Truncated, Branched, and/or Cyclic Analogues of Neuropeptide Y: Importance of the Pancreatic Peptide Fold in the Design of Specific Y_2 Receptor Ligands†

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Truncated, branched, and/or cyclic neuropeptide Y (NPY) analogues were tested for their ability to bind to the neuroblastoma cells, SK-N-MC (Y_1 receptor) and SK-N-BE(2) (Y_2 receptor). The design of such analogues was inspired by models of NPY based on the crystal structure of avian pancreatic polypeptide. The minimum length of the backbone was investigated using the following truncated analogues [binding affinity (nM) for Y_1 and Y_2 receptor subtypes respectively are given in parentheses]: des-AA$^{10-17}$[D-Ala^9]NPY (100, 0.9), des-AA$^{7-23}$[D-Ala^6]NPY (>1000, 1.2), des-AA$^{4-26}$[D-Ala^3]NPY (>1000, 120), cyclo(7,20)-des-AA$^{10-17}$[Glu^7,D-Ala^9,D-Dpr^{20}]NPY (100, nd), cyclo(2,27)-des-AA$^{7-23}$[Glu^2,D-Ala^6,D-Dpr^{27}]NPY (>1000, 3.6), cyclo(2,30)-des-AA$^{7-23}$[Glu^2,D-Ala^6,-D-Dpr^{30}]NPY (>1000, nd), cyclo(1,30)-des-AA$^{4-26}$[Glu^1,D-Ala^3,D-Dpr^{30}]NPY (>1000, >1000). A new family of branched NPY analogues corresponding to the partial deletion of the polyproline helix with conservation of the N-terminus was also examined: des-AA$^{7-23}$[(Ac-NPY$_{14-22}$)-ε-D-Lys^6]NPY (>1000, 2.1), des-AA$^{7-23}$[(Ac-NPY$_{7-22}$)-ε-D-Lys^6]NPY (>1000, 5.1), des-AA$^{7-23}$-[(Ac-LEALEG-NPY$_{14-22}$)-ε-D-Lys^6]NPY (>1000, 4.8). Finally, the role played by the flexible tail (residues 32-36) was studied with the following cyclic analogues: cyclo(30,34)-[Lys^{30},Glu^{34}]NPY$_{18-36}$ (>1000, 360), cyclo(30,34)-[Orn^{30},Gly^{34}]NPY$_{18-36}$ (>1000, 950), cyclo(30,34)-[Dpr^{30},Glu^{34}]NPY$_{18-36}$ (>1000, 590), cyclo(33,36)-[Lys^{33},Glu^{36}]NPY (>1000, >1000), cyclo(33,36)-[Lys^{33},Glu^{36}]NPY$_{18-36}$ (>1000, >1000). These results suggest that the Y_1 receptor is highly discriminatory since deletion of residues 10-17, shown to have little effect on Y_2 binding affinity, reduces Y_1 affinity 50-fold. Bridging sites and constructs have been identified that may serve as useful leads in the design of more potent and selective analogues. We have identified two positions (9 and 6) where the introduction of a D amino acid is not detrimental to binding affinity. Whether this modification leads to the stabilization of a yet unidentified turn compatible with high Y_2 receptor affinity will have to be determined by spectroscopic methods. Finally, stabilizing a putative α-helical conformation of the C-terminal heptapeptide of NPY$_{18-36}$ has a deleterious effect on the Y_1 and Y_2 receptors.

Introduction

Neuropeptide Y is an amidated 36 residue first discovered by Tatemoto et al. in 1982[1,2] using a purification scheme based on the detection of a C-terminal amide.[3]

NPY belongs to the pancreatic peptide (PP) family and shows 69% sequence homology with PYY and 50% with pPP (porcine PP). The crystal structure of avian pancreatic polypeptide (aPP) was first elucidated by Blundell et al.[4,5] and consists of a polyproline type II-like helix (residues 1-8), a β-turn (residues 9-13), an α-helix (residues 14-32), and a flexible tail (residues 33-36). The two helices are antiparallel and packed through hydrophobic interactions. All members of the PP family are structurally related, and it is anticipated that they would all share the same secondary structure known as the PP fold if analyzed under the same experimental conditions.

Tyr-Pro-Ser-Lys-Pro^5-Asp-Asn-Pro-Gly-Glu^{10}-Asp-Ala-Pro-Ala-Glu^{15}-Asp-Leu-Ala-Arg-Tyr^{20}-Tyr-Ser-Ala-Leu-Arg^{25}-His-Tyr-Ile-Asn-Leu^{30}-Ile-Thr-Arg-Gln-Arg^{35}-Tyr-NH_2

porcine NPY

NPY is distributed in the central and peripheral nervous systems. NPY possesses potent vasoconstrictor properties, i.e. reduces heart rate and increases blood pressure and stimulates food intake when injected into animals.[6-9] Three

† Abbreviations: The abbreviations for the amino acids (one- and three-letter codes) are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *138*, 9-37). The symbols represent the L-isomer except when indicated otherwise. In addition: AA, amino acid; Boc, *tert*-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CZE, capillary zone electrophoresis; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, 1,3-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; DMF, dimethylformamide; Dpr, diaminopropionic acid; EDT, ethanedithiol; Fmoc, *N*-[(9-fluorenylmethyl)oxy]carbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance chromatography; MBHA, methylbenzhydrylamine. NMR, nuclear magnetic resonance; NPY, neuropeptide Y; OFm, fluorenylmethyl ester; PP, pancreatic polypeptide; PYY, peptide YY; SEM, standard error of the mean; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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Table I. Target Structures of Truncated and/or Cyclic NPY Analogues

no.	analogues	structural modifications
1	NPY	native, see Figure 1
2	des-AA$^{10-17}$[D-Ala^9]NPY	see Figure 1
3	des-AA$^{7-23}$[D-Ala^6]NPY	see Figure 1
4	des-AA$^{4-26}$[D-Ala^3]NPY	see Figure 1
5	c(7,20)-des-AA$^{10-17}$[Glu^7, D-Ala^9, D-Dpr^{20}]NPY	see Figure 1 (cycle not shown)
6	c(2,27)-des-AA$^{7-23}$[Glu^2, D-Ala^6, D-Dpr^{27}]NPY	see Figure 1 (cycle not shown)
7	c(2,30)-des-AA$^{7-23}$[Glu^2, D-Ala^6, D-Dpr^{30}]NPY	see Figure 1 (cycle not shown)
8	c(1,30)-des-AA$^{4-26}$[Glu^1, D-Ala^3, D-Dpr^{30}]NPY	see Figure 1 (cycle not shown)
9	des-AA$^{7-23}$[(Ac-NPY$_{14-22}$)-ε-D-Lys^6]NPY	see Figure 2
10	des-AA$^{7-23}$[(Ac-NPY$_{7-22}$)-ε-D-Lys^6]NPY	see Figure 2 (altered side chain)
11	des-AA$^{7-23}$[(Ac-LEALEG-NPY$_{14-22}$)-ε-D-Lys^6]NPY	see Figure 2 (altered side chain)
12	NPY$_{18-36}$	see Figure 3
13	c(30,34)-[Lys^{30},Glu^{34}]NPY$_{18-36}$	see Figure 3 (cycle not shown)
14	c(30,34)-[Orn^{30},Glu^{34}]NPY$_{18-36}$	see Figure 3 (cycle not shown)
15	c(30,34)-[Dpr^{30},Glu^{34}]NPY$_{18-36}$	see Figure 3 (cycle not shown)
16	c(33,36)-[Lys^{33},Glu^{36}]NPY	see Figure 1 (cycle not shown)
17	c(33,36)-[Lys^{33},Glu^{36}]NPY$_{18-36}$	see Figure 3 (cycle not shown)

main receptors subtypes have been proposed, Y_1, Y_2, and Y_3 receptors (post- and prejunctional);[10,11] however the difference in potencies observed with NPY analogues in various systems has suggested the existence of multiple subtypes of NPY receptors.[12] Since it is well accepted that both termini are essential for the binding to the Y_1 receptor and that the C-terminus alone suffices to bind to the Y_2 receptor,[10] we sought to further define the structural elements responsible for receptor recognition and binding. We report here the design, synthesis, and characterization of three classes of NPY analogues: truncated and/or cyclic analogues (smaller and/or constrained through side chain–side chain amide bonds) similar to those reported earlier by Krstenansky;[13-16] truncated analogues where part of the polyproline-like helix has been removed; and cyclic analogues of NPY$_{18-36}$, made in an attempt to stabilize the C-terminal heptapeptide (Table I). The underlying goal was to obtain short and constrained bioactive analogues of NPY using the X-ray structure of aPP and the structure of NPY deduced by homology,[17] as templates. Next, we tried to identify a putative turn that would bring the N- and C-termini in close proximity. Peptides were tested for their binding affinity to Y_1 and Y_2 receptors.

Results

Synthesis, Purification, and Characterization (Table II). NPY analogues were synthesized manually by standard solid phase synthesis on a *p*-methylbenzhydrylamine-resin (MBHA-resin) using previously described protocols.[18] Briefly, *tert*-butoxycarbonyl (Boc) was used for N-terminal protection and deblocked with trifluoroacetic acid (TFA) in the presence of ethanedithiol (EDT). Most couplings employed 1,3-diisopropylcarbodiimide (DIC); however, some recouplings (when required through monitoring by the Kaiser test) were accomplished using 1-hydroxybenzotriazole (HOBt) and (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) in DMF in the presence of an excess of diisopropylethylamine (DIPEA). Coupling time was 1 h with recouplings done as needed. Usually Arg, His, and Asn (at positions 25, 26, and 29, respectively) needed to be recoupled. Washes included 2-propanol containing 1% EDT after TFA cleavage, DCM, and methanol. Triethylamine (TEA) in DCM was used for neutralization of the peptide resin after TFA deblocking. Side chains to be cyclized were protected with the Ofm/Fmoc (fluorenylmethyl ester/*N*-[(9-fluorenylmethyl)oxy]carbonyl) protecting groups as suggested by Felix et al.[19] After deblocking of these groups with piperidine, cyclization was achieved with BOP–HOBt–DIPEA. The protected peptide-resin was cleaved in anhydrous HF in the presence of a scavenger, precipitated, extracted, and lyophilized. The crude peptides were purified by reversed-phase HPLC[20] in two to four steps to yield highly purified compounds. The analytical techniques used for the characterization of NPY analogues included HPLC, optical rotation, capillary zone electrophoresis (CZE), amino acid analysis, and mass spectrometry. Results from these studies support the identity of the intended structures.

Circular Dichroism Spectra (Figure 3). The residue molar ellipticities in the wavelength range 190–250 nm of des-AA$^{7-23}$[(Ac-LEALEG-NPY$_{14-22}$)-ε-D-Lys^6]NPY (**11**) and cyclo(30–34)-[Lys^{30},Glu^{34}]NPY$_{18-36}$ (**13**) in aqueous

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Table II. Physical Values of NPY Analogues and Affinities for Both Y_1 and Y_2 Receptors

no.	CE, %	HPLC purity, %	HPLC t_R[a]	MH^+ (calcd)[a]	MH^+ (measd)[a]	$[\alpha]_d$, deg (c = 1, AcOH 1%)	Y_1, SK-N-MC: K_d, nM	Y_2, SK-N-BE(2): K_d, nM,
1		97				-58	see legend	see legend
2	97	99	48.9	3426.78	3426.93	-70	100[b]	0.9[c]
3	95	90	38.3	2388.34	2389.29	-61	>1000[d]	1.2[e]
4	99	98	46.9[f]	1669.92	1670.02	-40	>1000[d]	120[e]
5	90	-[g]	44.8	3345.77	3346.83	-61	100[b]	nd[i]
			50.5					
6	99	99	31.0	2325.31	2325.93	-49	>1000[h]	3.6[e]
7	99	97	31.5	2375.28	2376.46	-41	>1000[b]	nd
8	99	98	12.7	1590.85	1590.87	-35	>1000[h]	>1000[e]
9	99	95	45.3	3556.90	3557.90	-54	>1000[b]	2.1[c]
10	85	84	46.6	4237.18	4238.27	-53	>1000[b]	5.1[c]
11	89	83	57.4	4169.22	4170.56	-57	>1000[b]	4.8[c]
12		>97				-46	780	1.8
13	96	97	48.4[f]	2454.33	2455.40	-51	>1000[h]	360[c]
14	98	99	39.2	2439.30	2441.13	-52	>1000[h]	950[e]
15	97	99	42.8[f]	2412.28	2413.39	-34	>1000[h]	590[e]
16	85	98	61.6	4172.10	4174.4	-60	>1000[b]	>1000[c]
17	97	97	49.2	2376.31	2378.2	-59	>1000[b]	>1000[c]

[a] MH^+ is the monoisotopic mass. t_R = retention time in percent of solvent B. HPLC analysis were made using the buffer system: A = TEAP 2.5 and B = 60% CH_3CN/A. Flow was 1.5 mL/min, silica gel column was a Vydac C_{18} (0.46 × 25 cm, 5-μm particule size, 30-nm pore size) with a UV detector at 210 nm. In all cases gradient slope was 1% B/min. [b] NPY had a K_d of 2.5 nM in this assay. [c] NPY had a K_d of 0.38 nM in this assay. [d] NPY had a K_d of 2.9 nM in this assay. [e] NPY had a K_d of 0.28 nM in this assay. [f] Flow was 0.5 mL/min. [g] Conformation modification seen on the HPLC. [h] NPY had a K_d of 2.1 nM in this assay. [i] nd = not determined.

buffer (0.01 M sodium phosphate, 0.05 sodium chloride, pH 7.40) shown in Figure 3a suggest a strong random coil component for both peptides under these conditions. The lack of any positive elipticity below 200 nm for either peptide 11 or 13 argues strongly against any helical content, and the position and intensity of the maximum of negative elipticity of 13 below 200 nm is suggestive of a turn component. The spectra of 11 and 13 in 2,2,2-trifluoroethanol (TFE, Figure 3b) show that both peptides become highly helical under these conditions.

Binding Assay (Table II). The binding assay is based on the competition between NPY analogues at varying concentrations with a ^{125}I-PYY tracer in the presence of human neuroblastoma SK-N-MC cells (Y_1 receptors) or SK-N-BE(2) cells (Y_2 receptors). Most peptides showed no affinity for the Y_1 receptor, except analogues having the longest backbone: des-AA$^{10-17}$[D-Ala^9]NPY (K_d = 100 nM) and cyclo(7,20)des-AA$^{10-17}$[D-Ala^9]NPY (K_d = 100 nM). For the Y_2 receptor, des-AA$^{7-23}$[D-Ala^6]NPY (K_d = 1.2 nM), des-AA$^{10-17}$[D-Ala^9]NPY (K_d = 0.89 nM), cyclo(2,27)des-AA$^{7-23}$[Glu^2,D-Ala^6,D-Dpr^{27}]NPY (K_d = 3.6 nM), des-AA$^{7-23}$[(Ac-NPY$_{14-22}$)-ε-D-Lys^6]NPY (K_d = 2.1 nM), des-AA$^{7-23}$[(Ac-NPY$_{7-22}$)-ε-D-Lys^6]NPY (K_d = 5.1 nM), and des-AA$^{7-23}$[(Ac-LEALEG-NPY$_{14-22}$)-ε-D-Lys^6]-NPY (K_d = 4.8 nM) showed affinity for the Y_2 receptor within 1 order of magnitude of that of NPY and comparable to that of NPY$_{18-36}$.

Discussion

Three-dimensional models of NPY[17] derived from the crystal structure of aPP determined by X-ray[4] have been widely used for the successful design of NPY analogues.[13-16,21-25] This structure is supported by circular dichroism studies;[26] however NMR studies[27] in aqueous solution at pH 3.1 show that the amphipathic α-helical character of the C-terminal region extends from residue 11 to 36 and that the N-terminal fragment containing three prolines in both cis and trans conformations assumes no defined structure. Biological data[10] suggest that the PP fold is important for receptor recognition by bringing the C- and N-termini in close proximity.

In the absence of purified Y_1 and Y_2 receptors, structural studies investigating the folding of NPY analogues in the presence of their receptors are not yet possible. By designing selective and rigid analogues of NPY with high affinity, we hope to gain a better understanding of the mechanism by which specific ligands bind to and activate their receptors as well as gain an appreciation of the role played by the PP fold in that interaction. To answer these questions, three series of analogues were designed and synthesized.

The first series consists of truncated and/or cyclic analogues with structural features comparable to, but more rigid than, those reported by Krstenansky et al.[13-16] and Beck-Sickinger et al.[22-25] The former group designed NPY analogues where portions of the central sequence were removed and replaced by a flexible spacer (8-aminooctanoic acid) while the two helices (polyproline and α-helices) were bridged through the side chains of two cysteines. Similarly, Beck-Sickinger et al.[22-25] reported that a series of deletion analogues using ε-aminocaproic

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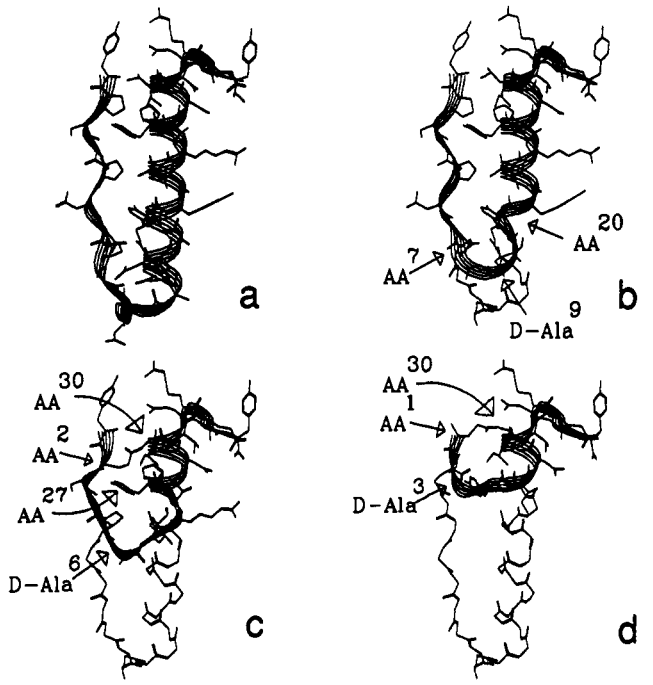

Figure 1. Comparison of structures of cyclized and des-AA analogue families of NPY with model of NPY based on homology with X-ray crystallographic structure of aPP.[4] In each figure the backbone of the NPY model is present with no ribboning and the analogue is highlighted with ribboning. All of the figures have been superimposed to present the same viewing aspect. (a) Porcine NPY, (b) des-AA$^{10-17}$[D-Ala^9]NPY; (c) cyclo-(2-30)-des-AA$^{7-23}$[Glu^2,D-Ala^6,DDpr^{30}]NPY, (d) cyclo-(1-30)-des-AA$^{4-26}$[Glu^1,D-Ala^6,D-Dpr^{30}]NPY.

Table III. Cα–Cα Distances between Prolines of the Polyproline Helix and Possible Bridgehead Residues of the C-Terminal α-Helix of the Three-Dimensional Model of NPY Based on the Crystallographic Structure of aPP

Cα–Cα pair	distance, Å
Pro^2–Tyr^{27}	6.18
Pro^2–Leu^{30}	7.70
Pro^5–Tyr^{20}	7.07
Pro^5–Leu^{24}	6.33
Pro^5–Tyr^{27}	7.64
Pro^8–Leu^{17}	6.95
Pro^8–Tyr^{20}	7.11

acid as a spacer between N- and C-termini would be unlikely to assume defined conformations in solution.

We first examined the role played by the prolines at positions 2, 5, and 8 as to whether they were part of a polyproline helix or whether they initiated turns. We hypothesized that if the introduction of the sequence Pro^n-D-Ala^{n+1} (with n being positions 2, 5, and 8) generated potent analogues, we would have identified a turn compatible with NPY's bioactive conformation (analogues **2–4**). To confer additional stability to our construct, we have introduced bridges in between the helices at the positions shown in Figure 1 (analogues **5–8**) and Table I. According to the crystal structure of aPP, positions showing the best contacts between the α-helix and the polyproline-like helix in the NPY structure were those shown in Table III. A systematic search of the Glu D-Dpr side chain amide bridge[29] conducted over the allowed side chain torsions found that the Cα–Cα distances span the range 4.72–7.74 Å with an unweighted average value of 6.72 Å, indicating that the Glu D-Dpr bridge should be suitable to stabilize the two helical arms. With the bridge length established, the optimal configuration of each residue was established by inspection; L-Glu and D-Dpr at the N- and C-termini, respectively, were determined to least perturb the three-dimensional model.

Among these analogues only **2** and **5** show affinity for the Y_1 receptor (K_d values are 100 nM), which is consistent with previous work done on this size of truncated analogues.[13-16,22-25,30] The similar affinity of the cyclic and the noncyclic analogues suggests that the bridge between positions 7 and 20 has no major influence on the relative position of both termini. Analogues **3** and **4** show no affinity for the Y_1 receptor, suggesting that the entire backbone of the molecule is important for binding to that receptor in addition to both termini. Different affinities are observed for these analogues on the Y_2 receptor. Analogue **2** binds with a K_d of 0.9 nM and **3** with a K_d of 1.2 nM: these are only about three times less potent than NPY itself on the Y_2 receptor. Compound **4**, however, binds to the Y_2 receptor with an affinity 10^{-3} times that of NPY. In this series, restriction of the conformation by bridging the two helices slightly lowered affinity for **6** as compared to **3** while affinity was lost in the case of **8** as compared to **4**.

The three-dimensional model of NPY indicating that the Cα–Cα distance between residues 7–20 was 9.3 Å suggested to Krstenansky et al.[13] the possibility of using these positions as bridgeheads with D-Cys^7 and Cys^{20}. Only minimal disruption of the secondary structural features of the analogue was predicted. The resulting analogue (C7-NPY) was found to be equipotent to NPY in the systems tested. In our hands, analogue **5** was found to be 40 times less potent in its ability to bind to the Y_1 receptor.

In the case of analogues **6** and **7**, distances between Cα carbons of residues 2–27 and 2–30 were calculated to be 6.18 Å (positions 2–27) and 7.70 Å (positions 2–30), respectively (see Table III). While both analogues had no affinity for the Y_1 receptor at the highest concentration tested (10^{-6} M), **6** was found to be 12 times less potent than NPY on the Y_2 receptor.

These results suggest at least two possibilities: the bridge which we chose (δ-Glu to β-D-Dpr) is conformationally disruptive, or functional amino acids were eliminated in these constructs. However as one of these constructs (analogue **6**) induces selectivity for binding to the Y_2 receptor subtype, one may infer that NPY folds differently while binding to the two receptors.

As the binding affinity for the Y_1 receptor was lost for small analogues (**3** and **4**), our strategy next focused on what structural elements can be added back to these molecules to restore activity. The second series of peptides was designed using analogue **3** as a template because of its reduced size and because of its significant affinity for the Y_2 receptor. D-Ala^6 was substituted by D-Lys^6, the side chain of which was elongated via the ε-amino group with various fragments of NPY's core: (ac-NPY$_{14-22}$, **9**; Ac-NPY$_{7-22}$, **10**; and Ac-LEALEG-NPY$_{14-22}$, **11**). As shown in Figure 2, the formation of an ε-amide from D-Lys^6 to

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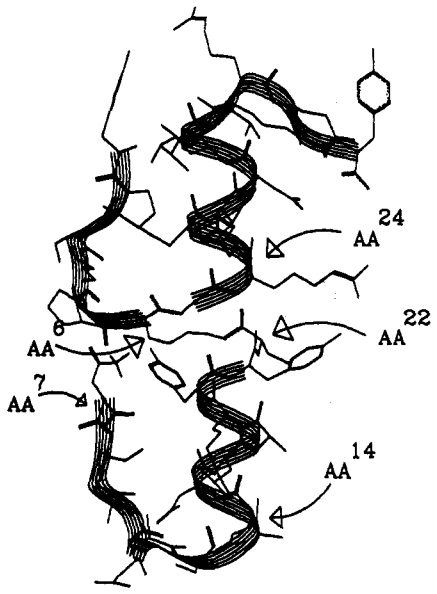

Figure 2. Ribbon illustration of des-AA$^{7-23}$-[(Ac-NPY$_{14-22}$)-ε-D-Lys^6]NPY constructed by mutation of the model of NPY. The ribbon follows the backbone of the analogue. Sections of the ribbon between residues 6 and 24 and along the D-Lys^6 side chain leading to the C-terminus of residue 22 have been removed for clarity.

Ser^{22} can be accommodated with a minimal disruption of the overall PP fold. The α-helical Ac-LEALEG sequence[31] was introduced to test whether any extension of the α-helix beyond the predicted turn would be tolerated. Analogues **9**–**11** showed Y_2 selectivity with good binding affinity (K_d = 2.1, 5.1, 4.8 nM, respectively). Results suggest that the deleted part of the polyproline helix is not vital for the peptide to bind to the Y_2 receptor, but is for the Y_1. This is not surprising in view of the fact that C-terminal fragments missing elements of the N-terminus do bind with good affinity to the Y_2 receptor and have no affinity for the Y_1 receptor.[16,32-33] Adding longer branches (**10** and **12**) makes no difference for the affinity for both receptors, molecule **10**, containing the whole backbone of NPY with different connections, is probably too flexible to fold correctly for Y_1 recognition. A possible explanation is that despite the fact that this molecule has the same composition as NPY, it cannot assume the proper conformation for Y_1 binding. In aqueous solution, the CD spectrum of **11** (Figure 3a) is principally random, with very little suggestion of a helical component. This spectrum resembles that of NPY$_{18-36}$ under aqueous conditions[34] and is distinct from that of native NPY which shows a positive ellipticity below 200 nm. As observed with NPY and NPY$_{18-36}$, but not observed with NPY$_{1-19}$, analogue **11** becomes highly helical in TFE (Figure 3b).

The third series was designed to stabilize the C-terminal heptapeptide (residues 30–36) by bridging the side chains

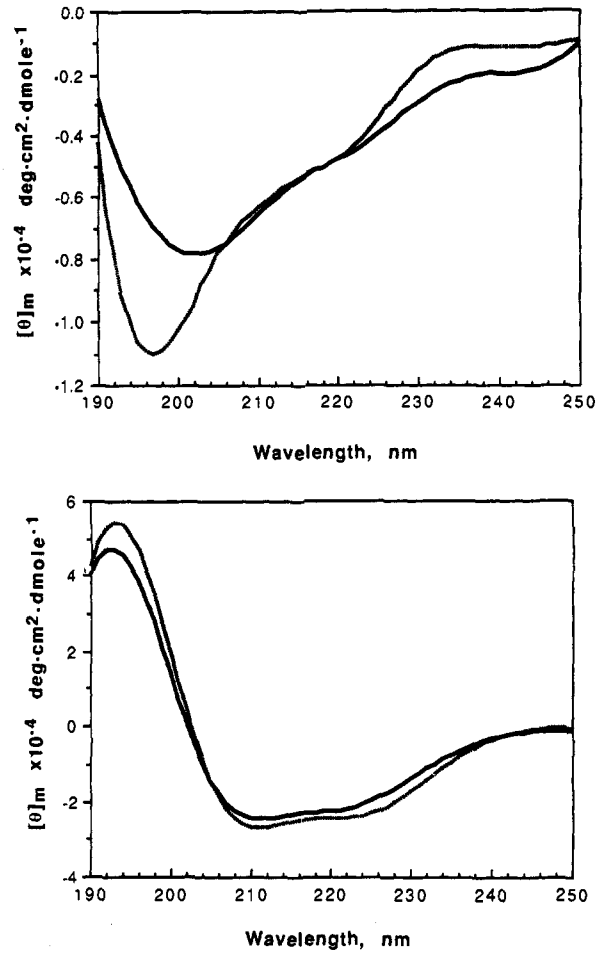

Figure 3. (a, top) CD spectra of **11** (des-AA$^{7-23}$-[(Ac-LEALEG-NPY$_{14-22}$)-ε-D-Lys^6]NPY, solid line) and **13** (cyclo-(30–34)-[Lys^{30},Glu^{34}]NPY$_{18-36}$, broken line) in 0.01 M sodium phosphate, 0.05 M sodium chloride, pH 7.40 buffer. (b, bottom) CD spectra of **11** (solid line) and **13** (broken line) in TFE.

of amino acids at positions 30–34 and 33–36 (see Figure 4). While NMR studies showed that the C-terminus of NPY adopts an α-helical conformation from residues 11–36, crystallographic data[4] implies that a turn at residues 33 and 34 allows the C-terminus to be oriented away from the α-helix axis. Analogues **13** to **17** (listed in Table I) with side chain to side chain bridges shown in growth hormone and corticotropin releasing factors to stabilize α-helices[35,36] were synthesized. In theory, these peptides should have been active if they were to assume an α-helical conformation when interacting with the Y_2 receptor despite the fact that NPY analogues with modifications (D-AA and other substitutions) in this area of the molecule had been shown to be less potent than NPY.[26,29] Introduction of the 30–34 bridge in analogue **13** may have introduced a minor turn component as judged by the CD spectrum in aqueous solution (Figure 3a). However, as shown in Figure 3b, in TFE this fragment becomes highly helical. Because NPY$_{18-36}$ (C-terminal half of NPY) has strong

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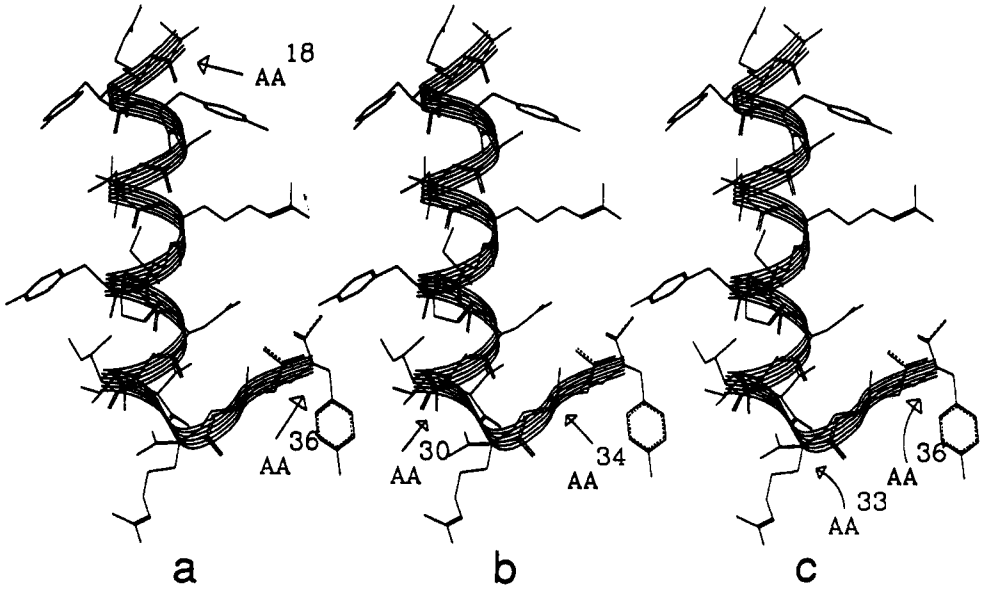

Figure 4. Illustration of NPY_{18-36} analogues showing positions of C-terminal bridgehead residues. (a) NPY_{18-36}, (b) a bridge between the side chains of residues 30 and 34 is the key structural feature of analogues 13–15, (c) a bridge between the side chains of residues 33 and 36 is the key structural feature of analogues 16 and 17.

affinity (K_d is 1.8 nM) for the Y_2 receptor, affinities of 13–15 can be compared to that of this nonadecapeptide. These peptides did not bind to the Y_1 receptor as expected (NPY_{18-36} has a K_d of 780 nM in that system). Data presented in Table II on these analogues suggest that their affinity for the Y_2 receptor can be modulated by changing the positions and the length of bridges. These modifications, however, result in an important loss of affinity, and K_d values cannot be easily correlated with the length of the bridge. In the absence of a purified receptor, only partial conclusions can be drawn on such a limited number of analogues and that more definitive understanding will only be derived from further investigations in this area. We could conclude that the introduction of a limited set of bridges at the C-terminus will likely result in a total loss of affinity for the Y_1 receptor and to a lesser extent to a lowering of the affinity for the Y_2 receptor subtype. On the other hand, [Leu[31],Pro[34]]NPY[28] with substitutions in that portion of the molecule has no affinity for the Y_2 receptor but is a Y_1 agonist. Stabilization of a putative C-terminal α-helix by the introduction of a bridge at positions 33 and 36 (16 and 17) also yielded analogues with poor affinity for both Y_1 and Y_2 receptors. One reason could be that any modification of residue 36 was shown to result in inactive compounds (K_d >1000 nM), which confirms the necessity of the phenolic group at that position.[23]

In conclusion, three classes (truncated and/or cyclic as well as branched) of NPY analogues were studied. Results from the binding studies confirmed earlier reports by us[29] and others[13,23] that the Y_1 receptor is highly discriminatory since deletion of residues 10–17 (2) shown to have almost no effect on Y_2 binding affinity reduces Y_1 affinity 40-fold. We have also identified bridging opportunities and constructs (6 and 9, respectively) that may serve as useful leads for more potent and selective analogues. We have identified (2, 3, 5, 6) two positions (9 and 6) where the introduction of a D amino acid is not detrimental. Whether this modification leads to the stabilization of a yet unidentified turn compatible with high Y_2 receptor affinity or is just irrelevant will have to be determined by spectroscopic methods. Finally, introducing side chain to side chain bridges in the C-terminal heptapeptide of NPY_{18-36} (13–17) has a deleterious effect on the Y_1 and Y_2 receptors. Attempts at stabilizing portions of the α-helix in different truncated NPY analogues[37,38] by the introduction of lactam bridges between side chains of residues spanning positions 14–32 resulted in compounds having lower affinity for both receptors than NPY.

Experimental Section

All reagents and solvents were reagent grade (Aldrich Chemical Co., Milwaukee, WI; Fisher Scientific, Springfield, NJ) and were used without further purification except TFA (Halocarbon, Hackensack, NJ) and TEA (Aldrich Chemical Co., Milwaukee, WI), which were distilled for use in the preparation of chromatographic buffers.

Peptide Synthesis. Peptides were manually synthesized on 2 g of *p*-methylbenzhydrylamine-resin (MBHA-resin, substitution 0.5 mmol/g) prepared by the method of Rivier et al.[18] from polystyrene cross-linked with 1% divinylbenzene (Biobeads SX-1, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA). α-Boc-amino acids (Bachem, Torrance, CA) in 2.5-fold excess were coupled via 1,3-diisopropylcarbodiimide in DCM and/or DMF. Asn and Gln were coupled in the presence of a 1.5-fold excess of 1-hydroxybenzotriazole. Side-chain protection of the α-Boc-amino acids utilized were as follows: Arg(Tos), Asp(O-chx) Glu(O-Bzl), His(Tos), Lys(2ClZ), Lys(Fmoc), Orn(Fmoc), Dpr(Fmoc), Ser(Bzl), Thr(Bzl), and Tyr(2BrZ). Deblocking was accomplished with 50% TFA in DCM in the presence of 1% EDT for 20 min. Cyclization were performed using OFm/Fmoc for side-chain protection of the carboxyl and amino functions, respectively.[19] After deblocking with 20% piperidine in DMF for 10 min, cyclization was made with BOP-HOBt-DIPEA (1:1:3) in DMF overnight, and then if cyclization was not completed (Kaiser test), the procedure was repeated until completed. The protected peptide-resins were cleaved in anhydrous HF in the presence of 3% anisole at 0 °C for 1.5 h. The crude peptides were precipitated with anhydrous diethyl ether and separated from ether-soluble nonpeptide materials by filtration. The peptides were extracted from the resin with water and the aqueous solutions were lyophilized.

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Peptide Purification. Crude peptides were purified by preparative reverse-phase HPLC.[20,39] Gradient conditions for preparative HPLC were inferred from the analytical results. Analytical studies were made on either a Hitachi 655 analytical system comprising a 655A-11 pump, a 655A-71 proportioning valve, a Rheodyne 7125 injector, a Vydac C_{18} column (0.46 × 25 cm, 5-μm particle size, 30-nm pore size), a 655-61 system processor, or a Waters Associates HPLC system comprising a Waters automated gradient controller, two pumps Waters Associates Model 501 pumps, a tunable Waters Associates 486 UV detector, an injector Rheodyne 7125, a servocorder SR 6253 Datamark, and a Vydac C_{18} column (0.46 × 25 cm, 5-μm particle size, 30-nm pore size). The preparative HPLC system used comprised a modified Waters Associates Prep LC 3000 System, a Waters Associate 600E System Controller, a Shimadzu SPD-6A spectrophotometric variable-wavelength detector (detection was at 230 nm), and a Fisher (Lexington, MA) Recordall series 5000 chart recorder. The cartridges used were hand-packed, in house, with Waters polyethylene sleeves and frits and Vydac bulk C_{18} material, 15–20-μm particle size, 30-nm pore size.

Peptide Characterization. Purified peptides were checked for purity on (a) CZE using a Na_2HPO_4 100 mM buffer pH 3.0 on a Beckman P/ACE 2000 Capillary Electrophoresis system controlled by an IBM computer using a SpectraPhysics ChromJet integrator, and (b) an automated Waters Associates HPLC comprised of two pumps Model 6000A, a WISP Model 712 data processor and system controller, a WISP 710B automatic injector and a Kratos Spectroflow 773 UV variable detector. Amino acids analysis of the peptides, following hydrolysis in 4 N methanesulfonic acid at 110 °C for 24 h, was performed on a Perkin-Elmer LC system comprised of two series 10 LC pumps, a ISS-100 sample injector, a RTC 1 column oven, a Kratos Spectroflow 980 fluorescence detector, and a LCI-100 integrator. A Pierce ion-exchange column was maintained at 60 °C and post column derivatization with o-phthalaldehyde was performed at 52 °C. Samples containing the internal standard γ-aminobutyric acid were injected and 5 min after injection were subjected to a gradient of 0–100% B in 25 min and then 100% B for 15 min with a flow rate of 0.5 mL/min. Buffers A and B were Pierce Pico buffer (pH 2.20) and Beckman Microcolumn sodium citrate buffer (pH 4.95), respectively.

Optical Rotations. Optical rotations were measured in 1% acetic acid (c = 1.0; i.e. 10 mg/mL of peptide uncorrected for TFA counterions or water present after lyophilization). Values were obtained from the means of 10 successive 5-s integrations determined at room temperature (about 23 °C) on a Perkin-Elmer 241 polarimeter (using the D line of Na emission) and are reported as uncorrected specific rotations.

LSIMS. Spectra were measured using a JEOL JMS-HX110 double-focussing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. An accelerating voltage of 10-kV and Cs^+ gun voltage of 25-kV was employed. The samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix.

Circular Dichroism Spectra. Circular dichroism (CD) measurements were conducted using an Aviv Model 62DS spectropolarimeter (Aviv Associates, Lakewood, NJ) with 0.5-mm cuvettes by signal averaging four scans in the range 190–250 nm. Data were collected at 1.0-nm intervals with a 2.0-s integration time and a spectral bandwidth of 2.0 nm. Spectra of compounds 11 and 13 were collected under two sets of conditions: (1) in 0.01 M sodium phosphate, 0.05 M sodium chloride (pH 7.40) at a concentration of 100 μM for each peptide, and (2) in TFE at concentrations of 103 and 100 μM, respectively. Concentrations were based on the calculated molecular weight of the TFA salt of the purified lyophilized peptide assuming a water current of 7% and were used for the calculation of residue molar ellipticities.

Molecular Modeling. Molecular modeling and other computational procedures were conducted with the DISCOVER and Insight II packages[40] on Silicon Graphics workstations. A full valence force field[41] was used to calculate potential energies. The analytical forms and parameters of this force field have been reported previously.[42,43] In order to build the initial NPY model, the aPP heavy atom crystallographic coordinates at a nominal resolution of 1.4 Å were extracted from the Brookhaven Protein Data Bank and hydrogen atoms were explicitly added. Minimization was conducted with the backbone atoms tethered to initially relax the molecule and eliminate bad steric contacts. This aPP structure was then mutated at the 16 residues which differ between aPP and porcine NPY with common side chain torsional angles retained wherever possible. This initial NPY structure was then minimized with backbone atoms tethered to a convergence of 1.0 kcal mol^{-1} $Å^{-1}$ maximum derivative.

Binding Assays. Cell Culture. Receptor binding assays were performed with the human neuroblastoma SK-N-MC cells (Y_1 receptors) and the SK-N-BE(2) cells (Y_2 receptors). Cells were grown to confluency in 100-mL plates, trypsinized, and then plated into 24 microwell plates (10^5 cells per well). Media for SK-N-MC cells was MEM Eagles (500 mL) buffer, containing L-glutamine (10 mL, 200 mM), penicillin/streptomycin (5 mL, 10 mg/mL), non-essential amino acids (5 mL, 100X Irvine Scientific), Na + pyruvate (5 mL, 11 mg/mL), fetal calf serum (50 mL, 10%), 500 μL of fungiyorl. Media for SK-N-BE(2) cells was DME: F12 (500 mL) buffer, containing L-glutamine (10 mL, 200 mM), penicillin/streptomycin (5 mL, 10 mg/mL), fetal calf serum (50 mL, 10%), 500 μL of fungiyorl. After 1 day for the SK-N-BE(2), and 2 days for the SK-N-MC, the cells had grown to the appropriate density and were used for binding assays.

Binding Experiments. Peptides to be tested were prealiquoted at 100 μg/mL in 100 μL solution in Milli-Q water (Millipore, USA). Solutions of peptides in different concentrations (10^{-6} to 10^{-10} M) were prepared in binding buffer (0.30 M sucrose, 10 mM HEPES, 0.1% BSA, and pH is adjusted to 7.4 with NaOH). Microwell plates were precoated with p(Lys-Ala) (100 μL of 0.2 g/L) in ddH_2O at 37 °C for 1 min followed by replacement with 400 μL of binding buffer per well and stored at 37 °C until all plates had been pretreated. Tracer (^{125}I-PYY from NEN) was diluted to 15 000 cpm/50 μL. Each peptide solution (50 μL) was tested in triplicate in the presence of 50 μL of tracer. After 45 min of incubation at 37 °C the cells were washed with 250 μL of ice cold binding buffer and lyzed with lysis buffer (8 M urea, 3 M acetic acid, 2% triton). For each well, buffer was transferred to a tube for counting. The content of each well was counted using a micromedic gamma counter. Data were prepared on a program called SCAPRE and were reduced with the program SCAFIT, part of the LIGAND system.

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