Gonadotropin-Releasing Hormone Antagonists: Novel Members of the Azaline B Family†,‡,§

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Received May 4, 1994®

A series of antagonists of gonadotropin-releasing hormone (GnRH) homologous to azaline B ([Ac-DNal[1],DCpa[2],DPal[3],Aph[5](Atz),DAph[6](Atz),ILys[8],DAla[10]]GnRH) was synthesized, characterized, and tested in a rat antiovulatory assay (AOA). Selected analogues were also tested in both an *in vitro* dispersed rat pituitary cell culture assay for inhibition of GnRH-stimulated luteinizing hormone release and an *in vitro* histamine release assay. The duration of action of some of the most potent and safest analogues in those assays was also determined in the castrated male rat in order to measure the extent (efficacy and duration of action) of inhibition of luteinizing hormone release. Structurally, this series of analogues has novel substitutions (X and Y) in the structure of the azaline B precursor: [Ac-DNal[1],DCpa[2],DPal[3],-Aph[5](X),DAph[6](Y),ILys[8],DAla[10]]GnRH. These substitutions were designed to confer increased hydrophilicity as compared to that of azaline B (determined by relative retention times on a C_{18} reverse phase column using a triethylammonium phosphate buffer at pH 7.3) or to make them more easily accessible synthetically. Some bulky substituents were introduced in order to probe the spatial limitations of the receptor's cavity. These substitutions include acylated 4-aminophenylalanine at positions 5 and/or 6 (29 analogues), N^{α}-methylated backbone substitutions (six analogues), N^{ω}-isopropylaminophenylalanine at position 8, and hydrophilic amino acids at position 1. Out of 20 novel analogues tested for long duration of action in this series, only seven ([Ac-DNal[1],DCpa[2],DPal[3],Aph[5],DAph[6],ILys[8],DAla[10]]GnRH, [Ac-DNal[1],DCpa[2],DPal[3],Aph[5](For),DAph[6](For),ILys[8],DAla[10]]GnRH, [Ac-DNal[1],DCpa[2],DPal[3],Aph[5](Ac),DAph[6](Ac),-ILys[8],DAla[10]]GnRH (acyline), [Ac-DNal[1],DCpa[2],DPal[3],Aph[5](Pio),DAph[6](Pio),ILys[8],DAla[10]]GnRH, [Ac-DNal[1],DCpa[2],DPal[3],Aph[5](Atz),DAph[6](Ac),ILys[8],DAla[10]]GnRH, [Ac-DNalDCpa[2],DPal[3],Aph[5](Atz-βAla),DAph[6](Atz-βAla),ILys[8],DAla[10]]GnRH, [Ac-DNal[1],DCpa[2],DPal[3],Aph[5](Atz-Gab),DAph[6](Atz-Gab),ILys[8],DAla[10]]GnRH) had relative potencies and/or duration of action comparable to those of azaline B. The others were one-half to one-tenth as effective as azaline B. N^{α}-Methylated backbone substitutions at position 5 yielded analogues that were significantly more hydrophilic presumably because of the breakage of the NHα-Tyr[5] to Arg[8]–CO hydrogen bond reported to stabilize a β-turn encompassing residues 5–8 and which favored β-sheet formation as shown earlier by Haviv et al.[2] This substitution resulted, however, in an increased potency in the histamine release assay and in significantly shorter duration of action.[3] Similarly, attempts at replacing isopropyllysine in position 8 by either isopropyl-4-aminophenylalanine or isopropyl-4-(aminomethyl)phenylalanine resulted in loss of potency in the AOA. Changes in chirality at position 1 or 10 resulted in analogues that were one-tenth and one-half as potent, respectively, as acyline. Introduction of a relatively hydrophilic acetylated residue in position 1 (Ac-4-aminophenylalanine, Ac-2-quinolylalanine, Ac-3-quinolylalanine) also resulted in potent analogues in the AOA in the latter two cases (yet very short acting in the case of ([Ac-D2Qal[1],DCpa[2],DPal[3],Aph[5](Atz),DAph[6](Atz),ILys[8],DAla[10]]-GnRH). Introduction of either mesityl, (2-chlorophenyl)isourea, or (3-chlorophenyl)isourea as a substituent on the 4-amino function at residues 5 and 6 of the azaline B precursor was considerably less successful. In this article, we describe in details, improved synthetic protocols for all novel amino acis, N^{α}-methylation of amino acids on the resin, and elimination of the undesired N^{ω}-methylation of pyridylalanine at position 3 as the result of base treatment (piperidine or hydrazine) during the deprotection of the Fmoc group or formation of the triazole moiety in the presence of CH_2Cl_2.

Introduction

Gonadotropin-releasing hormone (GnRH) antagonists are now recognized as potential drugs for the management of sex steroid-dependent pathophysiologies, induction of ovulation, and male contraception.[4–8] As of now, most available long term studies were carried out with the Nal-Glu antagonist ([Ac-DNal[1],DCpa[2],DPal[3],Arg[5],4-(*p*-methoxybenzoyl)-D-2-Abu[6],DAla[10]]GnRH)[9] which, although very potent in inhibiting gonadotropin secretion, also stimulates the release of histamine and is relatively short acting. These preliminary studies, however, suggested that a GnRH antagonist may ultimately be used for the management or treatment of endometriosis, infertility, ovulation induction in women with chronic anovulation (i.e., PCO), precocious puberty, uterine myoma, ovarian hyperandrogenism and hirsutism, premenstrual syndrome (PMS), and controlled induction of ovulation in *in vitro* fertilization programs and may be a promising lead in the treatment of breast and gynecological cancers.[8] Most of these disorders were originally studied with long acting preparations of the

† These results were presented in part at the occasion of the 13th American Peptide Symposium held in Edmonton, June 1993.[1]

‡ This article is dedicated to Dr. Marvin Karten, Ph.D.

§ Abbreviations. IUPAC rules are used for nomenclature except for the following: 2Cpi, (2-chlorophenyl)urea; 2Qal, 2-quinolylalanine; 3Cpi, (3-chlorophenyl)urea; 3Qal, 3-quinolylalanine; Ac, acetyl; Acr, acrylyl; Ahx, 6-aminohexanoyl; Apc, 3-amino-4-pyrazolecarboxyl; Aph, 4-aminophenylalanine; Aph(Atz), 4-[*N*-[5′-(3′-amino-1*H*-1′,2′,4′-triazolyl)]amino]phenylalanine; 3Aph(Atz), 3-amino-Phe(Atz); Atc, 3-amino-1,2,4-triazole-5-carboxyl; Atz, [5′-(3′-amino-1*H*-1′,2′,4′-triazolyl)]; But, *n*-butyryl; Cac, chloracetyl; Cpa, 4-chlorophenylalanine; DCM, dichloromethane; For, formyl; Fpa, 4-fluorophenylalanine; Gab, γ-aminobutyryl; Iac, imidazoleacetyl; IAmp, 4-[(*N*-*p*-isopropylamino)methyl]-phenylalanine; ILys, N^{ϵ}-isopropyllysine; Lys(Atz), N^{ϵ}-[5′-(3′-amino-1*H*-1′,2′,4′-triazolyl)]lysine; Mes, methanesulfonyl; Nal, 3-(2-naphthyl)alanine; NMP, *N*-methylpyrrolidone; Pal, 3-(3-pyridyl)alanine; Pca, 2-pyrazinecarboxyl; pGlu, pyroglutamic acid; Pio, *n*-propionyl; Ura, uraconyl.

® Abstract published in *Advance ACS Abstracts,* June 15, 1995.

0022-2623/95/1838-2649$09.00/0

Table 1. Physicochemical Properties and Activities of GnRH Analogues with R = DCpa^2-DPal^3

no.	compound	$[\alpha]^{20}_D$[a] (deg)	t_R[b] (min)	purity[c] (%)	MS[d]	AOA[e]		*in vitro* cell culture[f]	duration of action[g]
1	[Ac-DNal^1,R,Lys^5(Atz),DLys^6(Atz),ILys^8,DAla^{1}10]GnRH (azaline)	−34	13.8	>97	1544.6	2.0	(1/10)	0.23 (0.15−0.36)	short acting
2	[Ac-DNa1^1,R,Lys^5(Ac),DLys^6(Ac),ILys^8,DAla^{10}]GnRH	−31	16.9	>97	1465.09	2.5	(3/3)	0.36 (0.28−0.47)	very short acting
3	[Ac-DNal^1,R,Aph^5(Atz),DAph^6(Atz),ILys^8,DAla^{10}]GnRH (azaline B)	−33	15.8 ± 0.2	97	1612.8	1.0	(0/7)	1.3 (0.8−2.0)	long acting
4	[Ac-DNal^1,R,Aph^5(Atz),DAph^6(Atz),Phe^7,ILys^8,DAla^{10}]GnRH	−44	16.9	87	1646.3	1.0	(6/6)		intermediate
						2.5	(0/9)		
5	[Ac-DNal^1,R,Aph^5(mAtz),DAph^6(mAt),ILys^8,DAla^{10}]GnRH	−13	17.8	>97	1612.9	1.0	(4/6)	0.78 (0.53−1.2)	
						2.5	(0/7)		
6	[Ac-DNal^1,R,Aph^5,DAph^6,ILys^8,DAla^{10}]GnRH	−45	20.6	>97	1448.6	1.0	(7/8)	0.74 (0.5−1.1)	long acting
						2.0	(0/7)		
						2.5	(0/6)		
7	[Ac-DNal^1,R,Aph^5(For),DAph^6(For),ILys^8,DAla^{10}]GnRH	−38	18.5	94	1504.75	1.0	(3/9)		long acting
						2.5	(0/8)		
8	[Ac-DNal^1,R,Aph^5(Ac),DAph^6(Ac),ILys^8,DAla^{10}]GnRH (acyline)	−38	19.9	>97	1532.7	0.5	(6/7)	0.67 (0.41−1.1)	long acting
						1.0	(5/13)		
						2.5	(0/7)		
9	[Ac-Nal^1,R,Aph(Ac)^5,DAph(Ac)^6,ILys^8,DAla^{10}]GnRH	−19	21.4	>97	1532.85	10	(2/6)		
						25	(0/3)		
10	[Ac-DNal^1,R,Aph(Ac)^5,DAph(ac)^6,ILys^8,Ala^{10}]GnRH	−46	19.1	94	1532.78	1.0	(8/8)		intermediate
						2.5	(0/8)		
11	[Ac-DNal^1,R,Aph^5(Cac),DAph^6(Cac),ILys^8,DAla^{10}]GnRH	−38	24.5	92	1600.6	1.0	(5/5)		
						2.5	(4/8)		
12	[Ac-DNal^1,R,Aph^5(Pio),DAph^6(Pio),ILys^8,DAla^{10}]GnRH	−37	23.5	>97	1560.78	1.0	(4/5)		long acting
						2.5	(0/8)		
13	[Ac-DNal^1,R,Aph^5(But),DAph^6(But),ILys^8,DAla^{10}]GnRH	−38	24.6	91	1588.7	1.0	(6/6)		
						2.5	(0/8)		
14	[Ac-DNal^1,R,DAph^6(Ac),ILys^8,DAla^{10}]GnRH	−39	20.2	96	1491.8	0.5	(3/3)	2.8 (2.2−3.7)	
						1.0	(3/7)		
15	[Ac-DNal^1,R,Aph^5(Atz),DAph^6(Ac),ILys^8,DAla^{10}]GnRH	−26	17.9	>97	1572.8	1.0	(7/14)	0.82 (0.54−1.3)	long acting
						2.5	(0/8)		
16	[Ac-DNal^1,R,Aph^5(Iac),DAph^6(Iac),ILys^8,DAla^{10}]GnRH	−32	17.0	>97	1666.3	0.5	(3/3)	1.4 (1.0−1.9)	inter/long
						1.0	(3/8)		
17	[Ac-DNal^1,R,Aph^5(Ura),DAph^6(Ura),ILys^8,DAla^{10}]GnRH	−26	18.2	>97	1689.1	0.5	(4/4)	0.72 (0.44−1.2)	intermediate
						1.0	(4/8)		
18	[Ac-DNal^1,R,Aph^5(Atc),DAph^6(Atc),ILys^8,DAla^{10}]GnRH	−32	15.5	>97	1668.8	1.0	(11/15)	0.65 (0.44−1.0)	
						0.5	(4/4)		
19	[Ac-DNal^1,R,Aph^5(Pca),DAph^6(Pca),ILys^8,DAla^{10}]GnRH	−31	23.5	>97	1661.0	0.5	(8/8)		
						1.0	(4/8)		
20	[Ac-DNal^1,R,Aph^5(Ac-Gly),DAph^6(Ac-Gly),ILys^8,DAla^{10}]GnRH	−39	16.6	95	1648.9	0.5	(3/4)	0.85 (0.64−1.14)	short acting
						1.0	(5/8)		
21	[Ac-DNal^1,R,Aph^5(Atz-Gly),DAph^6(Atz-Gly),ILys^8,DAla^{10}]GnRH	−34	13.9	>97	1727.9	0.5	(3/4)	1.1 (0.8−1.5)	intermediate
						1.0	(5/8)		
22	[Ac-DNal^1,R,Aph^5(Atz-βAla),Daph^6(Atz-βAla),ILys^8,DAla^{10}]GnRH	−31	14.3	94	1755.9	1.0	(9/18)	0.52 (0.35−0.8)	long acting
						2.5	(0/6)		
23	[Ac-DNal^1,R,Aph^5(Atz-Gab),DAph^6(Atz-Gab),ILys^8,DAla^{10}]AnRH	−33	14.7	>97	1782.8	0.5	(10/14)	0.21 (0.12−0.4)	long acting
						1.0	(1/8)		
24	[Ac-DNal^1,R,Aph^5(Atz-Ahx),DAph^6(Atz-Ahx),ILys^8,DAla^{10}]GnRH	−30	16.5	>97	1838.9	0.5	(6/6)	0.48 (0.34−0.68)	intermediate
						1.0	(2/10)		
25	[Ac-DNal^1,R,Aph^5(Atz-Ala),DAph^6(Atz-Ala)ILys^8,DAla^{10}]GnRH	−41	15.2	93	1754.9	0.5	(4/4)	0.4 (0.29−0.56)	
						1.0	(1/8)		
26	[Ac-DNal^1,R,Aph^5(Atz-DAla),DAph^6(Atz-DAla),ILys^8,DAla^{10}]GnRH	−30	15.2	>97	1754.8	1.0	(2/8)	0.36 (0.26−0.49)	short acting

27	[Ac-DNal^1,R,Aph^5(pGlu),DAph^6(pGlu),ILys^8,DAla^{10}]GnRH	−46	*	>97	1668.6	1.0	(2/2)		
						2.5	(4/8)		
28	[Ac-DNal^1,R,Aph^5(Ser),DAph^6(Ser),ILys^8,DAla^{10}]GnRH	−29	16.1	>97$^+$	1622.6	1.0	(4/5)		
						2.5	(0/6)		
29	[Ac-DNal^1,R,Aph^5(Ac-Ser),DAph^6(Ac-Ser)ILys^8,DAla^{10}]GnRH	−48	15.5	>97	1706.7	1.0	(4/7)	0.4 (0.29−0.56)	
						2.5	(1/9)		
30	[Ac-DNal^1,R,Aph^5(DSer),DAph^6(DSer),ILys^8,DAla^{10}]GnRH	−33	16.1	>97$^+$	1622.8	2.5	(2/8)		
31	[Ac-DNal^1,R,Aph^5(Ac-DSer),DAph^6(Ac-DSer),ILys^8,DAla^{10}]GnRH]	−18	15.4	>97	1706.8	1.0	(2/7)		short acting
32	[Ac-DNal^1,R,Aph^5(Atz-Ser),DAph^6(Atz-Ser),ILys^8,DAla^{10}]GnRH	−36	13.0	>97	1786.8	1.0	(8/12)	0.45 (0.35−0.59)	short acting
33	[Ac-DNal^1,R,Aph^5(Ac-DHis),DAph^6(Ac-DHis),ILys^8,DAla^{10}]GnRH	−31	15.8	90	1806.6	0.5	(8/8)	0.47 (0.36−0.62)	short acting
						1.0	(4/15)		
						2.5	(0/2)		
34	[Ac-DNal^1,R,Aph^5(Ac-DAsn),DAph^6(Ac-DAsn)ILys^8,DAla^{10}]GnRH	−25	14.6	>97	1760.9	1.0	(4/6)		
						2.5	(2/8)		
35	[Ac-DNal^1,R,Aph^5(Ac-DCit),DAph^6(Ac-DCit),ILys^8,DAla^{10}]GnRH	−24	14.3	>97	1847.0	1.0	(6/7)		
						2.5	(4/8)		
36	[Ac-DNal^1,R,Aph^5(Atz),DAph^6(Atz),N$^\alpha$MeILys^8,DAla^{10}]GnRH	−36	16.4	>97	1626.8	2.5	(4/4)		
						5.0	(2/6)		
						10	(0/7)		
37	[Ac-DNal^1,R,N$^\alpha$MeAph^5(Atz),DAph^6(Atz),ILys^8,DAla^{10}]GnRH (azaline C)	−43	16.0	95	1626.9	1.0	(2/9)	1.9 (0.85−2.2)	short acting
38	[Ac-DNal^1,R,N$^\alpha$MeAph^5(Atz),DAph^6(Atz),Ile^8,DAla^{10}]GnRH	−65	19.2	92	1569.8	25	(2/8)		
						100	(0/4)		
39	[Ac-DNal^1,R,N$^\alpha$MeTyr^5,DAph^6(Atz),ILys^8,DAla^{10}]GnRH	−52	18.1	>97	1545.9	2.5	(2/7)		
40	[Ac-DNal^1,R,Aph^5(Atz),DAph^6(Atz),IAph^8,DAla^{10}]GnRH	−21	22.0	>97	1646.9	50	(4/7)		
41	[Ac-DNal^1,R,N$^\alpha$MeAph^5(Atz),DAph^6(Atz),IAph^8,DAla^{10}]GnRH	−38	22.0	>97	1660.8	10	(2/2)		
						50	(4/8)		
						100	(0/4)		
42	[Ac-DNal^1,R,Aph^5(Atz),DAph^6(Atz),IAmp^8,DAla^{10}]GnRH	−20	17.2	>97	1660.8	2.5	(3/7)	1.1 (0.73−1.8)	
43	[Ac-DNal^1,R,N$^\alpha$MeAph^5(Atz),DAph^6(Atz),IAmp^8,DAla^{10}]GnRH	−37	17.5	>97	1674.8	2.5	(4/4)		
						5.0	(0/6)		
44	[Ac-DAph^1,R,Aph^5(Atz),DAph^6(Atz),ILys^8,DAla^{10}]GnRH	−21	7.3	94	1557.7	10	(1/6)		
45	[Ac-D2Qal^1,R,Aph^5(Atz),DAph^6(Atz),ILys^8,DAla^{10}]GnRH	−21	10.3	97	1613.8	1.0	(9/12)		very short acting
46	[Ac-D3Qal^1,R,Aph^5(Atz),DAph^6(Atz),ILys^8,DAla^{10}]GnRH	−20	10.6	87	1613.7	0.5	(4/4)		
						1.0	(3/11)		
47	[Ac-DNal^1,R,Aph^5(2Cpi),DAph^6(2Cpi),ILys^8,DAla^{10}]GnRH	−43	36.9	>97	1754.8	50	(4/4)	0.1 (0.07−0.14)	
48	[Ac-DNal^1,R,Aph^5(3Cpi),DAph^6(3Cpi),ILys^8,DAla^{10}]GnRH	−35	38.7	>97	1754.9	250	(4/4)		
49	[Ac-DNal^1,R,Aph^5(Mes),DAph^6(Mes),ILys^8,DAla^{10}]GnRH	−35	21.3	80	1432.6	10	(5/8)		
						25	(0.6)		

[a] Corrected to $c = 1$ in 50% AcOH/H_2O. [b] Retention times under gradient conditions; buffer system A; TEAP, pH 7.30, buffer system B, 60% CH_3CN/40% A. Compound **2** was used as an internal standard throughout the study. [c] Purity by HPLC as previous+ = purity derived from CZE or HPLC under acidic conditions; * = no chromatogram could be obtained under neutral conditions. [d] MS observed values using a JEOL HX-110 MS spectrometer with a Cs^{2+} gun. [e] AOA−antiovulatory assay; dosage in mg (rats ovulating/total). [f] Relative potency compared to [Ac-Δ^3Pro^1,DFpa^2,DTrp3^6]GnRH = 1. [g] Castrated male rat assay (see Figure 1). Long duration of action = fully active after 72 h; intermediate = fully active at 48 h but not at 72 h; short acting = fully active at 24 h but not at 48 h; very short acting = inactive at 24 h.

superagonists which desensitize the gonadotrophs after approximately 2 weeks of treatment. An antagonist will likely clinically displace the agonists because it avoids the initial upregulation of the gonadotropin–gonadal axis, leads to rapid and predictable recovery, permits flexibility of the degree of gonadal suppression, and can be used as a diagnostic test of gonadotropin-dependent gonadal dysfunction. If GnRH antagonists are to be used successfully in humans, they need to be extremely potent and long acting (for reasons of economics) and exhibit negligible side effects such as stimulating histamine release (for safety reasons).

Azaline ([Ac-DNal[1],DCpa[2],DPal[3],Lys[5](Atz),DLys[6](Atz),-ILys[8],DAla[10]]GnRH, **1**) and azaline B ([Ac-DNal[1],DCpa[2],DPal[3],Aph[5](Atz),DAph[6](Atz),ILys[8],DAla[10]]GnRH, **3**) are two closely related antagonists of GnRH with high potency in an antiovulatory assay (AOA), an *in vitro* pituitary cell culture assay, and the castrated male rat assay.[3,10,11] Both analogues have low histamine-releasing activities, thus making members of this family particularly attractive for clinical investigation. For poorly understood reasons which may derive from the introduction of an aromatic side chain at positions 5 and 6 (as compared to the aliphatic side chain of the two lysine residues at positions 5 and 6 in azaline), azaline B is considerably longer acting than azaline, especially after intravenous administration.[3] Although azaline B is among the most potent of the GnRH antagonists, there is still a need for a more potent analogue which would meet rigorous criteria such as ease of formulation for acute or slow release and economical synthesis, two properties that may be best fulfilled by closely related analogues. Here we describe a series of azaline B precursor derivatives (Ac-DNal-DCpa-DPal-Ser-Aph(X)-DAph(Y)-Leu-ILys-Pro-DAla-NH_2) whereby the ω-amino functions of the 4-aminophenylalanine at positions 5 and 6 (X, Y) were acylated with different carboxylic acids and amino acids, N-methylation of residue 5 was used to reduce propensity of the peptides to form β-sheets, and hydrophilic aromatic amino acids were introduced in position 1 to increase potency and solubility in aqueous buffers and decrease the propensity of azaline B and congeners to form gels under certain conditions (high concentrations and presence of inorganic salts).[4] Acyline, selected because of its high efficacy in the castrated male assay, was also tested in the normal male rhesus monkey, and its ability to inhibit testosterone was compared to that of the Nal–Glu antagonist and azaline B with full description of the results to be presented elsewhere.

Synthesis, Purification, and Chemical Characterization (Table 1)

Analogues were synthesized by the SPPS methodology on a *p*-methylbenzhydrylamine resin (MBHA-resin) using protocols previously described[3] or shown below. In most cases, analogues were obtained by introduction of each of the substituents at positions 5 and 6 on the partially deprotected, fully built peptide-resin or after the introduction of either Boc-Aph(Fmoc) at position 5 or Boc-Ser(Bzl) at position 4. We have described a general method consisting of reacting a free amino function with diphenyl cyanocarbonimidate (PCI) followed by reaction of the *O*-phenyl isourea formed with a primary amine or hydrazine for the incorporation of modified N^{ω}-cyanoguanidino and aminotriazole moieties on selected amino functions during solid phase peptide synthesis.[3,11,12] This modification was used mostly in the design of novel gonadotropin-releasing hormone antagonists, one of which we synthesized in relatively large quantities.[13] This two-step reaction usually leads to clean products; however, a side reaction (10–20%) occurred on occasion, resulting in the formation of significant amounts of a closely associated impurity having a mass 14–15 amu higher than expected. This contaminant, which is usually not resolved under standard HPLC conditions (TEAP 2.25 or 0.1% TFA), is readily detected by CZE or HPLC under neutral conditions (TEAP 7.3). Both the HPLC and the electrophoretic behavior are consistent with the introduction of a positive charge into the molecule which was shown to arise from the piperidine/hydrazine-mediated methylation of the pyridyl moiety at position 3 in the presence of DCM. This deleterious side reaction, first recognized by Mills et al.,[14,15] occurred during either the piperidine-mediated removal of the Fmoc protecting group or the hydrazine treatment needed to generate the triazolyl moiety by reaction of the amines with DCM. NMR results have shown that the impurity which we have isolated is indeed the N^{ω}-methylpyridylalanine derivative. Of particular interest is the fact that this analogue (most likely because of the charge introduced at position 3) is not only less potent in the AOA but also is significantly more potent at releasing histamine. Two strategies could be applied to avoid this side reaction. The first one consisted in removing the Fmoc protecting group with concomitant derivatization prior to the introduction of the pyridylalanine, while the second consisted in eliminating DCM as a solvent. The protected peptide-resins were cleaved in anhydrous HF in the presence of a scavenger, precipitated, extracted, and lyophilized. The crude peptides were purified by reverse phase HPLC.[13] The analytical techniques used for the characterization of the analogues included HPLC with two different solvent systems (acidic and neutral, the latter allowing the easy detection of any N^{ω}-methylated pyridyl-containing peptide), amino acid analysis, optical rotation, capillary zone electrophoresis (CZE), and LSIMS. Results from these studies support the identity of the intended structures (Table 1).

While some syntheses were described in earlier publications[3] or references therein, others deserve further comments. Of all the analogues reported here, **2** is the simplest and was made for comparison studies. The fact that it is only one-half to one-third as potent as **1** in the AOA is particularly telling and suggested to us that the Atz function, although conferring unique properties to this family of analogues, was not necessarily unique as demonstrated later in the Aph-containing series (see **3** and **8** in particular). Compound **5**, for example, necessitated the synthesis of both D and L isomers of α-Boc-3-amino(Fmoc)phenylalanine. D- and L-N^{α}-Boc-N^{ω}-Fmoc-3Aph were prepared via condensation of 3-nitrobenzyl chloride with diethyl acetamidomalonate followed by HCl hydrolysis, N-acteylation, esterification (ethyl ester), resolution with α-chymotrypsin, and complete hydrolysis to yield the isolated 3-nitro-D- and 3-nitro-L-phenylalanines. Both amino acids were independently protected with the Boc group and hydrogenated to yield α-Boc-3-amino-D- and L-phenylalanines. The last step was the introduction of

the Fmoc protecting group on the ω-amino function (see the Experimental Section for complete details; preliminary details on the synthesis of this amino acid can be found in Jiang et al.[1]). Formylation of the 4-amino function of phenylalanine at positions 5 and 6 (**7**) used standard protocols applied to SPPS (excess formic acid in the presence of acetic anhydride). Whereas acylation of the side chain amino functions was achieved with the same protocol used for the capping of residue 1[3,10] with acetic anhydride (**8**–**10**, **14**, **15**, **20**, **29**, **31**, **33**–**35**), chloroacetyl chloride (**11**), propionic anhydride (**12**), and butyryl chloride (**13**), acylation with imidazole-4-acetic acid (**16**), urocanic acid (**17**), 3-amino-1,2,4-triazole-5-carboxylic acid (**18**), and 2-pyrazinecarboxylic acid (**19**) could only be achieved after dissolution of the unprotected carboxylic acids in hot DMSO and addition of 20% DCM to the cooled solution prior to coupling with DIC in the absence (**16**, **17**) or presence (**18**, **19**) of pentafluorophenol or HOBt. Introduction of the aminotriazolyl functional group on the N-terminus of glycine, β-alanine, and others (**21**–**26**, **32**) was no different from that of this functional group on the side chains of lysine or 4-aminophenylalanine.[11]

N^{α}-Methylation of **36**–**39**, **41**, and **43** was achieved by two methods: from the corresponding amino acid via N^{α}-methylation of *N*-Boc-phenylalanine,[16] nitration, hydrogenation, and Fmoc protection of the 4-amino group as described earlier by Theobald et al.[11] (see the Experimental Section for details) or from direct methylation on the α-nitrogen on the resin as described in the Experimental Section using protocols derived from the work of Kaljuste et al.[17]

The synthesis of 4-(aminomethyl)-D,L-phenylalanine was first described by Nutt et al.[18] He et al.[19] and Liu et al.[20] used this amino acid as an intermediate for the synthesis of ω-substituted 4-(aminomethyl)phenylalanines. Their strategy involved the chloromethylation of Ac-L-phenylalanine ethyl ester. Using this procedure, we prepared both 4-(thiomorpholinomethyl)-D- and L-phenylalanine[1] starting from the bromomethylated derivative. Synthesis of L-4-(aminomethyl)phenylalanine was later reported by Hartman and Halczenko[21] starting from Boc-4-iodophenylalanine; Stokker et al.[22] later developed a simple and inexpensive synthetic scheme for 4-(aminomethyl)-L-phenylalanines. We report here on a further improvement of this latter strategy (see the Experimental Section). Briefly, Stokker et al. reacted *N*-(hydroxymethyl)trichloroacetamide with L-phenylalanine[22] to obtain L-4-[(trichloroacetamido)methyl]phenylalanine. At this point they achieved complete deprotection using acid hydrolysis and selective N-protection using the copper complex route. Instead, we took advantage of the trichloroacetyl or trifluoroacetyl as a temporary protecting group on the ω-amino function to block the α-amino function with the base stable Boc group. The trichloro- or trifluoroacetyl groups were replaced by the Fmoc groups using standard protocols after base hydrolysis.

Introduction of the isopropyl group on α-Boc-4-(aminomethyl)phenylalanine (used for **42** and **43**) and α-Boc-lysine was achieved using $NaBH_4$[23,24] or $NaBH_3CN$[25] as previously described, although yields could be improved when monitoring the reductive amination by HPLC to ensure completeness of the reaction and the homogeneity of the final product. Because of the less basic character of the aromatic amino function in α-Boc-4-aminophenylalanine (**40**, **41**), the reductive step could, in our hands, only be driven to completion in the presence of molecular sieves.

D2Qal was synthesized by the malonic acid condensation of 2-(chloromethyl)quinoline with diethyl sodioacetamidomalonate. The acetylated ethyl ester was resolved with α-chymotrypsin using standard procedures and after complete acid hydrolysis was N-protected with the Boc group (see the Experimental Section). Boc-D3Qal was obtained from the Contraceptive Development Branch of the NIH. The introduction of these residues in the growing peptide on SPPS did not present any problem and ultimately yielded **45** and **46**. Analogues **47** and **48** were prepared by reacting the corresponding chlorophenyl isocyanates with the 4-aminophenyl side chains of the peptide resins. Sulfonation of the 4-amino function of Aph with methane sulfonyl chloride gave the desired mesityl-containing analogue **49**.

Biological Characterization (Table 1 and Figure 1)

In vitro, the peptides were tested for their ability to inhibit GnRH-mediated LH secretion by cultured pituitary cells[26] or release histamine by rat mast cells.[5,27] The AOA was carried out as described by Corbin and Beattie[28] using an aqueous (1-2% DMSO) vehicle unless stated otherwise. Results are expressed in terms of the dosage in μg/rat (rats ovulating/total number of treated rats). Measurement of circulating LH levels in castrated rats treated subcutaneously with the peptides was carried out over a period extending up to 120 h as reported earlier.[3,29–31]

Results and Discussion

We describe here solutions to challenges associated with the synthesis of novel amino acids, the low solubility of some of the reagents, the occurrence of unexpected side reactions (methylation of the pyridylalanyl ring), and the characterization of the final products. Should there be a need for large batches of any of the novel peptides described here, the synthesis of none would compare in difficulty with that of the "Nal-Glu" antagonist.[13,32]

The rationale for the synthesis of the different analogues presented in Table 1 will be presented below in detail. One of the main considerations was to obtain one or several GnRH analogues that would have all the outstanding characteristics of azaline B (potent at inhibiting gonadal function, exceptionally long acting and safe when it comes to its inability to release histamine). In addition, unlike presently available antagonists, this desired analogue would have to be readily soluble in aqueous buffers and not form gels at concentrations below 10 mg/mL upon administration. At this point, however, it is not known whether this propensity to form gels is directly or indirectly responsible for extended duration of action. We found that administration of peptides in different carriers resulted in different potencies in the AOA (Table 2); hydrophilic peptides (azaline B) administered in aqueous buffers would be very potent just as more hydrophobic peptides (antide, [Ac-DNal^1,DCpa^2,DPal^3,Lys^5(Nic),DLys^6(Nic),-ILys^8,DAla^{10}]GnRH) would be in corn oil. Invert vehicles (hydrophilic peptides such as azaline B admin-

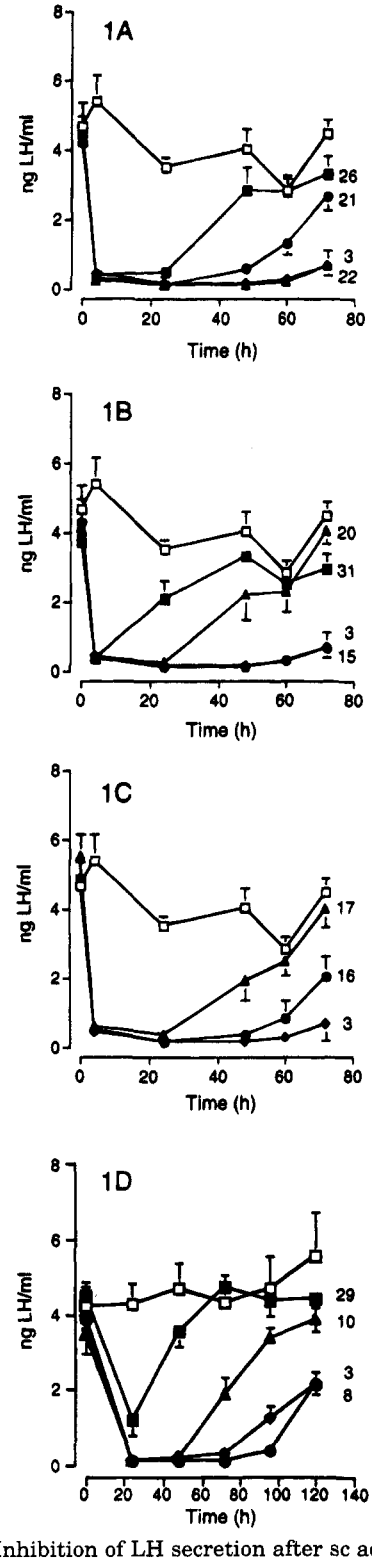

Figure 1. Inhibition of LH secretion after sc administration of analogues (50 μg) at one injection site. Blood samples were collected at the times shown on the abscissa. The SEMs, where not appearing, are encompassed within the size of the symbols in the graphs; $n = 5$. Compound numbers as well as unique symbols are used to identify each response; □ is vehicle alone.

istered in corn oil or the converse such as administering antide in an aqueous buffer) and both compounds appear to be as many as 5-fold less potent than one may have originally thought, suggesting excipient-dependent differences in bioavailability. In a limited number of experiments reported in an earlier publication, we had shown that among the peptides injected subcutaneously

Table 2. Effect of Vehicle and Time of Injection on Potency in the AOA

peptide	AOA[a]	vehicle[b]	time[c]
azaline B	0.5 (8/8)	aq	proestrus/noon
	1.0 (0.8)		proestrus/noon
	1.0 (0/5)		proestrus/8 a.m.
	1.0 (8/8)	co	proestrus/noon
	3.0 (5/8)	co	proestrus/noon
	1.0 (3/8)		proestrus/8 a.m.
	3.0 (0/8)	co	proestrus/8 a.m.
antide	1.0 (1/8)	co	proestrus/noon
	2.0 (0/8)	co	proestrus/noon
	2.0 (6/8)	aq	proestrus/noon
	4.0 (4/8)	aq	proestrus/noon
	8.0 (2/8)	aq	proestrus/noon
acyline	1.0 (5/8)	aq	proestrus/noon
	2.5 (0/7)	aq	proestrus/noon
	1.0 (0/6)	aq	proestrus/8 a.m.
	1.0 (1/8)	co	proestrus/noon
	2.5 (0/8)	co	proestrus/noon

[a] Dose in μg (rats ovulating/injected rats). [b] Vehicle: aq is ca. 2% DMSO in bacteriostatic water; co = corn oil. [c] Time of injection.

in the castrated male rat assay in comparatively large volumes (50 μg in 1 mL as compared to the standard 50 μg in 200 μL), azaline B was, of the peptides tested, the only one to retain long duration of action.[3] This suggests a mechanism of action independent of the gelling effect. Administering azaline B in corn oil on the morning of proestrus rather than at noon also shows efficacy. Of interest is our ability to modulate this property: acyline was not as potent as azaline B in aqueous buffer or as antide in corn oil but was more potent than azaline B in corn oil or antide in aqueous buffers. In contrast, it was more efficacious than antide[3] and at least as efficacious as azaline B in the castrated male rat assay.

Clearly, the relative solubility of some of these analogues correlates with both retention times and enhanced bioavailability (antide had a retention time of 13.6 min under gradient conditions at pH 7.30 where azaline B eluted after 9.0 min). Similarly, the most hydrophilic analogues pertaining to somewhat different series tend to release histamine as shown earlier.[10] Navigating the waters of compromise in search for better analogues may be the best definition of the content of this study. The availability of reasonably well-established tests (pharmacological, physicochemical, biological), although limited in an academic environment, was deemed sufficient for the identification of promising compounds for clinical investigation. Several observations gathered from earlier studies guided this research.

First, the histamine-releasing property of GnRH analogues was shown to be correlated to both the hydrophobic character of the N-terminus and the presence of strongly basic side chains such as arginines at positions 5 and 8 of the "Nal-Glu" antagonist ([Ac-DNal[1],DCpa[2],DPal[3],Arg[5],4-(*p*-methoxybenzoyl)-D-2-Abu[6],DAla[10]]GnRH) ($ED_{50} = 2.0$ μg/mL) or, even worse, the presence of arginines at positions 6 and 8 of the "Nal-Arg" antagonist ([Ac-DNal[1],DFpa[2],DTrp[3],DArg[6]]-GnRH) ($ED_{50} = 0.17$ μg/mL).[9] This deleterious activity was essentially abolished in antide which had weakly basic acylated lysine residues (Lys(Nic)) at positions 5 and 6,[33,34] an isopropyl lysine in position 8,[33] DAla in position 10,[35] and a hydrophobic N-terminus (Ac-DNal-DCpa-DPal-). Antide was found to have a desirable therapeutic ratio (high potency in the AOA when

administered in corn oil and an *in vitro* histamine release ED_{50} greater than or equal to 300 μg/mL when a value of ca. 150 μg/mL was found for GnRH itself).[5] Unfortunately, all these substitutions also resulted in a peptide which was difficult to formulate for clinical investigations. A major goal therefore became finding ways to increase the solubility of this family of antagonists without altering their desirable properties. We synthesized similar analogues substituted at positions 5 and 6 with cyanoguanidino and *N*-aminotriazolyl moieties[11] which were more readily soluble in aqueous solutions at pH 6.4 yet were relatively short acting.[10]

Second, only when substituted lysine residues at positions 5 and 6 in azaline (**1**) by 4-aminophenylalanines did we make the next important observation. This substitution conferred long duration of action to azaline B (compare properties of **1** and **2** to those of **3** and **8**, respectively, in Table 1) with additional improvement of the therapeutic ratio (AO potency over that of releasing histamine) since **3** and **8** are at least 2 times if not more potent as **1** and **2** in the AOA, 5 times as potent in the *in vitro* pituitary cell culture assay (Table 1), and equipotent in the histamine release assay. Of interest is the fact that the unsubstituted parent compound **6** is itself long acting, although marginally less so than azaline B or acyline.

Third, the observation that **2** had properties not much different from those of antide[33,34,36] or azaline (**1**) (see Table 1) incited us to pursue more systematically the study of analogues with simple acylated moieties on the ω-amino function of aminophenylalanines at positions 5 and 6, the rest of the molecule remaining constant: Ac-DNal-DCpa-DPal at the N-terminus and ILys-Pro-DAla-NH_2 at the C-terminus. Some variants on that theme are also reported.

We first ascertained that a simple substitution (Phe in position 7 (**4**)) that improved the biological stability of selected GnRH analogues[37] and potency in the AOA[38] (although results by other groups were not as favorable[39,40]) would not alter potency. We had shown that the introduction of an NMe-Leu at position 7 of closely related analogues did not seem to significantly influence potency in the AOA while resulting in an increased ability of the peptide to release histamine (ED_{50} = 34 ± 2.1)[3] and a significant loss in duration of action. Because of the apparent sensitivity of the receptor for the substitution at position 7, we were therefore not surprised that the Phe substitution resulted in a ca. 2-fold decrease of potency in the AOA. As a result, we did not investigate further the effect of that substitution on other biological parameters.

Our second concern was to ascertain that, of the two most readily available isomers of aminophenylalanine, i.e., the 3-amino and the 4-amino, the 3-amino isomer **5** was not considerably more potent. In fact, it was found to be less potent both *in vitro* (pituitary cell culture assay) and *in vivo* (AOA) than the 4-amino isomer **3**.

Our third control compound was the unsubstituted aminophenylalanine-containing analogue **6** which was also found to be less potent in the AOA than the parent azaline B (**3**). Of some interest is the observation that **6** eluted significantly later (20.6 min) than azaline B (15.8 min) under the conditions shown in Table 1 and therefore could be considered more hydrophobic. As mentioned earlier, we did not expect **6** to be long acting.

We then investigated the effect of the size of simple acylating agents such as formyl (**7**), acetyl (**8**), chloroacetyl (**11**), propionyl (**12**), and butyryl (**13**) on biological parameters. All analogues tested with the exception of **11** were fully active at 2.5 μg in the AOA and partially active or inactive at 1.0 μg when administered in an aqueous solution. It is evident that the bulkier or more hydrophobic the alkylating agent, the less potent is the corresponding analogue in that assay in aqueous solution. As shown in Table 2, the effect of the excipient is crucial if one looks at a 2-fold difference in potency. What is particularly interesting is that **8**, tested in the castrated male rat model, is as efficacious as azaline B (see Figure 1D) and considerably easier to make. We show that **8** is less potent than azaline B *in vitro* and in the AOA when administered in aqueous buffers on proestrus at noon but not on proestrus at 8 a.m. while equipotent with antide when administered in corn oil. On the other hand, acyline is clearly more efficacious than antide when compared after administration in aqueous buffers or than azaline B after administration in corn oil at the times that were investigated. Its effectiveness in the castrated male rat comparable to that of azaline B suggests that subtle and yet unidentified parameters, possibly timing of the injection and nature of the excipient (see table 2 for examples) and bioavailability (subtle differences in solubility), may influence the efficacy of an analogue in the AOA as it is being presently performed in our laboratories. We acknowledge that analogues with potencies in the AOA comparable to that of **8** have not generally been examined further in our screening program. This suggests that we may have failed to identify very efficient analogues in the past. Attempts at testing the most potent members of any new analogue series in the castrated male rat assay are shown in Figure 1 and Table 1. In summary, compounds **6**–**8**, **12**, **15**, **22**, and **23** were found to be long acting by our definition (Table 1), while we have no good answer as to why, for example, **20**, **26**, and **31**–**33** are short lived since there does not seem to be any correlation with relative hydrophilicity, at least in the way it was measured. Because out of all the long acting analogues **8** was synthetically the most accessible and possibly the longest acting, it was also tested in the intact male rhesus monkey where it was found to be slightly more potent than azaline B and 5–10 times more efficacious than the Nal-Glu antagonist (details to be reported elsewhere). Other examples including peptides with intermediate duration of action such as **4** (where the only difference is a Phe at position 7 versus a Leu), **10** (where an Ala replaces a DAla at position 10), and others shown below suggest that minor modifications of structure yield dramatic changes in biological efficacy, the possible result of subtle differences in bioavailability and affinity for both the receptor and other yet to be defined sites including blood proteins (see Figure 1 and descriptions below). The results obtained for histamine release were more predictable and are as follows with an order of potency for the analogues tested: (EC_{50}) **3** = **8** = **22** < GnRH = **1** < **2** = **37** < **7** < **16** = **33**, with **16** being approximately 15 times more potent than GnRH at releasing histamine. On the basis of the limited number

of compounds tested, we can suggest that most members of the azaline and azaline B family, but not of the azaline C family (N^{α}Me substitution at position 5), will have ED_{50}s equal to or greater than that of GnRH and that the introduction of polar side chains such as imidazoleacetic acid (**16**), but not 4-[*N*-[5′-(3′-amino-1*H*-1′,2′,4′-triazolyl)]amino] (**3**) or acetyl (**8**), may be somewhat deleterious.

Our last two control compounds had the corresponding L-amino acid at the N- (**9**) and C- (**10**) temini and were shown to be approximately 10- and 2-fold less potent, respectively, in the AOA than **8**. As described above, **10** was still very efficacious in the castrated male assay although less so than azaline B, possibly due to its more likely vulnerability to carboxypeptidases. The first one had longer retention time in our HPLC system than acyline, while substitution with L-Ala at position 10 resulted in an analogue slightly more hydrophilic.

In order to refine our understanding of the role of aminotriazolyl moiety at positions 5 and 6 of azaline B, we synthesized **14**, where tyrosine at position 5 was conserved and the aminotriazolyl moiety replaced by an acetyl group, and **15**, where the aminotriazolyl moiety remained at position 5 and was replaced by an acetyl group at position 6. While both analogues were equipotent in the AOA, **14** was 3 times as potent as **15** in the pituitary cell culture assay. Earlier studies had shown that the presence of Tyr at position 5 resulted in comparatively short duration of action compared to that of azaline B.[3] On the other hand, introduction of Aph(Atz) in position 5 with DAph(Ac) in position 6 resulted in **15** that is as efficacious as azaline B in the castrated male rat assay (see Figure 1B). Although this observation helps us further define the structural requirements for long duration of action, it is not clear whether substitutions at the 5 position of the kind shown here (Aph(Atz), Aph(Ac), Aph(Atz-βAla)) are indeed solely responsible for such property.

Compounds **16**–**19** were synthesized to investigate the possibility of replacing the aminotriazolyl moiety by imidazoleacetyl, uraconyl, aminotriazolecarboxyl, and 2,4-pyrazolylcarboxyl moieties, respectively. We expected to better define the receptor's requirements in terms of relative basicity of the different substituents. Neither of these substituents yielded dramatic changes in terms of biological potency in any of the biological systems in which they were tested (except perhaps in the histamine release assay), although these substitutions could be considered quite different in their steric and electronic properties as reflected by the diverse chromatographic behavior of these analogues on C_{18} silica gel at close to physiological pH. In this series, only **16** was tested for histamine release, while **16** and **17** were tested for duration of action. While Iac substitution resulted in increased histamine-releasing activity, it also resulted in a slight shortening of the duration of action (Figure 1C).

Compounds **20**–**35** are analogues which contain two additional amino acids coupled to the ω-amino functions at positions 5 and 6. These additional amino acids were selected for their simplicity and variable bulk (Gly (**20**, **21**), β-Ala (**22**), Gaba (**23**), aminohexanoic acid (**24**), their steric properties (Ala and DAla (**25**, **26**, respectively), pGlu (**27**), and additionally their potential increased hydrophilicity (Ser and DSer (**28**–**32**), DHis (**33**), DAsn (**34**), DCit (**35**)). Most analogues were either acetylated (**20**, **29**, **31**, **33**–**35**), blocked with the aminotriazolyl functional group (**21**–**26**, **32**), or free (**28**, **30**). As shown in Table 1, our goal of generating "hydrophilic" analogues as determined by retention times of HPLC was quite successful since most analogues have retention times either equal to or shorter than that of our original reference point azaline B. Overall, data are consistent because incremental increase in the size of the side chain (as in **21**–**24**) resulted in a parallel increase in retention times. Less expected was the observation that, in comparison with **28** and **30**, acetylation of Ser (**29**) or DSer (**31**) resulted in analogues with shorter retention times, suggesting that the free amino functions of these analogues (**28**, **30**) are not protonated at pH 7.30. The homologous series (**21**–**24**) is of particular interest because *in vitro* potencies varied at most by a factor of 5. In the AOA, results were essentially identical, but at 50 μg in the castrated male rat, **22** and **23** were more efficient than either **21** or **24**. Figure 1A indicates that the SEM for **22** is tighter than that of our reference **3** at 72 h, although not by much, while rats treated with **21** started to recover at that time point. It is also clear that animals treated with **21** (and **24**, data not shown) consistently escape at 48 h, whereas **3** and **22** reach that point approximately 24 h later. Also of interest is the fact that neither acetylation (**31**, **33**) nor blocking of the amino groups of analogues **26** and **32** with the aminotriazolyl moiety resulted in extended duration of action. An attempt at rationalizing these limited results may be premature at this time. It is likely that long duration of action is the result of unusual stability, distribution, and/or sequestration of the peptides in the body/circulation. Comparison of this study and an earlier one from our laboratories[3] indicates that AOA results obtained by us and others cannot be relied upon for the identification of the most effective (to be distinguished from most potent) analogues and of potential candidates for clinical application.

Less successful were series where we introduced α-methyl substitutions at positions 5 and 8 with or without IAmp or IAph substitution for the Ilys residue in position 8. The introduction of the α-methyl substitutions was caused by the observation[2] that the introduction of a methyl group on the backbone nitrogen of residue 5 resulted in an analogue that is significantly more soluble in aqueous buffers. This observation was confirmed by Rivier et al.[4] who introduced that modification in **37** (azaline C; see Table 1). It is generally accepted that the solubility of a compound in a particular solvent (g/L) is a constant. In the case of peptides, this notion can be challenged. Indeed, a peptide may be very easily soluble in an aqueous buffer and may remain in solution for an indefinite period of time. However it is not unusual (as is the case for many of the GnRH antagonists developed so far) that with time the peptide, which first assumed a random conformation in solution, slowly rearranges to yield a significant and critical concentration of an ordered and less soluble conformer which then gels. In the case of GnRH, it was suggested that the stabilized form consisted of β-sheets which aggregate and are less soluble.[2] The introduction of a backbone methyl group at position 5 would prevent formation of such β-sheets. Because of the presence of acetonitrile in our HPLC systems (a denaturing solvent),

it is not surprising that this increased solubility in aqueous media is not reflected by a shortening in retention times in the HPLC system. It is unfortunate that this favorable modification which increases solubility also results in the loss of some potency in the AOA, the loss of long duration of action, and significantly greater histamine-releasing property.

Compounds **40**–**43** were synthesized to test the hypothesis that since the substitution of Phe for Lys at positions 5 and 6 yielded antagonists with long duration of action,[3] a similar substitution at position 8 may also result in a favorable biological property. It becomes clear that the receptor favors a strong positive charge at position 8 since the aniline derivatives **40** and **41** are considerably less potent than the homologous (isopropylamino)methyl derivatives **42** and **43**.

Compounds **44**–**46** each have a primary (**44**) or aromatic (**45**, **46**) amine at the N-terminus. It was hoped that the introduction of an ionizable functional group at the N-terminus would have a positive effect on both solubility and histamine release activity. As suspected from earlier results,[41] the primary amino function in **44** has a significant and deleterious effect on potency in the AOA. The less basic Ac-2Qal and Ac-3Qal substitutions lead to compounds that were reasonably potent in the AOA. Compound **45** tested in the castrated male rat assay for long duration of action was however found to be inactive at the 24 h time point (data not shown).

Introduction of novel moieties such as ureas (**47**, **48**) or sulfonamides (**49**) led to analogues with low potency in the AOA (**47** and **48** were inactive at the doses tested, 50 and 250 μg/rat, respectively, while **49** was partially active at 10 μg). It is difficult at this time to explain the deleterious effect of these functionalities/substitutions in view of the fact that groups at least as bulky where successfully introduced at these positions (**31**–**35**).

Conclusions

The data presented herein, although limited, suggest that greater hydrophilicity most often translates into shorter duration of action and greater propensity to release histamine. The search for the optimum compromise between hydrophilicity (dependent on the p*I* of the molecule), histamine-releasing potency, and long duration of action seems to have been reached with **3**, **8**, **15**, and **22** which have retention times at pH 7.3 varying from 14.3 (**22**) to 19.9 (**8**) min. In all other analogues, it is apparent that some factor(s) bring about a conformational or compositional element that leads to shorter duration of action or greater propensity in releasing histamine. It is also unfortunate that factors that optimize solubility in aqueous buffers will also be those that result in increased potency in the histamine release assay as well as in a decrease in duration of action. Variation on the theme presented here is unlikely to unravel the structure of analogues with statistically more desirable properties that those uncovered for azaline B and compounds **6**–**8**, **12**, **15**, **22**, or **23**. Of the eight long acting analogues identified here, **22** is the most hydrophilic as measured by retention times on RP-HPLC (it also gives a more fluid solution in an aqueous solution containing 3% mannitol and 5% ethanol than does **8**); on the other hand, **8** is synthetically more accessible than any of the other analogues identified here, was shown to be at least as efficacious as azaline B in the rat and the intact male rhesus monkey, and therefore may be the most desirable analogue for further investigations including studies in the human.

Experimental Section

Instruments. Preparative RP-HPLC was accomplished using a Waters Assoc. (Milford, MA) Prep LC/System 500A and Model 450 variable wavelength UV detector, a Fisher (Lexington, MA) Recordall Model 5000 strip-chart recorder, and a Waters Prep LC 500A preparative gradient generator. Analytical RP-HPLC was run on a system using two Waters M-45 pumps, a Shimadzu Chromatopac EIA integrator, and a Rheodyne Model 7125 injector. The peptide synthesizer used was Beckman Model 990. Optical rotations were determined with a Perkin-Elmer Model 241 polarimeter. All melting points are uncorrected. TLC was run in the following solvent systems: (A) EtOAc:hexane = 1:1, (B) CH_3Cl:MeOH = 9:1, (C) *n*-butanol:AcOH:H_2O = 4:1:2, (D) CH_3Cl:MeOH:AcOH = 95:5:3. Plates were visualized with UV, I_2, and ninhydrin spray.

Starting Materials. Amino acid derivatives Boc-D-Ala, Boc-Arg(Tos), Boc-Leu, Boc-Orn(Fmoc), Boc-D-Orn(Fmoc), Boc-Lys(Fmoc), Boc-D-Lys(Fmoc), Boc-Pro, Boc-Ser(Bzl), Boc-D-Trp, Boc-D-Cit, and Boc-Tyr(2-BrZ) were obtained from Bachem Inc. (Torrance, CA). Boc-D-2Nal, Boc-D-Cpa, Boc-D-3Pal, Boc-D-Qal, Boc-I-Lys(Z)·DCHA were synthesized at the Southwest Foundation for Biomedical Research (under NIH Contract NO1-HD-6-2928) and made available by the Contraceptive Development Branch, Center for Population Research, NICHD. 1,1-Dichloro-1,1-diphenoxymethane, diphenyl cyanocarbonimidate (PCI), N^{α}-Boc-4-nitro-L- and -D-phenylalanine, N^{α}-Boc-4-amino-L- and -D-phenylalanine, and N^{α}-Boc-4-[*N*-(9-fluorenylmethoxycarbonyl)amino]-L- and -D-phenylalanine were synthesized according to previously published procedures.[11] The methylbenzhydrylamine resin used for peptide synthesis was obtained according to published procedures.[9] Resins with substitutions varying from 0.4 to 0.7 mequiv/g were used. All solvents were reagent grade or better.

Novel Amino Acid Synthesis or Derivatization. α-Boc-3-amino(Fmoc)phenylalanine: Diethyl(3-Nitrobenzyl)-acetomidomalonate. Sodium metal (12.64 g, 0.55 mol) was dissolved in dry ethanol (1150 mL); diethyl acetamidomalonate (108.61 g, 0.50 mol) was added to the stirred sodium ethoxide solution and reacted for 30 min; dry (high vacuum for 24 h), 3-nitrobenzyl chloride (85.79 g, 0.50 mol) was added through a powder funnel. The mixture was slowly heated and refluxed for 18 h. The cooled (room temperature) solution was poured into a beaker containing 2.3 L of cold water. The resulting precipitate was filtered, washed with ice water (3 × 200 mL), and dried: yield of crude product 101.2 g (0.287 mol, 57%); mp 61–65 °C; R_f 0.39 (A).

D,L-3-Nitrophenylalanine Hydrochloride. The crude condensation product (95.0 g, 0.27 mol) was suspended in 6 N hydrochloric acid (2 L) and refluxed for 18 h. The resulting clear solution was rapidly filtered to eliminate a small amount of insoluble materials and cooled in an acetone-dry ice bath to yield white needles: 61.8 g (0.25 mol, 93% yield); mp >200 °C; R_f 0.48 (C); ^{1}H NMR (DMSO-d_6, TMS) δ 3.40 (d, 2H, βCH$_2$), 4.27 (m, 1H, αCH), 7.50–8.23 (m, 4H, ArH), 8.80 (br s, 2H, NH_2).

D,L-*N*-Acetyl-3-nitrophenylalanine. D,L-3-Nitrophenylalanine hydrochloride (60.9 g, 0.247 mol) was dissolved in 3 N sodium hydroxide solution (413 mL, 1.24 mol) and cooled to −5 °C in an ice-salt bath. Acetic anhydride (74 mL, 1.13 mol) was added, and the mixture was stirred at 0 °C overnight. The precipitate was filtered, washed with cold water (2 × 150 mL), and dried: yield 59.0 g (0.234 mol, 95%); mp 155–157 °C; R_f 0.72 (C); ^{1}H NMR (CD_3OD, TMS) δ 1.90 (s, 3H, $COCH_3$), 3.27 (m, 2H, βCH$_2$), 4.72 (m, 1H, αCH), 7.50–8.20 (m, 4H, ArH).

D,L-*N*-Acetyl-3-nitrophenylalanine Ethyl Ester. With the temperature kept at −20 °C, thionyl chloride (32 mL, 0.44 mol) was added dropwise (ca. 30 min) to a solution of D,L-*N*-

Ac-3-nitrophenylalanine (58.0 g, 0.23 mol) in anhydrous ethanol (850 mL). The mixture was stirred at −20 °C for an additional 2 h and overnight at room temperature. After solvent removal, the resulting oil was taken up in ethyl acetate (400 mL), washed with saturated sodium hydrogen carbonate (2 × 100 mL) and brine (2 × 100 mL), dried over magnesium sulfate, and evaporated to yield light yellow crystals: 48.0 g (0.17 mol, 74.5%); mp 87−88 °C; R_f 0.19 (A), 0.74 (B), 0.90 (C); ^{1}H NMR (CD_3OD, TMS) δ 1.23 (t, 3H, CH_2CH_3), 1.90 (s, 3H, $COCH_3$), 3.17 (m, 2H, βCH_2), 4.20 (q, 2H, CH_2CH_3), 4.73 (m, 1H, αCH), 7.50−8.20 (m, 4H, ArH).

D-*N*-Acetyl-3-nitrophenylalanine Ethyl Ester and L-*N*-Acetyl-3-nitrophenylalanine. D,L-*N*-Acetyl-3-nitrophenylalanine ethyl ester (81.3 g, 0.29 mol) was dissolved in acetonitrile (500 mL) and diluted with water (1 L); temperature was maintained at 37−42 °C, and α-chymotrypsin (500 mg) was added; pH 7.0 was maintained constant by the addition (autotitrator) of 0.5 N sodium hydroxide solution. Base uptake stopped after ca. 5 h. The mixture was concentrated to remove the acetonitrile. The precipitate was filtered, washed with water several times, and lyophilized to yield the desired D-*N*-acetyl-3-nitrophenylalanine ethyl ester: 38.3 g (0.14 mol, 94%); mp 107.5−108.5 °C; R_f 0.19 (A), 0.74 (B); $[\alpha]_D$ = −14.5° (*c* = 1, MeOH, *T* = 20.0 °C); ^{1}H NMR (CD_3OD, TMS) δ 1.22 (t, 3H, CH_2CH_3), 1.90 (s, 3H, $COCH_3$), 3.17 (m, 2H, βCH_2), 4.20 (q, 2H, CH_2CH_3), 4.73 (m, 1H, αCH), 7.50−8.20 (m, 4H, ArH).

The filtrate was cooled (ice bath) and acidified to pH 2.0 with 6 N hydrochloric acid yielding L-*N*-acetyl-3-nitrophenylalanine as a white precipitate which was filtered, washed with cold water, and dried: 35.7 g (0.14 mol, 97%), mp 161−162 °C; R_f 0.72 (C); $[\alpha]_D$ = +32.2° (*c* = 1, MeOH, *T* = 20.0 °C); ^{1}H NMR (CD_3OD, TMS) δ 1.90 (s, 3H, $COCH_3$), 3.20 (m, 2H, βCH_2), 4.76 (m, 1H, αCH), 7.50−8.20 (m, 4H, ArH).

D- and L-3-Nitrophenylalanine Hydrochloride. D-*N*-Acetyl-3-nitrophenylalanine ethyl ester (21.8 g, 77.8 mmol) was suspended in 6 N hydrochloric acid (400 mL) and refluxed for 10 h to yield crystalline D-3′-nitrophenylalanine hydrochloride: 17.3 g (70 mmol, 90%); mp >200 °C; R_f 0.48 (C).

L-*N*-Acetyl-3-nitrophenylalanine (17.0 g, 67.4 mmol) was hydrolyzed in the same way to yield L-3′-nitrophenylalanine hydrochloride: 15.5 g (62.8 mmol, 93%); mp >200 °C; R_f 0.48 (C).

D- and L-*N*-Boc-3-nitrophenylalanine. To a solution of D-3-nitrophenylalanine hydrochloride (19.1 g, 77.8 mmol) in water (140 mL) and *tert*-butyl alcohol (140 mL) was added dropwise di-*tert*-butyl dicarbonate (20.4 g, 93.4 mmol) dissolved in *tert*-butyl alcohol (80 mL) while keeping the pH at 8.5 with 1 N sodium hydroxide solution. After 3 h of stirring at room temperature, the mixture was evaporated under reduced pressure to remove the *tert*-butyl alcohol. The resulting water solution was extracted with hexane (2 × 200 mL) and then acidified with saturated sodium hydrogen sulfate to pH 2.0. Usual workup (ethyl acetate extraction (3 × 150 mL), washing with brine (1 × 100 mL), drying over magnesium sulfate, solvent removal, and trituration with petroleum ether) yielded crystalline D-*N*-Boc-3-nitrophenylalanine: 19.25 g (62.0 mmol, 80%); mp 120−122 °C dec; R_f 0.85 (C); $[\alpha]_D$ = −16.0° (*c* = 1, MeOH, *T* = 19.5 °C); ^{1}H NMR (CD_3OD, TMS) δ 1.35 (s, 9H, $C(CH_3)_3$), 3.14 (m, 2H, βCH_2), 4.04−4.53 (m, 1H, αCH), 7.51−8.18 (m, 4H, ArH).

L-3-Nitrophenylalanine hydrochloride (16.6 g, 67.4 mmol) was protected in the same way to yield crystalline L-*N*-Boc-3-nitrophenylalanine: 16.52 g (53.2 mmol, 85%); mp 121−123 °C dec; R_f 0.85 (C); $[\alpha]_D$ = +16.8° (*c* = 1, MeOH, *T* = 19.5 °C) lit.[42] $[\alpha]_D$ = +18.35° (*c* = 1, MeOH, *T* = 25 °C); ^{1}H NMR (CD_3OD, TMS) δ 1.36 (s, 9H, $C(CH_3)_3$), 3.14 (m, 2H, βCH_2), 4.05−4.53 (m, 1H, αCH), 7.50−8.17 (m, 4H, ArH).

L-N^{α}-Boc-3-aminophenylalanine. L-*N*-Boc-3-nitrophenylalanine (12.4 g, 40 mmol) was dissolved in a mixture of ethanol (260 mL) and acetic acid (4.56 mL, 80 mmol). The mixture was purged with nitrogen for 10 min, and then Pd/C 10% (0.7 g) was added. Hydrogenation reaction was carried out in a 500 mL Parr hydrogenation vessel under a starting pressure of 42 psi; 3 h later, H_2 pressure had leveled at 32 psi. The mixture was filtered to remove Pd/C and washed with ethanol (2 × 15 mL). The filtrate was concentrated to ca. 10 mL. The desired product crystallized upon addition of anhydrous ether (60 mL): yield 9.2 g (32.8 mmol, 82%); mp 149−151 °C dec; R_f 0.13 (D, positive with ninhydrin); $[\alpha]_D$ = +9.0° (*c* = 1, MeOH, *T* = 24.0 °C); ^{1}H NMR (CD_3OD, TMS) δ 1.38 (s, 9H, $C(CH_3)_3$), 2.87 (m, 2H, βCH_2), 4.30 (m, 1H, αCH), 6.53−7.17 (m, 4H, ArH).

D-*N*-Boc-3-aminophenylalanine. D-*N*-Boc-3-nitrophenylalanine (15.5 g, 50 mmol) was reduced in the same way as the L isomer: yield 9.6 g (34.2 mmol, 68%); mp 150−152 °C dec; R_f 0.13 (D, positive with ninhydrin); $[\alpha]_D$ = −10.8° (*c* = 1, MeOH, *T* = 24.0 °C); ^{1}H NMR (CD_3OD, TMS) δ 1.38 (s, 9H, $C(CH_3)_3$), 2.86 (m, 2H, βCH_2), 4.30 (m, 1H, αCH), 6.54−7.16 (m, 4H, ArH).

L-N^{α}-Boc-N^3-Fmoc-3-aminophenylalanine. A solution of Fmoc-OSu (13.0 g, 38.5 mmol) in THF (100 mL) was added over a 30 min period to a solution of L-N^{α}-Boc-3-aminophenylalanine (9.0 g, 32.1 mmol) dissolved in THF/H_2O (1:1, 200 mL, pH 9.0 with 1 N NaOH). pH 9.0 was kept constant overnight with a pH-stat delivering 1 N sodium carbonate at room temperature. The solution was concentrated to eliminate the THF. The heterogeneous aqueous suspension was washed with ether (4 × 100 mL) to remove excess Fmoc-OSu and side products and then centrifuged to separate the precipitated L-N^{α}-Boc-N^3-Fmco-3-aminophenylalanine sodium salt. Acidification and extraction with ethyl acetate (3 × 60 mL) yielded a first crop of the desired L-N^{α}-Boc-N^3-Fmoc-3-aminophenylalanine: 6.3 g. The water layer from the centrifugation step was washed with additional ether (2 × 50 mL), acidified, and extracted with ethyl acetate to give a second crop of product (4.5 g): total yield 10.8 g (21.5 mmol, 67%); mp 100−104 °C; R_f 0.50 (D); $[\alpha]_D$ = +5.6° (*c* = 1, MeOH, *T* = 24.0 °C); ^{1}H NMR (CD_3OD, TMS) δ 1.35 (s, 9H, $C(CH_3)_3$), 2.98 (m, 2H, βCH_2), 4.03−4.50 (m, 4H, Fmoc-CH + Fmoc-CH_2 + αCH), 6.73−7.82 (m, 12H, Phe-ArH + Fmoc-ArH).

D-N^{α}-Boc-N^3-Fmoc-3-aminophenylalanine. D-N^{α}-Boc-3-aminophenylalanine (9.0 g, 32.1 mmol) was treated with Fmoc-OSu as described above for the L isomer: yield 12.8 g (25.5 mmol, 79%); mp 96−100 °C; R_f 0.50 (D); $[\alpha]_D$ = −6.0° (*c* = 1, MeOH, *T* = 19.5 °C); ^{1}H NMR (CD_3OD, TMS) δ 1.38 (s, 9H, $C(CH_3)_3$), 3.00 (m, 2H, βCH_2), 4.03−4.52 (m, 4H, Fmoc-CH + Fmoc-CH_2 + αCH), 6.80−7.82 (m, 12H, Phe-ArH + Fmoc-ArH).

L-N^{α}-Boc-N^4-Fmoc-4-(aminomethyl)phenylalanine (L-N^{α}-Boc-N^4-Fmoc-4Amp): *N*-Hydroxymethyl Trichloroacetamide. Sodium carbonate (2.4 g) and formaldehyde (36.5%, 50 mL, 0.66 mol) were added (pH 10.5) to trichloroacetamide (97.5 g, 0.60 mol) suspended in water (120 mL). After manual stirring (30 min), a white precipitate formed, which after cooling in an ice−water bath for 30 min was filtered, washed with a small amount of cold water (2 × 50 mL), and dried overnight on a lyophilizer to yield 96.0 g (84%) of product: mp 85−88 °C.

L-*N*-Boc-4-[(trichloroacetamido)methyl]phenylalanine. Powdered L-phenylalanine (60.0 g, 0.363 mol), first, and powdered *N*-hydroxymethyl trichloroacetamide (72.0 g, 0.374 mol), second, were added under vigorous stirring to concentrated sulfuric acid (250 mL) maintained at a constant temperature of 20−25 °C with an ice−water bath (10 min). The yellow-green clear solution was then stirred at room temperature (25 °C) for 4 h. The mixture was poured on ice (3.5 L), the pH was adjusted to 5.5 with 8 N sodium hydroxide while keeping the temperature below 20 °C, and the mixture was left to rest overnight at room temperature. The precipitate was filtered and washed with cold water (2 × 50 mL) to give the crude (ca. 50% pure by HPLC) product which still contained some sodium sulfate: mp 149 °C dec.

This wet solid was suspended in a mixture of *tert*-butyl alcohol and water (1:1, 400 mL). The pH was adjusted to 8.0, and di-*tert*-butyl dicarbonate (79.2 g, 0.363 mol) in *tert*-butyl alcohol was added in portions, keeping the pH at 8.0 by the use of an autotitrator delivering 1 N sodium carbonate. The mixture was then stirred at room temperature overnight. The mixture was concentrated under vacuum to a volume of 300 mL. The resulting white precipitate (confirmed to be the sodium salt form of the para-substituted product by ^{1}H NMR)

was separated by filtration and washed with petroleum ether (30–60 °C, 3 × 100 mL) and cold water (50 mL). The precipitate was suspended in a mixture of EtOAc and water (1:1, 300 mL); pH was brought to 2.5 with saturated sodium hydrogen sulfate. After EtOAc extraction (3 × 150 mL), removal of the solvent, and addition of petroleum ether, a white solid product was obtained: 30.7 g (69.8 mmol, 19.2% yield); mp 115 °C dec, 135–139 °C; $[\alpha]_D$ = +24.0° (*c* = 1, MeOH, *T* = 20 °C); MS *m/e* $(M + 1)^+$ 438.95 (mono 438.05); HPLC assay (column C_{18}, 0.46 × 25 cm, buffer A 0.1% TFA in H_2O, buffer B 0.1% TFA in 60% MeCN/40% H_2O, gradient condition 40–90% buffer B over 50 min at a flow rate of 2 mL/min, UV detection 0.1 AUFS at 214 nm) t_R = 23.7 min, purity >96%; ^{1}H NMR (DMSO-d_6, TMS) δ 1.32 (s, 9H, $C(CH_3)_3$), 3.00 (m, 2H, βCH_2), 4.33 (m, 1H, αCH), 4.73 (s, 2H, NH-CH_2-Phe), 7.11 (br s, 4H, ArH).

L-N^{α}-Boc-4-(aminomethyl)phenylalanine. L-*N*-Boc-*p*-[(trichloroacetamido)methyl]phenylalanine (1.1 g, 2.5 mmol) was dissolved in a mixture of 20% sodium hydroxide in EtOH/H_2O (v/v = 1:1, 10 mL). The reaction mixture was stirred at room temperature for 30 min and then neutralized with 1 N HCl. The mixture was evaporated to remove ethanol, and the remaining aqueous solutions was acidified to pH 2.5 with 1 N HCl and extracted with *n*-butanol (2 × 50 mL). The combined organic layers were washed with water (2 × 20 mL) and then vacuum-evaporated to give the foamy hydrochloride salt. This material was dissolved in water (50 mL) and the pH adjusted to 5.5 with 1 N ammonium hydroxide. The resulting precipitate was collected by filtration and washed with cold water: yield 300 mg (41%); mp 236–237 °C dec; $[\alpha]_D$ = +6.2° (*c* = 1, AcOH/H_2O = 1:1, *T* = 20 °C); MS *m/e* $(M + 1)^+$ 295.1 (mono 294.2); HPLC assay (performed as described previously except gradient was 10–90% B over 40 min) t_R = 12.9 min, purity >98%; ^{1}H NMR (CD_3COOD, TMS) δ 1.40 (s, 9H $C(CH_3)_3$), 3.10 (m, 2H, βCH_2), 4.20 (s, 2H, NH-CH_2-Phe), 4.50 (m, 1H, αCH), 7.33 (br s, 4H, ArH). Scaleup of this procedure was easily achieved.

L-N^{α}-Boc-N^4-Fmoc-4-(aminomethyl)phenylalanine. L-*N*-Boc-*p*-[(trichloroacetamido)methyl]phenylalanine (22.0 g, 50 mmol) dissolved in a mixture of 20% sodium hydroxide in EtOH/H_2O (v/v = 1:1, 200 mL) was stirred at room temperature for 30 min, neutralized (pH 7.0) with cold 3 N HCl (temperature was kept at 10–20 °C), concentrated to about 400 mL to eliminate all ethanol, and diluted with THF (200 mL). Fmoc-OSu dissolved in THF (150 mL) was slowly added to the mixture over 30 min, at constant pH (8.5) with the use of an autotitrator delivering 1 N sodium carbonate. The mixture was stirred at room temperature overnight. THF was removed under vacuum. A white precipitate comprising a mixture of excess Fmoc-OSu and the sodium salt of the desired product was obtained. EtOAc (200 mL) was added to the suspension (600 mL) and thoroughly shaken. The suspension was centrifuged (20 min, 5000 rpm) to yield a white precipitate and two liquid layers which were discarded. The solid was washed twice with a mixture of EtOAc (200 mL) and H_2O (100 mL), centrifuged (20 min, 5000 rpm), and resuspended in a mixture of H_2O (300 mL) and EtOAc (150 mL). The suspension was acidified to pH 2.5 with saturated sodium hydrogen sulfate and extracted with additional EtOAc (3 × 150 mL). The combined organic layers were washed with a saturated NaCl solution (2 × 100 mL) and dried over anhydrous sodium sulfate. After evaporation of EtOAc and addition of petroleum ether, a white precipitate was obtained: 22.5 g (87.1% yield); mp 94–97 °C; $[\alpha]_D$ = +10.3° (*c* = 1, MeOH, *T* = 20 °C); MS *m/e* $(M + 1)^+$ 517.1 (mono 516.2); HPLC assay (performed as described previously) t_R = 39.5 min, purity > 98%; ^{1}H NMR (DMSO-d_6, TMS) δ 1.33 (s, 9H, $C(CH_3)_3$), 2.95 (m, 2H, βCH_2), 4.03–4.43 (m, 6H, NH-CH_2-Phe + Fmoc-CH + Fmoc-CH_2 + αCH), 7.13 (br s, 4H, Phe-ArH), 7.20–7.93 (m, 8H, Fmoc-ArH).

L-N^{α}-Boc-N^4-Cbz-4-(isopropylamino)phenylalanine (L-N^{α}-Boc-N^4-Z-4IAph). L-N^{α}-Boc-4-aminophenylalanine (11.2 g, 40 mmol) was dissolved in acetone (200 mL), and molecular sieves (6.0 g, 4 Å) were added to the solution. The mixture was purged with N_2 for 10 min, and then Pd/C 10% (600 mg) was added. The reductive alkylation reaction was monitored by HPLC and carried out in a 500 mL Parr hydrogenation vessel for 26 h. After the filtration of the catalyst and molecular sieves and the evaporation of the solvent, the desired intermediate L-N^{α}-Boc-4-(isopropylamino)phenylalanine was obtained as a red oil. The oil was Cbz-protected using benzyl chloroformate (Z-Cl; 8.6 mL, 60 mmol) in a mixture of THF/H_2O (1:1, 200 mL) at pH 9.5. Usual workup yielded L-N^{α}-Boc-N^4-Cbz-4-(isopropylamino)phenylalanine as an oil, which turned to a solid by trituration with petroleum ether and cooling in a freezer: 11.0 g (24 mmol, 60.0%); mp 115–120 °C; $[\alpha]_D$ = +4.9° (*c* = 1, MeOH, *T* = 20 °C), −2.7° (*c* = 1, EtOAc, *T* = 20 °C); MS *m/e* $(M + 1)^+$ 457.0 (mono 456.2); HPLC assay (performed as described previously) t_R = 37.5 min; ^{1}H NMR ($CDCl_3$, TMS) δ 1.07 (d, 6H, $CH(CH_3)_2$), 1.38 (s, 9H, $C(CH_3)_3$), 3.10 (m, 2H, βCH_2), 4.30–4.73 (m, 2H, αCH + $CH(CH_3)_2$), 5.06 (s, 2H, Cbz-CH_2), 7.18 (s, 4H, Phe-ArH), 7.28 (br s, 5H, Cbz-ArH).

L-N^{α}-Boc-N^4-Cbz-4-[(isopropylamino)methyl]phenylalanine (L-N^{α}-Boc-N^4-Z-4IAmp). L-N^{α}-Boc-4-(aminomethyl)-phenylalanine (15.3 g, 52 mmol) was isopropylated under reductive conditions and Cbz-protected using procedures similar to that used for L-N^{α}-Boc-N^4-Z-4IAph to yield the desired L-N^{α}-Boc-N^4-Z-4IAmp as a foam: 17.5 g (37 mmol, 71.4%); mp 39–42 °C; $[\alpha]_D$ = +5.2° (*c* = 1, MeOH, *T* = 20 °C); MS *m/e* $(M + 1)^+$ 471.1 (mono 470.2); HPLC assay (performed as described previously) t_R = 40.5 min; ^{1}H NMR ($CDCl_3$, TMS) δ 1.09 (d, 6H, $CH(CH_3)_2$), 1.38 (s, 9H, $C(CH_3)_3$), 3.05 (m, 2H, βCH_2), 4.17–4.60 (m, 4H, αCH + N-CH_2-Phe + $CH(CH_3)_2$), 5.12 (s, 2H, Cbz-CH_2), 7.08 (s, 4H, Phe-ArH), 7.25 (br s, 5H, Cbz-ArH).

Boc-3-(2-quinolinyl)-D-alanine (Boc-D2Qal): Diethyl Acetamido(2-quinolinylmethyl)malonate. Sodium metal (5.4 g, 0.23 mol) was dissolved in dry ethanol (300 mL, refluxed and distilled from magnesium turnings (10 g)), and diethyl acetamidomalonate (25.2 g, 0.117 mol) was added to the reaction mixture. Solid 2-(chloromethyl)quinoline (25 g, 0.117 mol) that had been suspended in benzene (300 mL), concentrated under vacuum to remove residual water, and further dried overnight under high vacuum was added to the sodiomalonate solution and refluxed overnight. Upon cooling, the sodium salt was removed by filtration and the filtrate evaporated to dryness. The residue was dissolved in water (200 mL) and refrigerated overnight. The crude product was collected by filtration, washed with ice water to remove some color, and dried under high vacuum to yield the crude product (30.6 g, 0.085 mol, 73%): mp 121 °C dec.

***N*-Acetyl-3-(2-quinolinyl)-DL-alanine Ethyl Ester.** *N*-Acetyl-3-(2-quinolinyl)-DL-alanine ethyl ester was prepared from diethyl acetamido(2-quinolinylmethyl)malonate (30.6 g, 85 mmol) as described by Berger et al.[42] for *N*-acetyl-3-(6-quinolinyl)-DL-alanine ethyl ester to yield 22 g (78 mmol, 91%): mp 84 °C.

***N*-Acetyl-3-(2-quinolinyl)-D-alanine Ethyl Ester.** *N*-Acetyl-3-(2-quinolinyl)-D-alanine ethyl ester was prepared by the selective enzymatic hydrolysis of the corresponding L isomer using α-chymotrypsin as previously described by Berger et al.[42] for *N*-acetyl-3-(6-quinolinyl)-D-alanine ethyl ester to yield 10.1 g (36 mmol, 90%): mp 89–91 °C dec; $[\alpha]_D$ = +18.9° (*c* = 0.5, MeOH).

3-(2-Quinolinyl)-D-alanine. 3-(2-Quinolinyl)-D-alanine was prepared by acid hydrolysis of *N*-acetyl-3-(2-quinolinyl)-D-alanine ethyl ester as described for *N*-acetyl-3-(6-quinolinyl)-D-alanine ethyl ester to yield 7.2 g (33.6 mmol, 93%): mp 216–219 °C dec; $[\alpha]_D$ = −114.4° (*c* = 0.5, 1 N HCl).

Boc-3-(2-quinolinyl)-D-alanine. Boc-3-(2-quinolinyl)-D-alanine was prepared from 3-(2-quinolinyl)-D-alanine using a procedure described by Rivier et al.[9] for Boc-3-(3-pyridyl)-D-alanine to yield 9.1 g (28.9 nmol, 86%): mp 144 °C dec; $[\alpha]_D$ = −14.8° (*c* = 0.7, EtOAc).

L-N^{α}-Boc-N^4-Fmoc-N^{α}-methyl-4-aminophenylalanine (L-N^{α}-Boc-N^4-Fmoc-N^{α}Me4Aph). L-N^{α}-Boc-phenylalanine (40 g, 0.15 mol) was N-methylated using known conditions[16] to yield L-N^{α}-Boc-N^{α}-methylphenylalanine (40.1 g, 0.142 mol, 95%): mp 71 °C dec; $[\alpha]_D$ = −93.6° (*c* = 1, MeOH) (lit.[1b] oil). The Boc group was cleaved using conditions described by Olsen[43] to yield L-N^{α}-methylphenylalanine (15.5 g, 0.086 mol, 61%): mp 253–256 °C dec (lit.[43] mp 255–260 °C); $[\alpha]_D$ = +15.4° (*c* = 0.96, 1 N HCl) (lit.[43] $[\alpha]_D$ = +15.2 (*c* = 1, 1 N HCl)).

L-N^{α}-Methyl-4-nitrophenylalanine. The nitration of L-N^{α}-methylphenylalanine (11.6 g, 65 mmol) was carried out as described by Pless et al.[44] to yield L-N^{α}-methyl-4-nitrophenylalanine (8.7 g, 39 mmol, 60%): mp 254–257 °C dec (lit.[44] mp 155 °C); $[\alpha]_D = +32.8°$ ($c = 1$, 1 N HCl) (lit.[44] $[\alpha]_D = +31°$ ($c = 1$, 1 N HCl)).

L-N^{α}-Boc-N^{α}-methyl-4-nitrophenylalanine. L-N^{α}-Methyl-4-nitrophenylalanine (8.5 g, 39 mmol) was treated with *tert*-butyl dicarbonate (9.4 g, 12 equiv) as described by Pless et al.[44] to yield the desired protected amino acid (11.5 g, 35 mmol, 92%) as an oil: $[\alpha]_D = -73.6°$ ($c = 0.8$, HOAc), $-90.4°$ ($c = 0.5$, MeOH) (lit.[44] $[\alpha]_D = -73.9°$ ($c = 1.1$, HOAc)).

L-N^{α}-Boc-N^{α}-methyl-4-(*N*-Fmoc-amino)phenylalanine. L-N^{α}-Boc-N^{α}-methyl-4-nitrophenylalanine (11 g, 34 mmol) was reduced with 10% Pd/C following the method of Theobald et al.[11] and reacted with Fmoc-OSu as described[11] to give L-N^{α}-Boc-N^{α}-methyl-4-(*N*-Fmoc-amino)phenylalanine (15.9 g, 31 mmol, 91%) as a white foam: $[\alpha]_D = -43.8°$ ($c = 1.2$, EtOAc), $-47.6°$ ($c = 1.1$, MeOH); LSIMS (M + 1) 514.6 (base peak).

Peptide Synthesis. The resin-bound peptides incorporating the Fmoc-protected amino functions were synthesized by SPPS methodology[45] on a Beckman 990 peptide synthesizer with use of previously described protocols on the methylbenzhydrylamine (MBHA) and (*N*-ethylamino)methyl (NEAM) resins (approximately 1 g of starting resin/peptide) using *tert*-butyloxycarbonyl groups for N^{α}-amino protection. TFA treatment was extended to 2 × 15 min. Coupling time was 90–120 min followed by acetylation (excess acetic anhydride in CH_2Cl_2 for 15 min); 2–3-fold excess protected amino acid was used based on the original substitution of the resin. N-Terminal acetylation was performed using the same protocol as that used for capping (excess acetic anhydride in DCM). Compounds **1** and **2** were synthesized and purified as previously described.[3,11]

Most analogues were synthesized using the following protocol. The fully protected [Boc-Aph(N^{ω}-Fmoc)-DAph(N^{ω}-Fmoc)-Leu-Lys(N^{ϵ}-isopropyl,N^{ϵ}-Z)-Pro-DAla]MBHA-resin was synthesized either manually or automatically on a methylbenzhydrylamine resin (0.76 mequiv of NH_2/g) using solid phase peptide synthesis (SPPS) techniques[46] and an N^{α}-Boc strategy with the following side chain protecting groups: Aph(Fmoc), Lys(N^{ϵ}-isopropyl,N^{ϵ}-Z). The individual amino acids were incorporated in a sequential manner utilizing diisopropylcarbodiimide-mediated activation of the carboxyl group. The extent to which individual couplings had proceeded was qualitatively determined by the ninhydrin test as described by Kaiser et al.[47] The *tert*-butyloxycarbonyl (Boc) group was removed after each coupling cycle by treatment of the growing peptide-resin with 60% TFA in DCM in the presence of 1% ethanedithiol. This protected, resin-bound hexapeptide was treated with 20% piperidine in DMF (5 and 25 min) to liberate the ω-amino function of the otherwise fully protected MBHA-bound Boc-Aph(N^{ω}-Fmoc)-DAph(N^{ω}-Fmoc)-Leu-Lys(N^{ϵ}-isopropyl,N^{ϵ}-Z)-Pro-DAla. Acylation of the free N^{ω}-amino functions with a variety of acylating agents (**12**, **20–25**, **31**) and introduction of an aminotriazole moiety (most analogues) were then carried out using standard protocols. Removal of the α-Boc on Aph[5], followed by the addition of the four N-terminal amino acids and acetylation, gave the fully protected resin-bound peptide. HF treatment (anhydrous) at 0 °C in the presence of anisole (10% v/v) yielded the desired analogue (15–30% based on original substitution of the resin) after elimination of HF under vacuum, ether treatment, extraction in dilute acetic acid, and lyophilization. This method deviates from our original protocols in the fact that ω-functionalities at positions 5 and 6 are introduced prior to the completion on the resin of the whole sequence or more specifically prior to the introduction of the pyridylalanine residue at position 3. This eliminates methylation of the pyridyl ring (up to 20%) for which there is precedent.[14,48] It was suggested that since the introduction of the Atz moiety required both piperidine-mediated Fmoc removal and treatment with hydrazine, the introduction of the methyl group was arising from either piperidine or hydrazine reacting with a formaldehyde equivalent (such as DCM) followed by reaction of the imine formed with the pyridyl nitrogen. Of particular interest is the fact that this contaminant, which is usually not resolved under standard chromatographic conditions (TEAP 2.25 or 0.1% TFA, i.e., probably most acidic systems used except heptafluorobutyric acid), is readily detected by CZE.[13] Furthermore, although significantly less potent than the corresponding non-methylated analogue in the AOA, the methylated peptide is considerably more potent at releasing histamine than is the desired analogue (unpublished results). Although the actual mechanism is still unclear, we have found that this reaction can be very significant (although inconsistent). An alternative to the improved strategy described above was the elimination of both DCM and DMF by thorough washes of the peptide-resin with NMP prior to the use of either piperidine or hydrazine.

N^{α}-Methylation on the Resin. A general procedure for the introduction of a methyl group onto the N^{α}-amino function of a nascent peptide chain was recently described.[17] We have used this methodology for the synthesis of some of the analogues presented here (**36**, **38**). In short, the analogue was built on the solid phase up to and including the amino acid that was ultimately contain the N^{α}-methyl group. Removal of the α-Boc group (60% trifluoroacetic acid in DCM, 20 min) provided the unprotected N^{α}-amino function. Alkylation of this primary amino group with 4,4′-dimethoxybenzhydryl chloride[49] in the presence of triethylamine gave the corresponding N-terminal secondary amino function now containing the TFA-labile 4,4′-dimethoxybenzhydryl group. Methylation of this secondary amine by treatment with 36% aqueous formaldehyde in *N*-methylpyrrolidone (30:70) in the presence of excess sodium cyanoborohydride (2 × 40 min), followed by treatment with 60% trifluoroacetic acid in DCM (2 × 20 min) to remove the 4,4′-dimethoxybenzhydryl group, provided the corresponding N^{α}-methylated peptide. Further elongation of the chain could then proceed. In the case of **37**, **39**, **41**, and **43**, we introduced the readily made amino acid derivative (see earlier in the Experimental Section) into the nascent peptide chain using standard SPPS techniques.

Peptide Purification. Peptides were subsequently purified by RP-HPLC. The lyophilized crude peptides (0.5–1.5 g after HF cleavage) were dissolved in 0.25 M triethylammonium phosphate (200 mL), pH 2.25 (TEAP 2.25), and loaded onto a 5 × 30 cm preparative reverse-phase HPLC cartridge packed in our laboratory using a Vydac C_{18} silica gel column (330 Å pore size, 15–20 mm particle size). The peptide was eluted with use of a flow rate of 90–100 mL/min on a Waters Prep 500 System column with a mixture of A (TEAP 2.25) and B (60% CH_3CN, 40% A) with an appropriate gradient (90 min) such that retention time was ca. 45 min. The collected fractions were screened by use of analytical reverse phase HPLC under isocratic conditions, 0.1% TFA/H_2O at a flow rate of 2.0 mL/min (Vydac C_{18} column, 5 mm, 300 Å pore size, 4.5 × 250 mm). Appropriate fractions were then combined (diluted 1:2 with water) and desalted using a 0.1% TFA/acetonitrile gradient, 20% B (10 min) followed by a 20 min gradient to 90% B (where B contains 60% or 80% acetonitrile depending on the hydrophobicity of the analogue). See refs 50 and 51 for further details. In the majority of cases, significant quantities of the desired analogues (>50 mg) were obtained. In cases when methylation of the pyridyl ring had occurred, purification using the TEAP buffer at pH >6.0 and <7.3[51] allowed ready separation of the methylated analogue.

Peptide Characterization. Analytical RP-HPLC. Purity of the peptides was assessed using RP-HPLC and CZE under conditions reported in Table 1.

Amino Acid Analysis. 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, a TRC 1 column oven, a Kratos Spectroflow 980 fluorescence detector, and an LCI-100 integrator was used as described earlier.[52] Amino acids that were not detected in that system include Pal, IAph, IAmp, N^{α}MeAph, and all Atz-containing amino acids. Aph coelutes with isopropyllysine and was not quantitated. All other amino acids gave the expected ratios.

LSIMS. Spectra were measured using a Joel JMS-HX110 double-focusing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. An accelerating voltage of 10 kV and a Cs^+ gun voltage of 25–30 kV were employed. The samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix.

Optical Rotations. Optical rotations were measured in 1% acetic acid (c = 1.0; i.e., 10 mg/mL 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).

Biological Testing. *In vitro*, the peptides were tested for their ability to inhibit GnRH-mediated LH secretion by cultured pituitary cells. Potencies of GnRH antagonists were expressed relative to a standard ([Ac-Δ^3Pro^1,DFpa^2,DTrp^{3,6}]-GnRH).[26] In binding studies, the K_D for the potent agonist [DAla^6,NMeLeu^7,Pro^9-NHEt]GnRH (taken as standard) was determined from a Scatchard analysis to be approximately 0.3 nM. All the other K_D values were calculated from the potencies of the analogues (relative to the standard) determined from displacement data.[53] The assay for histamine release by rat mast cells has been reported previously.[5,27] The AOA was carried out as described by Corbin and Beattie[28] using an aqueous vehicle unless stated otherwise; cycling rats (250–300 g at the time of the assay) were injected sc with the peptides dissolved in saline or 5% DMSO in saline (200 μL) at noon on proestrus. Results are expressed in terms of the dosage in mg/rat (rats ovulating/total number of treated rats). Measurement of circulating LH levels was similar to that reported earlier.[29–31] Male Sprague–Dawley rats (220–240 g) were castrated under ether anesthesia 8 days prior to the start of the experiment. The peptides were first dissolved at a concentration of 1.0 or 10 mg/mL in bacteriostatic water and then further diluted in 0.04 M phosphate buffer containing 0.1% BSA. Antide was first acidified with 2 N HOAc until in solution. Subsequent dilutions were made in phosphate buffer. Blood samples (300 μL) were collected at the given times (see Figure 1). Sera (50 μL) were tested for LH levels in duplicate using reagents provided by the National Pituitary and Hormone Distribution Program of the NIDDK.[54]

Acknowledgment. This work was supported by NIH under Contracts NO1-HD-9-2903 and NO1-HD-0-2906 and in part by NIH Grant HD 13527 and the Hearst Foundation. We thank Dr. J. Reel for the histamine release data as well as for some of the antiovulatory data obtained at Bioqual Inc. under contract NO1-HD-1-3130 with the Contraceptive Development Branch, Center for Population Research, NICHD. Boc-D2Nal, Boc-DCpa, Boc-D3Pal, and Boc-ILys(Z) were synthesized at the Southwest Foundation for Biomedical Research (under NIH Contract NO1-HD-1-3137) or purchased from Synthetech by NIH and made available by the Contraceptive Development Branch, Center for Population Research, NICHD. We are greatly indebted to Dr. M. Karten for constructive comments and constant support in coordinating the flow of supplies and data originating from different institutions. We thank C. Miller, R. Kaiser, D. Pantoja, S. Johnson, W. Low, T. Goedken, and Y. Haas for their outstanding technical contributions and R. Hensley and D. Johns for manuscript preparation.

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JM940285O