Structural Requirements for Neuropeptide Y^{18-36}-Evoked Hypotension: A Systematic Study†

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It has been shown that NPY and select C-terminal fragments of NPY that evoke a hypotensive response upon intraarterial administration in the rat also cause mast cell degranulation and histamine release in vitro. Additionally, elevation of plasma histamine levels has been observed concomitant with the hypotensive effect of NPY and various C-terminal fragments. In order to investigate whether the hypotensive response to NPY^{18-36} is correlated to this observed elevation of histamine in vivo, we sought to characterize the structural requirements for each activity. We conducted a systematic replacement of each amino acid in NPY^{18-36} by its D-isomer. Additionally, various modifications were made to the N- or C-terminii of NPY^{18-36}. The following rank order of potency was obtained for the hypotensive action of these analogues of NPY^{18-36} relative to NPY^{18-36}. Only one analogue ([D-Tyr^{21}]NPY^{18-36}) exhibited significantly enhanced potency. Eleven analogues of NPY^{18-36}, ([D-Thr^{32}]-, [D-Arg^{35}]-, [D-Ile^{31}]-, [D-Leu^{30}]-, [D-Tyr^{27}]-, [D-Ser^{22}]-, [D-Tyr^{36}]-, [D-Gln^{34}]-, [D-Asn^{29}]-, [D-Ala^{23}]-, and [D-Arg^{33}]NPY^{18-36}) were equipotent with NPY^{18-36}. Four analogues ([D-His^{26}]-, [D-Ile^{28}]-, and [D-Ala^{18}]NPY^{18-36} and -NPY^{18-27}) had reduced potency (10–80%) while eight analogues ([D-Arg^{19}]-, [D-Tyr^{20}], [D-Leu^{24}]-, [D-Arg^{25}]-, [Ac-Ala^{18}]-, [Me-Ala^{18}]-, [desamino-Ala^{18}]NPY^{18-36} and NPY^{18-36} free acid) failed to produce a significant hypotensive response (<10%) at the doses tested. The sensitivity of NPY^{18-36} to chiral inversion of single residues or other modifications at the N-terminus suggested the presence of a conformationally well defined N-terminal pharmacophore. Additionally, five NPY^{18-36} analogues were tested for elevation of plasma histamine levels. The rank order of potency ([D-Thr^{32}]NPY^{18-36} = [D-Tyr^{21}]NPY^{18-36} ≫ NPY^{18-36} > [D-Ala^{18}]NPY^{18-36} > [Ac-Ala^{18}]NPY^{18-36}) was correlated with each analogue's potency at evoking a hypotensive response. In contrast, NPY^{1-36} failed to evoke an elevation in plasma histamine levels despite its hypotensive effects. Hence, we conclude that the magnitude of the hypotensive response evoked by an NPY^{18-36} analogue is primarily a function of its ability to elevate plasma histamine levels. However, the mechanism underlying NPY^{1-36}-evoked hypotension appears to be different.

Introduction

Neuropeptide Y (NPY) is a C-terminally amidated 36 amino acid peptide originally isolated from porcine brain.[1] NPY has a primary sequence highly homologous (70%) with that of the gut peptide peptide YY (PYY).[2] Both NPY and PYY interact with two receptors (Y_1 and Y_2) identified to date.[3] A third receptor subtype has recently been reported that preferentially binds NPY over PYY.[4,5] Binding of NPY or PYY to Y_1 or Y_2 receptors is disrupted by subtle modifications of the last few C-terminal residues.[6]

NPY administered intraarterially (IA) exerts a dose-dependent biphasic pressor–depressor response on arterial pressure (AP).[7,8] The initial pressor phase of the biphasic response has been reported to be mediated through the Y_1 receptor.[9] Various C-terminal fragments of NPY (e.g., NPY^{18-36}), which exhibit low affinity for the Y_1 receptor, do not increase arterial pressure, but evoke a hypotensive response of similar magnitude and duration as that seen for NPY^{1-36}.[10,11] The mechanisms underlying this depressor response are not yet fully elucidated. Recently, we and others have demonstrated that the depressor effect is not mediated by either the Y_1 or Y_2 receptor subtypes.[12] Additionally, the hypotensive response has been shown to involve elevation of plasma histamine levels, and is dependent upon histamine's action at the H_1 histamine receptor.[3,13] NPY, PYY, and NPY fragments have recently

† Abbreviations: The abbreviations for the amino acids are in accord with the recommendations of the IUPAC-IUUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *138*, 9–37). The symbols represent the L-isomer except when indicated otherwise. In addition, NPY, neuropeptide, Y; PYY, peptide YY; PP, pancreatic polypeptide; AP, arterial pressure; IA, intraarterial; SEM, standard error of the mean; SD, standard deviation; Ac, acetyl; Me, methyl; Boc, *tert*-butoxycarbonyl; EDT, ethanedithiol; DIC, 1,3-diisopropylcarbodiimide; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; OcHx, cyclohexyl ester.

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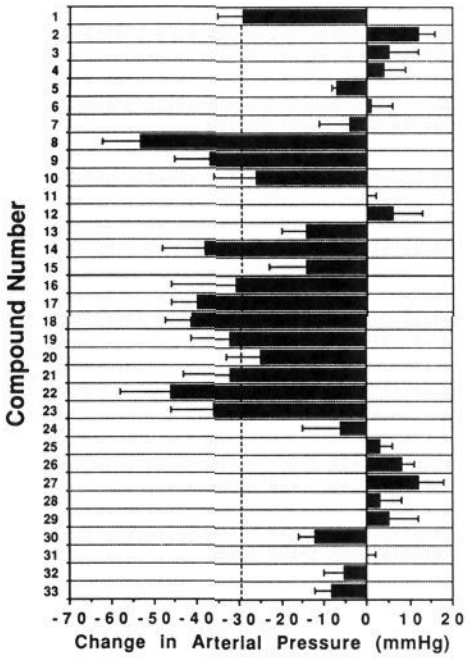

Figure 1. Change in mean arterial pressure (ΔMAP) in mmHg measured 5 min following administration of 100 μg of compound numbered according to Table I. Bars represent SEM ($n \geq 3$). The vertical dashed line gives the value for NPY^{18-36} for comparison.

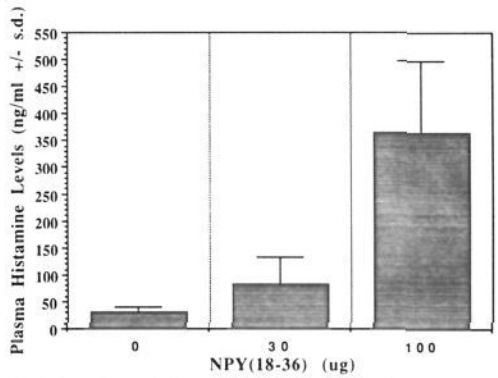

Figure 2. Elevation of plasma histamine levels (ng/mL) following IA administration of 0, 30, and 100 μg of NPY^{18-36}.

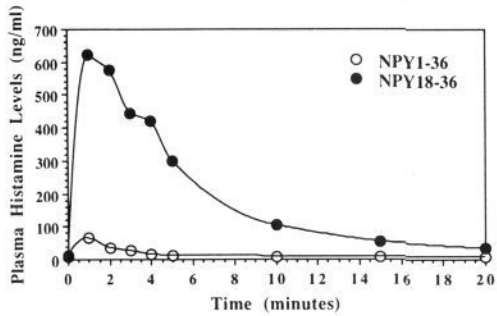

Figure 3. Time course of plasma histamine elevation following IA administration of either 10 μg of NPY^{1-36} or 100 μg of NPY^{18-36}; n = 1 for each.

been shown to release histamine from rat peritoneal mast cells in vitro.[14,15] However, the source and mechanism of release of plasma histamine participating in the depressor effect on the vasculature remains to be determined.

In the present study, we examined a variety of NPY^{18-36} analogues to determine the structural requirements for evoking a hypotensive response. Additionally, we examined the changes of plasma histamine levels following intraarterial (IA) administration of NPY^{1-36}, PYY^{1-36}, and various analogues of NPY^{18-36}. We present here results suggesting that the mechanisms underlying NPY^{1-36} versus NPY fragment-evoked hypotension may be different.

Results

Peptide Synthesis and Characterization. Peptide analogues were synthesized using a *p*-methylbenzhydrylamine-resin.[16,17] Briefly, *tert*-butoxycarbonyl (Boc) was used for N-terminal protection with N-terminal deblocking performed with TFA–CH_2Cl_2 (3:2) in the presence of 1% ethanedithiol (EDT) for 20 min. Coupling was mediated using 1,3-diisopropylcarbodiimide (DIC) or dicyclohexylcarbodiimide (DCC) in CH_2Cl_2 (DCM) or 50% DCM–dimethylformamide (DMF), depending on the solubility of the Boc-amino acids. Difficult couplings were accomplished using 1-hydroxybenzotriazole (HOBt) plus DCC or HOBt and (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP, 1:1) in DCM or 50% DMF–DCM in the presence of excess diisopropylethylamine. The protected peptide-resin was cleaved in anhydrous HF in the presence of anisole and methyl sulfide as scavengers. Crude peptides were extracted from the resin with dilute acetic acid and lyophilized.

Crude peptides were purified by preparative reverse-phase HPLC.[18-20] Purified peptides were subjected to HPLC analysis in 0.1% TFA/MeCN (see Table I). Purity of the peptides, synthesized over a 3-year period, were

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Table I. HPLC Isocratic Retention Times, Purities, Mass Spectrometry Data, Specific Rotations, and Changes in Mean Arterial Pressure Following a 0.4 mg/kg Dose of NPY^{18-36} or Analogs

no.	NPY^{18-36} analog or fragment	iso RT @ % MeCN[a]	purity %[b]	calcd[c] mass	obsd[c] mass	$[\alpha]^{25}_D$, deg[c]	amino acid anal.	ΔMAP @ 5 min (0.4 mg/kg)[d]	SEM ($n \geq 3$)
1	NPY^{18-36}	3.4 @ 30.0	>99	2456.34	2456.3	−46.0	Asp 1.02 (1.00), Thf 0.94 (1.00), Ser 0.88 (1.00), Glu 1.00 (1.00), Ala 2.00 (2.00), Ile 1.88 (2.00), Leu 2.06 (2.00), Tyr 4.00 (4.00), His 1.02 (1.00), Arg 4.36 (4.00)	−28.55	6.44
2	[Ac-Ala^{18}]	4.3 @ 33.0	>98	2498.35	2498.6	−47.7	Asp 0.71 (1.00), Thr 0.85 (1.00), Ser 0.96 (1.00), Glu 1.02 (1.00), Ala 2.01 (2.00), Ile 1.92 (2.00), Leu 2.00 (2.00), Tyr 4.23 (4.00), His 0.89 (1.00), Arg 4.27 (4.00)	12.00	3.85
3	[Me-Ala^{18}]	3.4 @ 30.0	>97	2470.36	2470.7	−46.4	Asp 0.96 (1.00), Thr 0.93 (1.00), Ser 0.94 (1.00), Glu 1.02 (1.00), Ala 1.06 (2.00), Ile 1.91 (2.00), Leu 2.00 (2.00), Tyr 4.13 (4.00), His 0.92 (1.00), Arg 3.51 (4.00), + NMe-Ala	5.25	6.92
4	[desamino-Ala^{18}]	4.5 @ 32.4	>98	2441.33	2441.7	−44.2	Asp 1.00 (1.00), Thr 0.96 (1.00), Ser 0.94 (1.00), Glu 1.04 (1.00), Ala 1.10 (1.00), Ile 1.84 (2.00), Leu 2.00 (2.00), Tyr 4.00 (4.00), His 0.97 (1.00), Arg 3.73 (4.00), + desamino Ala	4.40	5.11
5	[D-Ala^{18}]	4.3 @ 30.0	>97	2456.34	2456.6	−46.9	Asp 0.77 (1.00), Thr 0.91 (1.00), Ser 0.94 (1.00), Glu 1.05 (1.00), Ala 2.00 (2.00), Ile 2.08 (2.00), Leu 2.18 (2.00), Tyr 4.35 (4.00), His 1.02 (1.00), Arg 3.55 (4.00)	−7.00	1.47
6	[D-Arg^{19}]	4.0 @ 30.0	>96	2456.34	2456.6	−43.4	Asp 1.01 (1.00), Thr 0.99 (1.00), Ser 0.97 (1.00), Glu 1.02 (1.00), Ala 2.05 (2.00), Ile 1.86 (2.00), Leu 2.00 (2.00), Tyr 3.98 (4.00), His 0.97 (1.00), Arg 4.05 (4.00)	0.75	4.84
7	[D-Tyr^{20}]	3.7 @ 29.4	>96	2456.34	2456.4	−39	Asp 0.98 (1.00), Thr 0.92 (1.00), Ser 0.92 (1.00), Glu 1.05 (1.00), Ala 2.00 (2.00), Ile 1.80 (2.00), Leu 1.89 (2.00), Thr 3.84 (4.00), His 1.04 (1.00), Arg 4.27 (4.00)	−4.40	7.10
8	[D-Tyr^{21}]	4.1 @ 28.2	>99	2456.34	2456.6	−48	Asp 1.00 (1.00), Thr 0.93 (1.00), Ser 0.96 (1.00), Glu 1.08 (1.00), Ala 2.00 (2.00), Ile 1.86 (2.00), Leu 1.93 (2.00), Tyr 4.06 (4.00), His 1.35 (1.00), Arg 4.37 (4.00)	−53.00	9.29
9	[D-Ser^{22}]	4.0 @ 29.4	>91	2456.34	2456.5	−36	Asp 1.04 (1.00), Thr 0.96 (1.00), Ser 0.90 (1.00), Glu 1.03 (1.00), Ala 2.00 (2.00), Ile 1.90 (2.00), Leu 1.93 (2.00), Tyr 3.97 (4.00), His 0.94 (1.00), Arg 4.30 (4.00)	−36.67	8.19
10	[D-Ala^{23}]	4.4 @ 25.8	>93	2456.34	2456.54	−48	Asp 1.13 (1.00), Thr 0.90 (1.00), Ser 0.86 (1.00), Glu 0.921.00), Ala 2.00 (2.00), Ile 1.80 (2.00), Leu 1.87 (2.00), Tyr 3.85 (4.00), His 0.86 (1.00), Arg 3.61 (4.00)	−25.66	9.53
11	[D-Leu^{24}]	6.1 @ 24.6	>92	2456.34	2456.3	−41	Asp 1.11 (1.00), Thr 1.03 (1.00), Ser 0.92 (1.00), Glu 1.13 (1.00), Ala 2.00 (2.00), Ile 2.15 (2.00), Leu 1.99 (2.00), Tyr 4.27 (4.00), His 0.84 (1.00), Arg 4.35 (4.00)	0.00	2.00
12	[D-Arg^{25}]	4.5 @ 24.6	>98	2456.34	2456.4	−36	Asp 1.13 (1.00), Thr 0.92 (1.00), Ser 0.87 (1.00), Glu 0.97 (1.00), Ala 2.00 (2.00), Ile 1.84 (2.00), Leu 1.94 (2.00), Tyr 4.01 (4.00), His 0.89 (1.00), Arg 3.70 (4.00)	6.33	6.88
13	[D-His^{26}]	3.7 @ 24.6	>94	2456.34	2456.4	−38	Asp 1.16 (1.00), Thr 0.91 (1.00), Ser 0.87 (1.00), Glu 0.95 (1.00), Ala 2.00 (2.00), Ile 1.83 (2.00), Leu 1.88 (2.00), Tyr 4.00 (4.00), His 0.87 (1.00), Arg 3.61 (4.00)	−13.66	5.78
14	[D-Tyr^{27}]	3.7 @ 49.8	>96	2456.34	2456.4	−40	Asp 1.08 (1.00), Thr 0.89 (1.00), Ser 0.86 (1.00), Glu 0.94 (1.00), Ala 2.00 (2.00), Ile 1.79 (2.00), Leu 1.92 (2.00), Tyr 4.04 (4.00), His 0.91 (1.00), Arg 3.61 (4.00)	−38.00	9.85
15	[D-Ile^{28}]	4.7 @ 29.4	>90	2456.34	2456.4	−43	Asp 1.04 (1.00), Thr 0.99 (1.00), Ser 0.98 (1.00), Glu 1.14 (1.00), Ala 1.87 (2.00), Ile 1.86 (2.00), Leu 2.00 (2.00), Tyr 3.74 (4.00), His 1.24 (1.00), Arg 4.35 (4.00)	−14.00	8.58
16	[D-Asn^{29}]	3.9 @ 27.6	>99	2456.34	2456.4	−38	Asp 0.91 (1.00), Thr 0.92 (1.00), Ser 0.94 (1.00), Glu 1.02 (1.00), Ala 2.00 (2.00), Ile 1.88 (2.00), Leu 1.99 (2.00), Tyr 4.00 (4.00), His 0.95 (1.00), Arg 3.74 (4.00)	−30.60	15.06
17	[D-Leu^{30}]	4.3 @ 25.2	>96	2456.34	2456.4	−35	Asp 1.12 (1.00), Thr 0.92 (1.00), Ser 0.89 (1.00), Glu 0.95 (1.00), Ala 2.00 (2.00), Ile 1.84 (2.00), Leu 1.96 (2.00), Tyr 4.11 (4.00), His 0.90 (1.00), Arg 3.56 (4.00)	−40.00	6.08
18	[D-Ile^{31}]	4.2 @ 25.8	>97	2456.34	2456.3	−38	Asp 1.02 (1.00), Thr 0.97 (1.00), Ser 0.92 (1.00), Glu 1.14 (1.00), Ala 1.90 (2.00), Ile 1.90 (2.00), Leu 1.92 (2.00), Tyr 4.00 (4.00), His 1.30 (1.00), Arg 4.334.00)	−41.30	5.55
19	[D-Thr^{32}]	4.0 @ 25.8	>94	2456.34	2456.4	−35	Asp 1.17 (1.00), Thr 0.93 (1.00), Ser 0.90 (1.00), Glu 1.00 (1.00), Ala 2.00 (2.00), Ile 1.90 (2.00), Leu 1.99 (2.00), Tyr 4.07 (4.00), His 0.92 (1.00), Arg 3.77 (4.00)	−31.75	9.12

Table I (Continued)

no.	NPY^{18-36} analog or fragment	iso RT @ % MeCN[a]	purity %[b]	calcd[e] mass	obsd[e] mass	$[\alpha]^{25}_D$, deg[c]	amino acid anal.	ΔMAP @ 5 min (0.4 mg/kg)[d]	SEM ($n \geq 3$)
20	[D-Arg^{33}][f]	4.4 @ 28.8	>97	2456.34	2456.5	−39.4	Asp 1.08 (1.00), Thr 0.92 (1.00), Ser 0.92 (1.00), Glu 1.03 (1.00), Ala 1.92 (2.00), Ile 1.88 (2.00), Leu 2.00 (2.00), Tyr 4.09 (4.00), His 1.09 (1.00), Arg 3.94 (4.00)	−24.66	7.54
21	[D-Gln^{34}]	3.8 @ 28.8	>97	2456.34	2456.4	−40.3	Asp 1.04 (1.00), Thr 0.92 (1.00), Ser 0.94 (1.00), Glu 1.03 (1.00), Ala 1.93 (2.00), Ile 1.89 (2.00), Leu 2.00 (2.00), Tyr 4.10 (4.00), His 1.05 (1.00), Arg 3.90 (4.00)	−32.00	11.01
22	[D-Arg^{35}]	3.8 @ 28.2	>98	2456.34	2456.3	−41.0	Asp 1.10 (1.00), Thr 0.94 (1.00), Ser 0.91 (1.00), Glu 1.01 (1.00), Ala 1.96 (2.00), Ile 1.91 (2.00), Leu 2.00 (2.00), Tyr 4.02 (4.00), His 0.95 (1.00), Arg 4.07 (4.00)	−45.50	11.97
23	[D-Tyr^{36}]	4.1 @ 28.2	>98	2456.34	2456.5	−48.9	Asp 1.12 (1.00), Thr 0.96 (1.00), Ser 0.92 (1.00), Glu 1.04 (1.00), Ala 1.99 (2.00), Ile 1.92 (2.00), Leu 2.00 (2.00), Tyr 3.99 (4.00), His 1.08 (1.00), Arg 4.11 (4.00)	−36.00	10.06
24	[$Phe^{27,36}$]	3.9 @ 34.8	>96	2424.35	2424.6	−48.6	Asp 1.01 (1.00), Thr 0.98 (1.00), Ser 0.96 (1.00), Glu 1.04 (1.00), Ala 2.00 (2.00), Ile 1.91 (2.00), Leu 2.06 (2.00), Tyr 2.15 (2.00), Phe 2.01 (2.00), His 1.05 (1.00), Arg 4.11 (4.00)	−6.00	8.54
25	[$Phe^{20,27,36}$]	4.3 @ 36.6	>97	2408.36	2408.5	−48.5	Asp 0.98 (1.00), Thr 0.91 (1.00), Ser 0.94 (1.00), Glu 1.06 (1.00), Ala 2.00 (2.00), Ile 2.07 (2.00), Leu 2.15 (2.00), Tyr 1.09 (1.00), Phe 3.21 (3.00), His 0.97 (1.00), Arg 4.38 (4.00)	3.00	2.64
26	[$Phe^{21,27,36}$]	3.5 @ 36.6	>99	2408.36	2408.5	−51.7	Asp 0.88 (1.00), Thr 0.75 (1.00), Ser 0.60 (1.00), Glu 0.96 (1.00), Ala 2.00 (2.00), Ile 1.96 (2.00), Leu 1.97 (2.00), Tyr 0.71 (1.00), Phe 2.85 (3.00), His 0.93 (1.00), Arg 4.03 (4.00)	7.66	2.96
27	[$Phe^{20,21,27,36}$]	4.2 @ 40.8	>98	2392.36	2392.5	−52.4	Asp 1.06 (1.00), Thr 0.99 (1.00), Ser 0.96 (1.00), Glu 1.10 (1.00), Ala 2.00 (2.00), Ile 2.09 (2.00), Leu 2.20 (2.00), Phe 4.38 (4.00), His 1.10 (1.00), Arg 4.34 (4.00)	12.33	6.17
28	NPY^{18-24}	4.3 @ 13.8	>99	842.45	842.5	−25	Ser 0.86 (1.00), Ala 2.00 (2.00), Leu 1.06 (1.00), Tyr 2.17 (2.00), Arg 1.10 (1.00)	3.33	5.49
29	NPY^{18-25}	4.0 @ 13.8	>99	998.55	998.7	−29	Ser 0.86 (1.00), Ala 2.00 (2.00), Leu 1.04 (1.00), Tyr 2.19 (2.00), Arg 2.20 (2.00)	4.67	6.56
30	NPY^{18-27}	2.8 @ 16.2	>97	1298.68	1298.6	−38	Ser 0.84 (1.00), Ala 2.00 (2.00), Leu 1.06 (1.00), Tyr 3.18 (3.00), Arg 2.21 (2.00)	−12.00	3.61
31	NPY^{18-32}	4.4 @ 27.6	>94	1853.02	1852.9	−27	Asp 1.06 (1.00), Thr 0.89 (1.00), Ser 0.93 (1.00), Ala 2.02 (2.00), Ile 1.89 (2.00), Leu 2.00 (2.00), Tyr 2.99 (3.00), His 1.00 (1.00), Arg 2.13 (2.00)	0.00	2.00
32	$NPY^{18-36}OH$ 189-35-11	4.7 @ 27.6	>91	2457.33	2457.3	−47	Asp 1.04 (1.00), Thr 0.96 (1.00), Ser 0.95 (1.00), Glu 1.13 (1.00), Ala 2.00 (2.00), Ile 1.99 (2.00), Leu 2.02 (2.00), Tyr 4.18 (4.00), His 1.02 (1.00), Arg 4.14 (4.00)	−4.75	5.42
33	NPY^{18-37}[Tyr^{37}]OH	4.8 @ 28.8	>98	2620.39	2620.4	−45	Asp 1.10 (1.00), Thr 0.93 (1.00), Ser 0.93 (1.00), Glu 1.03 (1.00), Ala 1.91 (2.00), Ile 1.88 (2.00), Leu 2.00 (2.00), Tyr 5.13 (5.00), His 1.04 (1.00), Arg 3.93 (4.00)	−8.35	3.66

[a] Isocratic retention times in minutes at the specified % MeCN. [b] Consensus of values from three analytical determinations. [c] Determined at 25 °C in 1.0 M AcOH (concn ~ 0.5). [d] Change in mean arterial pressure (ΔMAP) measured at 5 min following administration of 100 μg of analog (means ± SEM ≥ 3). [e] [M + H] Monoisotopic mass.

found to be generally greater than 95% (Table I). The peptides were subjected to further characterization using mass spectrometry, amino acid analysis, optical rotation, and determination of in vitro relative potency (see Table I). Amino acid analysis gave results which were consistent with the expected structures (data not shown). Using a JEOL JMS-HX110 double focusing mass spectrometer fitted with a Cs^+ gun, we observed masses of the monoisotopic peaks corresponding to those calculated for $[M + H]^+$ of each analogue investigated (see Table I).

Biological Characterization. Peptide analogues were administered intraarterially to conscious and freely moving male Sprague–Dawley rats using arterial catheters. Changes in arterial pressure over time were calculated relative to control values measured prior to peptide administration. Figure 1 shows the change in arterial pressure (AP) measured 5 min following administration of a single dose of 100 μg of each analogue, numbered according to Table I. Data for effects on arterial pressure (AP) are expressed as ΔAP ± SEM. A partial dose–response curve for the NPY^{18-36}-evoked elevation of plasma histamine levels is presented in Figure 2. Representative time courses for the NPY^{1-36}- and NPY^{18-36}-evoked elevation of plasma histamine levels are presented in Figure 3.

In elucidating the structural requirements necessary for NPY^{18-36} to evoke a hypotensive response, each of the 19 amino acid residues were substituted. The following observations are grouped according to the nature of the modified residue, i.e., changes to basic, aromatic, and then hydrophobic amino acids.

Along the primary sequence of NPY^{18-36}, basic residues occur at positions Ala^{18} (primary amine, N-terminal), Arg^{19}, Arg^{25}, His^{26}, Arg^{33}, and Arg^{35}. N-acetylation, N-methylation, or desamination of the N-terminal Ala^{18} residue yielded inactive analogues, suggesting that the primary amine is essential for hypotensive activity. Similarly, [D-Ala^{18}]NPY^{18-36} exhibited markedly reduced hypotensive activity as compared to that of NPY^{18-36}.

Table II. Plasma Histamine Levels before and after Interarterial Administration of Peptide, and Hypotensive Response

compound	n	plasma histamine (ng/mL) ± SD			ΔAP ± SEM (mmHg)
		t = 0 min	t = 5 min	change	t = 5 min
NPY^{1-36}, 10 μg	7	24.1 ± 13.3	19.0 ± 7.0	−5.0 ± 10.1	−45.0 ± 4.0
PYY^{1-36}, 10 μg	6	13.7 ± 3.9	104 ± 24.6	+89.8 ± 25.0	+25.2 ± 2.5
NPY^{18-36}, 100 μg	5	29.9 ± 10.0	365 ± 134	+334 ± 130	−28.6 ± 6.4
[Ac]NPY^{18-36}, 100 μg	3	16.0 ± 11.2	31.1 ± 1.9	+15.1 ± 10.2	+12.0 ± 3.9
[D-Ala^{18}]NPY^{18-36}, 100 μg	3	7.0 ± 1.7	159 ± 13.3	+152 ± 13.4	−7.0 ± 1.5
[D-Tyr^{21}]NPY^{18-36}, 100 μg	3	15.9 ± 10.5	1230 ± 128	+1279 ± 134	−53.0 ± 9.3
[D-Thr^{32}]NPY^{18-36}, 100 μg	3	7.8 ± 1.0	1590 ± 128	+1585 ± 134	−31.8 ± 9.1

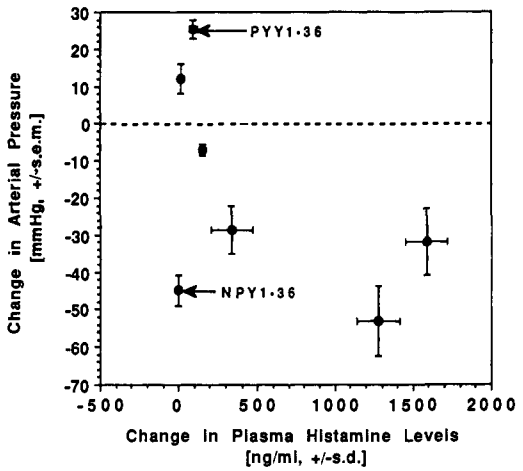

Figure 4. Changes in plasma histamine levels (±SD) and arterial pressure (±SEM) for NPY^{1-36}, PYY^{1-36}, and five NPY^{18-36} analogues, as presented in Table II.

Likewise, chiral inversion of residues, Arg^{19} or Arg^{25} abolished activity. Chiral inversion of the remaining basic residues, His^{26}, Arg^{33}, and Arg^{35} did not affect the hypotensive potency of NPY^{18-36}.

Aromatic amino acid residues are present in NPY^{18-36} at positions Tyr^{20}, Tyr^{21}, His^{26}, Tyr^{27}, and Tyr^{36}. [D-Tyr^{20}]NPY^{18-36} exhibited complete loss of depressor efficacy, while similar inversion of chirality at Tyr^{21} resulted in an increase in hypotensive potency. The hypotensive activity of NPY^{18-36} was relatively insensitive to the effects of residue chiral inversion at His^{26}, Tyr^{27}, and Tyr^{36}. However, [$Phe^{27,36}$]NPY^{18-36} was found to be inactive.

Hydrophobic residues occur along the primary sequence of NPY^{18-36} at positions Ala^{23}, Leu^{24}, Ile^{28}, Leu^{30} and Ile^{31}. [D-Ala^{23}]NPY^{18-36} retained full hypotensive potency while [D-Leu^{24}]NPY^{18-36} was inactive. D-amino acid substitutions of Ile^{28}, Leu^{30}, or Ile^{31} did not alter the biological potency of NPY^{18-36}.

Chirality inversion of the polar residues Ser^{22}, Asn^{29}, and Gln^{34} in NPY^{18-36} did not affect depressor potency. However, desamidation of the C-terminal amide to yield the free acid of NPY^{18-36} completely abolished any hypotensive effectiveness. This effect was partially counteracted by the addition of an additional tyrosine residue at position 37 which yielded an analogue with a depressor activity approximately 30% that of NPY^{18-36}. To further evaluate the structural requirements for NPY^{18-36}-induced hypotension, experiments were performed using N-terminal fragments of NPY^{18-36}. Among these fragments (NPY^{18-32}, NPY^{18-27}, NPY^{18-25}, and NPY^{18-24}) only NPY^{18-27} retained significant hypotensive activity.

Having established a significant body of data concerning the structural requirements of NPY^{18-36} evoking a hypotensive response, analogues with different potencies were selected for evaluation of their effects on plasma histamine levels. Those selected were NPY^{1-36}, PYY^{1-36}, NPY^{18-36}, [Ac-Ala^{18}]NPY^{18-36}, [D-Ala^{18}]NPY^{18-36}, [D-Tyr^{21}]NPY^{18-36}, [D-Thr^{32}]NPY^{18-36}, and NPY^{18-36} free acid. The effect of IA administration of these compounds (10 μg NPY or PYY, 100 μg all other peptides) on plasma histamine levels is presented in Table II.

NPY^{1-36}, although producing a profound hypotension when administered at doses sufficient to produce the characteristic biphasic effect on AP, failed to elicit an elevation in plasma histamine levels. In contrast, PYY^{1-36} produced a substantial elevation in plasma histamine levels, despite any observable depressor effect following IA administration. Additionally, the direct vasoconstrictor effect of PYY^{1-36} was observed despite the elevation of plasma histamine levels.

Among the NPY^{18-36} analogues, elevation of plasma histamine levels generally paralleled hypotensive effectiveness. The most potent of the vasoactive analogues, [D-Tyr^{21}]NPY^{18-36}, produced an enormous (80-fold) elevation of plasma histamine levels. The fully active analogue [D-Thr^{32}]NPY^{18-36} evoked a similar increase in plasma histamine levels. NPY^{18-36}, although equipotent with [D-Thr^{22}]NPY^{18-36} in depressor effect, evoked a more modest but substantial increase in histamine levels. [Ac-Ala^{18}]NPY^{18-36}, [D-Ala^{18}]NPY^{18-36}, and NPY^{18-36} free acid exhibited low potency to decrease arterial pressure and to elevate plasma histamine levels.

Discussion

Structural requirements for NPY^{18-36}-induced hypotension have been characterized in this work. Furthermore, evidence has been collected that supports the concept that histamine release may be responsible in part for NPY^{18-36}-, but not NPY^{1-36}-evoked hypotension.

Previous studies have demonstrated that C-terminal fragments of NPY bind to the Y_2 receptor subtype.[21] However, NPY^{18-36} analogues with C-terminal modifications (e.g., [D-Thr^{32}]NPY^{18-36} or [D-Arg^{35}]NPY^{18-36}) exhibit low affinity for binding to the Y_2 receptor. Modifications to the N-terminus of NPY^{18-36} have little effect on the binding affinity of NPY^{18-36} for the Y_2 receptor.[12] In contrast, modifications of the N-terminal residues profoundly altered the hypotensive potency of NPY^{18-36}. These results suggest that the pharmacophore responsible for Y_2 receptor affinity is different from that responsible for NPY^{18-36}-induced lowering of AP.

The hypotensive activity of NPY^{18-36} was particularly sensitive to modifications at N-terminal basic and aromatic residues. On the basis of this observation, we may speculate that basic and aromatic side chains contribute to the hypotension-inducing pharmacophore. The positively charged N-terminal primary amine was important in NPY^{18-36}-induced lowering of AP since N-acetylation,

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N-methylation, or desamination of the primary amine abolished the hypotensive activity of NPY^{18-36}. Previous work has demonstrated that a variety of C-terminal NPY fragments (NPY^{17-36} and NPY^{19-36}) can evoke a hypotensive response, as long as their respective N-termini are neither blocked, deleted, nor alkylated.[12] However, the presence of the primary amine was by itself not sufficient to allow NPY^{18-36} to evoke a hypotensive response, as inverting the chirality of the N-terminal residue (e.g., [D-Ala^{18}]NPY^{18-36}) markedly decreased NPY^{18-36}'s hypotensive potency.

In addition to the positively charged primary amine of Ala^{18}, NPY^{18-36} contains four basic arginine residues at positions to 19, 25, 33, and 35. NPY^{18-36}'s evoked hypotension was particularly sensitive to chiral inversion of Arg^{19} and Arg^{25}. As was the case for [D-Ala^{18}]NPY^{18-36}, inversion of one or the other of these residues would be expected to have a substantial effect on the conformation and relative spatial orientation of the positively charged groups. Similarly, the profound effects of inverting Leu^{24} (inactive), or Tyr^{20} (inactive) versus Tyr^{21} (superactive) suggest a strong conformational dependence.

This evidence suggests a conformationally well defined pharmacophore, characteristic of ligands interacting with specific receptors. This is in contrast to speculations that NPY-evoked hypotension is a consequence of nonreceptor-mediated mast cell degranulation releasing histamine.[14,15] Such nonreceptor-mediated degranulation is characteristic of various highly positively charged peptides, such as tachikinins or poly-L-lysine.[22-24] Nonreceptor-mediated mast cell histamine release is dependent more upon the overall basicity and proximity and less upon the conformation of positively charged amino acids.

Two observations remain that might be considered inconsistent with the notion of a discrete, well-defined N-terminal pharmacophore. First, altering NPY^{18-36} by substituting phenylalanines for Tyr^{27} and Tyr^{36} results in an inactive analogue. This observation could be explained by the fact that phenylalanine is hydrophobic while tyrosine is ampipathic. The conformational consequences of solvent shielding an additional two bulky hydrophobic side chains could disrupt the remainder of the linear molecule sufficiently so as to distort the pharmacophore. Second, the conversion of the C-terminal amide of NPY^{18-36} to the free acid results in an inactive analogue. This alteration would affect the conformation of and add a negative charge to NPY^{18-36}. Additionally, because NPY^{18-36} contains six positively charged residues, the formation of a side chain to the main chain salt bridge is possible. This could have major conformational consequences.

To speculate on why NPY^{18-27} but not NPY^{18-32}, NPY^{18-25}, and NPY^{18-24} was the only fragment tested that was partially effective at evoking a hypotensive response, one must take the primary sequence of NPY^{18-36} into account. The fragment NPY^{18-32} must accommodate a hydrophobic C-terminus, with leucine and isoleucine at positions 30 and 31, respectively. The necessity of solvent shielding hydrophobic residues may disrupt the remaining amino acids, distorting the pharmacophore. In contrast, NPY^{18-27} has only Ala^{23} and Leu^{24} to shield, and with three polar residues extending on from 25 to 27, this could be easily managed. In this way, the N-terminus could remain in an active conformation. The shorter fragments (NPY^{18-24}, NPY^{18-25}) may be of insufficient size to maintain the necessary secondary structure at or around the putative pharmacophore.

How do the conformational requirements for NPY^{18-36}'s pharmacophore relate to that of NPY^{1-36}? If NPY^{1-36} assumes a conformation in solution similar to that observed for the crystalline state of APP (the PP fold[25,26]), the segment from residues 14 to 30 would be in an α helix. Additionally, no primary amine is present around Ala^{18}, as it is in NPY^{18-36}. It is likely that the pharmacophore for NPY^{1-36}-evoked hypotension resides elsewhere (from residues 1 to 18?). However, without a similar study of NPY^{1-36} analogues, it is not possible to say with any certainty that this is the case.

The prospect that NPY^{1-36} and NPY^{18-36} evoke similar effects on AP through different receptor- (and/or nonreceptor-) mediated mechanisms is further suggested by their differential effects on plasma histamine levels. Among the NPY^{18-36} analogues in Table II, elevation of plasma histamine levels is strongly correlated with the magnitude of their evoked depressor effects (Figure 2). Only [D-Thr^{32}]NPY^{18-36} deviates somewhat. However, when NPY^{1-36} and PYY^{1-36} are included, NPY^{1-36} lies far away from the observed correlation. Despite the magnitude of its depressor effect, NPY^{1-36} did not elevate plasma histamine levels. (This is in contrast to effects on isolated rat peritoneal mast cells, where NPY^{1-36} evoked a greater than 30% release of total mast cell histamine content).[14,15] Given the observed correlation, the magnitude of the elevation of NPY^{1-36}-evoked plasma histamine level is clearly insufficient to account for the marked depressor response to NPY^{1-36}. This result suggests that the mechanism underlying NPY^{1-36}-evoked hypotension is different from that underlying NPY^{18-36}-evoked hypotension.

Investigating the effects of IA-administered peptides raises two concerns. First, the physiologically relevant site of action of these peptides may be distant from the site of administration. As a result, extraneous actions of the peptides may complicate the resulting observations. Second, the local concentration of the endogenous peptide may be a function of its release in close proximity to its site of action. This may necessitate using a large IA dose of peptide to mimic the local concentration of the endogenous ligand. This work has demonstrated that IA administration of a dose of NPY^{1-36} sufficient to evoke a hypotensive response fails to elicit a concomitant elevation in plasma histamine levels. However, the possibility remains that the site of action responsible for NPY^{1-36}-evoked histamine release may be isolated from the site of administration (i.e. the plasma volume). Hence, the actual concentration of peptide at this site of action may be insufficient to evoke a release of histamine sufficient to elevate plasma histamine levels significantly.

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In comparing the effects of 10 μg of NPY^{1-36} and 100 μg of NPY^{18-36} (or analogues), the following consideration is noted. The observation that NPY^{1-36} (as opposed to NPY^{18-36}) failed to elevate plasma histamine levels may be a result of the difference in concentration of the peptides administered. However, 10 μg of NPY^{1-36} produced the same degree of (submaximal) hypotension as 100 μg of various NPY^{18-36} analogues. Hence, the conclusion that elevation of plasma histamine levels was neither causal to, nor a result of IA-administered NPY^{1-36}-evoked hypotension remains valid.

This work supports the conclusion that NPY^{18-36}-evoked hypotension is a consequence of a well-defined pharmacophore residing at the N-terminus of the fragment. At present, data suggest that this pharmacophore is composed of some or all of the following components: one primary amine, two tyrosine rings, and two arginine guanidinyl groups. The sensitivity of this ensemble of chemical groups to chiral inversion suggests that the pharmacophore is interacting with a conformationally well-defined receptor. Additionally, the magnitude of NPY^{18-36} analogue-evoked hypotension is highly correlated with elevation of plasma histamine levels. Thus, NPY^{18-36}-evoked hypotension appear to be a function of receptor-mediated histamine release. In contrast, the NPY^{1-36}-evoked decrease in arterial pressure does not correlate with the NPY^{1-36}-induced effects on plasma histamine levels. Hence, the mechanism underlying the depressor effects of NPY^{1-36} appear to differ markedly from that of NPY^{18-36} and remains to be fully characterized.

Methods

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

Peptide Synthesis. Peptides were manually synthesized using α-Boc amino protection on MBHA resins prepared in house by the method of Rivier et al.[16,17] or amino acid-CM resins prepared by the method of Horiki et al.[27] using KF and CM resins (Lab Systems, San Mateo, CA). α-Boc-amino acids (Bachem, Torrance, CA) were coupled via dicyclohexylcarbodiimide in CH_2Cl_2 and/or DMF. Asn and Gln were coupled in the presence of a 2-fold excess of 1-hydroxybenzotriazole. Side-chain protection of α-Boc amino acids was as follows: Arg(Tos), Asp(OcHx), Glu(OcHx), His(Tos), Lys(2ClZ), Ser(Bzl), and Tyr(2BrZ). Deblocking was accomplished with 50% TFA in CH_2Cl_2 in the presence of 1% EDT for 25 min. The protected peptide-resin was cleaved in liquid HF in the presence of 3% anisole at 0 °C for 45 min for CM resins and 60 min for MBHA resins. The crude peptides were precipitated with anhydrous diethyl ether and separated from ether-soluble nonpeptide material by filtration. The peptides were extracted from the resin with water, and the aqueous solutions were then lyophilized.

Peptide Purification. Crude peptides were purified by preparative reversed-phase HPLC, usually in two steps. This methodology has been described in detail previously.[18-20] Briefly, gradient conditions were established by analytical HPLC. Gradient conditions for preparative HPLC were inferred from the analytical results. A Hitachi 655 analytical system was used, composed of a 655A-11 pump, 655A-71 proportioning valve, Rheodyne 7125 injector, Vydac C_{18} column (0.46 × 25 cm, 5-μm particle size, 30-nm pore size), 655A variable wavelength detector (detection was at 210 nm), and 655-61 system processor. The preparative HPLC system used included a modified Waters 500A Prep LC, Waters preparative gradient mixer of Eldex Chomatrol 11 gradient former, Waters 450 variable wavelength detector (detection was at 230 nm), and Houston Instruments Omniscribe chart recorder. The cartridges used were hand-packed in-house, using Waters polyethylene sleeves and frits and Vydac buld C_{18} material, 15–20-μm particle size, 0.30-nm pore size. The crude lyophilized peptides (0.5–1.5 g) were dissolved in water, loaded on a C_{18} cartridge, and eluted with linear TEAP (pH 2.25)/MeCN gradients. Fifty to one-hundred milliliter fractions were collected and monitored by isocratic analytical HPLC. Appropriately enriched fractions (>95% pure) were pooled, diluted, and reloaded onto the preparative cartridge. The peptide was eluted with a linear gradient of 0.1% TFA/MeCN. Fractions were again collected, analyzed, and those containing the purified peptide were pooled and lyophilized. Final products were in the range of 90–99% pure by HPLC analysis. Final yields ranged from 3% to 17% of theory when based on resin substitution.

Peptide Characterization. Purified peptides were subjected to HPLC analysis in two mobile phase systems: 0.1% TFA/MeCN and TEAP (pH 2.25)/MeCn on a C_{18} stationary phase. The analytical HPLC system used was the same as that described in detail above. Additionally, analytical HPLC in the 0.1% TFA/MeCN system was performed on a Waters HPLC system, which comprised two M-45 pumps, WISP sample injector, Kratos Spectroflow 773 UV detector, and Waters data module integrator/recorder. The purities quoted in Table I are a consensus of values determined in the three analytical systems described. The retention times quoted in Table I were determined from isocratic analysis of each peptide using the Hitachi system described above. The mobile phase system used was 0.1% TFA/MeCN (percentages as specified).

Amino acid analysis of the peptides was performed following hydrolysis in 4 N methanesulfonic acid at 110 °C for 24 h. A Perkin-Elmer LC system composed of two Series 10 LC pumps, an ISS-100 sample injector, TRC 1 column oven, Kratos Spectroflow 980 fluorescence detector, and LCI-100 integrator, was used. A Pierce AA511 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 gradient 0 to 100% B in 25 min and then 100% B for 15 min was commenced 5 min after injection. The flow rate was 0.5 mL/min and A and B buffers were Pierce Pico buffer (pH 2.20) and Beckman Microcolumn sodium citrate buffer (pH 4.95), respectively.

Optical rotations of peptides were measured in 1.0 M acetic acid (concn 0.5, i.e., 5 mg of lyophilized peptide per mL uncorrected for TFA counterions or water present after lyophilization). Values were calculated from the means of 10 successive 5-sec integrations determined at 25 °C on a Perkin-Elmer 241 polarimeter (using the D line of Na emission) divided by the concentrations of the sample in g/dL and are quoted as uncorrected specific rotations.

LSIMS mass spectra were measured with 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 were employed. The samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix.

Conscious Rat Blood Pressure Assay. Male Sprague-Dawley rats (240–280 g, Bantin-Kingman Laboratories, Fremont, CA) were used. All procedures performed on these animals were in accordance with the guidelines of the University of California, San Diego Committee on Investigations Involving Animal Subjects. All animals, when used in experiments, were conscious and free moving. Arterial catheters were constructed by heat bonding of 1-cm lengths of PE 10 tubing to pieces of PE 50 tubing. The PE 10 segment was inserted into the iliac artery. The remaining PE 50 was routed subcutaneously and exteriorized between the scapulae. Catheter placement was performed on the day of the experiment under halothane anesthesia. There was at least a 2-h recovery period between the catheter placement and the start of the experiment. Saline solutions of the peptides were administered IA in a 100-μL bolus. Mean arterial pressure (MAP) and heart rate (HR) were measured with Gould-Statham P23Db pressure transducers and monitored with a Beckman R-611 dynograph or a Gould physiograph. A Cyborg A-D converter linked to an IBM-XT compatible computer was used for data collection. MAP

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was calculated as [(systolic - diastolic/3) + diastolic]. Zero points were determined prior to administration of peptides by injection of saline vehicle.

Histamine Assay. Histamine was assayed using a sensitive [^{125}I]histamine radio immunoassay (AMAC Inc., Westbrook, ME). The histamine determination makes use of the binding competition between the acylated test sample or histamine standard (0–5 ng/mL) and ^{125}I-acylated histamine to a mouse antihistamine mAb-coated test tube. An aliquot of 100 μL of standard (0.5 ng/mL histamine) or sample was acylated by adding the histamine standard or sample to a test tube containing 1 mg of a lyophilized acylating reagent and 50 μL of acylating buffer (borate buffer, pH 8.2). The acylating reagent was completely solubilized by vortexing each tube for 15–20 s. One mL of ^{125}I-acylated histamine (0.073 μCi/mL) was added to each 150 μL of acylated histamine standard or test sample and vortexed. Each histamine standard or sample was assayed in duplicate by adding 500-μL aliquots from the reaction mixture to the antihistamine mouse mAb-coated tubes and incubated at 4 °C for 18 h. After the 18-h incubation, all of the fluid was aspirated from each tube, and the empty tubes were counted in a gamma counter (Searle, Model 1185). Results are expressed as ng/mL histamine and extrapolated from the histamine standard curve. The assay has a sensitivity of 0.1 nM histamine, and intraassay coefficient of variation of 8.0 ± 0.4% and an interassay coefficient of variation of 9.9 ± 0.9%. The assay is also specific, as cross reactions with histidine or *N*-methylhistamine are very low ($<10^{-4}$).

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Preparation and Structure–Activity Relationships of Simplified Analogues of the Antifungal Agent Cilofungin: A Total Synthesis Approach

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The echinocandins are a well-known class of lipopeptides characterized by their potent antifungal activity against *Candida* species. The mechanism of action of the echinocandins is generally thought to be the inhibition of β-1,3-glucan synthesis, an important structural component in the cell wall of *Candida* species. Extensive structure–activity studies on the fatty acid side chain of echinocandin B (1) led to the preparation of the clinical candidate cilofungin (4). However, little is known about the cyclic peptide. We now report the preparation, by solid-phase synthesis, of a series of simplified analogs of cilofungin in which the unusual amino acids found in the echinocandins were replaced with more readily accessible natural amino acids. The solid-phase approach to the total synthesis of these analogs allowed us to conveniently explore structural modifications that could not be accomplished by chemical modification of the natural product. The simplest analog 5 showed no biological activity. Structural complexity was then returned to the system in a systematic fashion so as to reapproach the original cilofungin structure. Antifungal activity and the inhibition of β-1,3-glucan synthesis were monitored at each step of the process, thereby revealing the basic structure–activity relationships of the amino acids and the minimal structural requirements for biological activity in the echinocandin ring system. The results suggests that the 3-hydroxy-4-methylproline residue enhances activity but the L-homotyrosine residue is crucial for both antifungal activity and the inhibition of β-1,3-glucan synthesis.

Introduction

The echinocandins are a well known class of cyclic lipopeptides characterized by their potent antifungal activity against *Candida* species.[1] In addition, they are known inhibitors of β-1,3-glucan synthesis,[2] a common structural unit found in the cell walls of these fungi.[3] Extensive structure–activity studies of the fatty acid side chain of the echinocandin B ring system by other workers showed that the fatty side chain was essential for antifungal activity and ultimately led to the preparation of the clinical candidate cilofungin (4).[4] However, little is known about

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