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Optically pure, structural and fluorescent analogues of a dimeric Y_4 receptor agonist derived by an olefin metathesis approach.

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Abstract

The dimeric peptide **1** (BVD-74D, as a diastereomeric mixture) is a potent and selective Neuropeptide Y Y_4 receptor agonist. It represents a valuable candidate in developing traceable ligands for pharmacological studies of Y_4 receptors, and as a lead compound for anti-obesity drugs. Its optically pure stereoisomers along with analogues and fluorescently labelled variants were prepared by exploiting alkene metathesis reactions. The (2*R*,7*R*)-diaminosuberoyl containing peptide, (*R*,*R*)-**1** had markedly higher affinity and agonist efficacy than its (*S*,*S*)-counterpart. Furthermore the sulfo-Cy5 labelled (*R*,*R*)-**14** retained high agonist potency as a novel fluorescent ligand for imaging Y_4 receptors.

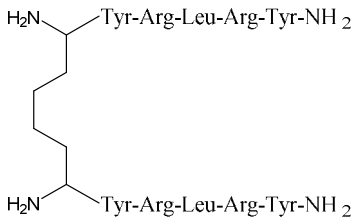
Introduction

The physiological functions of three polypeptides that form the NPY peptide family, neuropeptide Y (NPY)[1] and peptide YY (PYY),[2] and pancreatic polypeptide (PP),[3] are mediated by Y receptors, where four subtypes have been identified in human: Y_1R, Y_2R, Y_4R and Y_5R. All subtypes belong to the rhodopsin-like G_i coupled G-protein coupled receptor (GPCR) superfamily.[4] These Y receptor subtypes exhibit different binding affinity to the three members of NPY peptide family. It was found that Y_1R and Y_2R exhibit similar affinity to NPY and PYY, but poor affinity to PP. Y_4R is a PP-selective subtype with lower affinity for NPY and PYY. Lastly, all three peptides are equally potent at Y_5R.[5]

Activation of the PP/Y_4R signalling system induces satiety and promotes energy expenditure. This suggests that Y_4R agonists may become clinically useful anti-obesity drugs, while Y_4R antagonists may have potential as orexigenic agents to treat anorexia.[6-9] In developing such ligands, truncated peptide analogues are becoming increasingly popular. For example, [Nle^{30}]hPP_{25-36} and [Leu^{34}]$pNPY_{25-36}$ were found to be Y_4R selective partial agonists,[10] and a nonapeptide based on the C-terminal fragment of NPY, Ile-Asn-Pro-Ile-Tyr-Arg-Leu-Arg-Tyr-NH_2 exhibits moderate Y_4R agonism and Y_1R competitive antagonism with similar potency.[11-13] Its lactam-bridged dimeric variant *bis*(29/31', 29/31')[(Glu^{29}, Pro^{30}, Dpa^{31}, Tyr^{32}, Leu^{34})NPY_{28-36}], also known as 1229U91, showed enhanced potency at both receptor subtypes but is in particular the most potent known Y_1R antagonist.[13-17]

Another of several highly potent Y receptor ligands based upon dimeric C-terminal sequences is D/L-2,7-diaminooctanedioyl-bis(YRLRY-NH_2), **1** (BVD-74D)[18] (Figure 1). This peptide exhibited comparable Y_4R affinity with the native hPP (K_i = 0.05 nM vs. 0.08 nM) and showed 150-fold selectivity for Y_4R over Y_1R, and negligible affinity to Y_2R and Y_5R. In fasted rat subjects, **1** showed equally potent inhibitory effects on food intake as the endogenous PP.[18] A later study also reported that **1** significantly reduced food intake, water

intake and weight gain in mice fed with normal and high-fat diet.[19] However, the reported compound is, in fact, a mixture of diastereomers composed of the (*2S*,*7S*)- and (*2R*,*7R*)-diaminooctanedioyl-containing stereoisomers,[18] and they are inseparable by RP-HPLC. Therefore it was unclear which stereoisomer contributed to the *in vitro* and *in vivo* pharmacological activity.



1 (BVD-74D)

Figure 1: The BVD-74D peptide **1** reported by Balasubramaniam *et al.* is a diastereomeric mixture of the (2*S*,7*S*)- and (2*R*,7*R*)-diaminooctanedioyl stereoisomers.

We aimed to resolve this issue, and set a platform for the broader investigation of Y_4R pharmacology by the synthesis of optically pure stereoisomers of **1** and related analogues that probe the role of the bridging group and the role of the dimeric structure in facilitating high affinity. We hereby present our work on synthesis of a series of optically pure analogues of (*S*,*S*)-**1** and (*R*,*R*)-**1** along with their fluorescently labelled variants. We developed methodology for preparing the dimeric peptides utilising Grubbs metathesis, either in the synthesis of optically pure 2,7-diaminosuberic acid building blocks or the on resin metathesis of monomeric precursor peptides.[20-22] Those peptide analogues were analysed using cell based Y_4R competition binding assays and β-arrestin recruitment assays to identify the (*R*,*R*)-diastereomers as the high affinity constituent of **1** and the corresponding fluorescent analogues.

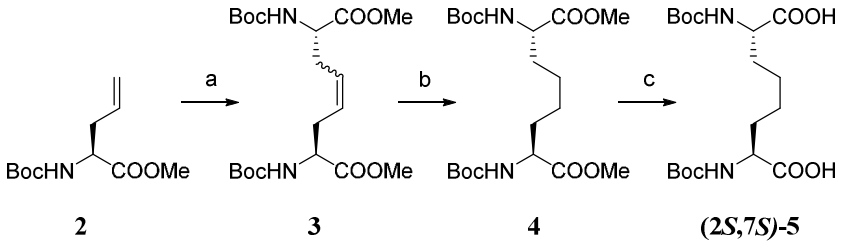
Results and Discussion

Chemistry

The main challenge in developing a convenient and robust strategy for synthesising optically pure dimeric **1** analogues was to identify an optimal condition for metathesis reactions. Two different approaches were attempted. The first approach involved pre-synthesis of the 2,7-diaminosuberic acid unit then bis-coupling to the linear peptidyl-resin, while the second involved a solid phase cross-metathesis between two completed linear N-terminal allylglycine containing peptides.

Synthesis of (*S,S*)-1 and (*R,R*)-1

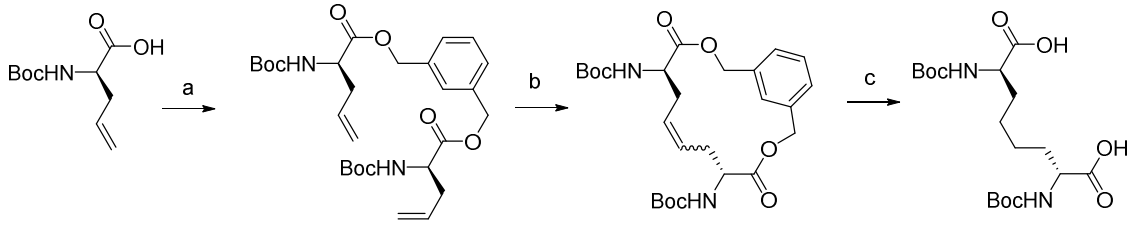
The synthesis of (2*S,*7*S*)-*N,N*-di-Boc-diaminosuberic acid[23] (2*S*,7*S*)-**5** via metathesis was achieved by adapting the methods of Nolen *et al.*[24] and Ward *et al.*[25] (Scheme 1) The (*S*)-*N*-Boc-allylglycine methyl ester (**2**) was treated with Grubbs catalyst 2nd generation in refluxing DCM overnight to obtain the desired alkene **3** in 95% yield. The intermediate **3** was then hydrogenated in presence of 10% Pd/C, which gave **4** almost quantitatively. Finally, the desired product (2*S*,7*S*)-**5** was generated by ester hydrolysis. Spectroscopic data for (2*S*,7*S*)-**5** were consistent with that previously reported,[23] including the determined (-) optical rotation in DMF.[26] Experimental details are provided in Supporting Information.



Scheme 1: Synthesis of (2*S*,7*S*)-*N,N*-di-Boc-diaminosuberic acid (2*S*,7*S*)-**5**. Reagents and conditions: a. Grubbs catalyst 2nd generation, DCM, reflux, overnight, 95%; b. 10% Pd/C, MeOH, H_2, RT, overnight; c. NaOH in H_2O (6 mg/ml), MeOH, reflux, overnight, 50% (from **3**).

To prepare the (2*R*,7*R*)-*N,N*-di-Boc-diaminosuberic acid, (2*R*,7*R*)-**5**, we investigated the use of 1,3-benzenedimethanol as a "template" to enable selective ring-closure metathesis as

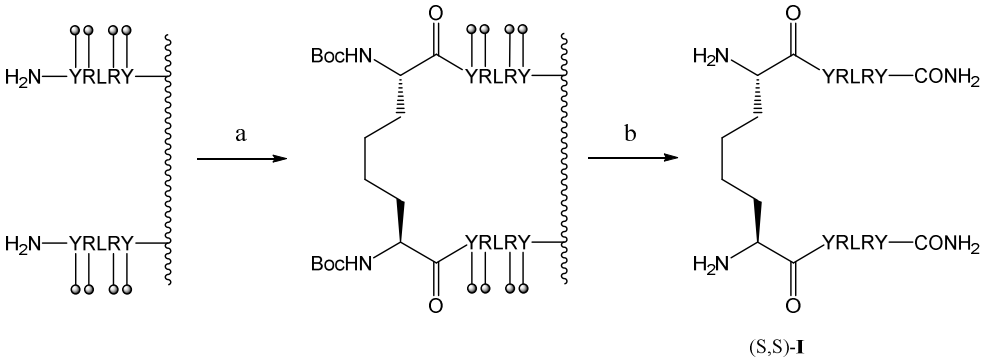
reported by previously.[27, 28] Esterification of Boc-D-allylglycine-OH (**6**) gave the diester **7** which was a substrate for RCM, and gave **8** in 54% yield. Finally one-pot reduction and hydrogenolysis of **8** was achieved by treating with hydrogen in the presence of 10% Pd/C affording (2*R*,7*R*)-**5**. Polarimetry confirmed the expected (+) optical rotation of the precursor.[26]



Scheme 2: Synthesis of (*2R,7R*)-*N,N*-di-Boc-diaminosuberic acid, (*2R,7R*)-**5**. Reagents and conditions: a. benzenedimethanol, EDCI, DMAP, DCM, RT, overnight, 56%; b. Grubbs catalyst 2nd generation, DCM, N_2, reflux, overnight, 54%; c. 10% Pd/C, MeOH, 1 atm H_2, 53%.

To further confirm the chiral integrity of the products, a chiral HPLC method was developed that showed that preparation of (2*S,*7*S*)-**5** and (2*R*,7*R*)-**5** was not accompanied by significant racemization either to each other or to the meso (2*R*,7*S*)-**5**[29] in these syntheses (see Supporting Information).

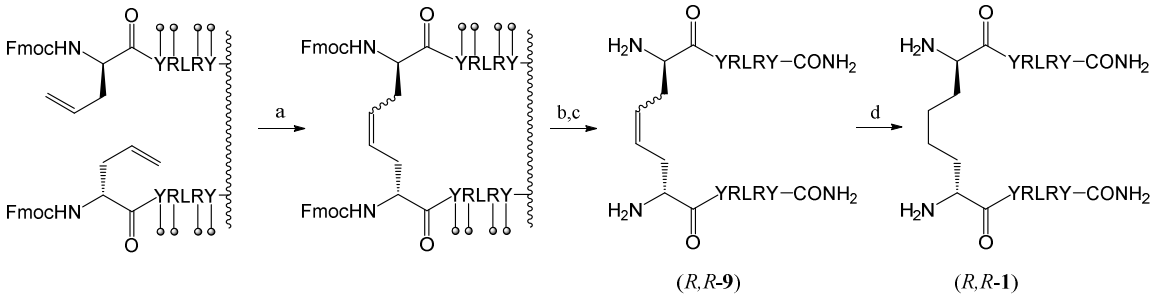
Having the protected building blocks in hand, dimeric peptide analogues (*S*,*S*)-**1** and (*R*,*R*)-**1** were prepared by conventional solid phase peptide synthesis (Scheme 3). After constructing the linear peptide chain on Rink amide resin, the coupling was carried out using 0.5 equivalents of the Boc-protected 2,7-diaminosuberic acid (*S*,*S*)-**5** or (*R*,*R*)-**5** activated with PyClock and overnight incubation. After cleavage, the desired peptides were obtained and readily purified by RP-HPLC.



Scheme 3: Synthesis of (*S,S*)-**1** using pre-synthesised (*2S,7S*)-*N,N*-di-Boc-diaminosuberic acid **5**. Reagents and conditions: a. (*2S,7S*)-**5**, PyClock, DIPEA, DMF, RT, overnight; b. Reagent K, RT, 3 h.

As an alternative approach, the synthesis of dimeric peptides was achieved by solid phase cross-metathesis of the corresponding resin-bound protected allylglycine containing peptides. (Scheme 4) The monomeric peptide chain containing either L- or D-allylglycine was first assembled following the standard Fmoc-based solid phase synthesis strategy, where the N-terminal Fmoc group was retained. The peptidyl-resin was then subject to cross-metathesis by treating with Grubbs catalyst 2nd generation under deoxygenated conditions and microwave heating in presence of LiCl as a chaotropic salt.[21] Fmoc deprotection followed by cleavage yielded the alkenyl peptides, (*S,S*)-**9** and (*R,R*)-**9**.

The synthesis of (*R,R*)-**1** was also achieved by hydrogenation of (*R,R*)-**9** in presence of 10% Pd/C in EtOAc (Scheme 4). While successful and operationally straightforward, the yield and purity of the crude peptide product was not as good as the same peptide made from pre-synthesised diaminosuberic acids as described above.



Scheme 4: Synthesis of dimeric peptides (*R,R*)-**9** and (*R,R*)-**1** via solid phase metathesis reaction. Reagents and conditions: a. Grubbs catalyst 2nd generation, LiCl in DMF, DCM, μwave 100˚C, 3 h; b. piperidine (20%) in DMF, RT, 5 min×2; c. Reagent K, RT, 3 h; d. Pd/C cartridge, H_2 (50 psi), EtOAc, 50˚C, 1 h.

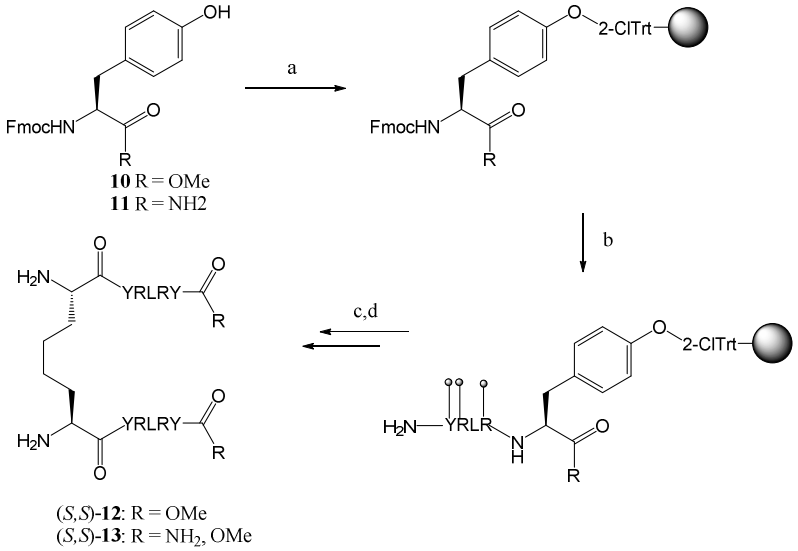
Synthesis of homo- and hetero-dimeric methyl esters of (*S*,*S*)-1

Having established efficient strategies for preparation of dimeric analogues with specified stereo-configuration, we investigated the role of the C-terminal amides in Y_4R interaction by replacing them as mono- or di-methyl esters. Our strategy was to utilise side-chain anchoring to the resin to allow manipulation of the terminal carboxylate. Peptide anchoring to resin via the side-chain of tyrosine esters has been described on both benzyl-type resins[30] and 2-chlorotrityl chloride resin.[31]

We first prepared the free phenolic tyrosine derivatives, Fmoc-Tyr-OMe (**10**)[32] and Fmoc-Tyr amide (**11**)[33](see Supporting Information). The di-methyl ester (*S*,*S*)-**12** was achieved by coupling **10** to 2-chlorotrityl chloride resin via the phenol group.[31] The remainder of the peptide sequence was assembled as described above with final coupling of (2*S*,7*S*)-**5** and standard acidolytic cleavage (Scheme 5).

To synthesise the heterodimeric mono-methyl ester (*S*,*S*)-**13**, **10** and **11** were anchored simultaneously to 2-chlorotrityl chloride resin as a 1:1 mixture. Standard SPPS was continued as described above and final coupling of (2*S*,7*S*)-**5** was followed by standard cleavage. The products were an approximately 2:1:1 mixture of the desired heterodimer (*S*,*S*)-**13** and the homodimers, the di-amide (*S*,*S*)-**1** and di-methyl ester (*S*,*S*)-**12** (Scheme 5). The three major

peptide products were readily resolved by RP-HPLC allowing isolation of the desired product. Notable in the syntheses of compounds (*S,S*)-**12** and (S,S)-**13** was an excellent recovery of peptide products, indicative of more efficient cleavage from the 2-chlorotrityl chloride resin than from Rink Amide resin.



Scheme 5: Synthesis of peptides (*S,S*)-**12** and (*S,S*)-**13**. Reagents and conditions: a. 2-chlorotrityl chloride resin, DIPEA, DCM, RT, overnight; b. standard solid phase synthesis; c. **5**, PyClock, DIPEA, DMF, RT, overnight; d. Reagent K, RT, 3 h.

Synthesis of fluorescently labelled diastereomers of (*S,S*)-1 and (*R,R*)-1

In order to develop fluorescently labelled Y_4R-targeting ligands for *in vitro* Y_4R studies, (*S,S*)-**1,** (*R,R*)-**1,** (*S,S*)-**II** and (*R,R*)-**II** were conjugated with either a rhodamine B (RhB) derivative[34] or the commercially available sulfo-Cy5 (sCy5) dye (Figure 2). Following our previously reported methods for mono-conjugation of dimeric peptides,[35] peptides (*S,S*)-**1** and (*R,R*)-**1** were treated with 0.7 molar equivalents of sCy5 in presence of PyClock and DIPEA to give the desired mono-labelled analogues (*S,S*)-**14** and (*R,R*)-**14** respectively after purification, and peptides (*S,S*)-**1,** (*S,S*)-**9** and (*R,R*)-**9** treated with the rhodamine B derivative to give (*S,S*)-**15**, (*S,S*)-**16** and (*R,R*)-**16** respectively (Scheme 6).

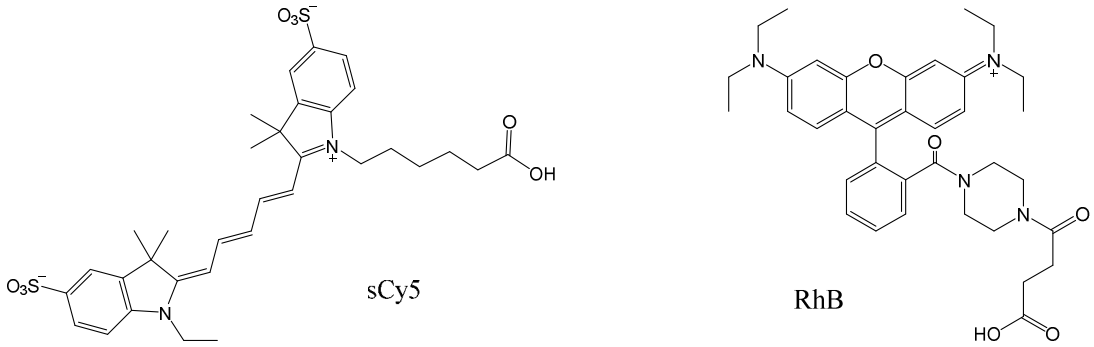
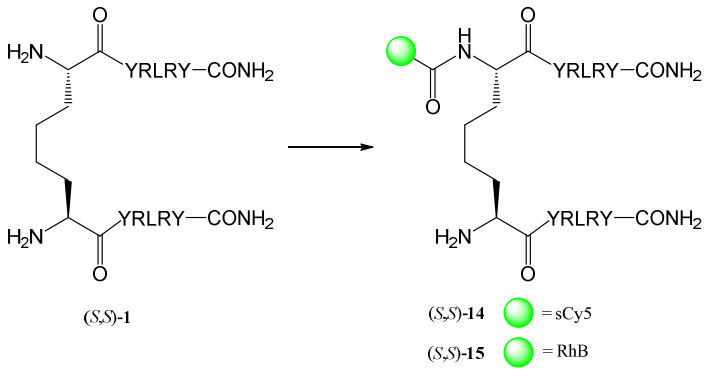


Figure 2: The structures of carboxy derivatised fluorophore reagents used: sulfo-Cy5 (sCy5) and rhodamine B (RhB).



Scheme 6: Example of fluorophore conjugation of (*S*,*S*)-**1**. Reagents and condition: sCy5 or RhB (0.7 *eq.*), PyClock, DIPEA in DMF, RT, overnight.

In summary, we have successfully developed unambiguous synthetic routes to prepare optically pure **1** and analogues through both solution and solid phase alkene metathesis reactions. Mono-labelled fluorescent analogues were conveniently prepared by standard solution phase coupling using limited molar equivalents of fluorophores. Utilising these strategies, 11 dimeric analogues were prepared and their analytical data are summarised in Table 1.

Table 1: **Synthesised dimeric peptides and their analytical data.**

Code	Sequence [a]	ESI-MS [b]	HPLC RT.[c] (min)
(*S,S*)-1	(*2S,7S*)-sub(YRLRY-NH_2)$_2$	569.75	11.21

(*R,R*)-1	(*2R,7R*)-sub(YRLRY-NH_2)$_2$	569.85	11.16
(*S,S*)-9	(*2S,7S*)-Δsub(YRLRY-NH_2)$_2$	569.05	11.19
(*R,R*)-9	(*2R,7R*)-Δsub(YRLRY-NH_2)$_2$	569.10	11.34
(*S,S*)-12	(*2S,7S*)-sub(YRLRY-OMe)$_2$	579.85	12.06
(*S,S*)-13	(*2S,7S*)-sub(YRLRY-NH_2)(YRLRY-OMe)	574.85	11.61
(*S,S*)-14	mono-sCy5-(*2S,7S*)-sub(YRLRY-NH_2)$_2$	782.70	12.38
(*R,R*)-14	mono-sCy5-(*2R,7R*)-sub(YRLRY-NH_2)$_2$	782.75	12.36
(*S,S*)-15	monoRhB-(*2S,7S*)-sub(YRLRY-NH_2)	767.40	13.37
(*S,S*)-16	monoRhB-(*2S,7S*)-Δsub(YRLRY-NH_2)$_2$	766.65	13.36
(*R,R*)-16	monoRhB-(*2R,7R*)-Δsub(YRLRY-NH_2)$_2$	766.70	13.26

a. Sub = 2,7-diaminosuberoyl linkage; Δsub = 2,7-diaminooctene-4-dioyl linkage.

b. ESI-MS base peak corresponds to $[M+3H]^{3+}$.

c. HPLC retention time using a Phenomenex Luna C-8 column (100Å, 3μm, 100×2.00mm). The gradient is composed of 100% H_2O (0.1% TFA) for 4 min, 0-60% acetonitrile in H_2O (0.1% TFA) over 10 min, and isocratic 60% acetonitrile in H_2O (0.1% TFA) for 1 min.

Pharmacology

The pharmacological characteristics of these dimeric peptides were assessed by receptor binding and functional assays using whole cell assay systems. This enabled binding to be assessed in physiological buffer in cells (expressing human Y_4R tagged with green fluorescent protein, GFP) rather than membranes, to provide equivalence with subsequent functional measurements.

Y_4R binding affinity data were obtained by competition binding against the sCy5-labelled peptide **17**, **(**[Lys^2(sCy5),Arg^4]BVD-15, 100 nM), analysed on a high content imaging platereader (Table 2, Figure 3).[36, 37] The endogenous reference ligand, human PP, showed

nanomolar affinity (IC_{50} = 2.31 nM) in this assay. Of all the peptides, (2*R*,7*R*)-**1** exhibited the highest Y_4R affinity (IC_{50} = 12.7 nM), and was 5.5-fold higher in affinity than (2*S*,7*S*)-**1**.

Table 2: Synthesised dimeric peptides and their pharmacological data.

Code	Sequence [a]	pIC_{50} [b]	R_{max} [c] (% 100 nM PP)	pEC_{50} [d]
PP		8.64±0.12	96.3±4.0	8.58±0.10
(*S*,*S*)-1	(*2S,7S*)-sub(YRLRY-NH_2)$_2$	7.16±0.10	57.1±10.9	7.08±0.30
(*R*,*R*)-1	(*2R,7R*)-sub(YRLRY-NH_2)$_2$	7.90±0.10	61.5±0.17	8.33±0.17
(*S*,*S*)-9	(*2S,7S*)-Δsub(YRLRY-NH_2)$_2$	6.88±0.09	44.5±4.8	7.52±0.20
(*R*,*R*)-9	(*2R,7R*)-Δsub(YRLRY-NH_2)$_2$	7.62±0.11	61.0±5.2	7.42±0.15
(*S*,*S*)-12	(*2S,7S*)-sub(YRLRY-OMe)$_2$	6.21±0.11	11.3±6.7[d]	N.D.
(*S*,*S*)-13	(*2S,7S*)-sub(YRLRY-NH_2)(YRLRY-OMe)	7.03±0.10	49.1±6.8	<6.5
(*S*,*S*)-14	mono-sCy5-(*2S,7S*)-sub(YRLRY-NH_2)$_2$	N.D.	56.6±11.8	7.02±00.33
(*R*,*R*)-14	mono-sCy5-(*2R,7R*)-sub(YRLRY-NH_2)$_2$	N.D.	65.6±5.7	7.48±0.16
(*S*,*S*)-15	monoRhB-(*2S,7S*)-sub(YRLRY-NH_2)	7.15±0.09	15.9±9.1[d]	N.D.
(*S*,*S*)-16	monoRhB-(*2S,7S*)-Δsub(YRLRY-NH_2)$_2$	7.16±0.10	29.2±4.6	8.26±0.37
(*R*,*R*)-16	monoRhB-(*2R,7R*)-Δsub(YRLRY-NH_2)$_2$	7.15±0.09	39.3±4.2	8.43±0.26

a. Sub = 2,7-diaminosuberoyl linkage; Δsub = 2,7-diaminooctene-4-dioyl linkage

b. Derived from competition binding assays using 100 nM **17** as the fluorescent ligand.

c. From Y_4R-β-arrestin2 recruitment assays.

d. In the absence of significant agonist activity, the effect at 1 μM peptide is reported. N.D. = not determined

The remaining analogues examined here have not been previously described (Table 2). In the alkenyl dimer series, a preference for the (*R,R*)-**9** was again observed compared to the (*S,S*)-diastereomer, but overall these compounds showed 2- to 3-fold lower affinity for Y_4R than

the corresponding diaminosuberic linked analogues **1**. It can be concluded that conformational restraint due to the presence of the alkene group is not favoured. Substitution of one C-terminal amide (*S,S*)-**13** for an ester moiety did not lead to a major loss of Y_4R affinity compared to the corresponding diamide (*S,S*)-**1** in these assays. However, replacement of both amides as in compound (*S,S*)-**12**, has a major impact with an order of magnitude drop in affinity. Mono *N*-terminal modification of (*R,R*)-**9** with rhodamine B giving (R,R)-**16** led to a 3-fold reduction in affinity, but rhodamine B addition was well tolerated in analogues (*S,S*)-**15** and (*S,S*)-**16**. The three rhodamine B containing peptides had essentially overlapping competition curves.

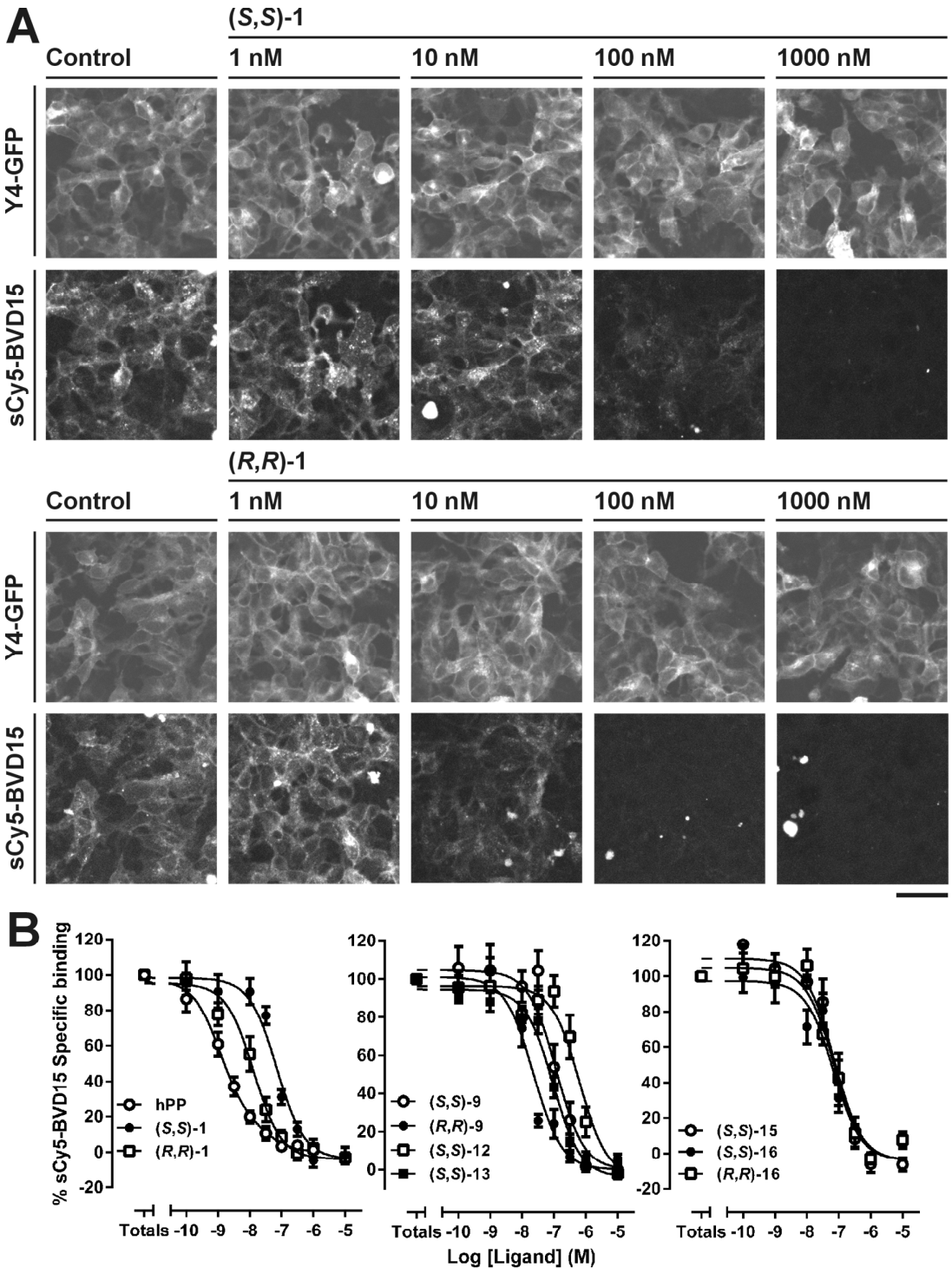


Figure 3: Y_4R competition binding assays using high content imaging. A. 293TR Y_4-GFP cells were incubated with 100 nM, **17** in the absence (control) or presence of increasing concentrations of stereoisomers of **1**. Following 30 min at 37°C, Y_4-GFP and fluorescent

ligand images were acquired on an IX Ultra platereader. The panels show 400×400 regions of interest from the 1000×1000 pixel original plate images; scale bar 50 μm. B. Specific binding of **17** was quantified and normalised using granularity analysis as described in the methods, to obtain competition curves for peptide analogues synthesised in the current study. Graphs show pooled competition data (n = 5), from which the pIC_{50} values in Table 2 were determined.

The selectivity of representative peptides **(*R,R*)-1**, (*S,S*)-**9** and (*S,S*)-**12** was measured by Y_1R-GFP whole cell competition binding (using the same fluorescent ligand **17** (10 nM), and confirmed at least 30-fold selectivity of representative for Y_4R over Y_1R, the Y receptor subtype most closely related in amino acid homology.[38] Y_1R pIC_{50} values were 6.44±0.14, 6.31±0.30 and 6.63±0.12 for analogues (*R,R*)-**1**, (*S,S*)-**9** and (*S,S*)-**12** respectively (n = 3).

The functional Y_4R agonism produced by the peptides was analysed using a β-arrestin2 recruitment assay to detect Y_4R activation (Figure 4), as previously described.[39, 40] One advantage of this assay is its limited receptor reserve, which improves correspondence between agonist potency (as EC_{50}, the concentration of agonist that produces 50% of its maximal response) and underlying functional receptor affinities, and also the likelihood that differences in agonist intrinsic efficacy are revealed through changes in relative maximum response (R_{max}).[41] Thus the reference agonist human PP stimulated β-arrestin2 association with an EC_{50} value (2.6 nM) very similar to its derived pIC_{50} in the whole cell Y_4R binding experiments.

Compared to PP, all the peptides were partial agonists in the Y_4R-arrestin recruitment assays, with typical R_{max} values 44-62% of that of PP (Table 2). The order of potency broadly reflected binding data in that (i) (*R,R*)-**1** (EC_{50} = 4.6 nM) was 20-fold more potent than the (*S,S*)-**1**, (Figure 4A), (ii) the alkenyl derivatives (*R,R*)-**9** and (*S,S*)-**9** were less potent overall than the dimers with saturated linkages (Figure 4B) and (iii) replacement of one or both C-

terminal amides with ester moieties, (*S,S*)-**13** and (*S,S*)-**12** respectively**,** resulted in a loss of potency and efficacy (Figure 4B).

The functional assay enabled assessment of both RhB- and sCy5-labelled analogues as Y_4R agonists. In general, maximum responses to rhodamine analogues were reduced compared to the respective parent compounds, with compound (*S,S*)-**15** (RhB derivative of (*S,S*)-**1**) being without significant effect in the assay (Table 2). In contrast, mono-labelling with sCy5 as in (*S,S*)-**14** and (*R,R*)-**14**, preserved the same level of maximal response exhibited by their parent peptides (Figure 4C). The sCy5-labelled (*R,R*)-**14** was more potent, with an EC_{50} value (34 nM) approximately 7-fold lower potency than (*R,R*)-**1**.

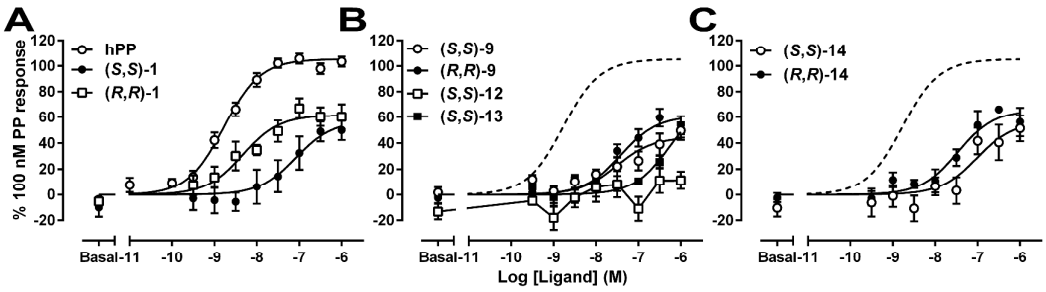


Figure 4: Y_4R agonism as assessed in the Y_4R β-arrestin2 recruitment assay. The HEK293 Y_4 β-arrestin2 BiFC cell line was stimulated for 60 min with human PP or synthesised compounds, and the development of complemented YFP fluorescence following Y_4R activation was imaged and quantified using granularity analysis. Panels A-C represent pooled data (n = 4 or greater), normalised to the 100 nM PP response, from which pEC_{50} and R_{max} values were estimated (Table 2).

The sCy5 derivatives (*S,S*)-**14** and (*R,R*)-**14** were explored further for their properties as novel Y_4R fluorescent ligands. Both compounds labelled Y_4R-GFP expressing 293TR cells in a concentration dependent manner (with (*R,R*)-**14** more potent), dependent on prior induction of receptor expression via tetracycline pretreatment (Figure 5). As anticipated for a ligand with agonist properties, both surface and intracellular distribution of sCy5 fluorescence was

observed, likely reflecting some co-internalisation of Y_4R ligand complexes from the plasma membrane following receptor activation. The presence of increasing concentrations of PP competed for the binding of (*S,S*)-**14** or (*R,R*)-**14**.

To confirm the utility of (*R,R*)-**14** in competition binding experiments, the fluorescence spectrum in physiological buffers was determined and found the lambda max to be the same as that of the underivatised sCy5-NHS dye (absorption max 656 nm, emission max 665 nm) with a relative quantum yield of 31% (see Supporting Information). We then used (*R,R*)-**14** to label cells with 100 nM (*R,R*)-**14**, and determined the PP pIC_{50} was 8.36±0.09 (IC_{50} = 4.31 nM, n = 3), consistent with the IC_{50} obtained using **17** (see Table 2).

The collected assay data from these studies raise interesting questions regarding the molecular mechanism governing the high potency of peptide (*R,R*)-**1** as compared to other analogues, and therefore how dimeric analogues impart enhanced affinity generally. Set against recent studies of the Y_4R-hPP interaction,[42] the simplest model is that the "first arm" of the dimer binds the receptor in the canonical fashion while the "second arm" contributes to the affinity, perhaps by mimicry of the hPP helical region 14-30. An alternate view might be that the ligand binding is driven by the doubling of the local concentration of the C-terminal binding motif. The influence of the flexible bridging ligand argues against this latter explanation although the clear preference for the presence of the (*R,R*)-configuration may imply a preference for a D-amino acid at that position of the truncated peptide that has not been tested in other analogues. The comparable affinity of (*S,S*)-**1** and the half ester, half amide (*S,S*)-**13** may also suggest that the two arms play different roles in receptor binding, where in the "second arm" the C-terminal carboxamide is not so critical. The development of synthetic routes to further interrogate the structure-activity of stereochemically defined homo- and heterodimers suggests a great opportunity for the much needed development of Y_4R ligands.

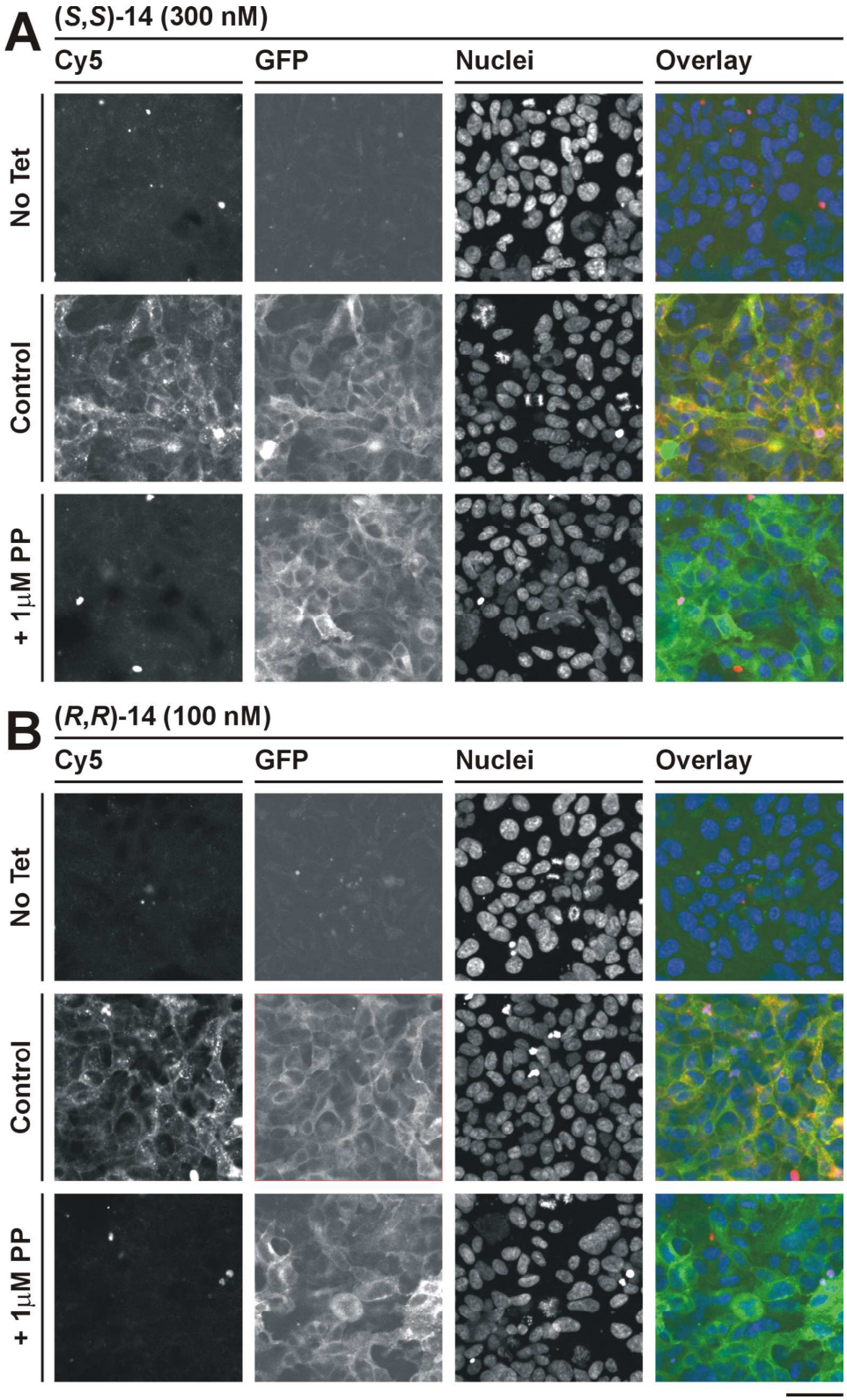

A
(S,S)-14 (300 nM)
Cy5
GFP
Nuclei
Overlay
No Tet
Control
+ 1μM PP
B
(R,R)-14 (100 nM)
Cy5
GFP
Nuclei
Overlay
No Tet
Control
+ 1μM PP

Figure 5: sCy5 labelled peptides as fluorescent Y_4R ligands. Representative images show binding and localisation of mono-sCy5 labelled (*S,S*)-**14** (300 nM) or (*R,R*)-**14** (100 nM) following 30 min pre-incubation with 293TR Y_4GFP cells. Panels illustrate the lack of fluorescent ligand binding in controls cells without Y_4R protein expression induced by tetracycline treatment (No Tet), or in the presence of competing ligand (1 μM PP). Examples are magnified regions of individual IX Ultra images, as described for **Figure 4**, representative of 3 independent experiments. Scale bar 50 μm.

Conclusion

Pharmacological observations support the conclusion that the (*R,R*)-**1** is the active principle of the original mixture of diastereomers **1**, and as for the native peptides, C-terminal amidation of these compounds is required for Y_4R agonist activity. As previous fluorescent ligand SAR studies have indicated,[43, 44] the choice of rhodamine or sCy5 fluorophore influences the result Y_4R properties of the labelled compounds, with mono-sCy5 labelled derivative (*R,R*)-**14** being identified as a novel nanomolar affinity fluorescent Y_4R agonist.

Experimental Section

Materials

N^{α}-Fmoc and N^{α}-Boc protected amino acids were purchased from Auspep, Chemimpex and Mimotopes. Unless otherwise specified, all amino acids used were of L-conformation. Rink amide resin (0.53 meq/g, 100-200 mesh), 2-chlorotrityl chloride resin (1.12 meq/g, 200-400 mesh), HCTU and PyClock were obtained from Chemimpex. TFA was purchased from Alfa Aesar. Thioanisole, 1,2-ethanedithiol, DIPEA, piperidine, Boc anhydride, triethylamine, EDCI, DMAP, 1,3-benzenedimethanol, Fmoc-OSu, pyridine, 1,4-dioxane and Grubbs catalyst 2nd generation were purchased from Sigma-Aldrich. Phenol, chlorotrimethylsilane and all solvents were obtained from Merck. The sCy5 fluorescence probe was purchased from W&J PharmaChem. The Rhodamine B analogue was purchased from Sigma-Aldrich

and modified as reported.[34] All solvents were of analytical grade, and all chemicals were used without further purification.

Molecular mass of the compounds were determined by ESI-MS using a Shimadzu LCMS2020 instrument, incorporating a Phenomenex Luna C-8 column (100Å, 3μm, 100×2.00mm). This system used 0.05% TFA in MilliQ water as the aqueous buffer, and 0.05% TFA in acetonitrile as the organic buffer. The eluting profile was a linear gradient of 0-60% acetonitrile in water over 10 min at 0.2 ml/min.

HRMS analyses were carried out on an Agilent 6224 TOF LC/MS Mass Spectrometer coupled to an Agilent 1290 Infinity (Agilent, Palo Alto, CA). All data were acquired and reference mass corrected via a dual-spray electrospray ionisation (ESI) source. Acquisition was performed using the Agilent Mass Hunter Data Acquisition software version B.05.00 Build 5.0.5042.2 and analysis was performed using Mass Hunter Qualitative Analysis version B.05.00 Build 5.0.519.13.

Crude peptides were purified on a Phenomenex Luna C-8 column (100Å, 10μm, 250×21.2mm) utilising a Waters 600 semi-preparative RP-HPLC that incorporates a Waters 486 UV detector. The wavelength was set at 230 nm. This system used 0.1% TFA in MilliQ water as the aqueous buffer, and 0.1% TFA in acetonitrile as the organic buffer. The eluting profile was a linear gradient of 0-80% acetonitrile in water over 60 min at 10 ml/min.

Peptide synthesis

The purity of all reported peptides are ≥ 95% according to the HPLC chromatographs produced by the ESI-MS method described above.

General synthesis

Linear peptide chains were synthesised on Rink amide resin or 2-chlorotrityl chloride resin (sequence dependent) using a 3-channel serial automated peptide synthesiser (“PS3”, Protein Technologies Inc.), which adopted standard Fmoc-based solid phase synthesis strategy. Fmoc

deprotection was performed by 20% v/v piperidine in DMF for 2×5 min. Fmoc protected amino acids (3 *eq.*) were coupled using DMF as solvent, and DIPEA in DMF (7% v/v) with HCTU (3 *eq.*) as the activating agent for 50 min.

Protected peptide-resins were cleaved by treating with Reagent K (5 ml) composed of TFA-H_2O-thioanisole-phenol-EDT (82.5%:5%:5%:5%:2.5%) for 3 h. The cleavage mixture was filtered, concentrated by a stream of N_2, precipitated in cold Et_2O and centrifuged at 3000 rpm for 5 min. The crude product was dissolved in water-acetonitrile mixture (50%:50%) and lyophilised.

(2*S*,7*S*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((*S*,*S*)-1)

The linear peptide chain was prepared by the general method described above on Rink resin (0.05 meq). The peptide resin was treated with (2*S*,7*S*)-*N,N*-di-Boc-diaminosuberic acid, (*S*,*S*)-**5** (10.0 mg, 0.5 *eq.*), PyClock (110 mg, 4 *eq.*), DIPEA (87.0 µl, 10 *eq.*) in DMF (5 ml) overnight. After cleavage and purification, the peptide was lyophilized to yield a white fluffy solid (3.5 mg). HPLC RT 11.21 min. ESI-MS: 569.75 $(M+3H)^{3+}$. HRMS (ESI) m/z calculated for $[C_{80}H_{124}N_{26}O_{16}+2H]^{2+}$, 853.4923, found: 853.4947.

(2*R*,7*R*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((*R*,*R*)-1)

The (*R*,*R*) diastereomer of **I** was prepared in the same fashion as the (*S*,*S*)-diastereomer, but on 0.05 mmol scale, and using (*R*,*R*)-**5** yielding a fluffy white sold (13.4 mg). HPLC RT 11.16 min. ESI-MS: 569.85 $(M+3H)^{3+}$. HRMS (ESI) m/z calculated for $[C_{80}H_{124}N_{26}O_{16}+3H]^{3+}$ 569.6660, found: 569.6683.

Alternatively, peptide (*R,R*)-**9** (16 mg) (see below) was dissolved in EtOAc (10 ml) then cycled through an H-Cube™ incorporating a 10% Pd/C cartridge at 50˚C at 1.5 ml/min under H_2 (50 psi). After 1h the solvent was removed *in vacuo* and the residue was purified by HPLC to yield a white fluffy solid (9.2 mg).

(2*S*,7*S*)-Diaminooct-4-enedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((*S*,*S*)-9)

The Fmoc protected linear peptide, Fmoc-Gly(All)-Tyr(OtBu)-Arg(Pbf)-Leu-Arg(Pbf)-Tyr(OtBu)-Rink amide resin was treated with LiCl in DMF (4.2 mg/ml, 200 µl), Grubbs catalyst 2nd generation (0.2 *eq.*) and DCM (4.5 ml) in a glass microwave vessel. The mixture was charged with N_2 and heated in a microwave reactor at 100˚C for 3 h. After the solvent was removed by filtration, Fmoc deprotection was performed using 20% v/v piperidine in DMF (5 ml) for 2×5 min, and the peptide was cleaved off resin as described above, to yield a white fluffy solid (10.0 mg), HPLC RT 11.19 min. ESI-MS: 569.05 $(M+3H)^{3+}$. HRMS (ESI) m/z calculated for $[C_{80}H_{122}N_{26}O_{16}+2H]^{2+}$ 852.9881.

(2*R*,7*R*)-Diaminooct-4-enedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((*R*,*R*)-9)

The peptide was prepared as for **(*S*,*S*)-9** above, but utilizing Fmoc-D-Gly(All) to yield a white fluffy solid (5.1 mg), HPLC RT 11.34 min. ESI-MS: 569.10 $(M+3H)^{3+}$.

(2*S*,7*S*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr dimethyl ester) ((*S*,*S*)-12)

A mixture of Fmoc-Tyr-OMe, **10** (2 *eq.*) and DIPEA (6 *eq.*) in DCM (5 ml) was added to 2-chlorotrityl resin and agitated overnight. The resin was filtered, washed with DCM×3, MeOH×1 and Et_2O×1, and dried *in vacuo*. The derivatised resin was subject to standard solid phase synthesis as described above and treated with (2*S*,7*S*)**-5** as described for (*S*,*S*)**-1** above**.** After cleavage and purification, the peptide was lyophilized to yield a white fluffy solid (40.5 mg). HPLC RT 12.06 min. ESI-MS: 579.85 $(M+3H)^{3+}$. HRMS (ESI) m/z calculated for $[C_{82}H_{126}N_{24}O_{18}+2H]^{2+}$ 868.4914, found: 868.4956.

(2*S*,7*S*)-Diaminooctanedioyl-(Tyr-Arg-Leu-Arg-Tyr methyl ester) (Tyr-Arg-Leu-Arg-Tyr amide) ((*S*,*S*)-13)

(*S,S*)**-13** was prepared as for (*S,S*)**-12** above, except that the 2-chlorotrityl chloride resin was treated with a 50%:50% mixture of Fmoc-Tyr-OMe (**10**) and Fmoc-Tyr-NH_2 (**11**). After cleavage and purification, the peptide was lyophilized to yield a white fluffy solid (7.5 mg). HPLC RT 11.61 min. ESI-MS: 574.85 $(M+3H)^{3+}$. Side products of (*S*,*S*)**-1** and (*S*,*S*)**-12** were

also identified in the product mixture. HRMS (ESI) m/z calculated for $[C_{81}H_{125}N_{25}O_{17}+2H]^{2+}$ 860.9920 found: 860.9956.

Fluorescent labelling of peptides

Peptides were treated with a mixture of the labelling agent (sCy5 or RhB, see Figure 2) (0.7 *eq.*), PyClock (2 *eq.*) and NMM (12 *eq.*) in DMF (2 ml). The mixture was stirred in darkness overnight and DMF was removed *in vacuo*. The crude product was washed with TFA (1 ml), precipitated with cold Et_2O and centrifuged at 3000 rpm for 5 min. The resulting precipitate was dissolved in water-acetonitrile (50%:50%) and lyophilised.

Mono-sCy5-(2*S*,7*S*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((*S*,*S*)-14)

According to the general method for fluorescent labelling described above, (*S*,*S*)-**1** (10 mg) was treated with sCy5-OH (0.7 eq.). After purification, (*S*,*S*)-**14** was obtained as a fluffy blue powder (1.3 mg). HPLC RT 12.38 min. ESI-MS: 782.70 $(M+3H)^{3+}$.

Mono-sCy5-(2*R*,7*R*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((*R*,*R*)-14)

According to the general method for fluorescent labelling described above, (*R,R*)-**1,** (5.9 mg) was treated with sCy5-OH (0.7 eq.). After purification, (*R*,*R*)-**14** was obtained as a fluffy blue powder (1.2 mg). HPLC RT 12.36 min. ESI-MS: 782.75 $(M+3H)^{3+}$.

Mono-RhB-(2*S*,7*S*)-Diaminooctanedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((*S*,*S*)-15)

According to the general method for fluorescent labelling described above, (*S*,*S*)-**1,** (10 mg) was treated with RhB-OH (0.7 eq.). After purification, (*S*,*S*)-**15** was obtained as a fluffy magenta powder (0.5 mg). HPLC RT 13.37 min. ESI-MS: 767.40 $(M+3H)^{3+}$.

Mono-RhB-(2*S*,7*S*)-Diaminooct-4-enedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((*S*,*S*)-16)

According to the general method for fluorescent labelling described above, (*S*,*S*)-**9,** (10 mg) was treated with RhB-OH (0.7 eq.). After purification, (*S*,*S*)-**16** was obtained as a fluffy magenta powder (1.2 mg). HPLC RT 13.36 min. ESI-MS: 766.65 $(M+3H)^{3+}$.

Mono-RhB-(2*R*,7*R*)-Diaminooct-4-enedioyl-bis(Tyr-Arg-Leu-Arg-Tyr-amide) ((*R*,*R*)-16)

According to the general method for fluorescent labelling described above, (*R*,*R*)-**9,** (10 mg) was treated with RhB-OH (0.7 eq.). After purification, (*R,R*)-**16** was obtained as a fluffy magenta powder (1.4 mg). HPLC RT 13.26 min. ESI-MS: 766.70 $(M+3H)^{3+}$.

Cell culture

HEK293T and 293TR cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum, and passaged when confluent by trypsinisation (0.25% w/v in Versene). Mixed population 293TR cell lines inducibly expressing Y receptors tagged with C-terminal GFP, and dual stable HEK293 cell lines expressing Y receptor-Yc and β-arrestin2-Yn (where Yc and Yn are complementary fragments of YFP) are as previously reported.[35, 39, 40]

Y_4R competition binding and imaging assays

293TR Y_4-GFP or Y_1-GFP cells were seeded at 20,000 cells / well in poly-D-lysine coated 96-well imaging plates (Greiner 655090), treated as required with 1 μg/ml tetracycline for 18-21 h and then used in experiments at confluence. Incubations were performed in HEPES-buffered saline solution (HBSS) including 0.1% BSA, the permeable nuclear dye H33342 (2 μg/ml, Sigma) and varying concentrations of competitor ligands (10^{-10} M to 10^{-5} M) for 2 min, prior to the addition of fluorescent ligand at the concentration indicated. After 30 min at 37°C the media was replaced with HBSS / 0.1% BSA and plates were immediately imaged (2 sites / well) on an IX Ultra confocal platereader (Molecular Devices, Sunnyvale CA, U.S.A.) using laser excitation / emission filter settings appropriate for H33342 (DAPI), Y receptor-GFP (FITC), and sCy5-labelled peptides. Bound ligand fluorescence was quantified by granularity analysis (2-3 μm diameter granules; MetaXpress 5.3, Molecular Devices), and normalised to positive (totals 100 %) and negative (0%, in presence of 100 nM PP) controls. pIC_{50} values were then determined from the pooled data using GraphPad Prism v6 (GraphPad software, San Diego, CA).

Y_4R-β-arrestin recruitment assays

Bimolecular fluorescence complementation (BiFC) based detection of Y receptor-β-arrestin2 association was performed as described previously.[39, 40] The Y_4R arrestin BiFC cell lines were seeded at 40,000 cells / well onto poly-D-lysine coated Greiner 655090 imaging plates, and experiments performed 24 h later. Stimulation with human PP (Bachem, St. Helens, U.K.) or other ligands was performed in HBSS / 0.1% BSA (10^{-10} M-10^{-6} M, duplicate wells) for 60 min at 37°C. Incubations were terminated by fixation with 3% paraformaldehyde in phosphate buffered saline (PBS, 10 min at 21°C), the cells were washed once with PBS and the cell nuclei were stained for 15 min with H33342 (2 μg ml^{-1} in PBS, Sigma). H33342 was then removed by a final PBS wash. Images (4 central sites / well) were acquired automatically on the IX Ultra confocal platereader, using 405 nm / 488 nm laser lines for H33342 and complemented YFP excitation respectively.

A granularity algorithm (MetaXpress 5.3) identified internal fluorescent compartments within these images of at least 3 μm diameter (range set to 3-1 μm), on the basis of granule intensity thresholds set with reference to the vehicle or 100 nM PP plate controls. The response for each data point was quantified as mean granule average intensity / cell, normalised to the reference agonist response. Concentration response curves were fitted to the pooled data by non-linear least squares regression (GraphPad Prism), yielding estimates of agonist potency as pEC_{50} and maximum response (R_{max}).

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Supporting Information Available

Experimental data relating to protected amino acid synthesis, chiral chromatography of (2*S*,7*S*)-**5** and (2R,7R)-**5,** HPLC spectra for all peptides and fluorescence spectra of (*R*,*R*)-**14**. This material is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations Used

BiFC	Bimolecular fluorescence complementation
DCM	dichloromethane
DIPEA	N,N-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDT	1,2-ethanedithiol
EtOAc	ethyl acetate
GFP	green fluorescent protein
HCTU	*O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*,*N*-tetramethyluronium hexafluorophosphate
MeOH	methanol
NMM	N-methylmorpholine
Pd/C	palladium on activated carbon
PyClock	(6-Chlorobenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate

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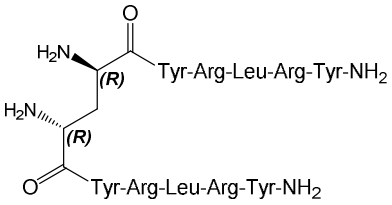
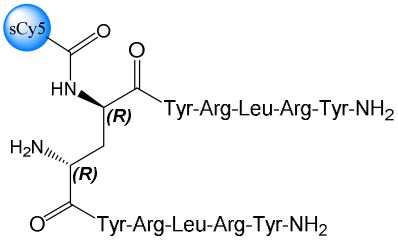


Y_4R agonist EC_{50} 4.6 nM, R_{max} 62%



Y_4R agonist
EC_{50} 34 nM,
R_{max} 66%