}-mtAEQ

cells in buffer I (HEPES, sodium-free) as used by Balasubramaniam et al.[15] to investigate the diastereomeric mixture **2**, revealed a K_d value of 0.67 ± 0.1 nM for both compounds, [^{3}H](2*R*,7*R*)-**10** and [^{3}H]**18**. The number of specific binding sites per cell, determined with [^{3}H](2*R*,7*R*)-**10** or [^{3}H]**18**, was 320,000 ± 20,000 and 340,000 ± 15,000, respectively. At concentrations around the K_d value, unspecific binding amounted to 10% ([^{3}H](2*R*,7*R*)-**10**) and 20% ([^{3}H]**18**), respectively, of the total binding. Association to the receptor was complete after 100 min (Figure 7B and Figure 7E). Dissociation from the receptor was biphasic (Figure 7C and Figure 7F) with a fast k_{off} (0.0329 min^{-1} for [^{3}H](2*R*,7*R*)-**10** and 0.0309 min^{-1} for [^{3}H]**18**) and a slow k_{off} (0.0024 min^{-1} for [^{3}H](2*R*,7*R*)-**10** and 0.0026 min^{-1} for [^{3}H]**18**).

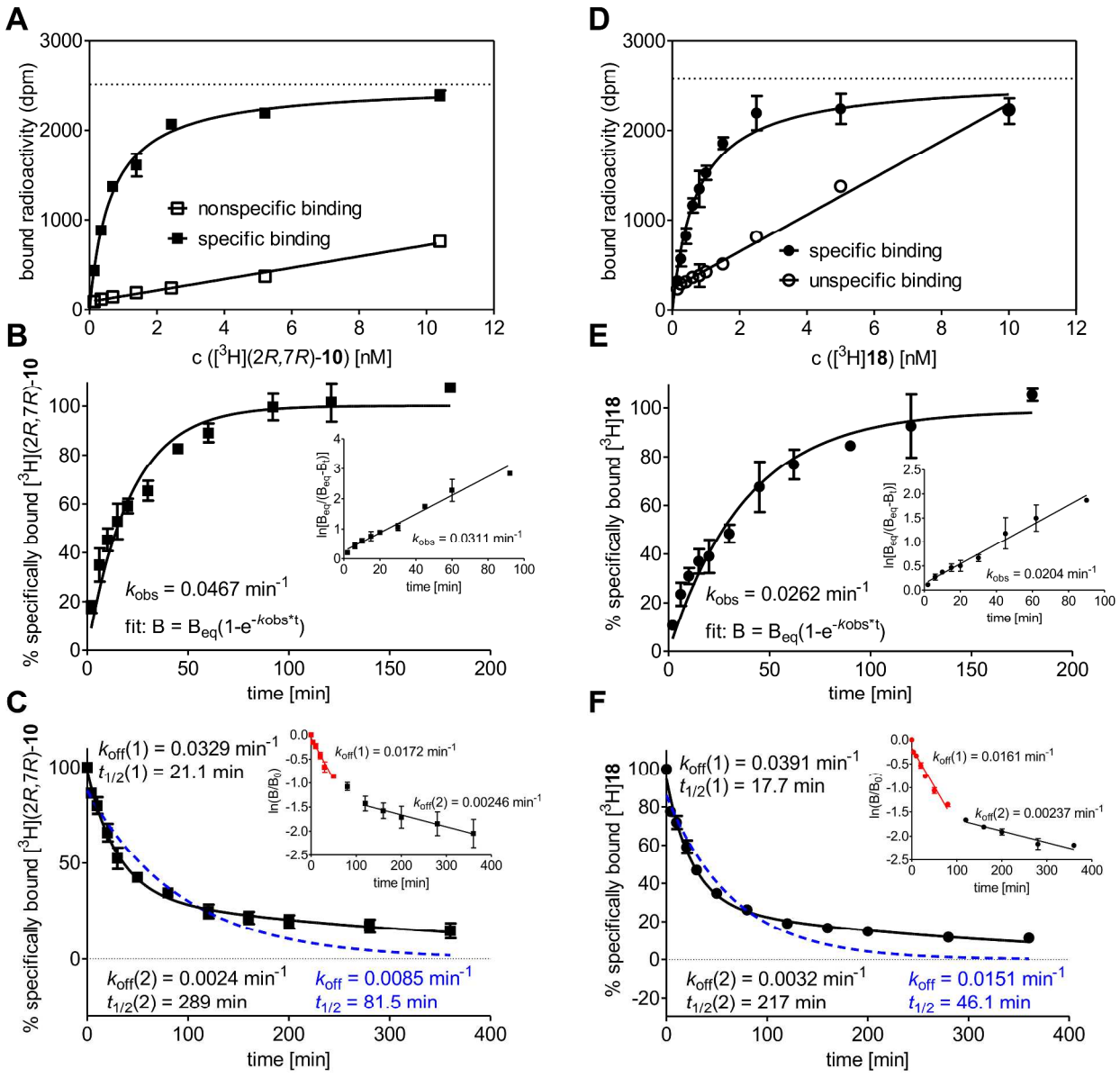


Figure 7. Y_4R binding characteristics of the radioligands [^{3}H](2*R*,7*R*)-**10** and [^{3}H]**18** determined at live CHO-hY_4-G_{qi5}-mtAEQ cells at 22 °C in buffer I (HEPES, sodium-free). (**A**) Representative saturation binding experiment with [^{3}H](2*R*,7*R*)-**10**. (**B**) Y_4R association kinetics of [^{3}H](2*R*,7*R*)-**10** (c = 1.5 nM). Inset: Linearization $\ln[B_{eq}/(B_{eq}-B_t)]$ versus time. (**C**) Y_4R dissociation kinetics of [^{3}H](2*R*,7*R*)-**10** (c = 1.5 nM, pre-incubation time: 2 h) determined in the presence of a 200-fold excess of (2*R*,7*R*)-**2**. Fitting of the data according to a biphasic exponential decay (black curve, $B = B_0(1) \cdot e^{-k_{off}(1)t} + B_0(2) \cdot e^{-k_{off}(2)t}$, $R^2 = 0.9532$) resulted in a

much better correlation than an analysis according to a monophasic exponential decay (blue dotted curve, $B = B_0 \cdot e^{-k_{off}t}$, $R^2 = 0.8321$). Inset: Linearization $\ln[B/B_0]$ versus time. (**D**) Representative saturation binding experiment with [^{3}H]**18**. (**E**) Y_4R association kinetics of [^{3}H]**18** (c = 1.5 nM). Inset: Linearization $\ln[B_{eq}/(B_{eq}-B_t)]$ versus time. (**F**) Y_4R dissociation kinetics [^{3}H]**18** (c = 1.5 nM, pre-incubation time: 2 h) determined in presence of 200-fold excess of **17**. Fitting of the data according to a biphasic exponential decay (black curve, $B = B_0(1) \cdot e^{-k_{off(1)}t} + B_0(2) \cdot e^{-k_{off(2)}t}$, $R^2 = 0.9819$) resulted in a much better correlation than an analysis according to a monophasic exponential decay (blue dotted curve, $B = B_0 \cdot e^{-k_{off}t}$, $R^2 = 0.9166$). Inset: Linearization $\ln[B/B_0]$ versus time.

Table 2. Binding data of [^{3}H](2*R*,7*R*)-**10** and [^{3}H]**18**.

	Saturation binding		**Binding kinetics**		
radioligand	K_d [nM]		k_{obs} [s^{-1}][c]	k_{off1} [s^{-1}][d]	k_{off2} [s^{-1}][e]
[^{3}H](2*R*,7*R*)-**10**	0.67 ± 0.1[a]	9.8 ± 2.5[b]	0.0467	0.0329	0.0024
[^{3}H]**18**	0.67 ± 0.1[a]		0.0262	0.0391	0.0032

[a]Equilibrium dissociation constant determined on CHO-hY_4-G_{qi5}-mtAEQ cells in buffer I; mean ± SEM from at least 4 independent experiments (performed in triplicate). [b]Equilibrium dissociation constant determined on CHO-hY_4-G_{qi5}-mtAEQ cells in Leibovitz' L-15 medium; mean ± SEM from at least 4 independent experiments (performed in triplicate). [c]Apparent association constant, mean from 3 independent experiments (performed in triplicate). [d]Fast dissociation constant, mean from 3 independent experiments (performed in triplicate), calculated according to a four-parameter equation describing a biphasic exponential decay. [e]Slow dissociation constant, mean from 3 independent experiments (performed in triplicate), calculated according to a four-parameter equation describing a biphasic exponential decay. Note: A kinetically derived dissociation constant $K_{d(kin)}$ and the association rate constant k_{on} was not calculated, as k_{off} was ambiguous due to a fast (k_{off1}) and a slow (k_{off2}) component.

Competition Binding Studies at NPY Receptor Subtypes. K_i values of the 'dimeric' pentapeptides were determined in competition binding experiments on live cells expressing human Y_1, Y_2, Y_4 or Y_5 receptors to determine the NPY receptor subtype selectivity. The unspecific binding at the respective NPY receptor expressing cells was low, and there was no hint to an internalization of the used radioligands, which might lead to a misinterpretation of the binding data. Therefore, investigations on a potential internalization, e. g. by acid wash experiments,[16] were not considered necessary.

Y_4R Binding. The binding data of the 'dimeric' pentapeptides and the reference compounds PP, **1** and **3**, were determined at the Y_4R, using the new radioligands [^{3}H](2*R*,7*R*)-**10** and [^{3}H]**18** (Figure 8). The K_i value of PP (0.65 nM) was in good agreement with published data (K_i: 0.50 nM,[16] 0.53 nM[25]), whereas the affinity of **3** (K_i = 660 nM) was slightly lower than reported (K_i = 130 nM[10]). In case of the 'cold' analogues of the radioligands, (2*R*,7*R*)-**10** (0.87 nM) and **18** (1.2 nM), the K_i values were in good agreement with the K_d values (cf. Figure 8, Table 2). Generally, the K_i values of the 'dimeric' pentapeptides at the Y_4R were by a factor of approximately 10 lower than the EC_{50} values determined in the functional assays (aequorin or luciferase assay, Table 1). Nevertheless, the Y_4R affinities correlated with the order of potency.

The diastereomeric mixture of **2** was previously reported[15] to show Y_4R agonism in the two-digit nM range (EC_{50} = 14.8 nM, cAMP assay), but much higher affinity (K_i = 0.05 nM, radioligand [^{125}I]PP) determined in sodium-free HEPES buffer (buffer I), which has a broad application in binding experiments in the NPY field. Experiments performed with different buffers in our laboratory revealed that the discrepancies between functional and binding data are, at least in part, caused by the absence (buffer I) or presence (Leibovitz' L-15 medium) of sodium ions. An exchange of the sodium-free buffer I with the Na^+ containing L-15 medium led to an

increase in the K_d value of [^{3}H](2*R*,7*R*)-**10** from 0.67 nM to 9.8 nM (cf. Table 2, footnote b). Similarly, K_i values determined in competition binding experiments using [^{3}H](2*R*,7*R*)-**10** in L-15 medium were by a factor of almost 10 higher (K_i values, (2*R*,7*R*)-**2**: 2.0 nM; (2*S*,7*S*)-**2**: 17 nM; (2*R*,7*R*)-**10**: 6.3 nM). It should be stressed that the binding constants determined in L-15 medium were in good agreement with the agonist potencies determined in the functional assays using Na^+ containing buffer III or DMEM.

Interestingly, the affinity of the Y_4R antagonist **3** was not affected in the same way by an exchange of the binding buffer. The K_i value of **3** in competition binding experiments using the L-15 medium was even lower (K_i = 150 nM) than in experiments using buffer I. These results are compatible with recent insights obtained from GPCR crystal structures. Several high-resolution crystal structures described Na^+ ions as allosteric stabilizers of inactive-state receptor conformations.[26,27] Therefore, Na^+ is expected to reduce the affinity of agonists binding to the active state.

As an alternative to [^{3}H](2*R*,7*R*)-**10**, the radioligand [^{3}H]**18** is also suited for competition binding. The K_i values determined by displacement of [^{3}H]**18** were on average slightly lower (Table 3).

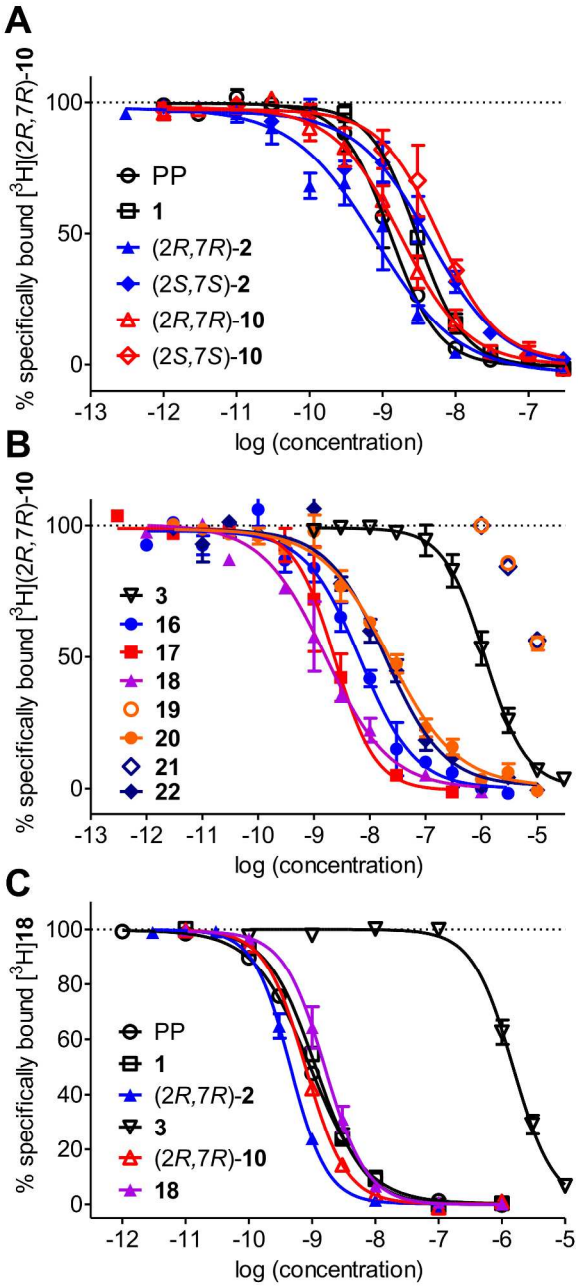


Figure 8. Radioligand displacement curves from competition binding experiments performed with [^{3}H](2*R*,7*R*)-**10** or [^{3}H]**18** at CHO-hY$_4$R-G$_{qi5}$-mtAEQ cells in buffer I. (**A**) and (**B**) Displacement of [^{3}H](2*R*,7*R*)-**10** (K_d = 0.67 nM, c = 0.6 nM). (**C**) Displacement of [^{3}H]**18** (K_d = 0.67 nM, c = 1 nM). Data represent mean values ± SEM of at least 3 independent experiments, each performed in triplicate.

NPY receptor subtype selectivity. The K_i values of (2*R*,7*R*)-**10** and **18**, the 'cold' analogues of the new Y_4R radioligands at the Y_1R, Y_2R and Y_5R were in the three-digit nanomolar range (Table 3). The 'heterodimeric' ligands **20** and **22** were comparable with (2*R*,7*R*)-**10** and **18** at the Y_1R and Y_5R, but only very weak binders at the Y_2R. K_i values were not determined for the 'homodimeric' ligand **21** at all NPY receptors due to low affinity. The same holds for compound **19** with exception of the Y_5R. Peptide dimers derived from the C-terminus of NPY such as **1** were among the first high affinity Y_1R antagonists described in literature.[13,28] Therefore, we selected (2*R*,7*R*)-**2** and **17** as examples for functional investigations at the Y_1R. In a standard Y_1R model, the Fura-2 calcium assay on HEL cells, both compounds revealed antagonism with K_b values of 9.5 nM and 25.3 nM, respectively (cf. Table 3, footnote, and Supporting Information, Figure S3). In case of (2*R*,7*R*)-**2** the result disagrees with published data for the diastereomeric mixture **2**, which was reported to be an agonist in a cAMP assay on Y_1R expressing HEK 293 cells.[15] It may be speculated, whether the opposing qualities of action are related to the specific repertoire of G_q and G_i coupling[29] in HEL cells, endogenously expressing the Y_1R, compared to genetically engineered HEK293 cells.

Table 3. NPY receptor binding data.

	Y_1R	Y_2R		Y_4R, K_i [nM]		Y_5R,
Compd.	K_i [nM][a]	K_i [nM][b]	[^{3}H](2*R*,7*R*)-**10**[c]	[^{3}H](2*R*,7*R*)-**10**[d]	[^{3}H]**18**[e]	K_i [nM][f]
PP	440[g]	>5000[g]	0.65 ± 0.13	-	0.37 ± 0.02	17[g]
1	-	-	1.6 ± 0.34	-	1.3 ± 0.78	-
2	7.5[h]	1100[h]	1.06 ± 0.47 (0.05[h])	-	-	>1000[h]

(2*R*,7*R*)-**2**	440 ± 81[i]	830 ± 250	0.45 ± 0.14	2.0 ± 0.04	0.18 ± 0.02	1500 ± 300
(2*S*,7*S*)-**2**	930 ± 270	950 ± 520	2.3 ± 0.16	17 ± 4.3	-	2100 ± 980
3	24[j]	920[j]	660 ± 140	150 ± 36	630 ± 80	>5000[j]
(2*R*,7*R*)-**10**	400 ± 46	420 ± 50	0.87 ± 0.16	6.3 ± 0.8	0.32 ± 0.02	710 ± 96
(2*S*,7*S*)-**10**	290 ± 110	750 ± 380	2.8 ± 0.44	-	-	400 ± 71
16	720 ± 100	1700 ± 70	3.5 ± 0.59[k]	-	-	280 ± 40
17	410 ± 150[l]	730 ± 150	1.2 ± 0.37[k]	-	-	140 ± 1.4
18	360 ± 46	1300 ± 760	1.2 ± 0.49[k]	-	0.66 ± 0.13	220 ± 40
19	>3000	>10000	>3000	-	-	550 ± 5.5
20	1400 ± 270	>3000	12 ± 1.6	-	-	220 ± 40
21	>10000	>10000	>3000	-	-	>10000
22	1600 ± 30	>3000	11 ± 1.4	-	-	460 ± 50

[a]Radioligand competition binding assay with [^{3}H]UR-MK136[30] (K_d = 6.2 nM, c = 4 nM) using MCF-7-hY_1 cells in buffer II.[31] [b]Radioligand competition binding assay with [^{3}H]propionyl-pNPY[32] (K_d = 1.4 nM, c = 1 nM) using CHO-hY_2-G_{qi5}-mtAEQ cells in buffer I.[33] [c]Radioligand competition binding assay with [^{3}H](2*R*,7*R*)-**10** (K_d = 0.67 nM, c = 0.6 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells in buffer I. [d]Radioligand competition binding assay with [^{3}H](2*R*,7*R*)-**10** (K_d = 9.8 nM, c = 10 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells in L-15 medium. [e]Radioligand competition binding assay with [^{3}H]**18** (K_d = 0.67 nM, c = 1 nM) using CHO-hY_4R-G_{qi5}-mtAEQ cells in buffer I. [f]Radioligand competition binding assay with [^{3}H]propionyl-pNPY (K_d = 4.83 nM, c = 4 nM) using HEC-1b hY_5R cells in buffer II.[34] [g]K_i value reported by Berlicki et al.[25] [h]K_i value reported by Balasubramaniam et al.;[15] these authors used [^{125}I]PYY (Y_1R expressed in HEK 293 cells, Y_2R or Y_5R expressed in CHO cells) and [^{125}I]PP (Y_4R expressing CHO cells) for the determination of the binding data. [i]Y_1R antagonism at HEL cells (Fura-2 calcium assay): K_b = 9.5 ± 0.12 nM (cf. Supporting Information, Figure S3); in contrast to data reported in ref.[15] for the diastereomeric mixture **2** (cAMP assay, Y_1R expressing HEK 293 cells) no Y_1R agonist activity. [j]K_i value reported by Keller et al.[10] [k]Ref.[16]: K_i values determined by flow cytometry using fluorescence-labeled K4-hPP in buffer I: **16**: 7.4 nM, **17**: 1.5 nM, **18**: 3.6 nM. [l] Y_1R antagonism at HEL cells (Fura-2 calcium assay): K_b = 25.3 ± 3.8 nM (cf. Supporting Information, Figure S3).

3. CONCLUSIONS

The synthesis of the enantiopure diaminosuberic acids (*R,R*)-**8** and (*S,S*)-**8**, accomplished in excellent yield via Grubbs' II catalyzed coupling, gave access to the individual diastereomers of compound **2** with (2*R,*7*R*)- or (2*S,*7*S*)-configuration in the linker. In the same way, octanedioic acid was used as an achiral linker to combine two identical or two different pentapeptide moieties, in which the Arg residues were either retained or replaced at one or two positions by Ala or by an amino-functionalized carbamoylated Arg. Interestingly, the latter turned out to compensate for the absence of the two amino groups in the connecting diacid with respect to affinity and potency (cf. compound **17**). Moreover, propionylation of one amino group in the linker of **2** (cf. compound **10**) or at the primary amino group in **17** gave access to the corresponding tritiated high affinity radioligands. [^{3}H](2*R*,7*R*)-**10** ([^{3}H]UR-KK193) and [^{3}H]**18** ([^{3}H]UR-KK200) are useful new pharmacological tools for the Y_4R. (2*R,*7*R*)-**2** was superior to (2*S,*7*S*)-**2** and, in contrast to published data for the mixture **2**, affinities and agonist potencies were essentially in the same range. As demonstrated by saturation and competition binding experiments, the composition of the buffer may account for discrepancies between functional and binding data. It should be kept in mind that the affinity of peptidic Y_4R agonists may be considerably higher in the absence of sodium ions, conditions widely used for the investigation of NPY receptor ligands.

4. EXPERIMENTAL SECTION

Chemistry: General Conditions. Chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise indicated. MeCN for HPLC

(gradient grade) was obtained from Merck (Darmstadt, Germany). DMF for peptide synthesis, NMP for peptide synthesis and HOBT hydrate were obtained from Acros Organics/Fisher Scientific (Nidderau, Germany). Fmoc-Sieber-PS resin (0.61 mmol/g), (*S*)-allylglycine, (*R*)-allylglycine, Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(*t*Bu)-OH and HBTU were purchased from Iris Biotech (Marktredwitz, Germany). Fmoc-Ala-OH, Fmoc-Leu-OH, dicyclohexylcarbodiimide, dioxane, sodium hydroxide and methanol were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid, dichloromethane, diethyl ether, ethanol and Triton X-100 were obtained from Sigma Aldrich (Deisenhofen, Germany), *N,N*-diisopropylethylamine (DIPEA) (99%) was from ABCR (Karlsruhe, Germany), di-tert-butyl dicarbonate (>97%) and 8-methoxy-8-oxooctanoic acid (98%) were from Alfa Aesar (Karlsruhe, Germany). Bovine serum albumin and bacitracin were purchased from Serva (Heidelberg, Germany), coelenterazin h was obtained from Biotrend (Cologne, Germany), human pancreatic polypeptide and porcine neuropeptide Y were synthesized by Prof. Dr. C. Cabrele (University Salzburg, Salzburg, Austria). Compound **1** was a gift from Dr. A. J. Daniels (Glaxo Wellcome, NC, USA). The synthesis of compounds **3**,[10] **11**,[16] **12**,[22] **13**,[16] **16-18**[16] and [^{3}H]propionyl-pNPY[32] was previously described. (4,5-DihydroIMES)(PCy_3)Cl_2Ru=CHPh (Grubbs' II catalyst) was synthesized according to literature procedure.[35] Millipore water was used throughout for the preparation of buffers and HPLC eluents. Polypropylene reaction vessels (1.5 or 2 mL) with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for the synthesis of the radioligands [^{3}H](2*R*,7*R*)-**10** and [^{3}H]**18**, for small scale reactions (e.g. the preparation of **16**), the investigation of chemical stabilities and for the storage of stock solutions. TL chromatography was performed on Merck silica gel 60 F_{254} TLC aluminum plates, silica gel 60 (40-63 μm, Merck) was used for column chromatography. Visualization was accomplished by UV light (λ = 254 nm or 366 nm) and ninhydrin/acetic acid

solution. NMR-spectra were recorded on a Bruker Avance 300 (7.05 T, ^{1}H: 300.1 MHz, ^{13}C: 75.5 MHz) or a Bruker Avance 600 instrument with cryogenic probe (14.1 T, ^{1}H: 600.3 MHz, ^{13}C: 150.9 MHz) (Bruker, Karlsruhe, Germany). ATR-IR spectroscopy was performed on a Biorad Excalibur FTS 3000 spectrometer (Biorad, Munich, Germany), equipped with a Specac Golden Gate Diamond Single Reflection ATR-System. Mass spectra were recorded on a Finnigan MAT95 (EI-MS 70eV) or a Finnigan MAT SSQ 710 A (CI-MS (NH_3)). For HRMS an Agilent Q-TOF 6540 UHD (Agilent, Waldbronn, Germany) equipped with an ESI source was used. Optical rotations (given as specific rotation $[\alpha]^{20}$, [° cm^3 dm^{-1} g^{-1}], concentrations (c) given as g/100 mL) were determined in an MCP 500 (Anton Paar, Ostfildern-Scharnhausen, Germany) or a 241 polarimeter (Perkin Elmer, Rodgau, Germany) at 589 nm in a 1.0-dm cuvette. Preparative HPLC was performed on a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Kinetex-XB C_{18}, 5 µm, 250 mm × 21 mm, Phenomenex, Aschaffenburg, Germany) at a flow-rate of 15 mL/min using mixtures of acetonitrile and 0.1% aqueous TFA solution as mobile phase. A detection wavelength of 220 nm was used throughout. The collected fractions were lyophilised using an alpha 2-4 LD apparatus (Martin Christ, Osterode am Harz, Germany) equipped with a RZ 6 rotary vane vacuum pump (vacuubrand, Wertheim, Germany). Analytical HPLC analysis was performed on a system from Merck-Hitachi (Hitachi, Düsseldorf, Germany) composed of a L-6200-A pump, a AS-2000A autosampler, a L-4000A UV detector, a D-6000 interface and a RP-column Kinetex-XB C_{18}, 5 µm, 250 mm × 4.6 mm (Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.8 mL/min. Mixtures of acetonitrile (A) and 0.1% aqueous TFA solution (B) were used as mobile phase. The following gradient was applied: A/B: 0-25 min: 10:90 - 60:40, 25-35 min: 60:40 - 95:5, 35-45 min: 95:5. Detection was performed at 220 nm, the oven temperature was 30 °C.

Compound Characterization. New non-peptide intermediates were characterized by ^{1}H- and ^{13}C-NMR Spectroscopy, HRMS and optical rotation (if applicable). New peptidic compounds (fully deprotected) were characterized by HRMS and RP-HPLC analysis. In addition, compounds **20** and **22** were characterized by ^{1}H-, ^{13}C- and 2D-NMR (^{1}H-COSY, HSQC, HMBC). Purity of tested compounds was >95% as determined by HPLC.

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. 5-ml or 20-ml Discardit II syringes (Becton Dickinson, Heidelberg, Germany) were equipped with 35 µm polyethylene frits (Roland Vetter Laborbedarf, Ammerbuch, Germany) and used as reaction vessels. DMF/NMP (8:2) was used as solvent. Protected standard *L*-amino acids (5 equiv) were pre-activated with HBTU (5 equiv)/HOBt (5 equiv)/DIPEA (10 equiv) for 5 min and added to the resin. 'Double' coupling at rt was performed for all standard amino acids for 45 min. After completed coupling of an Fmoc-aa, 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.

General procedure for cleavage of protected peptides from the resin. After the last coupling step and Fmoc deprotection, the resin was washed with DCM. Side-chain protected peptides were cleaved from the resin with CH_2Cl_2/TFA (97:3) (rt, 10 × 6 min). The peptides were separated from the resin by filtration. The combined filtrates were collected in a round bottom flask containing water (10 times the volume of the combined filtrates). The organic solvent was removed on a rotary evaporator; the aqueous phase was lyophilised followed by purification by preparative HPLC.

(2*R*,7*R*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) hexa(hydrotrifluoroacetate) ((2*R*,7*R*)-2). (*R,R*)-**8** (4.3 mg, 10.7 μmol), HBTU (8.5 mg, 2.1 equiv) and HOBt (3.5 mg, 2.1 equiv) were dissolved in anhydrous DMF (400 μL). DIPEA (10 μL, 5 equiv) was added and the mixture was stirred at room temperature for 5 min followed by the addition of a solution of **9** (40 mg, 2.5 equiv). The resulting mixture was stirred at 35 °C for 16 h. Water (10 mL) was added and the protected intermediate was extracted with DCM (2 × 10 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 2 mL) was added and the mixture was stirred at room temperature for 2.5 h. Water (100 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 10:90–35:65, t_R = 15.8 min). Lyophilisation of the eluate afforded (2*R,*7*R*)-**2** as a white fluffy solid (7.5 mg, 29.3%). HRMS (*m/z*): $[M+4H]^{4+}$ calcd. for $C_{80}H_{128}N_{26}O_{16}$, 427.2495; found, 427.2510. RP-HPLC (220 nm): 96% (t_R = 18.93 min, k = 5.6). $C_{80}H_{124}N_{26}O_{16} \cdot C_{12}H_6F_{18}O_{12}$ (1706.04 + 684.14).

(2*S*,7*S*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) hexa(hydrotrifluoroacetate) ((2*S*,7*S*)-2). (2*S,*7*S*)-**2** was prepared in analogy to (2*R,*7*R*)-**2** by using (*S,S*)-**8** (4.3 mg, 10.7 μmol). The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 10:90–35:65, t_R = 15.5 min). Lyophilisation of the eluate afforded (2*S,*7*S*)-**2** as a white fluffy solid (8.6 mg, 33.6%). HRMS (*m/z*): $[M+4H]^{4+}$ calcd. for $C_{80}H_{128}N_{26}O_{16}$, 427.2495; found, 427.2508. RP-HPLC (220 nm): 98% (t_R = 18.37 min, k = 5.4). $C_{80}H_{124}N_{26}O_{16} \cdot C_{12}H_6F_{18}O_{12}$ (1706.04 + 684.14).

(2*R*,7*R*)-Diethyl 2,7-bis[(*tert*-butoxycarbonyl)amino]oct-4-enedioate ((*R,R*)-6). According to literature procedure[36] in a flame-dried Schlenk flask (*R*)-**5** (106 mg, 0.43 mmol, 2 equiv) was dissolved in anhydrous DCM (8 mL) under N_2-atmosphere. Grubbs' II catalyst second generation (18.5 mg, 0.022 mmol, 0.1 equiv) was added and the reaction mixture was stirred under reflux for 11 h until all starting material had been consumed. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (PE/EtOAc 5:1) to obtain (*R,R*)-**6** (89 mg, 0.19 mmol, 89%) as colorless oil. R_f = 0.23 (PE/EtOAc 5:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.21 (t, 6H, *J* 7.1 Hz), 1.38 (s, 18H), 2.31 - 2.49 (m, 4H), 4.13 (qd, 4H, *J* 1.8 Hz, 7.1 Hz), 4.2 – 4.31 (m, 2H), 5.01 – 5.11 (m, 2H), 5.31 – 5.44 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ (ppm) 14.2, 28.3, 35.5, 53.1, 61.3, 79.8, 128.5, 155.2, 171.9. IR (neat) 3369, 2978, 2937, 1703, 1498, 1367, 1330, 1248, 1159, 1095, 1051, 1021, 969, 857, 779 cm^{-1}. $[\alpha]_D^{20}$ -27.0 (c 1, chloroform). LRMS (ESI): *m/z* $[M+H]^+$ 459.3, $[M+Na]^+$ 481.3, $[2M+Na]^+$ 939.5. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{22}H_{39}N_2O_8]^+$ 459.2701, found: 459.2711. $C_{22}H_{38}N_2O_8$ (458.55).

(2*S*,7*S*)-Diethyl 2,7-bis[(*tert*-butoxycarbonyl)amino]oct-4-enedioate ((*S,S*)-6). Compound (*S,S*)-(+)-**6** was prepared according to (*R,R*)-**6** from (*S*)-**5** (105 mg, 0.43 mmol, 2 equiv) as starting material to obtain the product as a colorless oil (47.3 mg, 0.1 mmol, 49%). The spectroscopic data were identical to those for the enantiomer (*R,R*)-(-)-**6**. $[\alpha]_D^{20}$ +27.9 (c 1, chloroform).

(2*R*,7*R*)-Diethyl 2,7-bis[(*tert*-butoxycarbonyl)amino]octanedioate ((*R,R*)-7). In a flame dried Schlenk flask under nitrogen atmosphere (*R*)-**5** (300 mg, 1.23 mmol, 2 equiv) was dissolved in anhydrous DCM (18 mL). Grubbs' II catalyst (52 mg, 0.06 mmol, 0.1 equiv) was added, and the reaction mixture was stirred under reflux for 12 h. After all starting material was consumed, the solvent was removed under reduced pressure, and EtOH (10 mL) was added. The mixture was transferred into an autoclave and stirred under hydrogen at 20 bar at room temperature for 15 h. The reaction mixture was filtrated, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (PE/EtOAc 3:1) to yield (*R,R*)-**7** (268 mg, 0.58 mmol, 95% over two steps) as colorless oil. R_f = 0.3 (PE/EtOAc 3:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 1.21 (t, 6H, *J* 7.1 Hz), 1.24 – 1.33 (m, 4 H), 1.37 (s, 18H), 1.46 – 1.79 (m, 4 H), 4.05-4.24 (m, 4H + 2H), 4.94 – 5.04 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ (ppm) 14.2, 24.9, 28.3, 32.6, 53.3, 61.3, 79.8, 155.4, 172.8. IR (neat) 3354, 2978, 2937, 2870, 1699, 1505, 1457, 1367, 1248, 1159, 1021, 861, 779 cm^{-1}. $[\alpha]_D^{20}$ -14.8 (c 1, chloroform). LRMS (ESI): *m/z* $[M+H]^+$ 461.3, $[M+Na]^+$ 483.3, $[2M+Na]^+$ 943.5. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{22}H_{41}N_2O_8]^+$ 461.2857, found: 461.2865. $C_{22}H_{40}N_2O_8$ (460.56).

(2*S*,7*S*)-Diethyl 2,7-bis[(*tert*-butoxycarbonyl)amino]octanedioate ((*S,S*)-7). Compound (*S,S*)-(+)-**7** was prepared according to (*R,R*)-**7** by using (*S*)-**5** (303 mg, 1.2 mmol, 2 equiv) as starting material to obtain the product as a colorless oil (212 mg, 0.46 mmol, 74% over two steps). The spectroscopic data were identical to those for its enantiomer (*R,R*)-(-)-7. $[\alpha]_D^{20}$ +16.9 (c 1, chloroform).

(2*R*,7*R*)-2,7-Bis[(*tert*-butoxycarbonyl)amino]octanedioic acid ((*R,R*)-8).[37] (*R,R*)-**7** (268 mg, 0.58 mmol, 1 equiv) was dissolved in EtOH (8 mL) and 1M NaOH solution (1.2 mL, 1.16 mmol, 2 equiv) was added dropwise. The reaction was stirred at room temperature for 21 h. When all the starting material had been consumed, the solution was adjusted to pH 2 by addition of 1M HCl solution. Water and EtOAc were added, and the phases were separated. The aqueous layer was extracted with EtOAc, and the combined organic phases were dried over $MgSO_4$, filtered and concentrated *in vacuo* to yield the product as a white solid (238 mg, 0.58 mmol, quant.). ^{1}H-NMR (300 MHz, CD_3OD): δ (ppm) 1.35 – 1.50 (m, 22H), 1.57 – 1.71 (m, 2H), 1.72 – 1.96 (m, 2H), 3.91 – 4.13 (m, 2H). ^{13}C-NMR (75 MHz, CD_3OD): δ (ppm) 26.5, 28.8, 32.7, 54.9, 80.5, 158.1, 176.5. IR (neat) 3295, 2978, 2933, 2363, 1789, 1707, 1680, 1509, 1449, 1394, 1370, 1312, 1244, 1159, 1077, 1047, 1017, 946, 846, 790, 758, 697 cm^{-1}. $[\alpha]_D^{20}$ +11.2 (c 1, DMF). LRMS (ESI): *m/z* = 403.2 $[M-H]^-$, 807.4 $[2M-H]^-$. HRMS (ESI): *m/z* $[M-H]^-$ calcd. for $[C_{18}H_{31}N_2O_8]^-$ 403.2086, found: 403.2096. $C_{18}H_{32}N_2O_8$ (404.46).

(2*S*,7*S*)-2,7-Bis[(*tert*-butoxycarbonyl)amino]octanedioic acid ((*S,S*)-8).[38] Compound (*S,S*)-(+)-**8** was prepared according to (*R,R*)-**8** by using (*S,S*)-**7** (212 mg, 0.46 mmol, 2 equiv) as starting material to obtain the product as a white solid (190 mg, 0.46 mmol, quant.). The spectroscopic data were identical to those for the enantiomer (*R,R*)-**8**. $[\alpha]_D^{20}$ -9.8 (c 1, DMF, ref.[38] -15.2, 5% in DMF).

(2*R*,7*R*)-2-Amino-7-(propionylamino)octanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) penta(hydrotrifluoroacetate) ((2*R*,7*R*)-10). (2*R,*7*R*)-**2** (4 mg, 1.67 μmol) was dissolved in 3% DIPEA in anhydrous DMF (400 μL). A solution of succinimidyl propionate in anhydrous DMF

(10 mg/mL) was added in small portions (a total of 17 µL (0.98 µmol)). To prevent two-fold acylation, the reaction was monitored by HPLC and stopped when approximately 35% of the starting material was consumed. Water (5 mL) was added and the product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 10:90–35:65, t_R = 17.2 min). Lyophilisation of the eluate afforded (2*R,*7*R*)-**10** as a white fluffy solid (1.1 mg, 0.47 µmol). HRMS (*m/z*): $[M+4H]^{4+}$ calcd. for $C_{83}H_{132}N_{26}O_{17}$, 441.2560; found, 441.2575. RP-HPLC (220 nm): 97% (t_R = 20.00 min, k = 6.0). $C_{83}H_{124}N_{26}O_{17}$ · $C_{10}H_5F_{15}O_{10}$ (1762.10 + 570.12).

(2*S*,7*S*)-2-Amino-7-(propionylamino)octanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) penta(hydrotrifluoroacetate) ((2*S*,7*S*)-10). (2*S,*7*S*)-**10** was prepared by analogy with (2*R,*7*R*)-**10** using (2*S,*7*S*)-**2** (4 mg, 1.67 µmol) as starting material. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–20 min: MeCN/0.1% aq TFA 10:90–35:65, t_R = 17.0 min). Lyophilisation of the eluate afforded (2*S,*7*S*)-**10** as a white fluffy solid (1.0 mg, 0.43 µmol). HRMS (*m/z*): $[M+4H]^{4+}$ calcd. for $C_{83}H_{132}N_{26}O_{17}$, 441.2560; found, 441.2577. RP-HPLC (220 nm): 97% (t_R = 19.56 min, k = 5.8). $C_{83}H_{124}N_{26}O_{17}$ · $C_{10}H_5F_{15}O_{10}$ (1762.10 + 570.12).

Tyr(*t*Bu)-Ala-Leu-Arg(Pbf)-Tyr(*t*Bu)-amide hydrotrifluoroacetate (14). The side chain protected pentapeptide **14** was synthesized according to the general procedure (0.1 mmol Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–24 min: MeCN/0.1% aq TFA 38:62–90:10, t_R = 11 min). Lyophilisation of the eluate afforded **14** as a white solid (51 mg, 49%). HRMS (*m/z*):

$[M+2H]^{2+}$ calcd. for $C_{54}H_{83}N_9O_{10}S$, 524.7986; found, 524.7999. $C_{54}H_{81}N_9O_{10}S \cdot C_2HF_3O_2$ (1048.36 + 114.02).

Tyr(*t*Bu)-Arg(Pbf)-Leu-Ala-Tyr(*t*Bu)-amide hydrotrifluoroacetate (15). The side chain protected pentapeptide **15** was synthesized according to the general procedure (0.1 mmol Fmoc-Sieber-PS resin (loading: 0.61 mmol/g)). Purification by preparative HPLC was performed with a Kinetex-XB C18 250 × 21 mm (gradient: 0–24 min: MeCN/0.1% aq TFA 38:62–90:10, t_R = 10.5 min). Lyophilisation of the eluate afforded **15** as a white solid (58 mg, 55%). HRMS (*m/z*): $[M+2H]^{2+}$ calcd. for $C_{54}H_{83}N_9O_{10}S$, 524.7986; found, 524.7999. $C_{54}H_{81}N_9O_{10}S \cdot C_2HF_3O_2$ (1048.36 + 114.02).

Octanedioyl-bis(Tyr-Ala-Leu-Arg-Tyr-amide) di(hydrotrifluoroacetate) (19). Compound **14** (16 mg, 13.8 μmol) was dissolved in anhydrous DMF/DIPEA (99:1, 600 μL). Compound **12** (2.5 mg, 6.78 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 DCM (2 × 10 mL). The combined organic layers were evaporated, 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 lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 10:90–62:38, t_R = 15.5 min). Lyophilisation of the eluate afforded **19** as a white fluffy solid (5.7 mg, 48.5%). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 0.82 (d, 6H, *J* 6.4 Hz), 0.86 (d, 6H, *J* 6.6 Hz), 1.03 (t, 4H, *J* 6.3 Hz), 1.15 (d, 6H, *J* 7.0 Hz), 1.26-1.34 (m, 4H), 1.38-1.56 (m, 10H), 1.56-1.63 (m, 2H), 1.63-1.71 (m, 2H), 1.94-2.03 (m, 4H), 2.56-2.63 (m, 2H), 2.67-2.73 (m, 2H), 2.81-2.88 (m, 4H), 3.04-3.12 (m, 4H), 4.14-4.21 (m, 2H),

4.24-4.32 (m, 6H), 4.38-4.45 (m, 2H), 6.57-6.64 (m, 8H), *ca* 6.8 (br s, 4H), 6.94-6.98 (m, 4H), 6.98-7.02 (m, 4H), 7.04 (s, 2H), *ca* 7.2 (br s, 4H), 7.31 (s, 2H), 7.43 (t, 2H, *J* 5.6 Hz), 7.65 (d, 2H, *J* 7.9 Hz), 7.88 (d, 2H, *J* 8.3 Hz), 7.91 (d, 2H, *J* 8.0 Hz), 8.02 (d, 2H, *J* 7.1 Hz), 8.11 (d, 2H, *J* 7.8 Hz), 9.13 (s, 4H). HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{74}H_{111}N_{18}O_{16}$, 502.6136; found, 502.6146. RP-HPLC (220 nm): 96% (t_R = 23.27 min, k = 7.1). $C_{74}H_{108}N_{18}O_{16} \cdot C_4H_2F_6O_4$ (1505.79 + 228.04).

Octanedioyl-1-(Tyr-Arg-Leu-Arg-Tyr-amide)-8-(Tyr-Ala-Leu-Arg-Tyr-amide) tri(hydrotrifluoroacetate) (20). Compound **13** (37.0 mg, 24.1 μmol), HOBt (3.69 mg, 1 equiv) and HBTU (9.14 mg, 1 equiv) were dissolved in 500 μL of DMF. DIPEA (14.5 μL, 3.5 equiv) was added, and the mixture was stirred for 5 min prior to the addition of a solution of **14** (30.6 mg, 1.1 equiv) in DMF (400 μL). The mixture was stirred at room temperature for 17 h, water (15 mL) was added, and the protected intermediate was extracted with DCM (2 ×20 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/H_2O (95:5, 3 mL) was added and the mixture was stirred at rt for 2.5 h. Water (150 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 10:90–62:38, t_R = 14.1 min). Lyophilisation of the eluate afforded **20** as a white fluffy solid (16.6 mg, 35.6%). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 0.83 (d, 6H, *J* 6.6 Hz), 0.86 (d, 6H, *J* 6.5 Hz), 1.00-1.07 (m, 4H), 1.19 (d, 3H, *J* 7.0 Hz), 1.25-1.34 (m, 4H), 1.36-1.55 (m, 13H), 1.56-1.72 (m, 5H), 1.95-2.03 (m, 4H), 2.55-2.63 (m, 2H), 2.67-2.74 (m, 2H), 2.81-2.89 (m, 4H), 3.01-3.12 (m, 6H), 4.16-4.23 (m, 2H), 4.23-4.35 (m, 6H), 4.37-4.44 (m, 2H), 6.57-6.65 (m, 8H), 6.94-7.02 (m, 8H), *ca.* 6.95 (br s, 6H), 7.04 (d, 2H, *J* 7.8 Hz), *ca.* 7.3 (br s, 6H), 7.34 (d, 2H, *J* 5.9 Hz), 7.53 (t, 1H, *J* 5.6 Hz), 7.56 (t, 2H, *J* 5.5

Hz), 7.74 (d, 1H, *J* 7.9 Hz), 7.77 (d, 1H, *J* 7.9 Hz), 7.85-7.95 (m, 5H), 8.01 (d, 1H, *J* 7.9 Hz), 8.11 (d, 1H, *J* 7.4 Hz), 8.15 (d, 1H, *J* 7.8 Hz), 9.16 (s, 4H). ^{13}C-NMR (150 MHz, [D_6]DMSO): δ 17.88, 21.37, 21.46, 23.07, 23.11, 24.12 (2 carb.), 24.79, 24.88, 25.02 (2 carb.), 25.06, 28.32 (2 carb.), 28.92, 28.96, 29.06, 35.14 (2 carb.), 36.53, 36.64, 36.75, 36.78, 40.41 (3 carb.), 40.53, 40.68, 48.06, 50.97, 51.07, 52.07, 52.09, 52.25, 53.92 (2 carb.), 54.03, 54.19, 114.75 (2 carb.), 114.77 (2 carb.), 114.85 (4 carb.), 127.48, 127.56, 127.90, 127.97, 129.97 (2 carb.), 130.01 (6 carb.), 155.69, 155.73, 155.79, 155.80, 156.68, 156.71 (2 quat. carb.), 170.76, 170.82, 171.13, 171.47, 171.86, 171.98, 171.99, 172.10, 172.25, 172.35, 172.68, 172.69. HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{77}H_{118}N_{21}O_{16}$, 530.9683; found, 530.9692. RP-HPLC (220 nm): 99% (t_R = 19.57 min, k = 7.0). $C_{77}H_{115}N_{21}O_{16} \cdot C_6H_3F_9O_6$ (1590.90 + 342.06).

Octanedioyl-bis(Tyr-Arg-Leu-Ala-Tyr-amide) di(hydrotrifluoroacetate) (21). Compound **15** (12 mg, 10.4 μmol) was dissolved in anhydrous DMF/DIPEA (99:1, 400 μL). Compound **12** (1.9 mg, 5.1 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 DCM (2 × 10 mL). The combined organic layers were evaporated, 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 lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 10:90–62:38, t_R = 15.3 min). Lyophilisation of the eluate afforded **21** as a white fluffy solid (3.7 mg, 41.8%). ^{1}H-NMR (600 MHz, [D_6]DMSO): δ 0.82 (d, 6H, *J* 6.7 Hz), 0.86 (d, 6H, *J* 6.5 Hz), 1.03 (t, 4H, *J* 6.5 Hz), 1.19 (d, 6H, *J* 7.1 Hz), 1.26-1.34 (m, 4H), 1.35-1.53 (m, 10H), 1.54-1.68 (m, 4H), 1.98 (t, 4H, *J* 7.3 Hz), 2.56-2.62 (m, 2H), 2.67-2.73 (m, 2H), 2.81-2.88 (m, 4H), 3.02-3.08 (m, 4H), 4.19-4.29 (m, 6H), 4.30-4.35 (m,

2H), 4.37-4.43 (m, 2H), 6.57-6.64 (m, 8H), *ca* 6.7 (br s, 4H), 6.94-6.98 (m, 4H), 6.99-7.02 (m, 4H), 7.03 (s, 2H), *ca* 7.2 (br s, 4H), 7.34 (s, 2H), 7.41 (t, 2H, *J* 5.7 Hz), 7.76 (d, 2H, *J* 7.9 Hz), 7.85-7.93 (m, 6H), 8.09 (d, 2H, *J* 7.2 Hz), 9.13 (s, 4H). HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{74}H_{111}N_{18}O_{16}$, 502.6136; found, 502.6143. RP-HPLC (220 nm): 97% (t_R = 22.17 min, k = 6.7). $C_{74}H_{108}N_{18}O_{16} \cdot C_4H_2F_6O_4$ (1505.79 + 228.04).

Octanedioyl-1-(Tyr-Arg-Leu-Arg-Tyr-amide)-8-(Tyr-Arg-Leu-Ala-Tyr-amide) tri(hydrotrifluoroacetate) (22). Compound **13** (28.1 mg, 18.3 µmol), HOBt (2.8 mg, 1 equiv) and HBTU (6.94 mg, 1 equiv) were dissolved in 500 µL DMF. DIPEA (11 µL, 3.5 equiv) was added, and the mixture was stirred for 5 min followed by the addition of a solution of **15** (23.2 mg, 1.1 equiv) in DMF (400 µL). The mixture was stirred at room temperature for 18 h, water (15 mL) was added and the protected intermediate was extracted with DCM (2 x 20 mL). The combined organic layers were evaporated, and the residue was dried *in vacuo*. TFA/water (95:5, 3 mL) was added, and the mixture was stirred at rt for 2.5 h. Water (150 mL) was added followed by lyophilisation. The product was purified by preparative HPLC (column: Kinetex-XB C18 250 × 21 mm; gradient: 0–25 min: MeCN/0.1% aq TFA 10:90–62:38, t_R = 14.2 min). Lyophilisation of the eluate afforded **22** as a white fluffy solid (12.5 mg, 35.3%). ^{1}H-NMR (600 MHz, [D$_6$]DMSO): δ 0.82 (d, 6H, *J* 6.5 Hz), 0.86 (d, 6H, *J* 6.6 Hz), 0.99-1.07 (m, 4H), 1.15 (d, 3H, *J* 7.1 Hz), 1.25-1.35 (m, 4H), 1.36-1.55 (m, 13H), 1.56-1.73 (m, 5H), 1.94-2.04 (m, 4H), 2.55-2.64 (m, 2H), 2.66-2.74 (m, 2H), 2.80-2.89 (m, 4H), 3.01-3.12 (m, 6H), 4.14-4.22 (m, 2H), 4.23-4.34 (m, 6H), 4.38-4.45 (m, 2H), 6.58-6.65(m, 8H), *ca.* 6.9 (br s, 6H), 6.93-7.02 (m, 8H), 7.04 (s, 2H), *ca.* 7.3 (br s, 6H), 7.31 (s, 1H), 7.35 (s, 1H), 7.52 (t, 1H, *J* 5.6 Hz), 7.53-7.57 (m, 2H), 7.66 (d, 1H, *J* 8.0 Hz), 7.74 (d, 1H, *J* 7.9 Hz), 7.85-7.95 (m, 4H), 7.99-8.05 (m, 2H), 8.11

(d, 1H, *J* 7.7 Hz), 8.15 (d, 1H, *J* 7.8 Hz), 9.16 (s, 4H). ^{13}C-NMR (150 MHz, [D$_6$]DMSO): δ 17.92, 21.36, 21.41, 23.09, 23.11, 24.10, 24.11, 24.87, 24.96, 25.02, 25.04 (2 carb.), 28.32 (2 carb.), 29.93, 28.97, 29.07, 35.15 (2 carb.), 36.56, 36.59, 36.71, 36.78, 40.42 (3 carb.), 40.59, 40.69, 48.38, 50.79, 50.95, 52.02, 52.08, 52.24, 53.91 (2 carb.), 54.07, 54.14, 114.75 (2 carb.), 114.76 (2 carb.), 114.82 (2 carb.), 114.85 (2 carb.), 127.47, 127.59, 127.89, 127.92, 129.98 (4 carb.), 130.01 (2 carb.), 130.05 (2 carb.), 155.70, 155.72, 155.76, 155.80, 156.67, 156.69, 156.70, 170.08, 171.06, 171.11, 171.64, 171.68 (2 quat. carb.), 171.98, 172.24, 172.33, 172.66, 172.67. HRMS (*m/z*): $[M+3H]^{3+}$ calcd. for $C_{77}H_{118}N_{21}O_{16}$, 530.9683; found, 530.9689. RP-HPLC (220 nm): 99% (t_R = 19.97 min, *k* = 6.0) $C_{77}H_{115}N_{21}O_{16} \cdot C_6H_3F_9O_6$ (1590.90 + 342.06).

Synthesis of the Radioligands [^{3}H](2*R*,7*R*)-10 and [^{3}H]18. The radiolabeled peptides [^{3}H](*R,R*)-**10** and [^{3}H]**18** were prepared by [^{3}H]propionylation of the precursor peptides (*R,R*)-**2** and **17**, respectively. A solution of succinimidyl [^{3}H]propionate (specific activity: 80 Ci/mmol, purchased from American Radiolabeled Chemicals, St. Louis, MO via Hartman Analytics, Braunschweig, Germany) (2.5 mCi, 5.5 µg, 32 nmol) in hexane/EtOAc (9:1) was transferred from the delivered ampoule into a 1.5 mL reaction vessel with screw cap, and the solvent was removed in a vacuum concentrator (30 °C, 30 min). A solution of the precursor peptide ((2*R,*7*R*)-**2**: 0.6 mg, 260 nmol, or **17**: 0.8 mg, 260 nmol) in anhydrous DMF containing 3% DIPEA (60 µL) was added, and the vessel was vigorously shaken at rt for 3 h. The mixture was acidified by addition of 2% aqueous TFA (80 µL) followed by addition of MeCN/H$_2$O (10:90) (400 µL). [^{3}H](2*R,*7*R*)-**10** and [^{3}H]**18** were purified using a HPLC system from Waters (Eschborn, Germany) consisting of two 510 pumps, a pump control module, a 486 UV/VIS detector and a Flow-one beta series A-500 radiodetector (Packard, Meriden, USA). A Luna C$_{18}$(2) column (3

μm, 150 × 4.6 mm, Phenomenex, Aschaffenburg, Germany) was used as stationary phase at a flow rate of 0.8 mL/min. Mixtures of acetonitrile supplemented with 0.04% TFA (A) and 0.05% aq TFA (B) were used as mobile phase. The following linear gradient was applied: A/B: 0–20 min: 8:92 – 33:67, 20–22 min: 33:67 – 95:5, 22–32 min: 95:5. For the purification of each radiolabeled peptide four HPLC runs were performed. The radioligands (t_R = 17.3 min ([^{3}H](2*R,*7*R*)-**10**) and t_R = 19.8 min ([^{3}H]**18**)) were collected in 2-mL reaction vessels with screw cap, and the volumes of the combined eluates were reduced by evaporation to approximately 600 μL. The same volume of ethanol (600 μL) was added, and the solution was diluted with 50% ethanol to a volume of 1500 μL for each radioligand. For the quantification of the radioligands, a four-point calibration was performed with (2*R,*7*R*)-**10** (0.15, 0.3, 0.45 and 0.6 μM; injection volume: 100 μL, UV-detection: 225 nm) or **18** (0.1, 0.2, 0.3 and 0.4 μM; injections volume: 100 μL, UV-detection: 225 nm) using the above mentioned HPLC system and gradient. Aliquots ([^{3}H](2*R,*7*R*)-**10**: 2 μL, [^{3}H]**18**: 2 μL) of the respective radioligand solutions were added to acetonitrile/0.05% aqueous TFA (10:90) (128 μL). 100 μL of the resulting solutions were analyzed by HPLC and 5 × 3 μL were counted in 3 mL of liquid scintillator (Rotiszint eco plus, Carl Roth, Karlsruhe, Germany) with a LS 6500 liquid scintillation counter (Beckman Coulter, Krefeld, Germany). To determine the radiochemical purity and to prove the identity of the radioligands, a solution of the radiolabeled peptide (100 μL, 0.35 μM), spiked with 'cold' (2*R,*7*R*)-**10** (5 μM) or **18** (5 μM), was analyzed using the same HPLC system as for quantification and simultaneous radiometric detection (Rotiszint eco plus/acetonitrile, 85:15 (v/v), flow rate: 4.0 mL/min). The radiochemical purities were > 99%. Analyses repeated after storage at -20 °C for 9 months revealed a radiochemical purities of 87% ([^{3}H](2*R,*7*R*)-**10**) and 90% ([^{3}H]**18**), respectively. Calculated specific activity: 0.960 TBq/mmol (25.94 Ci/mmol,

[^{3}H](2*R,*7*R*)-**10**) or 0.976 TBq/mmol (26.37 Ci/mmol, [^{3}H]**18**). The activity concentration was adjusted to 15.00 MBq/mL by the addition of 50% ethanol amounting to final concentrations of 15.6 µM [^{3}H](2*R,*7*R*)-**10**) and 15.4 µM ([^{3}H]**18**), respectively. Chemical yields: penta(hydrotrifluoroacetate) of [^{3}H](2*R,*7*R*)-**10**: 75.6 µg, 32.4 nmol, and tetra(hydrotrifluoroacetate) of [^{3}H]**18**: 64.7 µg, 28.1 nmol. Radiochemical yields: [^{3}H](2*R,7R*)-**10**: 0.842 mCi (31.14 MBq), 34%, [^{3}H]**18**: 0.741 mCi (27.39 MBq), 30%.

Investigation of the Chemical Stability. The chemical stability of 'cold' (2*R,*7*R*)-**10** and 'cold' **18** was investigated in buffer I at 22 ± 1 °C. The incubation was started by addition of 20 µL of a 1 mM aqueous solution of (2*R,*7*R*)-**10** or **18** to the buffer (780 µL or 580 µL) to give final concentrations of 25 µM ((2*R,*7*R*)-**10**) and 33.35 µM (**18**), respectively. After 0, 12 and 48 h, aliquots (100 µL) were taken, and 100 µL of 1% aqueous TFA were added. The resulting solutions were analyzed by RP-HPLC (cf. general experimental conditions).

Cells. The HEC-1B human endometrial cancer cell line and the MCF-7 (HTB 22) human breast cancer cells were from the American Type Culture Collection (Rockville, MD). A subclone of the MCF-7 cell line that shows higher Y_1R expression was established in our laboratory and used for binding experiments.[31] Human erythroleukemia (HEL) cells were kindly provided by Dr. M. C. Michel (Universitätsklinikum Essen, Germany). Human embryonal kidney cells (HEK-293 cells) and chinese hamster ovarian (CHO) cells were from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Genetically engineered cells, used for binding and functional assays, were generated and cultured as described (cf. brief description of protocols).

HEK293-hY$_4$-CRE Luc cells. HEK293 cells were stably co-transfected with the pGL4.29[luc2P/CRE/Hygro] plasmid (Promega, Mannheim, Germany) encoding hygromycin B resistance and the firefly luciferase, the transcription of which is controlled by the cAMP responsive element (HEK293-CRE Luc cells, ref.[24]) and pcDNA3.1 hY$_4$ vector (cDNA Resource Center; Bloomsberg, PA, USA) encoding the hY$_4$R (HEK293-hY$_4$-CRE Luc cells) and neomycin resistance. For transfection, the cells were seeded into a 24 well-plate (Becton Dickinson), so that they reached 60–70% confluency on the next day. The transfection mixture containing 2 µg of the DNA and 8 µL of FuGene HD transfection reagent (Roche Diagnostics, Mannheim, Germany) was prepared according to the manufacturer's protocol and added to the cells, followed by an incubation period of 48 h at 37 °C and 5% CO_2 in a water-saturated atmosphere.

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

Cell Culture. Cells were cultured 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,[30] HEL cells,[39] CHO-hY$_2$-G$_{qi5}$-mtAEQ cells[23] and CHO-hY$_4$-G$_{qi5}$-mtAEQ cells[23] were cultured as described previously. HEK293T-hY$_4$-CRE Luc cells were cultured in Dulbecco`s Modified Eagle Medium (DMEM) (Sigma-Aldrich, Deisenhofen, Germany) containing L-glutamine (Sigma-Aldrich), 4500 mg/L glucose, 3.7 g/L $NaHCO_3$ (Merck, Darmstadt, Germany), 110 mg/L sodium pyruvate (Serva, Heidelberg, Germany), 10 % fetal calf serum (FCS) (Biochrom, Berlin, Germany) and the selection antibiotics G418 (600 µg/mL) (Biochrom) and hygromycin B (250 µg/mL) (MoBiTec GmbH, Göttingen, Germany). HEC-1B cells expressing the human Y$_5$R were cultured as previously described. [34]

Buffers and Media Used in Binding and Functional Experiments. *Buffer I*. A sodium-free HEPES buffer (25 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) was used in binding experiments at the Y_2R and Y_4R. *Buffer II.* Binding experiments at the Y_1R and Y_5R were performed in a buffer containing 150 mM NaCl, 10 mM HEPES, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 5 mM KCl. *Buffer III*. For the aequorin assay on CHO cells stably expressing the Y_4R the buffer contained 120 mM NaCl, 25 mM HEPES, 1.5 mM $CaCl_2$, $MgCl_2$, 5 mM KCl, 10 mM D-glucose. *Leibovitz' L-15 medium* (containing 140 mM NaCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, amino acids, vitamins, pH 7.4) was used to perform additional binding experiments with selected compounds at the Y_4R. *DMEM* (without phenol red, containing 110 mM NaCl, 44 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.8 mM MgCl, 5.6 mM D-glucose, vitamins, amino acids, pH 7.4) was used to perform the luciferase assay.

Radioligand Binding Assay. Y_1R binding. Radioligand binding assays for all receptor subtypes were performed at 22 ± 1 °C. Competition binding experiments with the radioligand [^{3}H]UR-MK136 (K_d = 6.2 nM, c = 4 nM) were performed at intact MCF-7-Y_1 cells as previously described[30] with the following modifications: Experiments were carried out in 96-well plates with clear bottom (Corning Incorporated Life Sciences, Tewksbury, MA; Corning cat. no. 3610), and the volume per well was reduced to 100 µL. After incubation, the cells were washed with buffer II (200 µL) twice and covered with lysis solution (25 µL) consisting of urea, acetic acid and Triton-X-100 in water. The plates were shaken for 30 min prior to addition of liquid scintillator (Optiphase Supermix, PerkinElmer, Überlingen, Germany) (200 µL). The wells were sealed with a transparent film (permanent seal for microplates, PerkinElmer, prod. no. 1450–461). The plates were shaken to mix the scintillator and the aqueous phase and kept in the dark

for at least 30 min. Radioactivity (dpm) was measured with a MicroBeta2 plate counter (PerkinElmer, Rodgau, Germany).

Y_2R binding. Competition binding experiments were performed at CHO-hY_2R-G_{qi5}-mtAEQ cells[33] with [^{3}H]propionyl-pNPY (K_d = 1.4 nM, c = 1 nM).[32] One day prior to the experiment, the cells were adjusted to a density of approximately 170 000 per mL in Ham's F12 (Sigma Aldrich) supplemented with 10 % FCS. 200 µL per well were seeded into 96-well plates with clear bottom (Corning Incorporated Life Sciences, Tewksbury, MA; cat. no. 3610). The cells were allowed to attach overnight at 37 °C, 5% CO_2 in a water-saturated atmosphere. On the day of the experiment, the confluency of the cells was >90%. The culture medium was removed by suction, the cells were washed with buffer I (200 µL) and covered with binding buffer (buffer I supplemented with 1% BSA and 0.1 mg/mL bacitracin) (80 µL). For radioligand displacement experiments, binding buffer containing the competitor 10-fold concentrated (10 µL) and binding buffer containing the radioligand 10-fold concentrated were added. The cells were incubated for 90 min, the buffer was removed by suction, and the cells were washed twice with ice-cold buffer I (200 µL), before 25 µL of lysis solution (25 mM tricine; glycerol 10 % (v/v); EGTA, 2 mM; 1% (v/v) Triton X-100; $MgSO_4 \cdot 7\ H_2O$, 5 mM; DTT, 1 mM) were added. The plates were shaken for 30 min prior to addition of liquid scintillator (Optiphase Supermix, 200 µL). The sealed plates were processed as described above.

Y_4R binding. Binding assays with the radioligands [^{3}H](2*R*,7*R*)-**10** or [^{3}H]**18** were performed at intact CHO-hY_4R-G_{qi5}-mtAEQ cells.[23] For saturation and competition binding experiments, cells were grown to 80% - 100% confluency, scraped off the culture flask and centrifuged for 5 min at 300 g. The culture medium was discarded, and the cells were resuspended at a density of 500,000 cells/mL in buffer I or Leibovitz' L-15 medium, both supplemented with 1% BSA and

0.1 mg/mL bacitracin. Both, saturation and competition experiments were performed in a final volume of 200 µL in Primaria 96-well plates (Corning Life Sciences, Oneonta, NY). Saturation binding experiments were performed in a concentration range of 0.14 - 11 nM, when using buffer I, or 0.5 - 30 nM, when using L-15 medium. Competition binding experiments were performed with increasing concentrations of unlabeled compounds and radioligand concentrations in the range of the respective K_d value (0.6 nM in case of [^{3}H](2*R,*7*R*)-**10** in buffer I (sodium-free HEPES buffer), 10 nM of [^{3}H](2*R,*7*R*)-**10** in L-15 medium, 1 nM of [^{3}H]**18** in buffer I). Nonspecific binding was determined in the presence of a 200-fold excess of (2*R,*7*R*)-**2** (when using [^{3}H](2*R,*7*R*)-**10**) or **17** (when using [^{3}H]**18**), respectively. Incubation period was 90 min. Bound and free radioligand were separated by filtration through 0.3% polyethyleneimine pre-treated GF/C filters (Whatman, Maidstone, UK) using a Brandel Harvester (Brandel, Gaithersburg, MD, USA). Filter pieces for each well were punched out and transferred into 96-well plates 1450-401 (PerkinElmer) and scintillation cocktail (200 µL, Rotiscint eco plus) was added. After incubation in the dark for 60 min, radioactivity (dpm) was measured with a MicroBeta2 plate counter (PerkinElmer).

For kinetic experiments, the cells were adjusted to a density of approximately 170,000 per mL in Ham's F12 (Sigma Aldrich) supplemented with 10 % FCS. Cells were seeded in a volume of 200 µL per well into Primaria 96-well plates one day before the experiment. The cells were allowed to attach overnight at 37 °C, 5% CO_2 in a water-saturated atmosphere. On the day of the experiment, confluency of the cells was approximately 90%. The culture medium was removed by suction, the cells were washed with 200 µL of buffer I and covered with 160 µL of binding buffer (buffer I supplemented with 1% BSA and 0.1 mg/mL bacitracin) per well. Unspecific binding was determined in the presence of (2*R,*7*R*)-**2** in experiments with [^{3}H](2*R,*7*R*)-**10** or **17**

in experiments with [^{3}H]**18**. For association experiments, the radioligand concentration was 1.5 nM. The incubation of the cells was stopped after different periods of time (2 – 180 min) by removing the radioligand-containing medium, and cells were washed twice with ice-cold buffer I (200 µL). In case of dissociation experiments, cells were pre-incubated with [^{3}H](2*R,*7*R*)-**10** or [^{3}H]**18** at a concentration of 1.5 nM for 120 min. The solution was removed by suction and the cells were covered with binding buffer (200 µL) containing (2*R,*7*R*)-**2** (300 nM) or **17** (300 nM). After different periods of time (2 – 360 min) the cells were washed with ice-cold buffer I. After addition of lysis solution (25 µL), the plates were shaken for 30 min, and the content of the wells was transferred into 96-well plates 1450-401, followed by the addition of 200 µL of liquid scintillator (Optiphase Supermix). The plates were sealed and processed as described above.

Y_5R binding. Competition binding experiments were performed at HEC-1B-hY_5 cells[34] with [^{3}H]propionyl-pNPY (K_d = 4.8 nM, c = 4 nM).[32] One or two days prior to the experiment, the cells were seeded into 96-well plates with clear bottom (Corning cat. no. 3610). The cells were allowed to attach overnight at 37 °C, 5% CO_2 in a water-saturated atmosphere. On the day of the experiment, confluency of the cells was approximately 90%. The culture medium was removed by suction and the cells were washed with buffer II (200 µL) and covered with 80 µL of binding buffer (buffer II supplemented with 1% BSA and 0.1 mg/mL bacitracin). For displacement experiments, 10 µL of binding buffer containing the competitor (10-fold concentrated) and 10 µL of binding buffer containing the radioligand (10-fold concentrated) were added. The cells were incubated for 120 min. The solution was removed by suction and the cells were washed with ice-cold buffer II (2 × 200 µL) followed by the addition of lysis solution (25 µL). The plates

were shaken for 30 min followed by the addition of 200 µL of liquid scintillator (Optiphase Supermix). The plates were sealed and processed as described above.

Fura-2 Calcium Assay. The assay was performed for the functional characterization of selected ligands at the human Y_1 receptor using HEL cells and a LS-50B luminescence spectrometer (Perkin Elmer, Überlingen, Germany) as previously described.[39,40]

Aequorin Calcium Assay. The assay was performed on CHO-hY_4-G_{qi5}-mtAEQ cells in buffer III as previously described[23] 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). Luminescence data were normalized to the effect caused by 1 µM PP (measured in triplicate on every 96-well plate).

Luciferase Assay. The Luciferase assay was performed on HEK293-hY_4-CRE Luc cells. One day prior to the experiment, the cells were adjusted to a density of approximately 800,000 per mL in DMEM without phenol red supplemented with 5 % FCS. Cells were seeded in a volume of 160 µL per well into 96-well plates with clear bottom (Corning cat. no. 3610), and allowed to attach overnight at 37 °C, 5% CO_2 in a water-saturated atmosphere. A stock solution (10 mM) of forskolin (Sigma) in DMSO was used to prepare feed solutions in DMEM without phenol red (final DMSO concentration in the assay was 0.02%). After addition of 20 µL of forskolin solution (final concentration 2 µM), 20 µL of a 10-fold concentrated solution of the respective test compound were added. The cells were incubated at 37 °C in water saturated atmosphere containing 5 % CO_2 for 4.5 h. Afterwards, the medium was discarded, and 80 µL of lysis

solution (pH adjusted to 7.8 with hydrochloric acid) were added to each well. The plates were shaken at 600 rpm for 30 min. Afterwards, 40 µL of the lysate were transferred into white 96-well plates (Greiner, Frickenhausen, Germany). Luminescence was measured with a GENios Pro microplate reader. Light emission was induced by injecting 80 µL of the luciferase assay buffer (25 mM Gly-Gly; $MgSO_4 \cdot 7\ H_2O$, 15 mM; KH_2PO_4, 15 mM; EGTA, 4 mM; ATP disodium salt, 2 mM; DTT, 2 mM; D-luciferin potassium salt (Synchem, Felsberg, Germany), 0.2 mg/mL; pH was adjusted to 7.8 with hydrochloric acid). Luminescence [RLU] was measured for 10 s.

Data Analysis. All data are presented as mean ± SEM from at least 3 independent experiments performed in triplicate. Concentration response curves from the aequorin or luciferase assay and concentration displacement curves from radioligand binding experiments were analyzed by four-parameter sigmoidal fits (GraphPad Prism 5.0, San Diego, CA). Agonist potencies are given as EC_{50} values, maximal responses (efficacies) are expressed as α value referred to the effect of 1 µM hPP (α = 1.0).

K_i values were calculated from IC_{50} values using the Cheng-Prusoff equation.[41] Specific binding data from saturation binding experiments was plotted against the 'free' radioligand concentration and analyzed according to a two-parameter hyperbolic curve fit (Binding – Saturation: One site – Specific binding, GraphPad Prism 5.0, San Diego, CA, USA). The resulting B_{Max} value was used to calculate the number of binding sites per cell. Specific binding data from association experiments were analyzed with an equation describing a two-parameter exponential rise to a maximum (SigmaPlot 12.5, Systat Software Inc., Chicago, IL, USA) and gave the observed association constant k_{obs}. Specific binding data from dissociation experiments

were fitted according to a two-parameter or a four-parameter equation describing an exponential decay (SigmaPlot 12.5).

ASSOCIATED CONTENT

Supporting information

Synthesis of compounds (*R*)-**4**, (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, saturation and competition binding experiments with [^{3}H](2*R*,7*R*)-**10** performed in Leibovitz' L-15 medium, saturation binding experiments with [^{3}H]pNPY at the Y_2R and Y_5R, Fura-2 Ca^{2+} assay with (2*R*,7*R*)-**2** and **17** on Y_1R expressing HEL cells, purity control of all target compounds by HPLC, ^{1}H-NMR spectra of compounds (*R*)-**4**, (*S*)-**4**, (*R*)-**5**, (*S*)-**5**, (*R*,*R*)-**6**, (*R*,*R*)-**7**, (*R*,*R*)-**8**, **19**- **22** and ^{13}C-NMR spectra of compounds (*R*,*R*)-**6**, (*R*,*R*)-**7**, (*R*,*R*)-**8**, **20** and **22**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Bq, becquerel; br s, broad singlet; CHO, Chinese hamster ovary; cpm, counts per minute; DIPEA, *N*,*N*-diisopropylethylamine; dpm, disintegrations per minute; EtOAc, ethyl acetate; FCS, fetal calf serum; H_2, hydrogen gas; HBTU, 3-[bis(dimethylamino)methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate; HEC, human endometrial cancer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, hydroxybenzotriazole; hPP, human pancreatic polypeptide; MeCN, acetonitrile; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl; PE, petroleum ether; pNPY, porcine neuropeptide Y; PYY, peptide YY; RLU, relative

light unit; RP reversed phase; SPPS, solid phase peptide synthesis; $Y_{1,2,4,5}R$, NPY $Y_{1,2,4,5}$ receptor.

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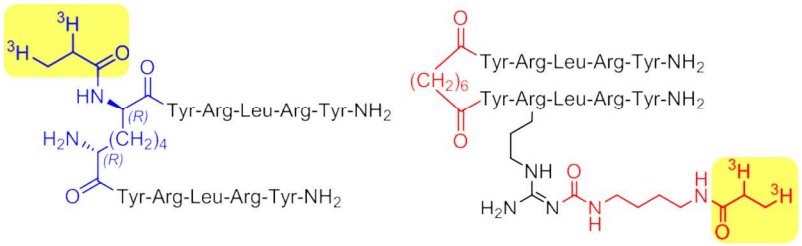

	K_d [nM]	K_i [nM]		
Radioligand	Y_4R	Y_1R	Y_2R	Y_5R
[^{3}H](2*R*,7*R*)-**10**	0.67	400	420	710
[^{3}H]**18**	0.67	360	1300	200