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Synthetic routes to the Neuropeptide Y Y1 receptor antagonist 1229U91 and related analogues for SAR studies and cell-based imaging†

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The potent Y_1 receptor antagonist, 1229U91 has an unusual cyclic dimer structure that makes syntheses of analogue series quite challenging. We have examined three new routes to the synthesis of such peptides that has given access to novel structural variants including heterodimeric compounds, ring size variants and labelled conjugates. These compounds, including a fluorescently labelled analogue **VIII** show potent antagonism that can be utilised in studying Y_1 receptor pharmacology.

Introduction

Neuropeptide Y (NPY) is a 36-amino acid C-terminal amidated polypeptide first isolated from porcine brain in 1982.[1] NPY shares a high degree of homology in amino acid sequence with pancreatic polypeptide (PP) and peptide YY (PYY). It is a peptide neurotransmitter implicated in various physiological processes at the central nervous system[2] (*e.g.* stimulation of feeding behaviour and inhibition of anxiety) and the peripheral nervous system[3] (*e.g.* vasoconstriction, insulin release, renal secretion, gastrointestinal secretion). These effects, together with those of the gastrointestinal hormones PYY and PP, are mediated in man by G-protein coupled receptor subtypes, Y_1, Y_2, Y_4 and Y_5.[4,5]

The important roles of NPY in both human physiology and pathophysiology have led to considerable efforts to develop subtype specific NPY receptor agonists and antagonists, which may be prospective clinical candidates for various indications such as cancer,[6] obesity[7] and epilepsy.[8] The utility of labelled ligands in imaging applications has also been recognized.[9,10]

Both small-molecule and peptide-based antagonists have been described for the Y_1 receptor however they are associated with a number of shortcomings. For example, the small-molecule ligand BIBP3226 possesses high selectivity and moderate Y_1 affinity but also has CNS toxicity.[11,12] It has been utilised as a pharmacological tool in over 100 studies.[13] Optimisation of BIBP3226 into the more active BIBO3304 gave a 10-fold increase in affinity towards Y_1-receptors however it is still burdened with cross-reactivity towards Neuropeptide FF receptors.[14,15]

Truncated NPY analogues have received increasing attention since 1995, when Leban *et al.* described the C-terminal decapeptide, Tyr-Ile-Asn-Leu-Ile-Tyr-Arg-Leu-Arg-Tyr-NH_2.[16] Based on this sequence the subsequent peptide (Ile-Asn-Pro-Ile-Tyr-Arg-Leu-Arg-Tyr-NH_2, known as BW1911U90 or BVD15), had a 10-fold increase in Y_1 activity and a 4-fold decrease in Y_2 affinity.[16] It also had agonist activity at Y_4 receptors with similar affinity to Y_1.[17,18] Other peptides similar to BW1911U90 have also been described recently such as the Y_1-selective agonist [Pro,[30]NIe,[31]Bpa,[32]Leu[34]]NPY(28–36),[19] the Y_1 selective [Lys(DOTA)[4]]BVD15[20] and analogous NOTA derivative[21] and the click chemistry radiolabelled analogue ^{18}F-ALK-BVD15.[22]

Another potent Y1 receptor antagonist known as 1229U91 (or GR231118) was described by Daniels in 1995.[23] It is a homodimer based on BW1911U90 whereby Glu^2 and Dap^4 have been included in order to form a lactam bridge between two sequences (Fig. 1). It has been demonstrated that 1229U91 exhibits a higher affinity and more potent competitive antagonism at Y1 receptors than BW1911U90. It also showed extended activity *in vivo* attributed to the stability of the cyclic peptide.[18,24] It was subsequently found to be an agonist at Y_4 receptors while showing a much weaker affinity towards Y_2 receptors.[18,25–27] Only a limited number of other dimer variants have previously been described.[17,23,28–30] They include modifications to the C-terminus residues and the use of disulfide bridges, diaminopimelic acid or other lactam bridge conformations to interconnect the monomer sequences.

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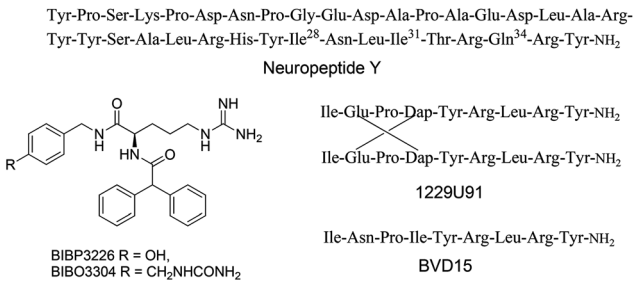


Fig. 1 Y1 receptor ligands.

The challenges associated with unambiguous synthesis of 1229U91 analogues are not trivial. The discovery of 1229U91 looks somewhat serendipitous as the product would normally be associated with a side-reaction in intramolecular cyclisation.[28,31] The original method to prepare 1229U91 was described by Daniels using Boc-based chemistry. The use of base sensitive side chain protecting groups 9-Fe and Fmoc on the Glu and Dap residues respectively allowed for selective deprotection and then on-resin cyclisation using BOP reagent.[28] Lew *et al.* described a solution phase cyclodimerisation of an *N*-Fmoc-protected (but side-chain deprotected) linear precursor yielding a 75 : 25 ratio of dimer to monomer.[31] The ability to achieve efficient and clean cyclisation in the absence of protecting groups for Tyr and Arg residues was a somewhat surprising but attractive element to this synthesis although Balasubramaniam reported that in their hands they found that this method was inferior to the original on-resin BOC method.[17] Note that both these approaches would best suit symmetrical cyclic dimers.

We identified a need for more versatile synthetic routes to 1229U91 analogues to explore structure activity relationships and/or incorporate labelling agents. Herein we report the development of such routes in preparing 1229U91 and a series of novel analogues. The methods have extended the existing solution phase and solid phase cyclodimerisation routes to allow for preparation of homo- and/or heterodimers in useful yields, but also an unambiguous synthesis of cyclic dimers that avoids concomitant competing intramolecular cyclisation.

These products have been tested in competition binding assays and functional studies, to yield high affinity functional antagonists of the Y_1 receptor, one of which incorporates a fluorescent rhodamine substitution that can be used in cell imaging studies.

Results and discussion

Chemistry

First Fmoc-based solid phase synthesis of 1229U91 and analogues. We first adapted the reported on-resin cyclisation method to Fmoc SPPS for the preparation of homodimers (Scheme S1†). An orthogonal protecting group strategy included Dap(Aloc) and Glu(OAll) residues while standard side chain protecting groups on Tyr and Arg residues were left intact. The N-terminal Ile was Boc-protected. The OAll and Aloc were selectively removed by $Pd(PPh_3)_4$ catalysed allyl transfer in $CHCl_3$–AcOH–NMM under N_2 for 2 h.[32] The cyclisation was then performed by treating the partially deprotected resin with PyClock/DIPEA in DMF for 6 h. Cleavage from the resin with TFA yielded the crude peptide. Under these conditions, the isolated yield was 5% and the cyclic dimer was almost exclusively favoured over the cyclic monomer. We also prepared the N-terminal truncated sequence **I** in this way obtaining a 5% overall yield.

While the solid phase route above is an efficient method for the synthesis of homodimeric cyclic peptides, it appeared limited from the perspective of generating heterodimers with mixed monomer sequences. To include those as possible products we turned to the solution phase route, to see if we could extend the utility of that pathway.

Solution phase synthesis of dimeric peptides. The first element of the syntheses that follow was the preparation of a series of partially protected monomeric, linear peptides that would become the substrates for solution phase cyclisation reactions. Some of these contain either modified amino acids or allow for later incorporation of the conjugates shown in Fig. 2. These syntheses were performed by conventional solid phase peptide synthesis on Rink Amide resin. The syntheses in general gave rise to the desired products with no identifiable deletion or side products. The isolated peptides are summarized in Table 1 (see also Fig. S4†).

We first utilised peptide **1** as monomer to examine the solution phase conditions described by Lew *et al.* We found that using PyBOP as cyclisation reagent and DIPEA as base we achieved the same ratio of cyclic dimer/monomer (80 : 20) as reported (Fig. 3a). The recoveries after cyclisation and then Fmoc-deprotection were quite poor, leading to overall a very low yield of 1229U91 (<1%). The yield was improved substantially by not isolating the Fmoc-protected cyclisation product, but treating reaction mixture directly with piperidine and then retrieving the final product directly by semi-preparative RP-HPLC. In this way yields of 4% (based on 0.1 mmol resin loading) could be obtained.

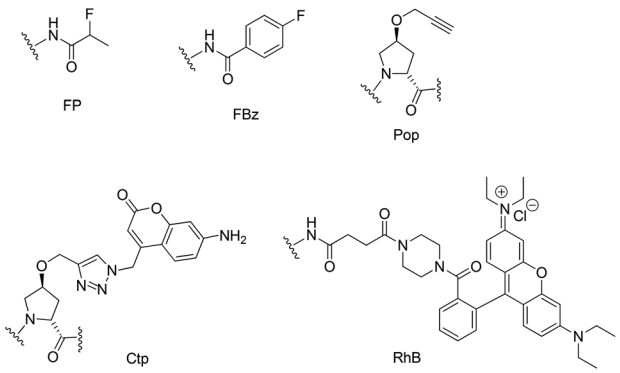


Fig. 2 Structures of conjugate groups.

Table 1 Protected linear monomer precursors

#	Sequence	$(M + 2H)^{2+}$
1	**Fmoc**-Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$	709.4
2	**Fmoc**-Ile-Glu-Pop-Dap-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$	736.5
3	**Fmoc**-Ile-Glu-Pro-Lys-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$	730.5
4	**FBz**-Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$	659.3
5	**Fmoc**-Ile-Glu(***O*-All**)-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$	729.4
6	**Fmoc**-Ile-Glu-Pro-Dap(**Alloc**)-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$	751.5
7	**Fp**-Ile-Glu(***O*-All**)-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$	655.4
8	**Fmoc**-Ile-Glu-Pro-Lys(**Alloc**)-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$	772.5
9	**Fmoc**-Ile-**Glu**(***O*-All**)-Pro-Lys-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$	750.2

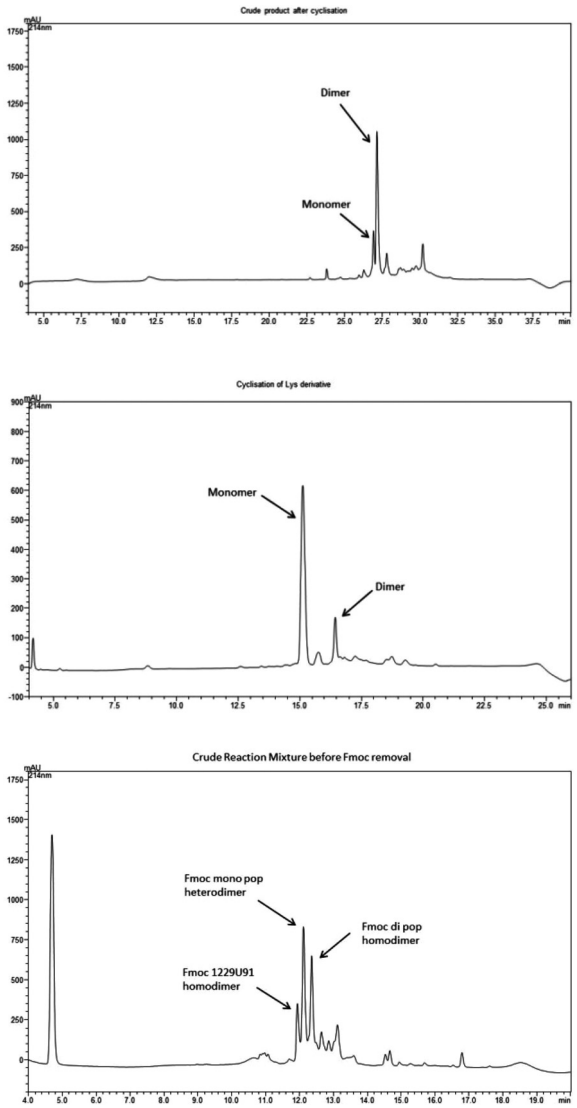


Fig. 3 HPLC traces of crude products from cyclisation reactions with PyBOP of (a) protected peptide **1**, (b) protected peptide **3** and (c) mixture of protected peptides **1** and **2**.

We examined other parameters to see if the ratio of dimer to monomer could be increased. Intramolecular and intermolecular amide bond formation will be competing events and should be influenced by changes to the coupling agent or base. No enhancement of the proportion of dimer was seen by changing the base from DIPEA to TMP (Fig. S2a†) (although the reaction mixture had fewer other impurities) or by replacing PyBOP with the slightly more reactive coupling reagent PyClock.

When the same reaction was attempted with peptide **2** where the proline residues had been replaced with an alkyne derivatised proline (Pop), the dialkynyl dimer **II** was obtained, with the 80 : 20 dimer/monomer ratio maintained. In contrast, using linear peptide **3** where the Dap residue was replaced with Lys, the proportion of the desired dimer **III** to the corresponding cyclic monomer **IIIa** was reversed (15 : 85) (Fig. 3b). This example showed the sequence dependence that can dictate the outcome of these competing reactions.

Synthesis of Heterodimers (non-orthogonal). This solution phase protocol was also used to prepare heterodimeric analogues of 1229U91. It was envisaged that a mixture of two analogous but independent sequences could be reacted under similar conditions to give a mixture of the heterodimer and the two possible homodimeric products. These could potentially be separated by HPLC.

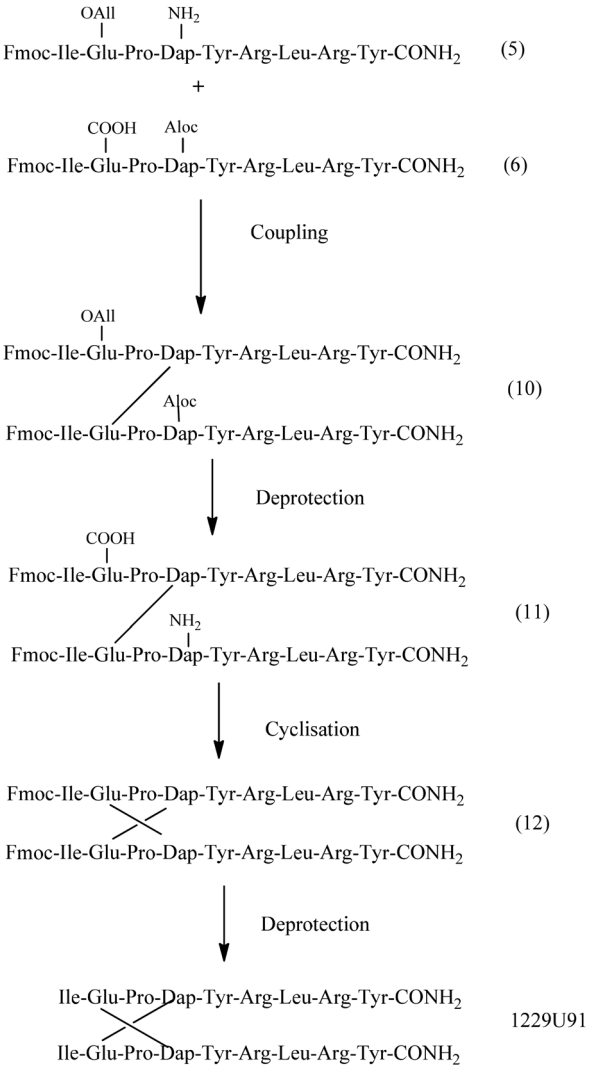
First, a mono-Pop containing analogue **IV** was prepared. A 1 : 1 mixture of the purified linear peptides **1** and **2** was treated with PyBOP and DIPEA yielding the expected mix of products (Fig. 3c). Deprotection of the Fmoc groups with piperidine and purification of the complex mixture allowed for isolation of the mono Pop heterodimer **IV** as well as the homodimer **II** by HPLC. Compound **IV** was then utilised as an intermediate in the synthesis of the fluorescently labelled product **IX** described later.

A second heterodimeric peptide was prepared by inclusion of an amino terminal fluorobenzoyl group in one of the monomers **4**. When monomer **4** and monomer **1** were coupled (Fig. S2b†) followed by deprotection, the mono- and di-labelled FBz derivatives **V** and **VI** were retrieved by HPLC.

In summary, the use of Fmoc-based solid phase synthesis with solution cyclisation can be used to retrieve useful amounts of both homo- and hetero-dimeric peptides.

Solution phase formation of cyclic dimers *via* orthogonal protection. Despite the improvements instituted in the syntheses above, these studies also identified a need for more chemoselective, sequence-independent methods if we were to expand our studies to include a variety of modified sequences, heterodimers or conjugates. The competition between cyclic dimer and monomer formation results from competition between an intermolecular coupling (followed by cyclic lactam formation) in the dimer case and intramolecular cyclisation for the cyclic monomer. In addition, with heterodimer formation we had competition between self- and hetero-coupling which may also be sequence dependent. We decided to examine orthogonal protection strategies to prevent these competing events.

Starting with the synthesis of 1229U91 itself (Scheme 1), two different protected peptides were prepared. In one the Glu side chain was protected with *O*-allyl ester (OAll) **5** and in the



Scheme 1 Strategy for orthogonal stepwise synthesis of 1229U91.

other the Dap was protected as the allyl carbamate (Alloc) **6**. The two sequences were then coupled by forming an amide bridge between the unprotected Dap and Glu side chains to give the branched intermediate **10**. This was in turn deprotected *via* Pd(0) catalysed allyl transfer, cyclised and Fmoc-deprotected to give 1229U91.

Note that the coupling of the two fragments was successful, but only after a key modification to the standard methods was made. It was necessary to use TMP as the base as it allowed for the acid fragment to be pre-activated without substrate degradation, as was observed in the case of DIPEA. The optimal conditions were that the acidic fragment peptide and PyClock (4 eq.) were dissolved in DMF. TMP (22 eq.) was added followed by the addition of the amino fragment (Final concentration 0.1 M in DMF). After 30 min, analysis by LCMS showed conversion to the desired side chain linked product (Fig. S2a†).

Where DIPEA was used only small amount of the desired bridged sequence was observed (Fig. S2b†). It was observed that **6** degraded under the reaction conditions. The same proved true for a protected test peptide Fmoc-Ile-Glu-Pro-Dap-(Boc)-$CONH_2$. Switching the base to TMP minimized this degradation.

To complete the synthesis, selective deprotection of both the Aloc and OAll groups was achieved using Pd(0) catalysed allyl transfer. The catalyst, $Pd(PPh_3)_4$, dissolved in $CHCl_3$–AcOH–NMM was added under a N_2 atm to the peptide and mixed for 2 h. A small amount of product contained incomplete removal of the OAll group. Cyclisation of the purified peptide was achieved using PyClock (3 eq.) and TMP (24 eq.) in DMF (1 mg mL^{-1}) followed by Fmoc deprotection to give the target peptide 1229U91.

The method above was then used to prepare two analogues of 1229U91. The first was a N-2-fluoropropyl substituted analogue **VII**. The Glu(OAll) protected peptide **7** was coupled to the Dap-protected fragment **6** (1 eq.) to give the branched product **13**. The allyl deprotection step was achieved again with $Pd(PPh_3)_4$ in $CHCl_3$–AcOH–NMM under N_2 atm for 2 h. Cyclisation of the purified peptide in DMF (1 mg mL^{-1}) using PyClock (3 eq.) and TMP (24 eq.) followed by Fmoc deprotection gave a 7% overall yield of **VII** after purification.

This method was also used to prepare the dimeric Lys-containing analogue **III** which was difficult to achieve by the conventional methods described above, due to preferential monomeric cyclisation. The linear peptide **8** (1 eq.) was activated with PyClock (3 eq.) in a solution of DMF and TMP (24 eq.) followed by the addition of the amino fragment **9** (1 eq.) (final peptide conc. in DMF, 66.5 mM) to give the coupled product **16** (Fig. S3a†). In this case, complete Pd catalysed removal of the protecting groups was best achieved using the conditions of Thiuret with phenyl silane (Fig. S3c†) as compared to $Pd(PPh_3)_4$ in $CHCl_3$–AcOH–NMM (Fig. S3b†). Cyclisation of the crude material was achieved using PyClock (3 eq.) and TMP in DMF. The solution phase Fmoc deprotection was performed using 10% piperidine in DMF, followed by preparative HPLC to give the desired product **III**. The 12% isolated yield was a improvement over the minority product (<2%) obtained *via* direct cyclisation above.

Post-synthesis modification. With the development of reliable methods for the synthesis of 1229U91 (and other derivatives) at reasonable scales labeling of these peptides has also been achieved as a "post-synthesis" step.

For example, the fluorescently labeled rhodamine derivative **VIII** was prepared by reacting purified 1229U91 with a limiting amount (*e.g.* 0.7 eq.) of an NHS-activated Rhodamine B derivative,[33] in a solution of DMF and DIPEA. The reaction was monitored by LCMS and the resultant mixture of the desired mono-labeled product, di-labeled product and unreacted 1229U91 was then purified by HPLC allowing for isolation of the mono-labeled derivative **VIII** in 26% yield.

Secondly, we were successful in introducing a triazolocoumarin to the peptide using click chemistry upon the propargyloxy derivative of 1229U91 **IV** to prepare **IX**. The reaction between the purified peptide and 7-amino-4-(azidomethyl)-2*H*-chromen-2-one[34] in a solution of DMF and H_2O was initiated

Table 2 1229U91 and analogues

Cmd #	Dimer sequence	ESI-MS[a]	IC_{50}/nM Y_2Y_4 KO[c]	95% Confidence limits
1229U91	Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr	823.5	0.10	0.49–0.021
I	Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr	748.1	7.32	2.9–16
II	Ile-Glu-Pop-Dap-Tyr-Arg-Leu-Arg-Tyr Ile-Glu-Pop-Dap-Tyr-Arg-Leu-Arg-Tyr	859.4	0.11	0.057–0.22
III	Ile-Glu-Pro-Lys-Tyr-Arg-Leu-Arg-Tyr Ile-Glu-Pro-Lys-Tyr-Arg-Leu-Arg-Tyr	851.6	0.12	0.049–0.30
IV	Ile-Glu-Pop-Dap-Tyr-Arg-Leu-Arg-Tyr Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr	841.4	n.d.	
V	FBz-Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr	864.1	0.13	0.039–0.44
VI	FBz-Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr FBz-Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr	904.8	4.12	0.82–21
VII	FP-Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr	848.1	0.53	0.094–3.0
VIII	RhB-Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr	766.2[b]	0.08	0.016–0.43
IX	Ile-Glu-Ctp-Dap-Tyr-Arg-Leu-Arg-Tyr Ile-Glu-Pro-Dap-Tyr-Arg-Leu-Arg-Tyr	913.4	19.2	8.3–44

[a] ESI-MS base peak corresponds to $[M + TFA + 3H]^{3+}$. Note $[M + 3H]^{3+}$ peaks were observed at lower intensity. See Fig. S5. [b] ESI-MS ion base peak corresponds to $[M + TFA + 4H]^{4+}$. [c] Inhibition of ^{125}I-NPY (25 pM) binding to brain membrane homogenates.

by standard CuAAC conditions. The reaction was complete in 3 h when 10 eq. of copper sulfate, sodium ascorbate and TBTA were used.

In summary, the work described above has provided us with methods that can serve for the synthesis of a wide variety of 1229U91 analogues shown in Table 2 (see also Fig. S5†). Collectively we now have the means to prepare compounds bearing multiple modifications with variation in ring size and unambiguous synthesis of heterodimers provided by the orthogonal protection of monomeric precursors.

Pharmacology

With the compounds described above in hand we were able to assess the influence of the various structural changes on Y_1 receptor affinity. To do this competition assays against [^{125}I]-PYY binding to brain homogenates from Y_2Y_4-receptor knockout mice were utilised. Such homogenates are a native tissue source of Y_1 receptors free from significant Y-receptor cross-reactivity.[35] The results are shown in Table 2.

The compounds assayed all showed high affinity for Y_1 receptors with IC_{50} values in the low nanomolar range or better. Notably, compounds **II**, **III**, **V** and **VIII** all show comparable affinity to 1229U91 itself. Some key results stood out for us from this work. Firstly, the equivalent affinities of **III** and 1229U91 is of interest as **III** is anticipated to adopt a markedly different ring structure, with 6 extra methylene units in the cyclic portion of the molecule. It was also of interest that the bis-Pop ligand **II** retained high affinity, suggesting that the ring structure could tolerate a range of changes.

Second, the tolerance for a range of prosthetic labeling groups was demonstrated, for example by inclusion of fluorobenzoyl (**V**) and 2-fluoropropyl (**VII**) as potential labeling conjugates for ^{18}F-radioimaging. The difference between **V** and **VI**, where a second label is detrimental to affinity suggests that care would need to be taken in generating such compounds as a final step in synthesis.

In the murine binding assay, in which low levels of native Y_1 receptor expression are limiting, we observed strong but inconsistent competition data with the rhodamine conjugate (**VIII**). However this compound was investigated successfully in transfected cell membranes and functional assays (see below). Disappointingly given the apparent tolerance for substitution by the propargyloxy groups in **II**, the "click" product **IX** had 100 fold reduced affinity compared to 1229U91.

Compounds **III** and **VIII** stood out as warranting further investigation; compound **VIII** because of the utility that a fluorescent ligand would have in studies of Y_1 pharmacology, and **III** because of potential to understand more of the SAR governing Y_1 binding and in particular selectivity with respect to Y4 receptors given the reported agonism at Y_4 shown by 1229U91.

These two compounds were thus studied in assays using rat Y_1- and human Y_4-transfected HEK293 cells. In [^{125}I]PYY competition binding studies using rat Y_1-GFP transfected cell membranes (as described in Kilpatrick *et al.*,[36] compound **III** was confirmed as a high affinity ligand with a K_i similar to 1229U91 itself (Table 2). Furthermore compound **VIII** also showed a clear concentration-dependent competition for specific [^{125}I]PYY binding, with a K_i in the low nM range, 24 fold lower affinity than 1229U91 (Table 2, ESI Fig. S7†). Nevertheless, compound **VIII** represents a novel template for Y_1 receptor fluorescent ligands, with equivalent affinity to previously reported NPY or argininamide (BIBP3226) analogues.[37–39]

Table 3 Studies of 1229U91, III and VIII in rat Y_1-transfected HEK293

	pK_i[a]	pK_b
1229U91	9.9 ± 0.06	9.5 ± 0.1
III	10.2 ± 0.12	8.4 ± 0.1
VIII	8.5 ± 0.02	8.6 ± 0.2

[a] Inhibition of [^{125}I]PYY (25 pM) binding to recombinant 293TR Y1 receptor-sfGFP cell line.

We used an assay of NPY-stimulated Y receptor association with β-arrestin2 to examine the functional effects of **III** and **VIII**, as we have previously reported for 1229U91.[40] Both **III** and **VIII** were Y_1 receptor antagonists in this assay (Table 3, ESI Fig. S7†), with estimated affinities in the nM range (pK_b 8.4–8.6; Table 3).

The fluorescently labelled compound **VIII** was also examined as a tracer for competition binding studies using live cell imaging with fluorescent platereaders.[41] **VIII** labelled Y_1-GFP transfected HEK293 cells using concentrations as low as 1 nM, with the ligand colocalised with plasma membrane Y_1-GFP fluorescence (Fig. 4). There was no evidence of significant ligand or receptor internalisation under the experimental conditions used. Specific binding of **VIII** to the Y_1 receptor was clearly demonstrated by its concentration dependent displacement using either an unlabelled agonist (NPY) or non-peptide antagonist (BIBO3304). NPY and BIBO3304 IC_{50} values were 27 and 14 nM respectively, consistent with expectations for a whole cell binding assay. In contrast, little fluorescent binding of compound **VIII** (100 nM) to Y_4-GFP cells was observed, demonstrating its relative selectivity for the Y_1 receptor.

When studied in the equivalent Y_4 receptor arrestin recruitment assay, no antagonism of PP activity was observed by

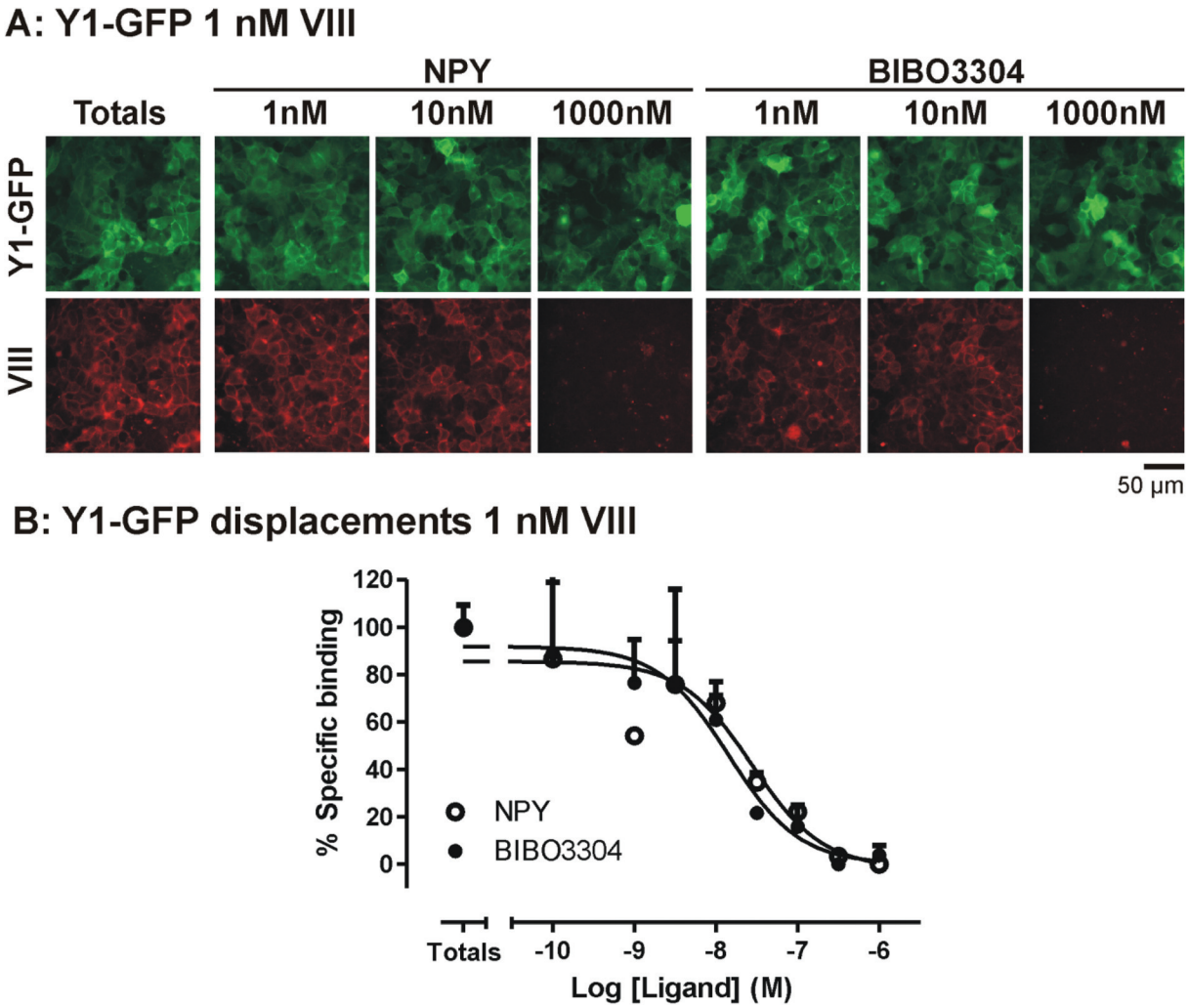


Fig. 4 Use of compound VIII as fluorescent ligand to label Y_1 receptors. Living 293TR cells expressing the Y_1-GFP receptor were incubated with 1 nM compound **VIII** in the absence (totals) or presence of increasing concentrations of NPY or BIBO3304, for 30 min at 37 °C. (A) illustrates representative images acquired on a Molecular Devices IX Micro platereader, monitoring localisation of the Y_1-GFP receptor (FITC channel) and bound compound **VIII** (TRITC channel). (B) represents a single representative experiment performed in triplicate, in which compound **VIII** binding and its displacement by NPY or BIBO3304 was quantified from the images using a granularity algorithm.

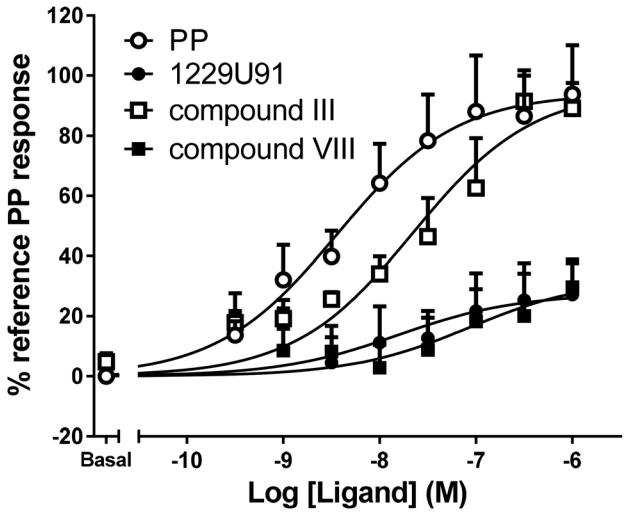


Fig. 5 Concentration response curve for Y_4 receptor agonist activity, measured in the β-arrestin2 recruitment assay. Pooled data are combined from 4 experiments.

these ligands, but rather agonist responses (Fig. 5). 1229U91 and fluorescent compound **VIII** were relatively low efficacy partial agonists, compared to human PP. The difference from previous reports of full 1229U91 agonism can be attributed to the absence of receptor reserve and lack of signal amplification when measuring receptor–arrestin interaction directly here, in contrast to downstream second messenger pathways (16, 17). However compound **III** was a full Y_4 agonist with an EC_{50} of 22 nM in this assay, just an order of magnitude less potent than PP itself (EC_{50} 3.6 nM). Thus in contrast to interactions with the Y_1 binding site, the markedly different ring structure adopted by **III** compared with 1229U91 appears to significantly enhance its ability to stabilise an active Y_4 receptor conformation.

Conclusions

By expanding the available synthetic approaches for the synthesis of side-chain bridged dimers related to 1229U91, we are in a position to fully interrogate the quite remarkable pharmacology of this ligand. As well as the apparent Y_1 potency and selectivity that has been identified over many years of study, the stability *in vivo* first identified by Hegde and co-workers places 1229U91 in a special category of pharmacologically-active peptides. In this work we have been able to develop syntheses that can accommodate the preparation of modified heterodimers, cyclic homodimers with altered ring size and/or conjugated derivatives. In doing so we have developed **VIII**, a rhodamine conjugated analogue of 1229U91 that shows very comparable Y_1 antagonist properties, and which can be used in Y_1 receptor imaging studies; and **III**, a Y_1 antagonist which also displayed enhanced Y_4 agonism. These compounds and their analogues could find application in future studies of Y receptor pharmacology.

Experimental section

N^{α}-Fmoc-protected amino acids were purchased from Auspep and ChemImpex. Rink amide resin and HCTU were purchased from ChemImpex. Piperidine, TFA and PyBOP were purchased from Auspep. DIPEA, phenylsilane, 4-methylmorpholine and tetrakis(triphenylphosphine)palladium were obtained from Sigma-Aldrich. DMF, DCM, chloroform, acetic acid, and PyClock were purchased from Merck. Fluorobenzoic acid was purchased from Alfa Aesar. Collidine was obtained from Ajax Chemicals. 4-Nitrophenyl-2-fluoropropionate was a gift from Peter McCallum Cancer Research Centre and 7-amino-4-(azidomethyl)-2*H*-chromen-2-one[34] was a gift from Dr Bim Graham (Monash Institute of Pharmaceutical Sciences). Fmoc-L-*trans*-4-propargyloxyproline (Pop) and the Rhodamine B derivative[33] were prepared in-house. All chemicals were used without further purification.

RP-HPLC was performed on a Phenomenex Luna C-8 column (100 Å, 10 μm, 250 × 50.0 mm) utilising a Waters 600 semi-preparative HPLC incorporating a Waters 486 UV detector. Eluting profile was a linear gradient of 0–80% acetonitrile in water over 60 min at a flow rate of 20 ml min^{-1}. Peptide identity and purity was confirmed by ESI-MS, using a Shimadzu LCMS2020 instrument, incorporating a Phenomenex Luna C-8 column (100 Å, 3 μm, 100 × 2.00 mm). Eluting profile was a linear gradient of 100% water for 4 min, followed by 0–64% acetonitrile in water over 10 min and isocratic 64% acetonitrile for 1 min, at a flow rate of 0.2 ml min^{-1}. All peptides assayed were of >95% purity.

Solid phase synthesis

Peptide syntheses were performed on a Protein Technologies PS3 synthesiser following the conventional Fmoc-based solid phase peptide synthesis strategy using Rink amide resin (*ca.* 0.7 meq g^{-1}, 100–200 mesh, 0.1 mmol scale). Fmoc-protected amino acids in 3-fold molar excess were coupled using DMF as solvent, 70 ml L^{-1} DIPEA in DMF with 3-fold molar excess of HCTU as the activating agent for 50 minutes. Fmoc deprotection was carried out by treatment with 20% piperidine in DMF for 10 minutes. Occasionally amino acids were incorporated into the sequence by a manual procedure. The amino acid (1.5 eq.) was dissolved in DMF and added to a suspension of HOBt (1.5 eq.) in DCM. After stirring for 2 min DIC (1.5 eq.) was added and the mixture stirred for further 10 min before adding to the vessel containing pre-swollen resin (1 eq.) and agitated for 2 h.

Peptide cleavage from resin was performed using a cocktail containing TFA–TIPS–DMB (92.5% : 2.5% : 5%) for 3 hours.[42] The cleavage mixture was filtered, concentrated by a stream of nitrogen, precipitated by cold diethyl ether and centrifuged. The resulting crude product was dissolved in water–acetonitrile (1 : 1) and lyophilised overnight.

The on-resin linear sequence used in the preparation of peptides **5** and **6** were N-terminus labelled by dissolving fluorobenzoic acid (3 eq.) in DMF and adding to a suspension of HOBt (3 eq.) in DCM. After stirring for 2 min DIC (3 eq.) was

added and the mixture stirred for further 10 min before adding to the vessel containing pre-swollen resin (1 eq.) and agitating for 2 h.

The on-resin linear sequence used in the preparation of peptide **7** was N-terminus labelled by dissolving 4-nitrophenyl 2-fluoropropionate (1.5 eq.) in DIPEA (12 eq.) and DMF and adding to the vessel containing pre-swollen resin (1 eq.) and agitating for 2 h.

Orthogonal deprotection methods

Mtt and O-2-PhiPr removal. Adapting the method originally described by Aletras,[43] the peptide-resin was allowed to swell in DMF, washed with DCM and then treated with 1% TFA and 5% TIPS in DCM for 10 × 2 min. The resin was then washed with DCM (×3), 10% DIPEA in DMF (×3) and DMF (×3).

Allyl and Aloc removal

Solid phase. Following the method described by Kates,[44] a solution of $Pd(PPh_3)_4$ (3 eq.) dissolved in $CHCl_3$–MeOH–NMM (37 : 2 : 1) under a nitrogen atmosphere was added to a flask containing the peptide-resin and shaken for 2 h. The resin was filtered, and washed with 0.5% DIPEA in DMF (×3) and sodium diethyldithiocarbamate (0.5% w/w) in DMF.

Solution phase. $Pd(PPh_3)_4$ (3–6 eq.) was dissolved in a mixture of $CHCl_3$–MeOH–NMM (37 : 2 : 1) under a nitrogen atmosphere and then added to a solution of the crude peptide in $CHCl_3$–MeOH–NMM (37 : 2 : 1) and stirred for 2 h. The solvent was removed *in vacuo*, the residue acidified with a small amount of TFA and the peptide precipitated with cold ether and isolated.

Solid phase. Following the method described by Thieriet,[32] the peptidyl resin was allowed to swell in DMF and was then washed and suspended in DCM under a nitrogen atmosphere. $PhSiH_3$ (24 eq.) in DCM was added to the resin suspension. A solution of $Pd(PPh_3)_4$ (0.25 eq.) dissolved in DCM under a nitrogen atmosphere was then added to the peptide solution and mixed for 30 min. The resin was washed with DCM (×3), DMF (×3) and DCM (×3). The resin was then suspended in DCM and the allyl deprotection step repeated.

Solution phase. The crude cleaved peptide was dissolved in MeOH, placed under a nitrogen atmosphere and $PhSiH_3$ (24 eq.) added. A solution of $Pd(PPh_3)_4$ (1 eq.) dissolved in DCM under a nitrogen atmosphere was then added to the peptide solution and mixed for 2 h. The solvents were removed *in vacuo*, the residue acidified with a small amount of TFA and the peptide precipitated with cold ether and isolated.

ivDde and ODmab removal. According to the method outlined by Chan,[45] the peptide-resin was allowed to swell in DMF, filtered, and then treated with 2% hydrazine monohydrate in DMF (3 × 3 min) and then washed with DMF.

Solid phase cyclisation methods

Method for 1229U91 on-resin. The linear protected peptide resin Boc-Ile-Glu(OAll)-Pro-Dap(Aloc)-Tyr(*t*Bu)-Arg(Pbf)-Leu-Arg-(Pbf)-Tyr(*t*Bu)-Rink resin was OAll/Aloc deprotected using the Thieret method as described above. The resin was then allowed to swell in DMF before a solution of PyClock (3 eq.) in DMF was added followed by DIPEA (10 eq.) The resin was agitated for 6 h and then washed with DMF (×3), MeOH (×3) and Et_2O (×3). Peptide cleavage from resin was performed as described above and the crude peptide purified by RP-HPLC.

Peptide **I** was prepared in the same way, except using Boc-Glu(OAll)-Pro-Dap(Aloc)-Tyr(*t*Bu)-Arg(Pbf)-Leu-Arg(Pbf)-Tyr(*t*Bu)-Rink resin. After Fmoc-based SPPS, the N-terminus of the unprotected Glu residue was Boc-protected by adding Boc anhydride (3 eq.), dissolved in DIPEA (6 eq.) and DMF, to the pre-swelled resin (0.1 eq.) and mixed for 2 h.

Solution phase cyclisation methods

1229U91 was prepared by treating linear peptide **1** (0.1 M) in DMF with PyBOP (2 eq.) and DIPEA (12 eq.) and the reaction mixture was stirred for 2 h. A solution of 20% piperidine in DMF was then added stirring continued for a further 30 min. The solvent was removed *in vacuo* and the residue triturated with cold ether after which the residue was purified by RP-HPLC or extracted with 1 : 1 ACN–H_2O and the extract purified by RP-HPLC.

In the same way, peptide **2** was reacted to yield peptide **II**. When peptide **3** was treated in this way compound **III** was obtained as a minor component. The cyclic monomeric peptide, cyclo(Glu,Lys)-Ile-Glu-Pro-Lys-Tyr-Arg-Leu-Arg-Tyr (**IIIa**) was obtained as the major component.

In the same way, an equimolar mixture of **1** and **2** was treated to give a mixture of products **IV**, **II** and **1229U91** which were isolated by RP-HPLC.

An equimolar mixture of **1** and **4** was treated to give a mixture of products **V**, **VI** and **1229U91** which were isolated by RP-HPLC.

Solution phase formation of cyclic dimers *via* orthogonal protection. The partially protected peptide **6** (1 eq.) and PyClock (4 eq.) were dissolved in DMF (100 mg mL^{-1}). TMP (24 eq.) was added followed by the partially protected peptide **5** (1 eq.). The reaction mixture was stirred at ambient temperature for 2 h. Volatile components were removed *in vacuo* and the resulting residue was treated with a small volume of TFA precipitated with cold Et_2O to yield crude peptide **10**. Selective deprotection of the OAll/Aloc groups was performed by the method of Thieret as described above to give peptide **11**. Cyclisation of **11** was achieved by dissolving the peptide in DMF (5 mg mL^{-1}) and TMP (24 eq.) and PyClock (4 eq.) were added and the mixture stirred for 6 h. Volatile components were removed *in vacuo* and the resulting residue was treated with a small volume of TFA and precipitated with cold Et_2O to yield crude peptide **12**. Finally peptide **12** was dissolved in a solution of 10% piperidine in DMF and mixed for 1 h. Volatile components were removed *in vacuo* and the resulting residue was treated with a small volume of TFA and crude peptide was precipitated with cold Et_2O. The precipitate was purified by RP-HPLC to give 1229U91.

In the same way, peptide **VII**, was prepared by coupling linear precursors **6** and **7** to give **13** followed by OAll/Aloc deprotection, and cyclisation and Fmoc-deprotection. Peptide **III** was prepared in the same way from linear peptides **8** and **9**.

Conjugation methods

Compound **VIII** was achieved by dissolving purified 1229U91 (1 eq.) in DMF and DIPEA (12 eq.) and adding a solution of the NHS-activated Rhodamine B derivative[33] (0.7 eq.) in DMF which was stirred for 2 h.

The click reaction to prepare peptide **IX** involved dissolving the purified peptide **IV** (1 eq.) in H_2O and adding a solution of the azidocoumarin[34] (4 eq.) in DMF to give a 1 : 3 ratio of H_2O to DMF. Copper sulfate (10 eq.), TBTA (10 eq.) and sodium ascorbate (10 eq.) were then added and the reaction mixed for 3 h.

Receptor binding methods

Preparation of membranes from mouse brain. To test the Y_1R affinity of the synthesised ligands, receptor binding assays (described below) were performed on crude membranes prepared from the brains of Y_2R- and Y_4R-deficient mice (Y_2-/-Y_4-/-), where Y_1R accounts for the majority of remaining Y receptors. Membranes were prepared following modified membrane extraction protocol published elsewhere.[46] In brief, fresh frozen Y_2-/-Y_4-/- mouse brains were cut into small cubes and homogenised in ice-cold homogenisation buffer (50 mM Tris-HCl, 10 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH = 7.4, supplemented with 1 mg mL^{-1} bacitracin (250 000 U; Calbiochem-Novabiochem., La Jolla, CA, USA) prior to use on ice with a glass homogeniser (Wheaton, USA) using 30 strokes. Subsequently, the homogenates were centrifuged at 32 000*g* for 15 minutes at 4 °C. The resulting pellet was re-suspended in ice-cold homogenisation buffer and re-homogenised using 30 strokes on ice, followed by centrifugation at 32 000*g* for 15 minutes at 4 °C to obtain the final pellet. The final pellet was re-suspended in ice-cold homogenisation buffer and flash frozen in liquid nitrogen. The protein concentration of the suspension was determined using Bradford protein assay (Quick Start™ Bradford Protein Assay, Bio-Rad Laboratories Pty., Ltd, Hercules, CA, USA).

Cell culture

HEK293 T and 293TR cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% foetal 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), have both been described elsewhere.[36,47]

[^{125}I]PYY radioligand binding assays

Competition assays were performed on Y_2-/-Y_4-/- mouse brain membrane preparations or 293TR Y1 receptor GFP membranes following procedures published previously.[36,46,47] Briefly, for mouse brain preparations, equal volumes (25 μL) of non-radioactive ligands and ^{125}I-human polypeptide YY (^{125}I-hPYY, 2200 Ci $mmol^{-1}$; PerkinElmer Life Science Products, Boston, MA, USA) were added into each assay. The final concentration of ^{125}I-hPYY in the assay was 25 pM. The binding of ^{125}I-hPYY was competed by Y_1R ligands of interest at increasing concentrations ranging from 10^{-12} M to 10^{-6} M. Non-radioactive human PYY (Auspep, Parkville, VIC, Australia) at 10^{-6} M was used as the non-specific binding control. The reaction was initiated by the addition of 50 μL of membrane suspension containing 30 μg of protein into the assay mixture and incubated for 2 hours at room temperature. After the incubation, each sample was layered with 200 μL of pre-cooled (4 °C) horse serum and centrifuged at 13 000*g* for 4 minutes to separate of bound from free ^{125}I-PYY. The supernatant solution was removed and resultant pellet was harvested and counted for radioactivity using a γ-counter (Wallac 1470 WIZARD® Gamma Counter; PerkinElmer Life Sciences, Turku, Finland).

Using membranes from the 293TR Y1 receptor-sfGFP cell line (after tetracycline induction, prepared as Kilpatrick[36,47]), competition binding assays were performed for 90 min at 21 °C in buffer (25 mM HEPES, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.1% bovine serum albumin, 0.1 mg ml^{-1} bacitracin; pH 7.4), increasing concentrations of unlabelled ligands (10^{-12} M to 10^{-6} M, duplicate) and [^{125}I]PYY (15 pM). Membrane bound radioligand was separated by filtration through Whatman GF/B filters soaked in 0.3% polyethyleneimine on a Brandel cell harvester, and retained radioactivity was quantified using a gamma-counter (Packard Cobra II, Perkin Elmer, Waltham MA, U.S.A.). Non-specific binding in these experiments comprised less than 5% of total counts, and was subtracted from the data.

In both sets of data, IC_{50} values were calculated from displacement curves (repeated 2–4 times for each peptide, fitted using non-linear least squares regression in GraphPad Prism 5.01 (Graphpad software, San Diego CA, U.S.A.). The assays using membrane preparations from Y2Y4 knockout animals gave a less uniform distribution of results than the recombinant cell assay data. The IC_{50} values and 95% confidence interval measure was selected as more suitable to describe the variability of this data set. In the recombinant cell assay data, the Cheng–Prusoff equation was used to convert IC_{50} measurements to pK_i values (±SEM).

Functional analysis of Y receptor–arrestin recruitment

This analysis used bimolecular fluorescence complementation (BiFC) based detection of Y receptor – β-arrestin2 association, as described previously (Kilpatrick refs). Y1 arrestin or Y2 arrestin BiFC cell lines were seeded at 40 000 cells per well onto poly-D-lysine coated 96 well black clear bottomed plates (655090, Greiner Bio-One, Gloucester, U.K.), and experiments were performed once cells reached confluence at 24 h. Medium was replaced with DMEM/0.1% bovine serum albumin (BSA), and if appropriate cells were pretreated for 20 min at 37 °C with 1229U91 analogues (3–100 nM). NPY, PP (Bachem, St. Helens, U.K.) or other ligands were then added for 60 min (10^{-11} M–3×10^{-6} M, triplicate wells). 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 the permeable dye H33342 (2 μg ml^{-1} in PBS, Sigma). H33342 was then removed by a final PBS wash. Images (4 central sites per well) were acquired automatically on an IX Ultra confocal platereader (Molecular Devices, Sunnyvale CA, U.S.A.), equipped with a Plan Fluor 40× NA0.6 extra-long working distance objective and 405 nm/488 nm laser lines for H33342 and sfGFP excitation respectively.

An automated granularity algorithm (MetaXpress 5.1, Molecular Devices) identified internal fluorescent compartments within these images of at least 3 μm diameter (range set to 3–18 μm). For each experiment, granules were classified on the basis of intensity thresholds which were set manually with reference to the negative (vehicle) or positive (1 μM NPY, or 100 nM PP) plate controls. The response for each data point was quantified as mean granule average intensity per cell, from assessment of 12 images (4 sites per well in triplicate), normalised to the reference agonist response. Concentration response curves were fitted to the pooled data by non-linear least squares regression (Graphpad Prism), and antagonist pK_b values were calculated from agonist curve shifts using the Gaddum equation ($pK_b = \log[CR - 1] - \log[B]$, where [B] is the antagonist concentration, and CR is the EC_{50} ratio for the agonist response in the presence and absence of antagonist).

Fluorescent imaging of compound VIII

293TR Y1-GFP or Y4-GFP cells were seeded at 20 000 cells per well in poly-D-lysine coated 96 well imaging plates (Greiner 655090), treated with 1 μg ml^{-1} tetracycline for 18–21 h and then used in experiments at confluence. Cells were incubated in HEPES-buffered saline solution (HBSS) including 0.1% BSA, H33342 (2 μg ml^{-1}) and varying concentrations of competitor ligands (10^{-10} M to 10^{-6} M) for 2 min, prior to the addition of compound VIII at a final concentration of 1 nM (Y1-GFP) or 100 nM (Y4-GFP). Incubations were continued for 30 min at 37 °C, after which the media was replaced with HBSS/0.1% BSA (to remove free compound VIII). The cells were immediately imaged (2 sites per well) on a Molecular Devices IX Micro epifluorescence platereader using excitation/emission filter sets appropriate for H33342 (DAPI), Y receptor-GFP (FITC), and the rhodamine ligand (TRITC). Read time was less than 10 min, and repeated "total" wells at the end of the read confirmed stable binding of the fluorescent ligand over this period. Bound ligand fluorescence was quantified by granularity analysis (2–3 μm diameter granules; count per cell using MetaXpress), and normalised to positive (totals 100%) and negative (0%, presence of 1 μM NPY) controls. NPY and BIBO3304 IC_{50} values were then determined using Graphpad Prism, as for radioligand binding.

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