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Rapid Parallel Synthesis Applied To the Optimization Of A Series Of Potent Nonpeptide Neuropeptide Y-1 Receptor Antagonists

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ABSTRACT:
This study describes the integrated application of parallel synthesis and computational chemistry to the design of potent nonpeptide antagonists for the neuropeptide Y-1 (NPY1) receptor. A lead molecule was modeled in the active site of the NPY1 receptor, and a potentially fruitful region for analog construction was identified. Synthesis of suitable scaffolds followed by solution phase generation of a small library of analogs produced a compound with 5-fold improvement in binding over the already potent lead. This new compound was shown to be an unanticipated side product of the parallel synthesis reaction.

During the past fifteen years, a multidisciplinary effort has been initiated to understand the chemistry, pharmacology, and potential clinical applications of agonists and antagonists of neuropeptide Y (NPY), the sequence of which was first reported in 1982.[1] NPY is synthesized in both the peripheral and central nervous systems, and has been implicated in the regulation of feeding, energy metabolism, vascular tone, learning and memory, and release of pituitary hormones .[2,3] Two related peptides, PYY and pancreatic polypeptide, are produced in the gut and pancreas, respectively.[4]

At least six receptors for members of the NPY family have been identified pharmacologically,[5] and five of these have been cloned.[6-12] The Y-1 receptor binds NPY and PYY, as well as an analog in which the glutamine residue of position 34 of NPY has been replaced with proline from the corresponding position of pancreatic polypeptide. In contrast to the Y-1 receptor, which does not bind the C-terminal fragment NPY13-36, the Y-2 receptor binds this fragment with reasonably high affinity. Conversely, the Y-2 receptor has poor affinity for [Pro^{34}]NPY. Although the Y-1 and Y-2 receptors bind NPY and PYY with equal affinity, a Y-3 receptor that recognizes NPY but not PYY has been characterized pharmacologically.[13] A pancreatic polypeptide receptor, denoted Y-4 or PP1, has recently been

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cloned.[8] A Y-5 receptor has been cloned which has peptide pharmacology closer to that of a feeding receptor.[9a] Finally, a Y-6 receptor has been cloned from several species, but is not functionally expressed in the human or rat.[9b]

Recently, there have been several reports of the synthesis of non-peptide Y-1 receptor antagonists (Figure 1).[14-18] These non-peptide antagonists should be important tools for revealing the roles of the Y-1 receptor. One such series, developed at Lilly, derives from a benzimidazole platform.[16,17] A member of this benzimidazole series, **II**, had a K_i of 6 nM in the Y-1 binding assay. Compound **II** was itself the result of extensive chemical modification of an initial screening lead with an affinity of 3 μM.[18] The purpose of the present study was to further optimize the sidechain pharmacophore of this series by integrating computational methods and parallel synthesis.

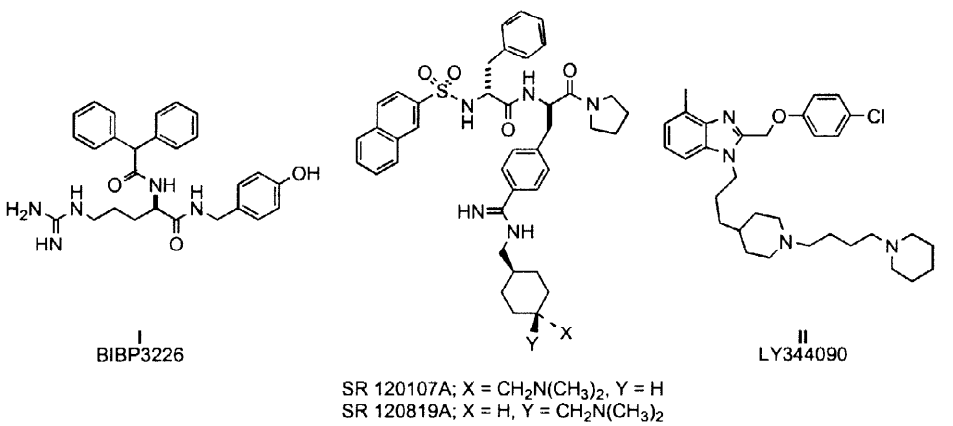


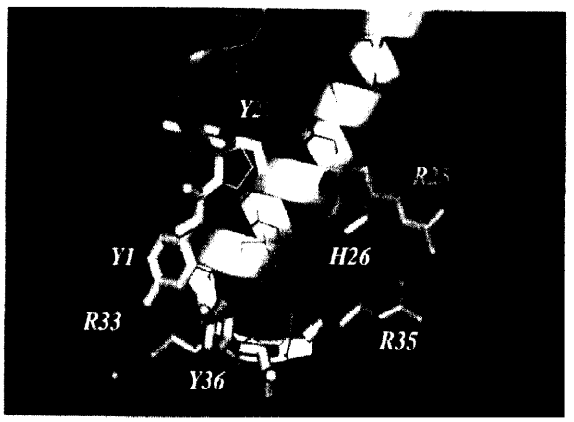
Figure 1. Nonpeptide neuropeptide Y-1 antagonists.

Results

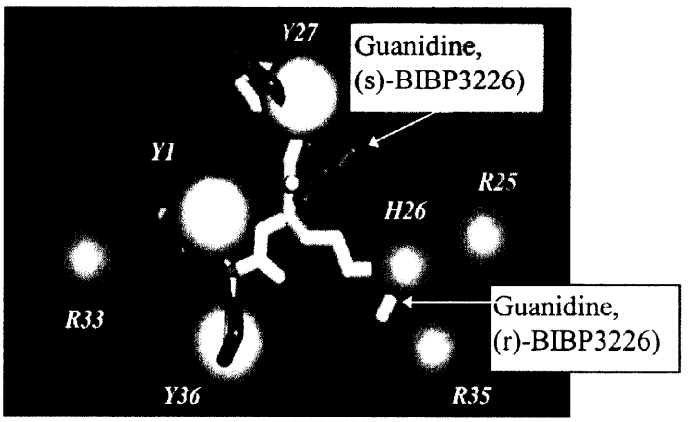
As a first step toward optimizing **II**, computer modeling studies were carried out to identify positions that warranted further optimization. A three-dimensional model of NPY was constructed based on the published crystal structure[19] of a related peptide, avian pancreatic polypeptide (figure 2a). The model focused on residues Y1, Y36, R33, and R35, since alanine scanning[20] suggested that these sidechains were important for receptor activity.

Our initial strategy was to determine whether the spatial arrangement of the these NPY sidechains could be overlaid on BIBP3226 (**I**, Figure 1), a small-molecule NPY-1 receptor antagonist with functional groups that resemble the side-chains from one arginine and two tyrosines of NPY. The differential affinities of the two enantiomers of **I** (R-isomer, 4 nM; S-isomer, > 1 μM) were used to test the validity of the superimposition. In agreement with binding data, when the benzhydryl moiety of **I** was aligned with Y1 and Y36 of NPY, the guanidinium group of the R-enantiomer of compound **I** aligned with a very positively charged region defined by R25, H26, and R35 of the NPY pharmacophore, while the S-isomer aligned its guanidinium group in the opposite direction, toward a hydrophobic region (figure 2b, see experimental section for details). Free energy calculations confirm this conclusion (Table 1).

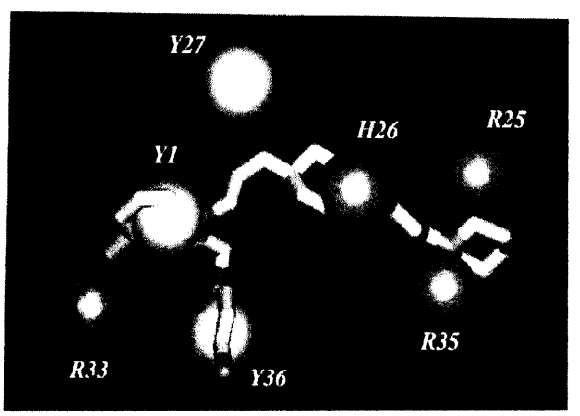
Figure 2. Computational Models of the NPY Pharmacophore and Small Molecule Mimetics BIBP3226 and LY344090



a. Model of the NPY Pharmacophore



b. BIBP3226 (**I**) Superimposed on the NPY Pharmacophore (R-enantiomer: light; S-enantiomer: dark)



c. LY344090 Superimposed on the NPY Pharmacophore

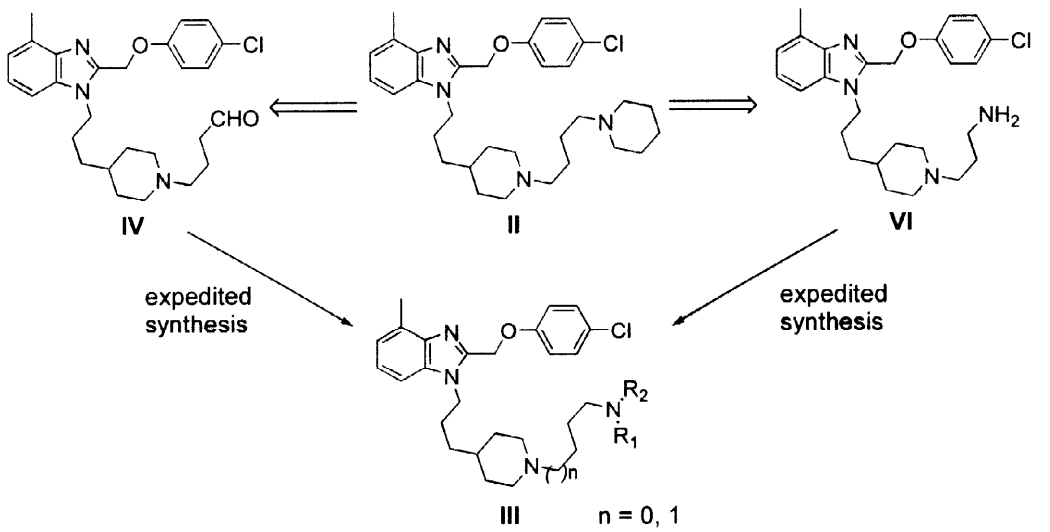
Table 1. Comparison of enantiomers of **II** to the NPY pharmacophore model

isomer	RMS deviation (Å)	ΔE_{relax} (kcal/mol)	Y-1 binding K_i (nM)
R	1.0	49	4
S	2.1	$>10^4$	>1,000

Benzimidazole compounds such as **II** have demonstrated equal binding affinity to **I**. As with **I**, the benzimidazole structure-activity relationships have emphasized the importance of aromatic moieties at the binding site, as well as cationic groups such as the two piperidine moieties in **II**. Because of these gross structural similarities, we superimposed **II** on the NPY model. In the best-fitting model, two of the phenyl rings of **II** projected into the space occupied by tyrosines 1 and 36 of NPY, while the two piperidine groups projected into the cationic region bounded by histidine 26 and arginines 25 and 35. We were intrigued with the idea that the distal piperidine group may not have been interacting with the receptor optimally within the three-dimensional structural constraints of the benzimidazole architecture, and that other nitrogen-containing functionalities might optimize this interaction.

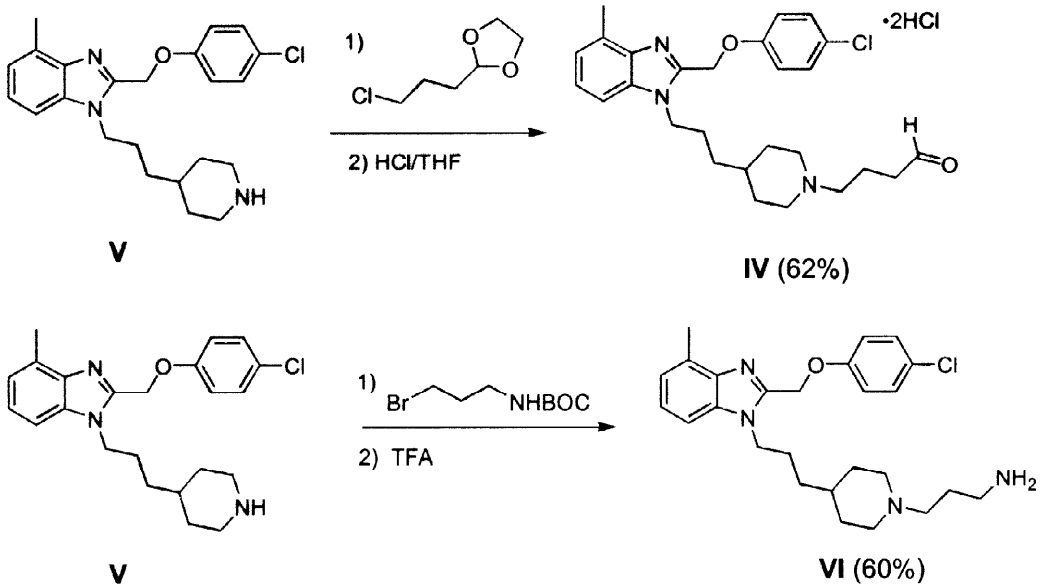
We set out to test this hypothesis by synthesizing a series of compounds of general structure **III** (Scheme 1), representing a library of molecules with varying chain lengths and varying substituents on the terminal nitrogen.

Scheme 1



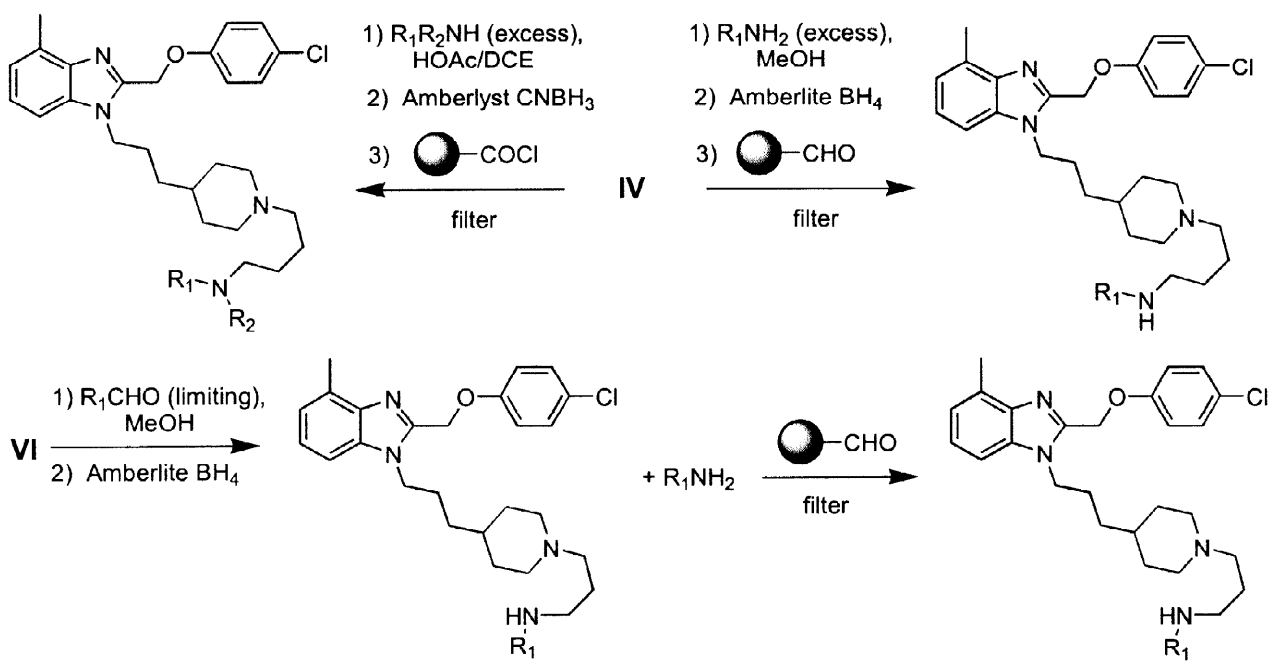
A straightforward and practical methodology for the rapid synthesis of amines was recently reported by our group.[21] Application of this methodology to the synthesis of molecules resembling structure **III** required the synthesis of aldehyde **IV** and amine **VI** as suitable substrates for expedited synthesis, as outlined in Scheme 2. Aldehyde **IV** was prepared in two steps from piperidine **V**, an intermediate in the synthesis of compound **II**, by alkylation with 1-(3-chloropropyl)dioxolane followed by hydrolysis of the acetal. Amine **VI** was synthesized by alkylation with N-Boc-3-bromopropylamine followed by deprotection.

Scheme 2



With these starting materials in hand, a small library of 84 secondary and tertiary amines of general structure **III** was synthesized and analyzed for affinity at the NPY-1 receptor. Either aldehyde **IV** was reacted with a 1.5 equivalents of primary amine, or 1.5 equivalents of amine **VI** was reacted with an aldehyde to form the corresponding Schiff base (Scheme 3).

Scheme 3



The Schiff base thus formed was then reduced with resin-bound borohydride to yield a mixture of product secondary amine and starting primary amine. The undesired starting material was then removed via scavenging with a resin bound aldehyde.[21]

Of the 84 compound synthesized, 65 compounds were synthesized by reductive alkylation of aldehyde **IV** with commercially available amines, and 19 compounds were synthesized via the reductive alkylation of amine **VI** with commercially available aldehydes. The commercial amine and aldehyde reactants were selected using molecular dissimilarity methods as described in Materials and Methods.

Several representative library members are included in Table 2. From this first library set, some interesting trends can be noted. Certain substitutions on the distal piperidine ring proved to have a dramatic effect on the activity of the molecule. Substitution at the piperidine 4-position was generally unfavorable, with a 3-fold loss in activity on substitution of a simple methyl substituent (entry **12**), and a 4-fold loss in activity for the 4-benzyl compound (entry **10**). Substitution at the piperidine 3-positions, however, gave molecules of widely varying activity (see entry **14** vs. entry **3**). When the distal piperidine was replaced by piperazine, an examination of various 4-alkyl substituents very large, inflexible R-groups or R-groups with attenuated basicity at the 4-position were generally less active (entries **2**, **4**, **6**, and **8**).

Although twenty three of the library members displayed Ki values less than 20 nM (ten of these are listed in Table 2), including seven amines with binding affinities below 10 nM, only one compound exceeded the potency of the lead structure: the compound derived from reaction of **IV** and N-methyl-2-aminoethylpyrrole, with a K_i at the Y-1 receptor of about 1 nM. This compound was selected for resynthesis for the purposes of further characterization and testing.

In the course of these studies, however, it became clear that the spectroscopic data were not consistent with structure **VII**, the anticipated product of reductive amination. This structure was unambiguously ruled out when it was found that aldehyde **IV** and N-methyl-2-aminoethylpyrrole yielded the same adduct with 1:1 acetic acid/methanol as solvent *in the absence of any reducing agent.* A reasonable alternative structure that was consistent with all the spectroscopic data is **VIII** (Scheme 4), resulting from a Pictet-Spengler cyclization[22] between the aldehyde and amine components.

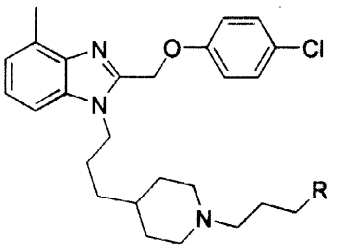
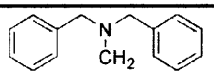
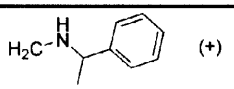
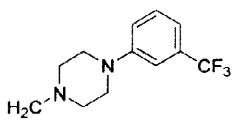
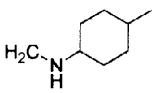
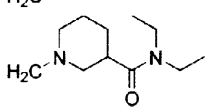
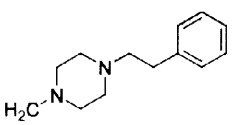
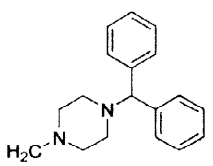
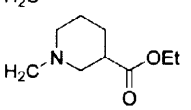
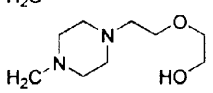
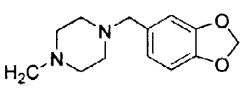
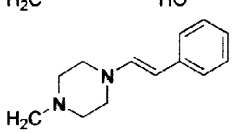
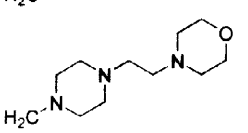
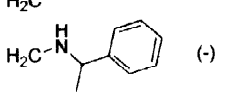
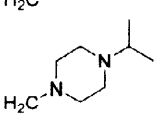
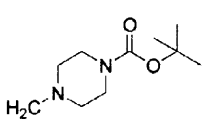
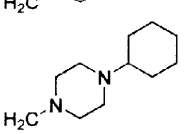
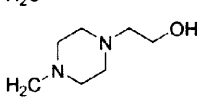
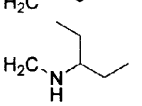
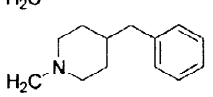
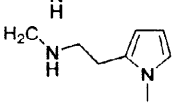


Table 2: Binding constants for Representative Library members

Entry	R	Ki (nM)	Entry	R	Ki (nM)
1		700[a]	11		19.9 ± 2.2
2		200[a]	12		19.5 ± 0.4
3		100[a]	13		20[a]
4		100[a]	14		14.3 ± 2.3
5		80[a]	15		14.1 ± 1.2
6		80[a]	16		11.8 ± 0.2
7		35.9 ±0.6	17		11.7 ± 0.0
8		40[a]	18		7.60 ± 0.85
9		30[a]	19		6.15 ± 0.17
10		25.8 ± 2.4	20[d]		1.20 ± 0.59

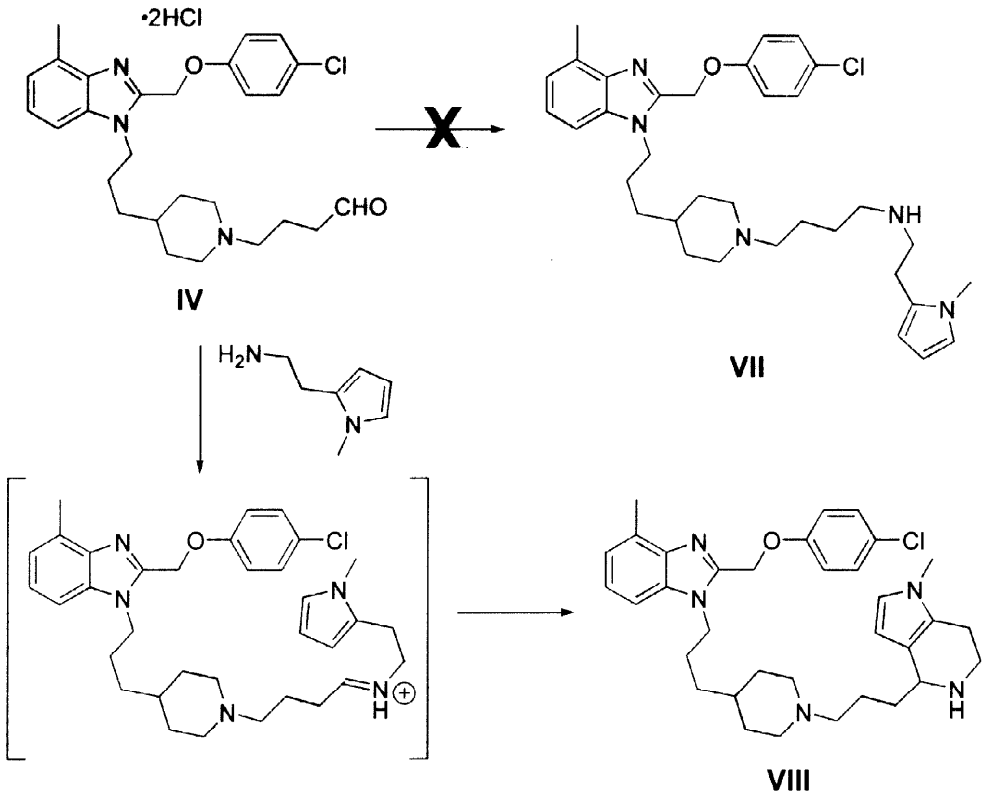
[a]3-point K_i determination

[b]synthesized from the amine tether

[c]initial lead structure synthesized for method verification

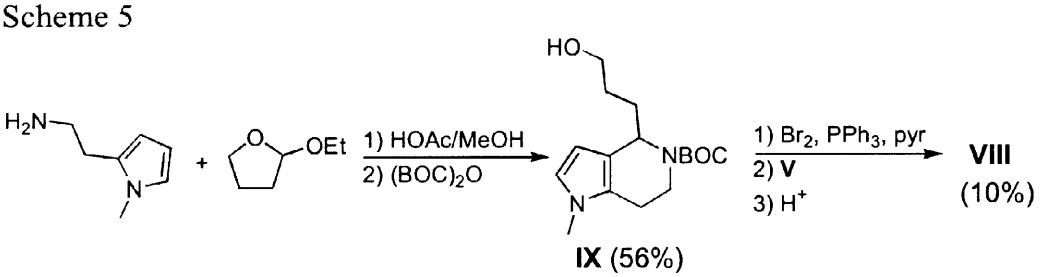
[d]actual structure shown to be Pictet-Spengler product (vide supra)

Scheme 4



Structure **VIII** was confirmed by an alternate synthetic route outlined in Scheme 5: N-methyl-2-aminoethylpyrrole was condensed with 2-ethoxytetrahydrofuran to give alcohol **IX** after BOC-protection of the amine nitrogen. This alcohol was converted to the corresponding bromide. Alkylation of piperidine **V** with this bromide followed by deprotection in neat TFA gave material that was spectroscopically identical to that obtained by direct condensation of aldehyde **IV** with N-methyl-2-aminoethylpyrrole. Receptor binding affinity was essentially the same as for **VIII** prepared by parallel synthesis.

Scheme 5



The original intended product **VII** was synthesized by an unambiguous route and found to have a K_i in Y-1 binding of 10 nM, about 10-fold weaker than **VIII**.

Functionally, **VIII** was an antagonist at the human Y-1 receptor, blocking the NPY-mediated inhibition of cyclic AMP accumulation in SK-N-MC cells with a K_i of 13 nM. For comparison, **II** had a K_i value of 45 nM in the same model.

Conclusion

A surprising finding of the present study was the observation that the only lead that surpassed the starting compound **II** in affinity was the result of an unanticipated side-reaction. Unanticipated cyclizations and other serendipitous reactions have led to affinity breakthroughs in several of our other neuropeptide programs.[23, 24] These unforeseen events can be especially valuable because they can move a chemical series out of an unproductive local minimum and into a different region that is more amenable to improvement. Computational chemistry assisted in the current study by suggesting a region of the molecule whose modification might provide for a boost in potency. Even when the chemistry goes as planned, the number and diversity of products from parallel synthesis increases the likelihood of a totally unanticipated breakthrough in structure-activity relationships. The present study shows that combinatorial chemistry can stack the deck in favor of unexpected good fortune.

Experimental

Molecular Modeling

A three-dimensional model of NPY was constructed based on its high degree of sequence homology with a structurally determined and related peptide, avian pancreatic polypeptide, deposited as **1app** in the Protein Databank.[19] After residue alignment and subsequent coordinate transfer, the NPY model was first energy minimized, and then subjected to 100 psec of Langevin molecular dynamics simulation at 300 °K in order to relieve backbone strain and optimize sidechain packing. QUANTA/CHARMm, Version 4.1 was used for sequence homology construction, energy minimization, and molecular dynamics simulations. Setup parameters used in these calculations were a 12 Å non-bonded cutoff distance, a RMS force of 0.01 kcal $mole^{-1}$ Å and 0.001 energy tolerance for determining minimization convergence, and a dielectric constant of 1.0, since the peptide was solvated with a 6.0 Å shell of TIP3 water molecules. The Cartesian forcing algorithm within the QUANTA molecular similarity module was used to calculate the best-fit free energy values. For the calculation of values in Table 1, the positions of each of the pharmacophore elements were defined as the guanidyl carbon for the arginines and the mid-points of the aromatic systems for the tyrosines. The NPY molecule was held rigid, and the similar functional elements from each of the BIBP3226 isomers were then constrained to the same space. As a result, each isomer was forced to adapt to the NPY

pharmacophore model. As a measure of similarity, relaxation energy (ΔE_{relax}) and root-mean-square (RMS) deviation of fit to the NPY model were calculated and compared with K_i values in Y-1 binding. The relaxation energy is defined as the difference in free energy between the best-fit and the fully minimized structures. The RMS deviation of fit is defined as the RMS difference between equivalent functional groups of NPY and each of the BIBP3226 isomers.

Selection of parallel synthesis reactants

Primary amines for the reductive amination were selected as follows. The ACD database of commercially available chemicals was searched for primary amines using the MACCS software package (MDL, San Leandro, CA). The 6,642 matches were reduced to 1,636 by eliminating compounds with carboxylic acids and those with with m.w. > 250. The list was further reduced to 577 by similarity clustering using the "leader" algorithm. Eliminating compounds with two primary amines (or other undesired features) reduced the list to 386, which was then reduced to < 100 by hand-selection. Secondary amines and aldehydes were selected in a similar manner.

Preparation of an NPY antagonist library

General procedure for reductive amination of an aldehyde and a primary amine: To a 4 ml screw cap glass vial is added 120 μl methanol, 30 μmol of a primary aliphatic amine, and 20 μmol of aldehyde IV as the dihydrochloride salt. The vial is sealed with a teflon backed cap and the solution then shaken for 2-3 hours to allow for imine formation, then treated with approximately 30 mg (2.5 mmol BH_4^-/g resin, 75 μmol) of Amberlite IRA-400 borohydride resin (Aldrich Chemicals). The slurry is then shaken an additional 24 hours to effect reduction to the secondary amine, then 150 μl methylene chloride and approximately 40 mg (1 mmol/g resin, 40 μmol) polystyrene-linked benzaldehyde resin is added to the vial and the mixture shaken overnight, then filtered through a cotton plug, and the residual solids rinsed with methanol. Evaporation under a flow of air or nitrogen, followed by drying for several hours at room temperature in a vacuum oven, yields a product of typically 80-95% purity in yields ranging from 50-99%.

*Reaction of **IV** with (-)-α-methylbenzylamine (Table 2, entry 7)*: ^{1}H NMR ($CDCl_3$): 6.9-7.4 (m, 12H); 5.42 (s, 2H); 4.3 (m, 2H); 2.8 (m, 1H); 2.9-3.0 (m, 2H); 2.4 (m, 2H); 2.8 (s, 3H); 1.3-2.1 (m, 20H). Ion spray MS: 574.4 (M+).

*Reaction of **IV** with 4-methylcyclohexylamine (Table 2, entry 12)*: ^{1}H NMR ($CDCl_3$): 6.9-7.2 (m, 7H); 5.15 (s, 2H); 4.0 (m, 2H); 2.9-3.0 (m, 1H); 2.7 (m, 2H); 2.5 (s, 3H); 2.0-2.2 (m, 2H); 0.7-1.7 (m, 29H). Ion spray MS: 565.2 (M+H).

*Reaction of **IV** with (+)-α-methylbenzylamine (Table 2, entry 11)*: ^{1}H NMR ($CDCl_3$): 6.9-7.4 (m, 12H); 5.42 (s, 2H); 4.3 (m, 2H); 2.8 (m, 1H); 2.9-3.0 (m, 2H); 2.4 (m, 2H); 2.8 (s, 3H); 1.3-2.1 (m, 20H). Ion spray MS: 573.2 (M+H).

*Reaction of **IV** with 2-aminopentane (Table 2, entry 19)*: ^{1}H NMR ($CDCl_3$): 6.9-7.2 (m, 7H); 5.28 (s, 2H); 4.2 (m, 2H); 2.75-2.9 (m, 2H); 2.62 (s, 3H); 2.5 (m, 1H); 2.2-2.4 (m, 4H); 1.1-1.9 (m, 19H); 0.8 (m, 6H). ion spray MS: 539.5 (M+H).

General procedure for reductive amination of an aldehyde and a secondary amine: To a 4 ml screw cap glass vial is added 30 μmol secondary amine, 20 μmol aldehyde IV as the dihydrochloride salt, and 120 μl 10% acetic acid in dichloroethane. The vial is sealed with a teflon-backed cap and shaken at room temperature for 1 hour, and 30 mg (3.3 mmol $CNBH_3^-$/g resin, 0.1 mmol) of cyanoborohydride resin is added. After shaking overnight, 20 mg (2.5 mmol Cl^-/g resin) of acid chloride resin and 150 μl of 10% acetic acid in dichloroethane are added and the vial is shaken for 5 hours. The reaction mixture is filtered through cotton and the solvent evaporated to provide the desired tertiary amine product. Traces of reduced aldehyde and boron salts which formed can be readily removed by solid phase extraction.

*Reaction of **IV** with dibenzylamine (Table 2, entry 1)*: ^{1}H NMR ($CDCl_3$): 7.0-7.4 (m, 17H); 5.35 (s, 2H); 4.21 (m, 2H); 3.55 (s, 4H); 2.8 (m, 1H); 2.69 (s, 3H); 2.4-2.5 (m, 1H); 2.2 (m, 1H); 1.1-1.9 (m, 18H). Ion spray MS: 649.2 (M+H).

*Reaction of **IV** with 4-trifluoromethylphenylpiperazine (Table 2, entry 2)*: ^{1}H NMR ($CDCl_3$): 7.0-7.4 (m, 11H); 5.35 (s, 2H); 4.2 (m, 2H); 3.2-3.3 (m, 4H); 2.9 (m, 2H); 2.70 (s, 3H); 2.6 (m, 4H); 2.3-2.5 (m, 4H); 1.1-2.0 (m, 15H). Ion spray MS: 682.2 (M+H).

*Reaction of **IV** with N, N-diethylnipecotamide (Table 2, entry 3)*: ^{1}H NMR ($CDCl_3$): 7.0-7.4 (m, 7H); 5.36 (s, 2H); 4.2 (m, 2H); 3.3-3.4 (m, 4H); 2.9-3.0 (m, 4H); 2.7 (s 3H); 2.1-2.4 (m, 5H); 1.1-2.0 (m, 28H). Ion spray MS: 636.4 (M+H).

Reaction of IV with 1-(diphenylmethyl)piperazine (Table 2, entry 4): ^{1}H NMR ($CDCl_3$): 7.0-7.4 (m, 17H); 5.32 (s, 2H); 4.2 (m, 2H); 2.9 (m, 2H); 2.7 (s, 3H); 2.2-2.5 (m, 10H); 1.8-2.0 (m, 4H); 1.4-1.7 (m, 8H); 1.1-1.35 (m, 6H). Ion spray MS: 703.9 (M+H).

Reaction of IV with 1-hydroxyethylethoxypiperazine (Table 2, entry 5): ^{1}H NMR ($CDCl_3$): 6.9-7.3 (m, 7H); 5.35 (s, 2H); 42. (m, 2H); 3.5-3.7 (m, 6H); 2.9 (m, 2H); 2.7 (s, 3H); 2.2-2.6 (m, 10H); 1.75-1.9 (m, 8H); 1.1-1.7 (m, 11H). Ion spray MS: 626.3 (M+H).

Reaction of IV with 1-styrylpiperazine (Table 2, entry 6): ^{1}H NMR ($CDCl_3$): 6.9-7.4 (m, 12H); 6.5 (m, 1H); 6.2-6.3 (m, 1H); 5.35 (s, 2H); 4.2 (m, 2H); 3.2 (m, 2H); 2.9-3.0 (m, 2H); 2.7 (s, 3H); 2.3-2.7 (m, 10H); 1.8-2.0 (m, 5H); 1.1-1.7 (m, 10H). Ion spray MS: 654.5 (M+H).

Reaction of IV with 1-(t-butoxycarbonyl)piperazine (Table 2, entry 8): ^{1}H NMR ($CDCl_3$): 6.9-7.4 (m, 7H); 5.35 (s, 2H); 4.2 (m, 2H); 3.4 (m, 4H);2.9 (m, 2H); 2.7 (s, 3H); 2.3-2.4 (m, 8H); 1.8-1.9 (m, 6H); 1.55-1.7 (m, 4H); 1.48 (s, 9H); 1.1-1.4 (m, 5H). Ion spray MS: 639.5 (M+H).

Reaction of IV with 1-hydroxyethylpiperazine (Table 2, entry 9): ^{1}H NMR ($CDCl_3$): 6.9-7.3 (m, 7H); 5.35 (s, 2H); 4.2 (m, 2H); 3.6 (m, 2H); 2.9 (m, 2H); 2.7 (s, 3H); 2.4-2.6 (m, 8H); 2.3 (m, 4H); 1.8-2.0 (m, 4H); 1.4-1.7 (m, 7H); 1.1-1.4 (m, 6H). Ion spray MS: 582.1 (M+H).

Reaction of IV with 4-benzylpiperidine (Table 2, entry 10): ^{1}H NMR ($CDCl_3$): 6.9-7.3 (m, 12H); 5.36 (s, 2H); 4.2 (m, 2H); 3.54 (m, 1H); 2.8-3.0 (m, 4H); 2.7 (s, 3H); 2.55 (m, 1H); 2.2-2.4 (m, 4H); 1.8-2.0 (m, 6H); 1.4-2.2 (m, 8H); 1.1-1.3 (m, 8H). Ion spray MS: 627.4 (M+H).

Reaction of IV with 1-phenethylpiperazine (Table 2, entry 13: ^{1}H NMR ($CDCl_3$): 6.9-7.3 (m, 12H); 5.35 (s, 2H); 4.2 (m, 2H); 2.75-3.0 (m, 4H); 2.7 (s, 3H); 2.4-2.6 (m, 8H); 2.2-2.35 (m, 4H); 1.8-2.0 (m, 6H); 1.4-1.7 (m, 6H); 1.1-1.4 (m, 5H). Ion spray MS: 642.5 (M+H).

Reaction of IV with ethylnipecotate (Table 2, entry 14): ^{1}H NMR ($CDCl_3$): 6.9-7.3 (m, 7H); 5.36 (s, 2H); 4.05-4.25 (m, 4H); 2.75-3.05 (m, 4H); 2.7 (s, 3H); 2.55 (m, 1H); 2.2-2.4 (m, 4H); 2.1 (m, 1H); 1.5-2.0 (m, 16H); 1.1-1.4 (m, 7H). Ion spray MS: 609.5 (M+H).

Reaction of IV with 1-piperonylpiperazine (Table 2, entry 15): ^{1}H NMR ($CDCl_3$): 6.9-7.3 (m, 7H); 6.82 (s, 1H); 6.7 (s, 2H); 5.92 (s, 2H); 5.34 (s, 2H); 4.2 (m, 2H); 3.4 (s, 2H); 2.9 (m, 2H); 2.68 (s, 3H); 2.2-2.5 (m, 8H); 1.8-2.0 (m, 6H); 1.4-1.7 (m, 8H); 1.1-1.4 (m, 5H). Ion spray MS: 672.3 (M+H).

Reaction of IV with 1-(1-morpholinoethyl)piperazine (Table 2, entry 16): ^{1}H NMR ($CDCl_3$): 6.9-7.3 (m, 7H); 5.34 (s, 2H); 4.2 (m, 2H); 3.7 (m, 4H); 2.9 (m, 2H); 2.68 (s, 3H); 2.2-2.6 (m, 10H); 1.7-2.0 (m, 8H); 1.4-1.6 (m,8H); 1.1-1.3 (m, 7H). Ion spray MS: 651.5 (M+H).

Reaction of IV with 1-isopropylpiperazine (Table 2, entry 17): ^{1}H NMR ($CDCl_3$): 6.9-7.3 (m, 7H); 5.34 (s, 2H); 4.2 (m, 2H); 2.9 (m, 2H); 2.7 (s, 3H); 2.5-2.6 (m, 8H); 2.2-2.4 (m, 4H); 1.8-2.2 (m, 5H); 1.5-1.7 (m, 6H); 1.1-1.4 (m, 5H); 1.05 (d, 6H). Ion spray MS: 580.2 (M+H).

Reaction of IV with 1-cyclohexylpiperazine (Table 2, entry 18): ^{1}H NMR ($CDCl_3$): 6.9-7.3 (m, 7H); 5.34 (s, 2H); 4.2 (m, 2H); 2.9 (m, 2H); 2.7 (s, 3H); 2.4-2.6 (m, 6H); 2.2-2.4 (m, 4H); 1.5-1.9 (m, 18H); 1.1-1.4 (9H). Ion spray MS: 620.3 (M+H).

N-BOC-3-bromopropylamine: 3-Bromopropylamine hydrobromide, 43.8 g (0.200 mole), and di-(tert-butyl) carbonate, 48 g (0.220 mole), were slurried in THF, 1L and chilled with ice / water to 5°C. To this was added 1N NaOH, 220 ml, over approximately 30 minutes, during which time a solution formed. The reaction was stirred overnight and allowed to warm to room temperature (RT). To the reaction mixture was added ethyl acetate and brine. The resulting layers were separated and the aqueous layer was again extracted with ethyl acetate. The combined organics were washed with brine, dried with sodium sulfate, filtered, and evaporated to a slowly crystallizing oil, 42 g (90%), mp 39 °C. FABMS, M+ = 238, calculated mw = 238.

3-(4-Piperidyl)-1-propanol: 3-(4-Pyridyl)-1-propanol, 500 g (3.65 mole) and 125 g 5% Rh/C in 3.4 L ethanol was treated with and hydrogen at 60 °C under 60 psi for 24 hrs. The catalyst was removed by filtration and the filtrate evaporated to form a slush of crystalline solid in liquid. The product, 3-(4-piperidyl)-1-propanol, was collected on a filter as an off-white crystalline solid, mp 65 °C, 447 g (85%). FDMS, M+ = 143, calculated mw = 143.

3-(4-(N-Boc-piperidyl))-1-propanol: 3-(4-Piperidyl)-1-propanol, 71.5 g (0.50 mole) was dissolved in 1.25 L THF and mixed with a solution of potassium carbonate, 82.5 g (0.60 mole) in 1.25 L water. To this solution at 5 °C was added di-(tert-butyl) carbonate, 131 g (0.60 mole) and the resulting mixture was stirred and allowed to come to RT over night. The reaction mixture was diluted with ethyl acetate and the layers were separated. The aqueous layer was again extracted with ethyl acetate and the combined organics were washed with brine, dried with sodium sulfate, filtered and evaporated to a thick oil. The crude product was purified over silica gel eluting with a gradient of 100% hexane to 1:1 hexane / ethyl acetate. The BOC protected product weighed 70 g (57%). FDMS, M+ = 243.1, calculated mw = 243.35. Elemental analysis, C13 H25 N O3, CHN.

3-(4-(N-Boc-piperidyl))-1-bromopropane: Triphenylphosphine, 75 g (0.280 mole) was dissolved in 600 ml fresh dichloromethane and chilled to 5 °C. To this solution was added bromine, 15 ml (0.290 mole) over approximately 20 minutes keeping the temperature below 10 °C. Near the end of the addition, the color of the reaction mixture retained a yellow/orange

color. Small portions of triphenylphosphine were added to the reaction mixture so as to cause dissipation of the color and yield a nearly white mixture. The 3-(4-(N-BOC-piperidyl))-1-propanol, 48.6 g (0.200 mole), was dissolved in 200 ml dichloromethane and to it was added pyridine, 22.6 ml (0.280 mole). The resulting solution was added to the bromine reagent over 10 minutes. The reaction was stirred overnight and the temperature was allowed to reach RT. The reaction was evaporated to a slush and mobilized by adding hexane and diethyl ether. The solids (triphenylphosphine oxide and pyridine hydrobromide) were removed by filtration and washed well with ether and hexane. The combined filtrate and washes were evaporated and the resulting oil was purified by silica chromatography eluting with a gradient of 100% hexane to 1:1 hexane / ethyl acetate. The product yield was 55.4 g (90%) of oil, which solidified on chilling. FDMS, M = 305.0, calculated mw 306.25. elemental analysis, C13 H24 Br N O2, CHN. 300 mHz nmr, $CDCl_3$, (m, 2H, 1.05 ppm), (m, 2H, 1.30 ppm), (s, 10H, 1.40 ppm), (d, 2H, 1.60 ppm), (m, 2H, 1.83 ppm), (t, 2H, 2.65 ppm), (t, 2H, 3.36 ppm), (m, 2H, 4.08 ppm).

1-(3-(4-(1-(N-BOC-3-aminopropyl)piperidyl))propyl)-2-(4-chlorophenoxymethyl)-4-methylbenzimidazole: 1-(3-(4-Piperidyl)propyl)-2-(4-chlorophenoxymethyl)-4-methyl benzimidazole 1.0 g (2.5 mMol), sodium bicarbonate, 295 mg (3.5 mMol), and DMF, 100 ml, were combined and heated to 40 °C for 30 minutes. To this was added N-BOC-3-bromopropylamine, 715 mg (3.0 mMol), and the reaction mixture was heated to 120 °C for 4 hours and then allowed to cool to RT. The majority of the DMF was removed by evaporation and the residue was dissoved in ethyl acetate and saturated sodium bicarbonate solution, 25 ml each. The layers were separated and the aqueous layer was extracted twice more with fresh ethyl acetate, 25 ml each. The combined organics were washed with brine, dried with sodium sulfate, filtered and evaporated to yield an oil. The crude oil was chromatographed on silica and eluted with a gradient of 100% hexane to 1:1 hexane / ethyl acetate to yield 893 mg oil (64%) as product. FDMS m+ = 554, calculated mw 554. 300 mHz nmr, $CDCl_3$, (s, 2H, 1.14 ppm), (m, 3H, 1.20 ppm), (s, 9H, 1.36 ppm), (m, 4H, 1.55 ppm), (m, 4H, 1.78 ppm), (T, 2H, 2.30 ppm), (s, 3H, 2.60 ppm), (d, 2H, 2.83 ppm), (m, 2H, 3.10 ppm), (t, 2H, 4.10 ppm), (s, 2H, 5.24 ppm), (m, 1H, 5.66 ppm), (d, 2H, 6.91 ppm), (m, 1H, 7.00 ppm), (m, 4H, 7.12 ppm).

1-(3-(4-(1-(3-Aminopropyl)piperidyl))propyl)-2-(4-chlorophenoxymethyl)-4-methylbenzimidazole (VI): The BOC protected amine, 800 mg (1.4 mmol), was treated with anhydrous TFA, 20 ml, at RT for 60 minutes. The TFA was removed in vacuo and the residue was dissolved in water and extracted with diethyl ether and dichloromethane. The aqueous layer was treated with saturated potassium carbonate solution and extracted with dichloromethane and ethyl acetate. The combined extracts from the basic layer were washed with brine, dried with anhydrous potassium carbonate, filtered, and evaporated to an oil, 600 mg, (94%). FDMS, M+ = 455, calculated mw = 455. 300 mHz nmr, CDCl3, (m, 4H, 1.14 ppm), (m, 2H, 1.26 ppm), (m, 1H, 1.35 ppm), (m, 4H, 1.56 ppm), (m, 4H, 1.80 ppm), (t, 2H,

2.30 ppm), (s, 3H, 2.66 ppm), (t, 2H, 2.68 ppm), (d, 2H, 2.85 ppm), (t, 2H, 4. 16 ppm), (s, 2H, 5.30 ppm), (d, 2H, 6.95 ppm), (m, 1H, 7.05 ppm), (m, 4H, 7.20 ppm).

2-(4-Chlorophenoxymethyl)-4-methylbenzimidazole: 2,3-Diaminotoluene, 25 g (0.205 mole) was dissolved in 700 ml methanol and to it was added cautiously a solution of anhydrous HCl, 38 g, in methanol, 700 ml. After the mixture cooled, the precipitated solid was collected on a filter and vacuum dried, 28 g. A second crop of 5 g was collected. The first crop material was used in the next step without further treatment.

4-Chlorophenoxy acetonitrile, 25 g (0.15 mole) was dissolved in methanol, 1.5 L, and the resulting solution was stirred at RT. To this was added in one portion, sodium methoxide,8.1 g (0.15 mole) which resulted in a cloudy mixture. This was stirred at RT under a nitrogen atmosphere for 45 minutes and then treated with the diaminotoluene dihydrochloride, 28 g (0.14 mole). After stirring overnight, the reaction mixture was diluted with water, 3 L, to give a tacky solid precipitate. The supernatant was removed by decantation and replaced with dichloromethane so as to dissolve most of the precipitate. The dark solution was filtered to remove talc-like insolubles and dried with sodium sulfate. A 30 g mass of reddish crystalline solid remained after the above solution was filtered and evaporated, mp = 145 °C. FDMS, M+ = 272, calculated mw = 272. 300 mHz nmr, $CDCl_3$, (s, 3H, 2.63 ppm), (s, 2H, 5.33 ppm), (d, 2H, 6.83 ppm), (d, 1H, 7.10 ppm), (m, 3H, 7.20 ppm), (d, 1H, 7.40 ppm), (bs, 1H, 9.30 ppm).

1-(3-(4-(N-BOC-piperidyl))propyl)-2-(4-chlorophenoxymethyl)-4-methylbenzimidazole: 2-(4-Chlorophenoxymethyl)-4-methylbenzimidazole, 9.55 g (0.035 mole), sodium hydride (60% in oil) 1.61 g (0.040 mole), and DMF, 200 ml, were combined and heated at 50 °C for 30 minutes. 3-(4-(N-Boc-piperidyl))-1-bromopropane 12.4 g (0.041 mole) in 40 ml DMF was added over 10 minutes to the benzimidazole / hydride mixture and then heated at 60 °C for 6 hours. Silica TLC, 1:1 hexane / ethyl acetate shows no remaining starting benzimidazole and two product spots: the lesser at R_f 0.40 and the greater (desired) at R_f 0.50. The reaction was evaporated slowly, overnight at RT under a stream of air. The residue was treated with saturated sodium bicarbonate solution and ethyl acetate. The layers were separated and the aqueous layer was extracted with ethyl acetate 3 X 50 ml. The combined organics were washed with brine 3X 50 ml, dried with sodium sulfate, filtered and evaporated. The residue, composed of the two isomers: the 7-methyl and the 4-methyl with respect to the BOC-piperidinopropyl moiety, was separated into the two components by silica gel chromatography using a gradient of 100% dichloromethane to 10% ethyl acetate in dichloromethane. The major component (and the desired product) eluted first, and 13 g (74%) of crystalline product were collected. FDMS, M+ = 497, calculated mw = 497. Elemental analysis for C28 H36 N3 O3 Cl, CHN.

1-(3-(4-Piperidyl)propyl)-2-(4-chlorophenoxymethyl)-4-methylbenzimidazole (V): 1-(3-(4-(N-BOC-piperidyl))propyl)-2-(4-chlorophenoxymethyl)-4-methylbenzimidazole, 8.0 g (0.016 mole), was added in portions to anhydrous trifluoroacetic acid, 100 ml, at RT. The resulting

solution was stirred at RT for 50 minutes and then evaporated to dryness. The residue was dissolved in water and ether. The layers were separated and the aqueous was extracted again with 30 ml ether. The aqueous was basified with 1 N NaOH to pH 11 and extracted with dichloromethane, 3x100 ml. The combined dichloromethane extracts were dried with anhydrous potassium carbonate, filtered and evaporated to yield a solid, 5.66 g (89%). FDMS, M+ = 397, calculated mw = 397. Elemental analysis for C23 H28 N3 O Cl, CHN. 300 MHZ MNR, $CDCL_3$, (m, 2H, 1.10 ppm), (m, 3H, 1.25 ppm), (d, 2H, 1.60 ppm), (m, 2H, 1.82 ppm), (t, 2H, 2.52 ppm), (s, 3H, 2.65ppm), (d, 2H, 3.04 ppm), (s, 1H, 3.80 ppm), (t, 2H, 4.16 ppm), (s, 2H, 5.30 ppm), (d, 2H, 6.96 ppm), (m, 1H, 7.08 ppm), (m, 4H, 7.20 ppm).

Dioxolane derivative of IV: The benzimidazole from the previous paragraph, 2.98 g (7.5 mMol), sodium bicarbonate, 655 mg (7.8 mMol) and dimethylformamide, 100 ml, and 2-(3-chloropropyl)-1,3-dioxolane, 2.26 g, 1.98 ml (15 mMol), were combined at RT and heated to 90 °C under nitrogen for 8 hours. An additional equivalent of the dioxolane material was added and the heating was continued for 4 hours. The DMF was removed by slow evaporation at RT and the residue was dissolved in ethyl acetate and saturated sodium bicarbonate solution. The layers were separated and the aqueous was extracted with ethyl acetate, 2x50 ml. The combined extracts were dried with sodium sulfate, filtered, and evaporated to give 3 g of thick oil. This material was chromatographed on silica gel using a gradient of 100% ethyl acetate to 10% methanol in ethyl acetate to give 2.4 g (62%) of slowly crystallizing oil. FDMS, M+ = 511, calculated mw = 512. Mp = 82 °C. 300 mHz nmr, $CDCl_3$, (m, 5H, 1.25 ppm), (m, 6H, 1.63 ppm), (m, 4H, 1.90 ppm), (m, 2H, 2.38 ppm), (s, 3H, 2.68 ppm), (d, 2H, 2.95 ppm), (m, 2H, 3.85 ppm), (m, 2H 3.95 ppm), (t, 2H, 4.20 ppm), (m, 1H, 4.85 ppm), (s, 2H, 5.35 ppm), (d, 2H, 6.98 ppm), (m, 1H, 7.10 ppm), (m, 4H, 7.24 ppm).

Tethered aldehyde (IV): The benzimidazole dioxolane, 1.07 g (2.09 mMol), and 200 ml THF were heated and stirred together to 60 °C. To the solution was added 6.27 ml 1 N HCl [3 eq. (the first 2 eq. formed the dihydrochloride salt which precipitated immediately)] and the resulting mixture was stirred at 60 °C for 7 minutes and then allowed to slowly cool to RT overnight. The resulting white crystalline product was removed by filtration, washed with THF and vacuum dried at 50 °C for 4 hrs and at RT overnight to give 1.08 g melting at 120 °C. Silica gel TLC: 85:15 CH2Cl2 revealed a new material with R_f 0.3 while the dioxolane had R_f 0.5. FDMS M+ = 468, calculated mw = 468 for the free base. IR showed no carbonyl peak but instead displayed a broad OH peak, suggesting that the desired aldehyde was stabilized as the hydrate. The nmr, CD_3OD, exhibited no aldehyde peak and displayed the loss of the ethylene ketal when compared to the NMR of the starting material.

1-Methyl-2-(2-aminoethyl)pyrrole: Commercially available 1-methylpyrrole-2-acetonitrile, 150 g (1.25 mole), was dissolved in absolute ethanol, 960 ml, and anhydrous ammonia, 375 ml. After cautious addition of Raney Nickel (15 g), the reaction was pressurized with hydrogen to

500 psi and heated at 80 °C for 8 hr. The catalyst was cautiously removed by filtration and carefully washed with ethanol. The combined filtrate and wash were evaporated to an oil and distilled at 60°C/0.2 torr to give the desired product, 138 g, 89% yield. The nmr, $CDCl_3$, shows expected peaks at: (s, 2H, 1.18 ppm), (t, 2H, 2.68 ppm), (t, 2H, 2.90 ppm), (s, 3H, 3.54 ppm), (m,1H, 5.94 ppm), (d, 1H, 6.08 ppm), (d, 1H, 6.56 ppm). FDMS: $m^+ = 125$, calculated mw = 124.

Preparation of LY353827 (VIII) by Pictet-Spengler cyclization: The dihydrochloride salt of aldehyde IV (271 mg, 0.5 mmol) and 1-methyl-2-(2-aminoethyl)pyrrole (IX, 63 mg, 0.5 mmol) were dissolved in 3 ml of a 1:1 mixture of acetic acid and methanol. This solution was stirred at room temperature for 5 hours, then poured over a SCX column (Varian, 5g SCX resin). The column was washed extensively with methanol, and the product was then eluted with 2M ammonia in methanol. The solvent was evaporated and the residual oil purified by flash chromatography (25:5:1 chloroform/methanol/ammonium hydroxide). The residual oil was dissolved in 2 ml 1:1 methanol/methylene chloride and treated with 200 mg (1 mmol/g resin, 0.2 mmol) polystyrene-linked benzaldehyde resin overnight to remove residual starting amine. The resin was then filtered through a cotton plug, rinsed with methanol, and the collected organics evaporated to yield 234 mg of clean VIII (82%). ^1H NMR ($CDCl_3$): 7.2-7.3 (m, 4H); 7.1 (m, 1H); 7.0 (d, 2H); 6.48 (d, 1H); 5.85 (d, 1H); 5.39 (s, 2H); 4.21 (t, 2H); 3.85 (m, 1H); 3.5 (s, 3H); 3.4 (m, 1H); 2.9-3.0 (m, 3H); 2.7 (s, 3H); 2.3-2.45 (m, 4H); 1.2-2.0 (m, 15H). ^{13}C NMR ($CDCl_3$): 156.5; 147.7; 135.0; 130.2; 129.5 (CH); 126.5 (CH); 126.5; 123.2 (CH); 122.6 (CH); 119.9 (CH); 116.0 (CH); 107.3 (CH); 103.5 (CH); 63.6; 59.0; 53.7; 53.6; 53.4 (CH_3); 44.6; 42.1; 35.5 (CH); 34.3; 33.7; 32.7 (CH_3); 32.2; 27.2; 23.4; 22.5; 16.6 (CH).

1-Methyl-4-(3-hydroxypropyl)-5-BOC-4,5,6,7-tetrahydro-5-aza-indole (IX): To a solution of 2-aminoethyl-1-methylpyrrole (124 mg, 1 mmol) in 5 ml 50% aqueous acetic acid was added 2-ethoxytetrahydrofuran (124 µl, 116 mg, 1 mmol). The resulting solution was stirred for twelve hours at room temperature, then poured over a Varian BondElut SCX column (5g SCX/20 ml column). The column was rinsed with methanol, dried, and the product was eluted with 2M anhydrous ammonia in methanol. Evaporation of the solvent gave 160 mg of crude product. This material was dissolved in 4 ml anhydrous methylene chloride and treated with BOC-anhydride (200 mg, 0.9 mmol). The resulting solution was stirred for 30 minutes, then diluted with 20 ml methylene chloride and 10 ml water. The organic layer was separated, dried over magnesium sulfate, and evaporated. The crude residue was purified by flash chromatography (2:1 ethyl acetate/hexane) to give 108 mg IX (56%).

1-Methyl-4-(3-bromopropyl)-5-BOC-4,5,6,7-tetrahydro-5-aza-indole: A solution of triphenylphosphine (10.1 g, 38.6 mmol) in 100 ml methylene chloride was cooled to 0 °C and treated with bromine (2.05 ml, 6.40 g, 40 mmol) until a persistent orange color was achieved. A small amount of triphenylphosphine was added to give a pale yellow solution, and a solution

of alcohol IX (8.1 g, 27.5 mmol) and pyridine (3.12 g, 38.6 mmol) in 25 ml methylene chloride was added at 0 °C. When addition was complete, the resulting solution was allowed to warm to room temperature overnight. The reaction was then evaporated to a slurry, then diluted with ether/hexane and filtered. The collected solids were washed thoroughly with ether/hexane, and the combined filtrate was evaporated. The resulting oil was purified by HPLC (0-50% ethyl acetate/hexane gradient) to give 8 g (81%) product bromide.

Unambiguous synthesis of VIII by alkylation of V: Amine V (2.72 g, 10 mmol) in 100 ml DMF was treated with sodium hydride (0.44 g of 60% suspension in mineral oil, 11 mmol) and heated at 35 °C for 30 minutes. The bromide prepared above (4.46 g, 12.5 mmol) was added, and the resulting reaction mixture was heated at 60 °C for 1 hour, then stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was partitioned between ethyl acetate and sodium bicarbonate. The layers were separated and the aqueous layer was extracted with additional ethyl acetate. The combined organics were washed with brine, dried over potassium carbonate and evaporated, and the residual oil purified by HPLC to give 3.7g (67%) of BOC-protected amine VIII. A small amount of this material (25 mg, 0.04 mmol) was dissolved in 500 µl methylene chloride. To this solution was added 1 ml TFA. The resulting dark purple solution was stirred for ten minutes at room temperature, then evaporated. The residue was partitioned between aqueous bicarbonate and ethyl acetate. The layers were separated, and the aqueous layer extracted with ethyl acetate. The combined organics were dried over sodium sulfate and evaporated to give a residue which was purified by flash chromatography to give 3.3 mg (16%) clean VIII, which was spectroscopically identical to previously prepared material, plus an additional 7.0 mg of slightly contaminated material.

Y-1 receptor binding: Binding assays were performed with crude membranes isolated from a stable cell line transfected with the human Y-1 receptor (AV12-Y1). The homogenate binding studies were conducted as previously described.[10] The cell pellets were resuspended using a Polytron (Brinkmann Instruments, Westbury, NY) in a 25 mM HEPES (pH 7.4) buffer containing 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 2 g/l bacitracin (Sigma, St. Louis, MO). Cell membranes were incubated in a final volume of 200 µl containing ~100 pM [^{125}I]pPYY (2200 Ci/mmol, DuPont-NEN, Boston, MA) for 2 hours at room temperature. Nonspecific binding was defined as the amount of radioactivity remaining bound after incubating in the presence of 1µM NPY. Incubations were terminated by rapid filtration through GF/C filters (Wallac, Gaithersburg, MD), which had been presoaked in 0.3% polyethyleneimine (Sigma), using a TOMTEC (Orange, CT) cell harvester. The filters were washed with 5 ml of 50 mM Tris (pH 7.4) at 4 °C and rapidly dried at 60 °C. The dried filters were treated with MeltiLex A (Wallac), melt-on scintillator sheets, and the radioactivity retained on the filters counted using the Wallac 1205 Betaplate counter. The results were analyzed using the Prism software package (Graphpad, San Diego, CA). K_i values for test agents were calculated by application

of the Cheng-Prusoff equation[25] based on a K_d for [^{125}I]pPYY of 100 pM. Parallel synthesis products were initially tested in triplicate at three concentrations: 10, 100, and 1000 nM. IC_{50} values were estimated from these three-point curves by nonlinear least-squares curve-fitting with the Hill coefficient fixed at unity. Full 11-point IC_{50} determinations were then carried out for the more potent compounds.

Cyclic AMP Accumulation: The SK-N-MC human neuroepithelioma cell line was obtained from the American Type Culture Collection, Rockville, MD. Three days before the experiment, two confluent 75 cm^2 flask of SK-N-MC cells were split into 17 6-well plates. Three hours before the experiment, medium was replaced with growth medium containing 2 μCi of [^{3}H]adenine per well. Incubations were initiated by removing the medium, washing once with 0.9% sodium chloride, and adding the test compound in a volume of 1 ml. Incubation buffer was Tyrode's balanced salt solution with 50 mM glucose, 15 mM HEPES (pH 7.4), 10 μM indolidan (from Lilly), 100 μM rolipram (from Schering AG, Berlin), 10 μM phosphoramidon (Sigma), and 100 μM bacitracin (Sigma). The order of incubations was randomized, and incubations were carried out in triplicate at ambient temperature in a laminar flow hood (~27 °C) for 10 minutes. Incubations were terminated by addition of 0.5 ml of 70% ethanol containing ~5000 dpm of [^{32}P]cyclic AMP (for determination of recovery). A 20 μl aliquot was taken to measure total [^{3}H]adenine incorporation, and the remainder of the sample was subjected to alumina chromatography to isolate [^{3}H]cyclic AMP.[26] [^{3}H]Cyclic AMP was expressed as parts-per-million (dpm per 10^6 dpm [^{3}H]adenine incorporation). A curve for inhibition by NPY of cyclic AMP accumulation in the presence of 1 μM forskolin was constructed for each experiment; maximum inhibition was usually about 90% and the EC_{50} for NPY about 150 pM. The IC_{50} for test compound was calculated by nonlinear least-squares curve-fitting from the concentration-response curve for blockade of the inhibition caused by 1 nM NPY. The IC_{50} was converted to a K_i value by application of the Cheng-Prusoff equation,[25] based on the EC_{50} of NPY in the same experiment.

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