Neuropeptide Y: Y_1 and Y_2 Affinities of the Complete Series of Analogues with Single D-Residue Substitutions†

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In an effort to gain insight into the bioactive conformation of neuropeptide Y upon interaction with its receptors, all single-point D-amino acid substituted NPY analogues were prepared, and their Y_1 and Y_2 receptor binding affinities were evaluated using the human neuroblastoma cell lines, SK-N-MC and SK-N-BE2, respectively. Solid-phase synthesis (Boc strategy) followed by preparative HPLC purification produced analogues of high purity that were characterized by RP-HPLC, AAA, LSIMS, CZE, and optical rotation. Of the 37 isomers (a naturally occurring glycine at position 9 was replaced by Ala and D-Ala), Y_1 receptor binding was most perturbed by chiral inversion of residues at the C-terminus (residues 20, 27, 29–35, $K_i \geq 300$ nM). Substitutions at residues 2–5, 28, and 36 had K_i values ranging from 40 to 260 nM. Substitutions at all other positions yielded analogues with affinities ranging from 1.5 to 20 nM. Binding affinities to the Y_2 class of receptors all measured in the low or sub-nanomolar concentrations, with the exception of C-terminally modified isomers (residues 30–35). Only [D-Arg[33]]- and [D-Gln[34]]NPY displayed no measurable binding affinity to Y_2 receptors at the highest concentration tested (1000 nM). Representative analogues were selected on the basis of their binding affinities and position in the sequence for structural analysis using circular dichroism (CD) spectroscopy. Of the nine peptide evaluated ([D-Pro[5]]-, [Ala[9]]-, [D-Ala[9]]-, [D-Glu[10]]-, [D-Asp[11]]-, [D-Ala[18]]-, [D-Tyr[20]]-, [D-Tyr[27]]-, and [D-Arg[33]]NPY), only [D-Tyr[27]]NPY expressed a definitive correlation between loss of binding affinity and disruption of secondary structure by having the propensity to form β-sheets at the expense of α-helical content. It was concluded that although the incorporation of a single D-amino acid within the sequence of NPY may confer a conformational perturbation, the receptor interaction was only affected when certain critical residues were modified, findings that provide a basis for the identification of the binding pharmacophore of NPY.

Introduction

Neuropeptide Y (NPY, Figure 1), a member of the pancreatic polypeptide (PP) family of peptides, is a 36 residue C-terminally amidated polypeptide.[1,2] NPY elicits numerous pharmacological effects including mediation of vasoconstriction, analgesia, feeding behavior, and hormone secretion.[3-7] NPY shows 69% sequence homology to the intestinal hormone peptide YY (PYY) and 50% homology to pancreatic polypeptide (PP).[8] Recent spectral studies as well as molecular dynamics simulation suggest that the tertiary structure of NPY may be characterized by having a polyproline-type II helix for residues 1–8, a β-turn through positions 9–14, an amphipathic α-helical segment extending from residues 15–32, and a C-terminal turn structure from residues 33–36.[8-11]

The physiological and pharmacological activities of NPY have suggested a multiplicity of receptor subclasses; the best-described subclasses of NPY receptors have been designated Y_1 and Y_2 receptors, though other forms have been proposed.[12-14] In the sympathetic nervous system, receptors of the Y_1 subtype exist predominantly postsynaptically to mediate vasoconstriction, while those of the Y_2 type act presynaptically in the regulation of catecholamine release. The Y_2 type appears to be the predominant form in the central nervous system. The pharmacological distinction between the two receptor subclasses is derived from differential binding properties of C-terminal fragments and modifications of NPY, especially the 13–36 fragment which binds only to Y_2 receptors and [Leu[31],Pro[34]]NPY which has been demonstrated to be a Y_1-selective agonist.[13,15,16] Recently, several cyclic and centrally truncated analogues of NPY that show high selectivity for a single class of receptors have been developed.[17-19] While X-ray crystallographic and NMR structural studies of peptides will allow prediction of the most stable conformations, SAR are necessary to provide a correlation with observed bioactivities. In the present study, we have investigated the effects of D-substitutions on Y_1 and Y_2 receptor binding affinities. Such a systematic study allows the quantitation of the relative contribution

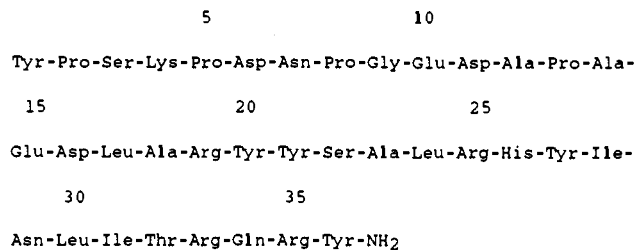

Figure 1. Primary sequence of porcine neuropeptide Y.

† Abbreviations: The abbreviations for the amino acids 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 where indicated otherwise. In addition: AAA, amino acid analysis; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CZE, capillary zone electrophoresis; DCM, dichloromethane; DIC, 1,3-diisopropylcarbodiimide; DMF, dimethylformamide; EDT, ethanedithiol; HOBt, 1-hydroxybenzotriazole; LSIMS, liquid secondary ion mass spectrometry; NMP, *N*-methylpyrrolidone; NPY, neuropeptide Y; PP, pancreatic polypeptide; PYY, peptide YY; RP-HPLC, reverse-phase high-performance chromatography.

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of residue orientation to the stabilization of secondary and tertiary structures. These substitution studies have been carried out in a number of bioactive peptides including bombesin,[20] growth hormone releasing factor,[21] somatostatin,[22] gonadotropin releasing hormone,[23] neurotensin,[24] and corticotropin releasing factor[25] among others. Recently, several D-substitution studies have been conducted on truncated NPY analogues.[26,27] We reported earlier the hypertensive potencies of a limited number of D-substituted, full-length NPY analogues;[16] in the present study we have evaluated the whole series for their ability to bind to both the Y_1 and Y_2 receptors. It was speculated that the systematic substitution of each residue by its D-isomer might yield insight as to which residues were most critical for maintenance of the bioactive conformation(s) by correlating structure (using CD spectroscopy) and binding affinity.

Results

Chemical Synthesis and Characterization. All peptides were assembled by standard solid-phase peptide synthesis (SPPS) techniques on *p*-methylbenzhydrylamine (MBHA) resin, using previously described protocols.[18] Briefly, *tert*-butyloxycarbonyl (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) as primary coupling reagent; more difficult cycles required the use of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) in *N*-methylpyrrolidinone (NMP) or dimethylformamide (DMF) in the presence of excess of diisopropylethylamine (DIPEA). Coupling times were generally 1–2 h, monitored by qualitative Kaiser test,[28] repeating coupling steps as necessary. After TFA deprotection, resin washes included 2-propanol (containing 1% EDT), triethylamine (10% in DCM) for neutralization of peptide, MeOH, and DCM. The protected peptide was cleaved from the resin by anhydrous HF in the presence of 10% anisole at 0 °C for 90 min. Volatiles were removed *in vacuo*. The crude peptides were precipitated with anhydrous ethyl ether and filtered to remove ether-soluble, nonpeptide materials, and extracted in water. After lyophilization, crude peptides were purified by preparative reverse-phase HPLC in two stages to yield final products of high purity. Each analogue was fully characterized by LSIMS, amino acid analysis (supplemental material), optical rotation, and analytical HPLC. Diastereomers were further analyzed by HPLC in four systems and capillary zone electrophoresis (CZE) in two systems to ensure their purity and uniqueness with the results presented elsewhere.[29] In summary, we were able to separate all diastereomers described in this report from NPY itself and we are confident that the biological data reported here for each analogue reflect the biological activity of that analogue and are not mitigated by the presence of significant amounts of NPY as a contaminant.

Receptor Binding Activity. Y_1 Binding Affinity Using SK-N-MC Human Neuroblastoma Cells (Refer to Table I and Figure 2). The receptor binding assay is based on the competition between NPY analogues (at a range of concentrations) with [^{125}I]PYY tracer in the presence of human neuroblastoma SK-N-MC cells (Y_1 type receptors). Of the 37 analogues, Y_1 receptor binding (K_i for NPY is 2.0 nM) was most perturbed by chiral inversion of residues at the C-terminus (residues 20, 27, 29–35, K_i ≥ 300 nM). Substitutions at residues 2–5, 28, and 36 had K_i values ranging from 40 to 260 nM. Substitutions at all other positions, including substitution of Gly^9 by L- or D-Ala (K_i = 4.5 and 2.9 nM, respectively) yielded analogues with affinities ranging from 1.5 to 20 nM (ranking order [D-Asp^{11}]- > [D-Ala^{14}]- > [D-Leu^{24}]- > [D-Arg^{25}]- > [D-Ser^{22}]- > [D-Glu^{10}]- = [D-Glu^{15}]- = [D-Ala^{18}]- > [D-Leu^{17}]- = NPY > [D-Ala^9]- > [D-Arg^{19}]- > [Ala^9]- > [D-Tyr^1]- > [D-Ala^{12}]- > [D-His^{26}]- > [D-Ala^{23}]- > [D-Asp^{16}]- > [D-Asp^6]- > [D-Pro^{13}]- > [D-Asn^7]- = [D-Pro^8]- > [D-Tyr^{21}]NPY.

Y_2 Binding Affinity Using SK-N-BE2 Human Neuroblastoma Cells (Refer to Table I and Figure 2). When testing for affinities at the Y_2 receptor, [D-Tyr^1]-, [D-Pro^2]-, [D-Ala^9]-, [D-Ala^{14}]-, [D-Asp^{16}]-, [D-Leu^{17}]-, [D-Ala^{18}]-, and [D-Leu^{24}]NPY all bound at sub-nanomolar concentrations (0.2–0.9 nM), as compared to NPY which displayed a K_i of 0.3 nM. All other analogues, excluding those modified in the C-terminal segment, bound at low nanomolar concentrations (1.0–8.7 nM, reflecting a 5–29-fold decrease in affinity). The D-isomers in positions 31, 32, and 35 produced analogues with greatly reduced affinities (19–190 nM), while such chiral substitutions of Arg^{33} and Gln^{34} were devoid of binding at the highest concentrations tested (1000 nM). The D-isomer of Tyr^{36} bound with 10% of the relative affinity of NPY. Note: because of the fewer number of Y_2 receptors per cell as compared to Y_1 receptors in SK-N-MC cells and the higher level of nonspecific binding to SK-N-BE2 cells, the observed error (% CV) associated with the K_i values was larger than those produced in the Y_1 assay.

Circular Dichroism Spectra (Figures 3 and 4 and Table II). The residue molar ellipticity in the wavelength range 190–240 nm of NPY and selected analogues, in aqueous buffer (0.01 M sodium phosphate, 0.05 M sodium chloride, pH 7.40) were measured each at a concentration of 100 μM. Of those analogues which bound to receptors with relatively high affinity, shown in Figure 3, all displayed spectra comparable to that of NPY with the exception of [D-Ala^{18}]NPY, which produced a spectrum with 13% β-sheet content when deconvoluted using the PROSEC program (Aviv Associates) which employs the reference spectrum of Yang et al.[30] Spectra of low-affinity binding analogues (Figure 4) suggested a strong α-helical component (28–35%) similar to that of NPY except for [D-Tyr^{27}]-NPY, which produced a spectrum with less than 8% α-helicity. Spectra of both [Ala^9]- and [D-Ala^9]NPY (not shown) were virtually identical to that of NPY.

Discussion

A three-dimensional conformation of NPY has been proposed through combined use of molecular modeling, NMR, and circular dichroism techniques.[8,10,11] Together, these studies suggest that the tertiary structure of NPY may be characterized by having a polyproline-type II helix for residues 1–8, a β-turn through positions 9–14, an amphipathic α-helical segment extending from residues 15 to 32, and a C-terminal turn structure from residues 33 to 36. It was expected that by systematically replacing each residue in the NPY sequence with the corresponding D-isomer the tertiary structure of the peptide backbone may be perturbed in such a way that could either disrupt or enhance binding to the receptor or allow discrimination between receptors. In most cases, the change in affinities at the Y_2 receptor type paralleled that at the Y_1 receptor,

Table I. Physical and Biological Properties of NPY Analogues

NPY analogue	purity[a]	RT[a]	obsd m/z[b]	$[\alpha]_D$[c] (deg)	Y_1K_i (nM)[d]	% CV	Y_2K_i (nM)[e]	% CV
NPY	>99	13.3	4252.1	-57	2.0	10	0.3	45
[D-Tyr^{1}]	>99	13.3	4252.0	-68	5.2	6.6	0.5	34
[D-Pro^{2}]	>99	13.5	4251.9	-47	42	6.5	0.7	25
[D-Ser^{3}]	>99	13.3	4251.8	-48	86	11	1.4	28
[D-Lys^{4}]	>99	13.3	4251.9	-54	160	14	2.4	23
[D-Pro^{5}]	>95	13.3	4251.9	-54	260	6.9	3.6	32
[D-Asp^{6}]	>96	13.0	4251.8	-56	9.5	6.5	4.2	33
[D-Asn^{7}]	>95	13.3	4252.2	-48	15	5.6	2.6	31
[D-Pro^{8}]	>99	12.8	4252.1	-54	15	6.1	2.5	28
[Ala^{9}]	>97	16.1	4265.9	-66	4.5	7.4	1.0	30
[D-Ala^{9}]	>99	15.1	4266.3	-61	2.9	9.2	0.8	29
[D-Glu^{10}]	>97	12.6	4252.0	-58	1.9	15	1.3	30
[D-Asp^{11}]	>99	12.1	4251.9	-52	0.9	7.9	0.5	32
[D-Ala^{12}]	>96	14.2	4251.8	-45	5.7	5.2	2.8	33
[D-Pro^{13}]	>99	12.8	4252.3	-52	14	9.1	2.2	29
[D-Ala^{14}]	>99	12.8	4252.0	-59	1.2	13	0.5	25
[D-Glu^{15}]	>99	11.2	4252.0	-58	1.9	16	1.2	31
[D-Asp^{16}]	>99	12.7	4252.0	-53	6.3	7.8	0.9	24
[D-Leu^{17}]	>96	11.0	4252.0	-65	2.0	12	0.7	27
[D-Ala^{18}]	>97	11.8	4251.9	-70	1.9	24	0.2	37
[D-Arg^{19}]	>99	10.5	4252.0	-74	4.0	7.8	2.6	28
[D-Tyr^{20}]	>98	14.2	4252.6	-71	390	12	4.2	34
[D-Tyr^{21}]	>96	11.4	4252.2	-70	19	14	1.2	37
[D-Ser^{22}]	>99	11.6	4252.3	-73	1.8	12	1.4	42
[D-Ala^{23}]	>99	10.1	4252.2	-75	6.2	6.9	3.6	29
[D-Leu^{24}]	>98	10.4	4252.2	-70	1.5	10	0.8	26
[D-Arg^{25}]	>97	7.5	4252.1	-65	1.6	20	4.6	28
[D-His^{26}]	>98	11.1	4252.2	-65	5.8	16	4.2	39
[D-Tyr^{27}]	>99	10.0	4252.2	-67	>1000	-	8.7	27
[D-Ile^{28}]	>95	6.1	4251.9	-65	49	7.6	7.6	51
[D-Asn^{29}]	>99	12.5	4252.4	-52	670	13	1.8	29
[D-Leu^{30}]	>99	9.7	4252.2	-64	>1000	-	18.7	25
[D-Ile^{31}]	>99	8.9	4252.2	-66	>1000	-	152	30
[D-Thr^{32}]	>99	10.5	4252.0	-54	>1000	-	190	42
[D-Arg^{33}]	>96	14.9	4251.9	-59	>1000	-	>1000	-
[D-Gln^{34}]	>99	14.0	4252.2	-56	440	14	>1000	-
[D-Arg^{35}]	>99	13.3	4252.3	-55	>1000	-	86	31
[D-Tyr^{36}]	>98	13.3	4252.2	-60	210	8.5	2.4	27

[a] Purities and retention times determined by analytical RP-HPLC using Vydac C_{18} column (5-μm particle size; 46 × 250 mm). Buffer system comprised of (A) = TEAP 2.25, (B) = 60% MeCN in buffer A. Gradient: 45–65% B in 20 min. Flow rate was 2.0 mL/min; detection: 0.1 AUFS at 210 nm. [b] Observed monoisotopic mass for $[M + H]^+$ measured with LSI-MS in glycerol and 3-nitrobenzyl alcohol (1:1) matrix; Cs ion source. Calculated mass was for NPY 4252.13 amu. [c] Optical rotations measured in 1% acetic acid, sodium D line. [d] Y_1 specific receptor binding affinity (K_i) measured on SK-N-MC human neuroblastoma cells. [e] Y_2 specific receptor binding affinity (K_i) measured on SK-N-BE2 human neuroblastoma cells.

although the magnitude of the changes was almost always greater at the Y_1 receptor. Six Y_2 receptor specific analogues ([D-Tyr^{27}]-, [D-Asn^{29}]-, [D-Leu^{30}]-, [D-Ile^{31}]-, [D-Thr^{32}]-, and [D-Arg^{35}]NPY) and one Y_1 receptor specific analogue ([D-Gln^{34}]NPY) were discovered in this scan, though most were of low affinity.

It has been shown that the incorporation of a single D-amino acid is disruptive to secondary structural integrity especially in regions in which the orientation of the residue sidechain is crucial to determining the formation of α-helices or turns.[21] The location of the glycine in the sequence of NPY and its absence of side-chain functionality permits this residue to act as a flexible hinge or bend, as seen in other peptides.[23] To probe the significance of this residue for conformational integrity, we replaced the naturally occurring glycine with the two stereoisomers of alanine. Reduced binding affinities to the Y_1 and Y_2 receptors by a factor of only two or three suggest that substitution by either L- or D-alanine may have little effect on conformation. Indeed, these substitutions had no significant effect on the CD spectra of these analogues, suggesting no significant deviation in secondary structure when compared to that of NPY.

The substitution of a single D-amino acid in the central segment (residues 9–32) containing both a putative β-turn (residues 9–14) and an α-helix (residues 15–32) would be expected to disrupt the natural conformation of the molecule and may thereby affect binding affinity and selectivity. Binding results of analogues with modifications in this region did not produce an overall trend, while substitutions at both ends (including part of the α-helix starting at residue 26) had more drastic effects (see below). This may be indicative of the fact that the role of the central region of the molecule is to bring together both N- and C-termini and that this can be achieved despite minor structural disturbances such as those resulting from the introduction of a D residue. When evaluated by CD spectroscopy, the relative amount of turn component was slightly favored by substitution of Glu^{10} and Asp^{11} by their corresponding D-enantiomers in the hypothetical turn region. Among these analogues evaluated by CD containing a single D-amino acid in the putative α-helical segment of NPY, [D-Ala^{18}]-, [D-Tyr^{20}]-, and especially [D-Tyr^{27}]NPY all displayed spectra with characteristically diminished α-helical content (25, 28, and 8%, respectively). The decrease in helicity was largely accounted for by a comparable increase in β-sheet formation. Because [D-Ala^{18}]NPY was a high-affinity analogue whereas [D-Tyr^{20}]- and [D-Tyr^{27}]NPY were low-affinity analogues, these results indicated that loss of α-helical content was not necessarily deleterious to receptor recognition. In the case of a molecule as large as NPY (590 atoms), a minor

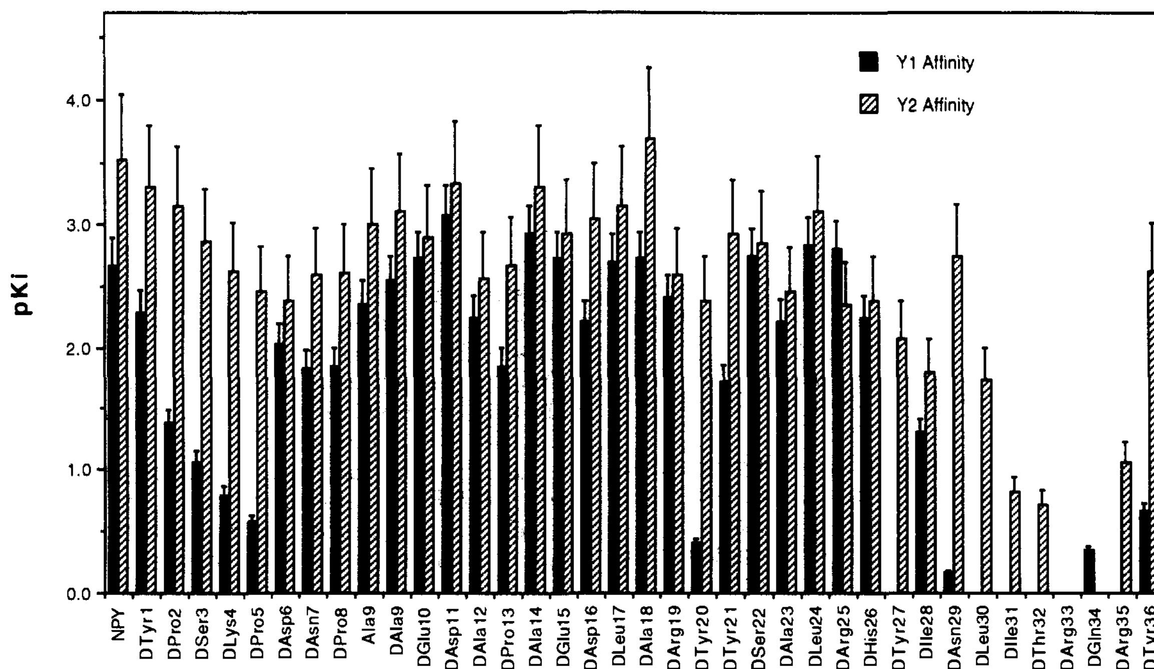

Figure 2. D-substituted analogues: graphical representation of Y_1 and Y_2 binding affinities of each analogue on SK-N-MC and SK-N-BE2 human neuroblastoma cells, respectively. Affinities expressed as pK_i values, where pK_i = $-\log K_i$ values with concentration given in μM.

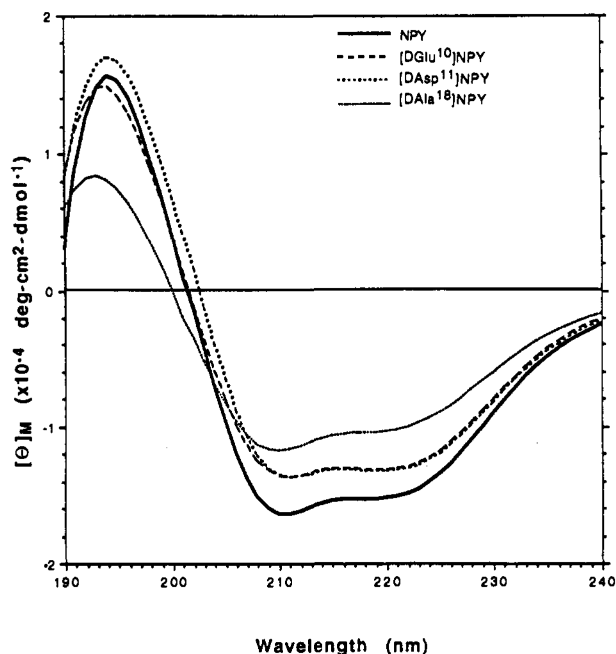

Figure 3. CD spectra of NPY and selected high affinity analogues (c = 100 μM) in aqueous buffer, pH 7.4. See the Experimental Section for additional details.

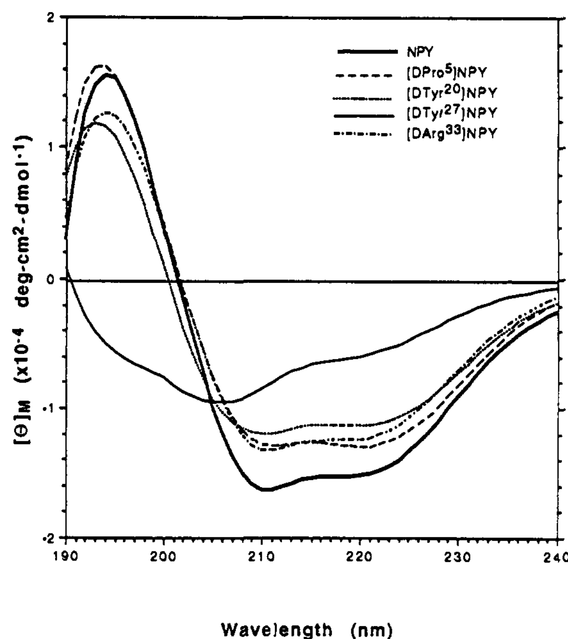

Figure 4. CD spectra of NPY and selected low affinity analogues (c = 100 μM) in aqueous buffer, pH 7.4. See the Experimental Section for additional details.

conformational disruption might be compensated by other stabilizing forces, thus minimizing the global effect of a single D-substitution. In this way, the naturally occurring tertiary structure of the molecule could be preserved through the formation of intramolecular salt bridges or hydrophobic interactions, producing analogues with comparable or even higher binding affinity despite the presence of a D-substitution, which may explain the lack of correlation between binding affinity and secondary structure exhibited by [D-Ala^{18}]NPY.

The modification of the two tyrosines at positions 20 and 27 produced analogues with substantial loss of both Y_1 and Y_2 binding affinity. The low affinity of [D-Tyr^{20}]-NPY in our systems has also been described by Martel et al., who concluded that it was the oxygen moiety on the benzyl ring together with appropriate stereochemistry that was responsible for adequate receptor affinity.[31] Molecular modeling indicated that Tyr^{27} may interact with the N-terminal tyrosine; an arrangement that is perhaps stabilized by the overlap of Π orbitals of the two aromatic rings. In addition, NMR data indicated that Tyr^{27} acted as a flexible hinge between two α-helices and showed strong interaction with other hydrophobic residues.[10] This hypothesis is supported by the present CD data in which the inverted orientation of residue 27 significantly perturbed secondary structure.

Table II. Calculated Secondary Structural Components[a] (%) for NPY and Selected Analogues in Aqueous Solution

compound	α-helix	β-sheet	turns	random
NPY	36	0	31	33
[D-Pro[5]]NPY	34	7	28	31
[Ala[9]]NPY	35	0	29	36
[D-Ala[9]]NPY	36	0	31	33
[D-Glu[10]]NPY	35	0	32	34
[D-Asp[11]]NPY	38	0	34	29
[D-Ala[18]]NPY	25	13	25	38
[D-Tyr[20]]NPY	28	12	24	36
[D-Tyr[27]]NPY	8	32	15	45
[D-Arg[33]]NPY	33	1	33	33

[a] Spectra were deconvoluted using the program PROSEC (Aviv Associates) which employs the reference spectrum of Yang et al.[30]

In correlating the binding data to *in vivo* hypertensive activities reported earlier,[16] we found that while substitutions by D-Glu[10], D-Asp[11], and D-Arg[25] in all cases slightly enhanced binding to SK-N-MC cells (Y_1 receptor), the same analogues produced hypertensive potencies equal to that of NPY for [D-Glu[10]]NPY while [D-Asp[11]]NPY and [D-Arg[25]]NPY had relative potencies of 20 and 70%, respectively. Such differences could be explained by the fact that in vitro studies are generally free from the various competing mechanisms of degradation, internalization, plasma binding, and systemic clearance as compared in *in vivo* experiments.

The central region of NPY in general has been shown to play a minor role in Y_2 binding activity, since large segments have been removed entirely without loss of potency.[18,26] Because of this, it was hypothesized that the central region of NPY acted only to spatially align and connect the C- and N-termini, a role that could be imitated by effective substitutions and the use of molecular constraints. This observation seems to be confirmed by the relatively high binding affinity of most analogues for Y_2 binding affinity (see Table I).

We have identified the entire C-terminal region as a whole (residues 29–36) to be crucial for high-binding affinity for both receptor subtypes. We therefore postulated that this region comprises the binding pharmacophore since even minor modifications within this hypothetical binding domain result in analogues with no measurable binding affinity or hypertensive activity.[16] This conclusion has been supported by numerous other binding studies as well as *in vivo* activity investigations, including those reported previously by our group.[16,19,27,31] The amidated C-terminus, though not adopting a specific secondary structure, nevertheless exists in a rigidly defined conformation when effectively interfacing with the receptors. Deletion or substitution of any kind in this region produces analogues devoid of hypertensive activity and without significant binding affinities at both receptor types. The presence of an intact C-terminus alone, however, is not sufficient to confer effective Y_1 receptor recognition since N-terminal deletions or modifications also produce analogues with loss of binding and cardiovascular potency, though binding to the Y_2 receptor tolerates such changes without significant loss of affinity.[12] The D-isomer of tyrosine at the N-terminus reduced Y_1 and Y_2 binding affinity by 3- and 1.7-fold in our assays, although this same analogue produced a 2-fold increase in hypertensive potency.[16] This apparent contradiction may be explained by a greater resistance to proteolytic degradation or elimination as a consequence of the D-substitution which offset any deleterious change in configuration engendered by the substitution. In the present study, substitution by D-amino acids in the N-terminal region in general produced analogues with decreased affinity for both receptors, with the greatest decrease measured when residues 4 and 5 were modified, findings that parallel hypertensive potencies. N-Terminally alanine-substituted analogues (from residues 1 to 10) reported by Forest et al. had greatly reduced binding affinities as well.[32] The low relative affinities of the Ala-substituted analogues on rat brain membranes compared to that of the D-substituted analogues (on SK-N-MC and SK-N-BE2 human neuroblastoma cells) can only suggest that the stability contributed by the N-terminus is more the result of specific side-chain functionality than that of the overall conformation of the backbone.

Early structure measurements of porcine neuropeptide Y by NMR indicated that this molecule exists in an α-helical conformation from the aspartic acid residue at position 11 to the tyrosine at position 36 with little interaction between the N- and C-termini;[33,34] similar results have been described for human NPY, which differs from the porcine sequence by the substitution of a methionine for the leucine at position 17.[35] It is apparent, therefore, that these predictions significantly deviate from the PP-fold model constructed by homology to the X-ray crystal structure of pancreatic polypeptide.[8] In fact CD analysis of [D-Arg[33]]NPY in the current study produced a spectrum with slightly decreased α-helical content, supporting the suggestion that the helical portion of NPY may extend well into the C-terminus. However, the bioactive conformation, as deduced from binding and other biological studies of truncated and cyclic analogues, defines the requirement for the antiparallel alignment of the termini for full activity and thus supports the original PP-fold model.[18,26,36] More recent NMR investigations of human NPY[10] and bovine PP[11] have supported this PP-fold model with minor modifications. Assuming, therefore, that the actual binding conformation is best described as being stabilized by the N-terminal polyproline helix, present results suggest greatest disruption of this fold is produced by inverting the chirality of residues 3–5. Since binding affinities, though reduced, were still measurable in analogues with changes made in the N-terminus, the present data together with earlier results[16] supports the hypothesis that the N-terminus stabilizes the conformation required to maintain high affinity to receptors but is not involved in receptor activation, a role reserved for the C-terminus.[31] Data from the present ligand/receptor binding study supported by CD spectral data provide the basis of a more definitive interpretation of the bioactive conformation of NPY at both receptor subclasses and support earlier conformational predictions based on the original PP-fold model and the most recent NMR data.

Experimental Section

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

Peptide Synthesis. All peptides were synthesized manually using Boc strategy on MBHA resins prepared in our laboratory by methods previously described.[18] Side-chain protection of α-Boc amino acids (Bachem, Torrance, CA) was as follows: Arg(Tos), Asp(β-OcHx), Glu(γ-OcHx), His(Tos), Lys(2ClZ), Ser-

(Bzl), Thr(Bzl), and Tyr(2BrZ). Asn and Gln were coupled in the presence of a 2-fold excess of 1-hydroxybenzotriazole. His-(Tos-DCHA salt) was coupled with a 2-fold excess of BOP. Removal of the Boc group was accomplished by treatment of the peptide-resin with 60% TFA in CH_2Cl_2 in the presence of 1% EDT. The protected peptide-resin was cleaved in anhydrous HF in the presence of 10% anisole at 0 °C for 1.5 h. Crude peptides were precipitated and washed with diethyl ether, then extracted from resin and other organic material with water, and lyophilized.

Purification. Crude peptides were purified by preparative reverse-phase HPLC[37,38] on a Waters DeltaPrep LC 3000 system equipped with a Waters 1000 Prep Pak Module and a Shimadzu SPD-6A variable-wavelength UV detector. The cartridges used were hand packed, in house, with Waters polyethylene sleeves and frits and Vydac C18 packing material (15–20 μm particle size, 30-nm pore size). The material was eluted first using a linear TEAP/60% MeCN (pH 2.25 or 5.2) buffer system gradient; acceptable fractions were pooled, reloaded onto the preparative cartridge, and desalted in 0.1% TFA. Final products were >95% pure by HPLC analysis.

Peptide Characterization. Purified peptides were subjected to HPLC analysis [Vydac C_{18} column, on a Perkin-Elmer Series 400 Liquid Chromatograph, Kratos Spectroflow 757 UV detector, and Hewlett-Packard Model 3390A integrator (for specific conditions, see Table I)], amino acid analysis [hydrolysis in 4 N methanesulfonic acid at 110 °C for 24 h, followed by ion-exchange chromatography and postcolumn derivativization with o-phthalaldehyde], and LSI-MS analysis measured with a JEOL JMS-HX110 double focusing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. Samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix. The unique identity of each stereoisomer was confirmed by multiple co-injection experiments by HPLC analysis performed on a Hewlett-Packard Series II 1090 liquid chromatograph with diode array detector and HPLC ChemStation. The C_{18} or phenyl column was Vydac from the Separations Group and was 0.21 × 15 cm. The buffer A was 0.1% TFA or TEAP. The TEAP was 0.1% phosphoric acid adjusted to the desired pH by addition of TEA. Buffer B was composed of 60% acetonitrile in buffer A. Analyses were performed at 40 °C at flow rate of 0.2 mL/min and detected at 210 nm. Four isomers could not be resolved by these techniques, and therefore were subjected to analysis by capillary electrophoresis performed on a Beckman P/ACE System 2050 with Spectra-Physics ChromJet SP440 integrator. Fused silica capillaries were 50 cm × 75 mm. The buffer was 100 mM phosphoric acid adjusted to pH 2.5 by addition of 2 M sodium hydroxide or 100 mM phosphoric acid adjusted to pH 2.5 by addition of triethylamine. Voltage ranged from 12 to 20 kV and produced from 66 to 100 mA. Full description of methods leading to characterization of each isomer have been presented elsewhere.[29]

Optical Rotation. Optical rotations of analogues were measured in 1% acetic acid (c = 1, i.e. 10 mg of lyophilized peptide/mL uncorrected for TFA counterions or water present after lyophilization). Values were calculated from the means of 10 successive 5-s integrations determined at 25 °C on a Perkin-Elmer 241 polarimeter (using the D line of Na emission) divided by the concentration of the sample in g/dL and are quoted as uncorrected specific rotations.

Binding Experiments. Binding experiments were carried out by Alanex Laboratories using SK-N-MC and SK-N-BE(2) human neuroblastoma cells (American Type Culture Collection, Bethesda, MD). The methodology used was identical to that reported by us in an earlier publication.[18]

Circular Dichroism Spectra. Circular dichroism (CD) measurements were obtained with an Aviv Model 62DS spectropolarimeter (Aviv Associates, Lakewood, NJ) under control of the manufacturer's operating system (60DS) using 0.5-mm cuvettes thermostated at 20.0 °C and signal averaging five scans in the range 190–240 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 all compounds were collected in 0.01 M sodium phosphate, 0.05 M sodium chloride (pH 7.40) to produce a peptide concentration of 100 μM. Concentrations were based on the calculated molecular weight of the TFA salt of the purified lyophilized peptide assuming a water content of 7% and were used for the calculation of residue molar ellipticity. Residue molar ellipticity were calculated based on the number of residues in each analogue. Spectra were deconvoluted using the program PROSEC (AVIV Associates), which employs the reference spectrum of Yang et al.[30]

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