GnRH Antagonists: A New Generation of Long Acting Analogues Incorporating *p*-Ureido-phenylalanines at Positions 5 and 6†

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A series of antagonists of gonadotropin-releasing hormone (GnRH) of the general formula Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph/4Amf(P)-D4Aph/D4Amf(Q)-Leu-ILys-Pro-DAla-NH_2 was synthesized, characterized, and screened for duration of inhibition of luteinizing hormone release in a castrated male rat assay. Selected analogues were tested in a reporter gene assay (IC_{50} and pA_2) and an in vitro histamine release assay. P and Q contain urea/carbamoyl functionalities designed to increase potential intra- and intermolecular hydrogen bonding opportunities for structural stabilization and peptide/receptor interactions, respectively. These substitutions resulted in analogues with increased hydrophilicity and a lesser propensity to form gels in aqueous solution than azaline B [Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph(Atz)-D4Aph(Atz)-Leu-ILys-Pro-DAla-NH_2 with Atz = 3′-amino-1*H*-1′,2′,4′-triazol-5′-yl, **5**], and in some cases they resulted in a significant increase in duration of action after subcutaneous (sc) administration. Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph(L-hydroorotyl)-D4Aph(carbamoyl)-Leu-ILys-Pro-DAla-NH_2 (acetate salt is FE200486) (**31**) and eight other congeners (**20**, **35**, **37**, **39**, **41**, **45**−**47**) were identified that exhibited significantly longer duration of action than acyline [Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph-(Ac)-D4Aph(Ac)-Leu-ILys-Pro-DAla-NH_2] (**6**) when administered subcutaneously in castrated male rats at a dose of 50 μg in 100 μL of phosphate buffer. No correlation was found between retention times on a C_{18} reverse phase column using a triethylammonium phosphate buffer at pH 7.0 (a measure of hydrophilicity) or affinity in an in vitro human GnRH report gene assay (pA_2) and duration of action. FE200486 was selected for preclinical studies, and some of its properties were compared to those of other clinical candidates. In the intact rat, ganirelix, abarelix, azaline B, and FE200486 inhibited plasma testosterone for 1, 1, 14, and 57 days, respectively, at 2 mg/kg sc in 5% mannitol (injection volume = 20 μL). Based on the information that **31**, **33**, **35** and **37** were significantly shorter acting than acyline or azaline B after intravenous administration (100 μg/rat), we surmised that the very long duration of action of the related FE200486 (for example) was likely due to unique physicochemical properties such as solubility in aqueous milieu, comparatively low propensity to form gels, and ability to diffuse at high concentrations in a manner similar to that described for slow release formulations of peptides. Indeed, in rats injected sc with FE200486 (2 mg/kg), plasmatic concentrations of FE200486 remained above 5 ng/mL until day 41, and the time after which they dropped below 3 ng/mL and plasma LH levels started rising until full recovery was reached at day 84 with levels of FE200486 hovering around 1 ng/mL. Additionally, FE200486 was less potent at releasing histamine from isolated rat mast cells than any of the GnRH antagonists presently described in preclinical reports.

Introduction

GnRH antagonists are now recognized as potential drugs for the management of sex steroid-dependent pathophysiologies, for induction of ovulation, and for male contraception.[1−4] Long term clinical studies were carried out first with cetrorelix[5] (**1**), the Nal-Glu antagonist[6] (**2**), and ganirelix[7] (**3**). Although these analogues are very potent in inhibiting gonadotropin secretion, they also stimulate the release of histamine in the rat, and these analogues are relatively short acting.[6,8] Cetrorelix, ganirelix, and abarelix[9] (**4**, also known as PPI-149), a short acting antagonist with low histamine-releasing activity, are available as long acting depot formulations using proprietary technologies. Animal and clinical studies presently suggest 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),

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precocious puberty, uterine myoma, ovarian hyperandrogenism and hirsutism, premenstrual syndrome, and may also be a promising lead in the treatment of breast and gynecological cancers.[4] Most of these disorders can be managed with long acting preparations of the superagonists which desensitize the gonadotrophs after approximately two weeks of treatment. An antagonist will likely become the preferred choice over the agonist in the clinic because it avoids the initial up-regulation of the gonadotropin−gonadal axis, leads to rapid and predictable recovery, achieves more profound inhibition of gonadotropins, and can be used as a diagnostic test of gonadotropin-dependent gonadal dysfunction. Although azaline B[10] (**5**) and acyline[11] (**6**) are among the most potent and long acting GnRH antagonists when administered in aqueous buffers or corn oil, there is strong evidence that their relatively low solubility and propensity to form gels result in an inverse relationship between concentration and bioavailability, limiting the use of azaline B to concentrations lower than or equal to 10 mg/mL. As a result, sufficient quantities of azaline B could not be administered in clinically acceptable volumes by the subcutaneous (sc) route in order to achieve inhibition of gonadotropins for four weeks. The same lack of solubility and bioavailability prevented other modes of formulation.[12] Because all reported GnRH antagonists, with the possible exception of abarelix (**4**), form gels at unacceptably low concentrations, the development of formulations for GnRH antagonists that would last more than a month for the treatment of sex hormone-dependent pathologies (prostate cancer, endometriosis, etc.)[4] remains a challenging goal. We concluded from these studies that adequate bioavailability for GnRH antagonists can only be attained with analogues that are significantly more soluble than the above and hypothesized that this property could be induced by increasing the number of hydrogen bonding sites on the peptide side chains. The work of Williams and co-workers[13,14] on the quantitation of factors responsible for ligand−receptor binding that includes structural considerations (constrained conformations), electrostatic and van der Waals forces, and forces resulting from hydrogen bond formation suggested an important role for the last. We tested this proposal with the introduction of a variety of urea functionalities on the side chains of selected amino acids at positions 5 and 6 of GnRH antagonists (Table 1). By increasing the number of possible hydrogen bonds between the peptide and its receptor, its environment, or for intramolecular structure stabilization, we aimed at increasing binding affinity to the GnRH receptor, bioavailability from the site of administration, and stabilization against enzymatic degradation and at delaying elimination. Taken together, each of these factors was hypothesized to result in increased duration of action.

Results and Discussion

Synthesis, Purification, and Chemical Characterization (Table 1). Analogues were synthesized by the SPPS methodology on a *p*-methylbenzhydrylamine resin (MBHA-resin, Boc strategy) or a TentaGel S RAM resin (Fmoc strategy) using protocols previously described[10,11] or shown below. In most cases, analogues were obtained by the introduction of each of the side chain substituents at positions 5 and 6 on the partially deprotected peptido-resin resulting from treatment with 20% piperidine (for example, after the introduction of Boc-DAph/DAmf(Fmoc) at position 6 and Boc-Aph/Amf(Fmoc) at position 5).

All alkylurea peptides were prepared by reaction of the resin-bound partially deprotected amino peptide with the appropriate isocyanate. The reaction took place either during elongation or after completion of the peptide chain. Peptides containing unsubstituted urea were prepared either by direct coupling reaction of individually synthesized *N*-ureido amino acids (see Experimental Section) or by the reaction of the resin-bound partially deprotected amino peptide with the following isocyanates: tosyl isocyanate, benzyl isocyanate, trimethylsilyl isocyanate, and *tert*-butyl isocyanate. The protecting groups of the resulting ureas were removed during final anhydrous HF cleavage except for trimethylsilyl, which was very acid labile and removed under TFA deprotection conditions (1% *m*-cresol in 50% TFA/DCM) during SPPS. All four isocyanates afforded the desired product after anhydrous HF cleavage. It was found that the presence of a base such as DIEA was deleterious when using tosyl or trimethylsilyl isocyanates as it caused overreaction of the isocyanate resulting in a biuret as byproduct. Reaction of amino peptido-resins with benzyl isocyanate yielded very clean products. However, removal of benzyl groups from benzyl-ureas required extended treatment of anhydrous HF (6 h at room temperature). *tert*-Butyl isocyanate also gave very clean products with easy removal of the *tert*-butyl group under routine HF cleavage conditions (1.5 h at 0−5 °C). Interestingly, *tert*-butyl groups on *tert*-butyl-ureas were very stable under the TFA deprotection conditions, and overnight treatment of 95% TFA in H_2O gave less than 5% of the desired hydrolysis product. Accordingly, *tert*-butyl isocyanate was routinely used for introduction of unsubstituted ureas during SPPS using the Boc strategy.

The protected peptido-resins were cleaved and deprotected in anhydrous HF in the presence of a scavenger. The crude peptides were purified by reverse-phase HPLC (RP-HPLC) in two steps and isolated as their TFA salts.[15] Selected peptides (**1**−**6**, **31**, and **35** when noted) were isolated as their acetate salts using the method of Hoeger et al.[16] The analytical techniques used for the characterization of the analogues included RP-HPLC with two different solvent systems (other than those used for purification) and capillary zone electrophoresis (CZE). With very few exceptions, all analogues were greater than 98% pure. Mass spectrometric analysis supported the identity of the intended structures.

The following reference compounds were synthesized using published protocols: cetrorelix[5] (**1**), Nal-Glu[6] (**2**), ganirelix[7] (**3**), abarelix[9] (**4**), azaline B[8] (**5**), and acyline[11] (**6**).

The synthesis of the other analogues deserves further comment. The syntheses of **8**, **9**, and **13** involved reaction of tosyl isocyanate with the unprotected ω-amino function of a variety of amino acids (α,β-diamino propionic acid, α,γ-diamino butyric acid, and *p*-aminophenylalanine) on the otherwise fully protected and assembled peptide resins. Similarly, **7** and **25** were synthesized by reaction of *tert*-butyl isocyanate (rather than tosyl isocyanate) with ω-amino function of

Table 1. Physicochemical Properties, Duration of Action, and Affinity of GnRH Analogues with [X = Ac-DNal[1],DCpa[2],DPal[3]] and [Y = ILys[8],DAla[10]][a]

no.	compound	TFA[b]	TEAP pH 7.0[c]	CE avg[d]	MS calcd[e]	MS obs[e]	duration[f]	RGA IC_{50},[g] nM	RGA pA_2[h]	HR% (at conc μg/mL)
1	[X,DCit[6],DAla[10]]GnRH Cetrorelix	>98	14.40 *	97	1430.1	1430.5	long	4.2	8.9	34 (1), 5 (3), 64 (10)
2	[X,Arg[5],DGlu(AA)[6],Y]GnRH Nal-Glu	>98	17.00 >98	>98	1485.7	1485.5	short	3.3	9.3	41 (1), 60 (3), 68 (10)
3	[X,DHar(Et_2)[6],Har(Et_2)[8],DAla[10]]GnRH Ganirelix	>98	15.80 >98	>98	1569.9	1569.7	intermediate	3.6	9.3	30 (10), 57 (30), 62 (100)
4	[X,N$^{\alpha}$-Me-Tyr[5],DAsn[6],Y]GnRH Abarelix	>98	NA >98	>98	1415.7	1415.6	very short	3.5	9.1	
5	[X,Aph(Atz)[5],DAph(Atz)[6],Y]GnRH Azaline B	>98	14.04 *	>98	1612.8	1612.7	long	6.9	8.8	17 (10), 70 (30), 93 (100)
6	[X,Aph(Ac)[5],DAph(Ac)[6],Y]GnRH Acyline	98	17.41 *	>98	1532.8	1532.7	long	6.8	8.6	43 (30), 56 (100) 87 (300)
7	[X,D/L-Agl(Me,Cbm)[5],DAph(Ac)[6],Y]GnRH	>98	16.60 *	>98	1457.7	1457.3	very short			
8	[X,Dap(Cbm)[5],DAph(Ac)[6],Y]GnRH	>98	16.17 *	>98	1457.7	1457.5	short			
9	[X,Dab(Cbm)[5],DAph(Ac)[6],Y]GnRH	>98	15.92 *	>98	1471.7	1471.5	short			
10	[X,Dap(EtCbm)[5],DAph(Ac)[6],Y]GnRH	>98	18.00 >98	>98	1485.7	1485.5	short			
11	[X,Dap(MeOPhCbm)[5],DAph(Ac)[6],Y]GnRH	>98	21.30 *	>98	1563.8	1563.4	short			
12	[X,Dap(Hor)[5],DAph(Ac)[6],Y]GnRH	>98	16.20 *	>98	1554.7	1554.4	very short			96 (150)
13	[X,Aph(Cbm)[5],DAph(Ac)[6],Y]GnRH	>98	16.22 *	95	1533.7	1533.6	intermediate	7.2		
14	[X,Aph(diureidoacetyl)[5],DAph(Ac)[6],Y]GnRH	96	13.70 >98	>98	1648.8	1648.5	intermediate	8.9		
15	[X,Aph(Dhp)[5],DAph(Ac)[6],Y]GnRH	73	18.76 *	86	1628.8	1628.5	short	8.2		
16	[X,Aph(D/L-Hya)[5],DAph(Ac)[6],Y]GnRH	>98	14.50 >98	>98	1630.8	1630.5	intermediate	8.1		
17	[X,Aph(Imz)[5],DAph(Ac)[6],Y]GnRH	>98	13.90 >98	>98	1602.8	1602.5	long	9.1	9.2	
18	[X,Aph(DImz)[5],DAph(Ac)[6],Y]GnRH	>98	NA >98	>98	1602.8	1602.9	intermediate	10.8		
19	[X,Aph(Oro)[5],DAph(Ac)[6],Y]GnRH	>98	14.41 *	97	1628.8	1628.6	very short	10.7		
20	[X,Aph(Hor)[5],DAph(Ac)[6],Y]GnRH	>98	15.54 *	>98	1630.8	1630.4	very long	4.3	8.9	54 (100)
21	[X,N$^{\alpha}$-Me-Aph(Hor)[5],DAph(Ac)[6],Y]GnRH	>98	14.60 >98	>98	1644.8	1644.6	short	8.1	9.1	50 (30), 124 (100)
22	[X,Aph(DHor)[5],DAph(Ac)[6],Y]GnRH	>98	16.00 >98	>98	1630.8	1630.8	long	8.6	9	
23	[X,Amf(Hor)[5],DAph(Ac)[6],Y]GnRH	>98	16.65 >98	>98	1644.8	1644.7	long	7.9	9.5	
24	[X,Aph(Ac)[5],DAph(Hor)[6],Y]GnRH	>98	14.00 >98	>98	1630.8	1630.5	intermediate			17 (30), 64 (100)
25	[X,Aph(Ac)[5],DAph(Cbm)[6],Y]GnRH	>98	16.46 >98	>98	1533.7	1533.6	long	6.1	9.3	
26	[X,Aph(Ac)[5],DAmf(Cbm)[6],Y]GnRH	>98	16.65 >98	>98	1547.8	1547.7	intermediate	6.4	9.2	
27	[X,Aph(Cbm)[5],DAph(Cbm)[6],Y]GnRH	96	14.76 *	>98	1534.7	1534.6	long	2.8	8.8	<10 (10), 75 (30), 153 (100)
28	[X,Aph(MeCbm)[5],DAph(MeCbm)[6],Y]GnRH	>98	15.60 >98	>98	1562.8	1562.8	intermediate			
29	[X,Aph(EtCbm)[5],DAph(EtCbm)[6],Y]GnRH	>98	20.33 *	>98	1590.8	1590.5	short	5	8.9	
30	[X,Aph(Hor)[5],DAph(Hor)[6],Y]GnRH	>98	13.80 *	>98	1728.8	1728.4	long	10.8	9	<10 (30), 11 (100)
31	[X,Aph(Hor)[5],DAph(Cbm)[6],Y]GnRH	>98	14.27 >98	>98	1631.8	1631.9	very long	3	8.8	54 (100), 68 (300)
32	[X,Aph(DHor)[5],DAph(Cbm)[6],Y]GnRH	>98	14.63 >98	>98	1631.8	1631.7	intermediate	8.8	9.2	
33	[X,Aph(Hor)[5],DAmf(Ac)[6],Y]GnRH	>98	15.71 >98	>98	1644.8	1644.5	long	9.5	9	
34	[X,Aph(DHor)[5],DAmf(Ac)[6],Y]GnRH	>98	15.73 >98	>98	1644.8	1644.7	long	7	8.8	

Table 1 (Continued)

no.	compound	TFA[b]	TEAP pH 7.0[c]	CE avg[d]	MS calcd[e]	MS obs[e]	duration[f]	RGA IC_{50},[g] nM	RGA pA_2[h]	HR% (at conc μg/mL)
35	[X,Aph(Hor)^5,DAmf(Cbm)^6,Y]GnRH	>98	14.66 >98	>98	1645.8	1645.9	very long	5.6	8.9	21 (30), 48 (100), 64 (300)
36	[X,Aph(DHor)^5,DAmf(Cbm)^6,Y]GnRH	>98	14.80 >98	>98	1645.8	1645.8	intermediate	7.7		
37	[X,Aph(Hor)^5,DAmf(MeCbm)^6,Y]GnRH	>98	15.76 >98	>98	1659.8	1659.8	very long	7.9	9.1	56 (100), 67 (300)
38	[X,Aph(Hor)^5,DCit^6,Y]GnRH	>98	13.71 >98	>98	1583.8	1583.7	long	5.8	9.1	
39	[X,Aph(Hor)^5,DPal^6,Y] GnRH	>98	15.82 >98	>98	1574.7	1574.7	very long	2.8	8.7	68 (100), 62 (300)
40	[X,Aph(Hor)^5,DNal^6,Y]GnRH	>98	18.34 >98	>98	1623.8	1623.7	short	13		
41	[X,Aph(Hor)^5,DAph(DHor)^6,Y]GnRH	>98	13.79 >98	>98	1728.8	1728.7	very long	7.7	9	69 (100), 64 (300)
42	[X,Lys(Hor)^5,DLys(Hor)^6,Y]GnRH	>98	12.00 >98	>98	1660.8	1660.5	very short			
43	[X,Aph(Hor)^5,DAph(Cbm)^6,ILys^8,Pro^9-NHEt]GnRH	>98	NA >98	>98	1588.8	1588.7	long			
44	[X,Aph(Hor)^5,DAmf(Cbm)^6,ILys^8,Pro^9-NHEt]GnRH	>98	NA >98	>98	1602.8	1602.9	intermediate	8.8	9.2	
45	[X,Aph(Hor)^5,DAmf(Cbm)^6,ILys^8,DAla-ol^{10}]GnRH	97	NA >98	>98	1632.8	1632.9	very long	7.8	9	
46	[X,Aph(Hor)^5,DAmf(Cbm)^6,ILys^8,Ala-ol^{10}]GnRH	>98	NA >98	>98	1632.8	1632.9	very long	5.5	9.2	
47	[X,Aph(Hor)^5,DAph(Cbm)^6,ILys^8,DAla-ol^{10}]GnRH	>98	NA >98	>98	1618.8	1618.9	very long			
48	[X,Aph(Hor)^5,DAph(Cbm)^6,ILys^8,Ala-ol^{10}]GnRH	>98	NA >98	>98	1618.8	1618.9	long			

[a] Characterization of GnRH antagonists by HPLC, CZE, and MS in a castrated male rat assay for the measure of duration of action, in a reporter gene assay (IC_{50} and pA2), and in an in vitro histamine release assay. [b] Purity by HPLC under gradient conditions (40% to 70% B over 30 min). Buffer system A: 0.1% TFA. Buffer system B: 0.1% TFA in 70% ACN/H_2O. [c] Retention time and purity by HPLC under gradient conditions (40% to 70% B over 30 min). Buffer system A: TEAP pH 7.0. Buffer system B: 70% ACN/40% A. An asterisk (*) indicates that poor resolution did not allow quantitation of impurities under neutral conditions. NA, not available. [d] Purity by capillary electrophoresis was performed on a Beckman model P/ACE 5510 instrument using a voltage of 15 kV, a 3 s injection with a 0.1 mg/mL peptide solution, and a 20 mM phosphate buffer, pH 2.5, containing 15% ACN. The value shown is the average of two values from two separate analyses. [e] MS calculated and observed values using a Finnigan LCQ ion-trap mass spectrometer with an ESI source using flow-injection at a rate of 5 μL/min with a 0.1 mg/mL peptide solution. [f] Castrated male rat assay (see Figure 1). Duration of action: very long = over 80% inhibition of LH release at 96 h; long = over 80% at 72 h but not 96 h; intermediate = over 80% at 48 h but not at 72 h; short = over 80% at 24 h but not 48 h; very short = no inhibition at 24 h. [g] Antagonism of GnRH-induced response was determined for each analogue (IC_{50}) in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a stably integrated alpha luciferase reporter gene. The values shown represent the average of two determinations performed in duplicate. [h] Competitive antagonism of GnRH-induced response in the reporter gene assay determined in the presence of increasing concentrations of each analogue (pA_2). The values shown represent the average of two determinatitions performed in duplicate.

N^{α}-Me-α-aminoglycine and *p*-aminophenylalanine on the otherwise fully protected resins. Compounds **10** and **11**, ethyl and 4-methoxyphenyl isocyanates, respectively, were reacted with the deprotected amino function at position 5 on the otherwise fully protected peptido-resins. The syntheses of **12**, **14**−**20**, and **22**−**24** were accomplished by *N*,*N*′-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) mediated coupling reactions of resin-bound partially deprotected *ω*-amino groups with L-hydroorotic acid (Hor, **12**, **20**, and **23**−**24**), D-hydroorotic acid (DHor, **22**), diureidoacetic acid (diureidoacetyl, **14**), 2,4-dihydroxy-pyrimidine-5-carboxylic acid (Dhp, **15**), hydantoin-5-acetic acid (D/L-Hya, **16**), L-2-imidazolindone-4-acetic acid (Imz, **17**), D-2-imidazolindone-4-acetic acid (DImz, **18**), and orotic acid (Oro, **19**).

The introduction of the N^{α}-methyl in **21** was achieved on partially assembled peptide resin using a published procedure by Kaljuste et al.[17] Further elongation of the peptide chain afforded the fully assembled peptide resin, [Ac-DNal-DCpa-DPal-Ser(Bzl)-N^{α}-Me-Aph(Fmoc)-DAph(Ac)-Leu-Lys(isopropyl,Cbz)-Pro-DAla]MBHA-resin, which was then treated with 25% piperidine in DMF and reacted with L-hydroorotic acid, DIC, and HOBt to give the fully protected **21** on the resin. The unsubstituted urea function in **26** was introduced on the resin by direct coupling of a synthesized novel amino acid, Boc-DAmf(Cbm)-OH (or Boc-DUmf-OH).

For the purpose of optimizing our synthetic methodologies with regard to the introduction of an unsubstituted urea functionality in peptides using the solid-phase approach, **27** was synthesized by both the Boc and Fmoc strategies. With the Boc strategy, the two urea groups were introduced simultaneously into positions 5 and 6 by reaction of the deprotected *ω*-amino groups (at positions 5 and 6) on the otherwise fully protected and assembled peptido-resin with benzyl-, tosyl-, or *tert*-butyl-isocyanates. All three isocyanates yielded the desired **27**, with *tert*-butyl isocyanate giving the best purity and yield. With the Fmoc strategy, we chose to synthesize the protected ureido-containing amino acids first (Fmoc-DUph-OH and Fmoc-LUph-OH). These were incorporated sequentially into the peptide on a TentaGel S RAM resin (Peptides International) using a Millipore 9050 Plus PepSynthesizer and DIC couplings. Compounds **28**−**30** and **42** were synthesized

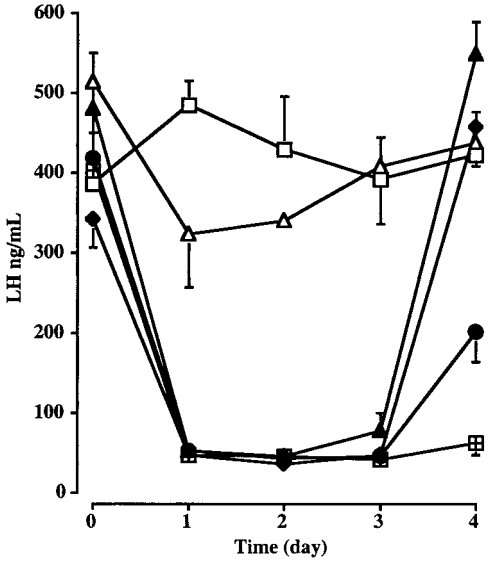

Figure 1. Inhibition of LH secretion after sc administration of analogues (**1**, **4**−**6**, **31**). Total dose was 50 μg in 100 μL of 40 mM phosphate buffer containing 0.1% or 0.6% DMSO. Blood samples were collected at the times shown on the abscissa. The SEM, where not appearing, are encompassed within the size of the symbols in the graph. Vehicle □; cetrorelix (**1**) ▲; acyline (**6**) ●; abarelix (**4**) △; FE200486 (**31**) (cross-hatched box); azaline B (**5**) ◆. Weight of rats (n = 5) was 210−240 g at end of experiment.

using methyl and ethyl isocyanates and L-hydroorotic acid using the Boc strategy described for **27**.

The syntheses of **31** and **32** included the coupling of Boc-D-Aph(*t*BuCbm)-OH at position 6 and reaction of L- and D-hydroorotic acids, DIC, and HOBt with the freed amino function at position 5 of the otherwise fully protected resin-bound peptides. Compounds **33**−**36** were prepared with a similar strategy. The syntheses of **33** and **34** involved coupling of Boc-D-Amf(Fmoc)-OH immediately followed by Fmoc removal and acetylation using acetic anhydride prior to chain elongation. The syntheses of **35** and **36** involved coupling of Boc-D-Amf-(*t*BuCbm)-OH. Boc-Aph(Hor)-OH was synthesized and used in the assembly of **37**−**41**.

The syntheses of **43**, **47**, and **48** involved the reactions of the fully deprotected peptide fragment, Ac-D-Nal-D-Cpa-D-Pal-Ser-Aph(Hor)-D-Aph(Cbm)-Leu-Lys(isopropyl)-Pro-OH, with ethylamine hydrochloride (**43**), D-alaninol (**47**), and L-alaninol (**48**) using the PyBOP reagent. The fragment was first synthesized manually on Merrifield resin using Boc-D-Aph(Cbm)-OH (or Boc-D-Uph-OH) and Boc-Aph(Hor)-OH at positions 6 and 5, respectively. After cleavage with anhydrous HF, the crude fragment was purified by RP-HPLC using a TFA buffer system. Peptides **44**−**46** were synthesized using the same strategy: Ac-D-Nal-D-Cpa-D-Pal-Ser-Aph(Hor)-D-Amf(Cbm)-Leu-Lys(isopropyl)-Pro-OH was first assembled, cleaved, deprotected, and purified before activation with isobutyl chloroformate and condensation with ethylamine hydrochloride to yield **44**. Compounds **45** and **46** were obtained after PyBOP coupling of the same purified peptide fragment with D-alaninol and L-alaninol, respectively.

Biological Characterization (Table 1 and Figure 1). Biological screening of the analogues may appear to be nonconventional in that peptides were tested in the castrated male rat first for duration of action. It was shown in earlier reports that there was no correlation between hydrophobicity or affinity of GnRH analogues and duration of action.[8,18] Our goal was to identify novel analogues with durations of action significantly greater than that of acyline or azaline B, which was reported to have longer duration of action after administration in either aqueous solution or oil than any other antagonist described in the literature.[8,11] We used the castrated male rat assay because it is highly reproducible and can accommodate the screening of as many as 10 analogues at one time. In short, test compounds were injected sc to castrated male rats at a standard dose of 50 μg/rat. Blood sampling was performed predose and then 24, 48, 72, 96, and in some cases, 120 h post-dose. The effects of the test compounds on the gonadotropic axis were determined by measurement of plasma LH levels.

A selection of analogues were also tested in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a stably integrated alpha luciferase reporter gene. The antagonism of the GnRH agonist-induced response was determined (IC_{50}). In addition, assays were conducted to establish whether the compounds were competitive antagonists by determining the dose−response relationships for GnRH in the absence or in the presence of increasing concentrations of analogue (pA_2).

Additionally, GnRH antagonists were tested for their ability to release histamine from isolated rat peritoneal mast cells as described.[19] Percent release of histamine is reported at given doses (Table 1).

Analogues reported here, **1**,[11] **2**,[6] **3**,[20] **4**,[9] **5**,[8,10] and **6**[11] were synthesized and tested for comparison purposes. By our definition, an analogue is very long acting when 50 μg injected sc in 40 mM phosphate buffer containing 0.1% BSA and 0.6% DMSO in a volume of 100 μL results in an inhibition of LH (>80%) that lasts for more than 120 h. Under the same conditions, an analogue that is long acting will inhibit LH secretion for at least 72 h, an analogue that is intermediate will inhibit LH secretion for at least 48 h, an analogue that is short acting will inhibit LH secretion for at least 24 h, and an analogue that is very short acting will not inhibit LH secretion at 24 h. This is illustrated in Figure 1 where we describe the time course of activity of five selected analogues [**1** (long acting), **4** (very short acting), **5** (long acting), **6** (long acting), and **31** (very long acting)].

Ten days after castration, rats (5 per group) were injected on Mondays with the excipient (100 μL of aqueous buffer), acyline (50 μg in 100 μL of aqueous buffer) as an internal standard, and the novel analogues (50 μg in 100 μL of aqueous buffer). Blood samples were collected daily for 4 days. Plasma LH levels were measured by radioimmunoassay.[21] Because this assay was highly reproducible in that acyline showed very consistent duration of action [average of 10 assays shows 84.8% (± 0.6) inhibition at 24 h, 88.1 (± 0.3) at 48 h, 84.4% (± 2.1) at 72 h, and 38.2% (± 8.6) at 96 h], it could be used reliably for comparative studies. Any substitutions that led to analogues with a duration of action equal to or longer than that of acyline in that assay were considered favorable, and any analogue that inhibited LH secretion for 96 h or more was studied more extensively.

The overall rationale for the synthesis of the different analogues was presented in our introduction. We further took into account the observation that the introduction of D-citrulline (carbamoyl-ornithine) at position 6 of cetrorelix yielded an unexpectedly long acting analogue.

One of the main considerations was to obtain one or several GnRH analogues that would have all of the outstanding characteristics of azaline B (potent at inhibiting gonadal function, very 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 would not form gels at concentrations below 10 mg/mL upon administration. Analogues with an N-Me substitution at position 5 first reported by Haviv et al.[22] were very water soluble because of the inability of these peptides to form stable β sheets known to be sparingly soluble. We had shown however, that such substitution also increased the propensity of these analogues to release histamine.[8] We were therefore very concerned with the possibility that any substitution that would yield more hydrophilic GnRH antagonists would also lead to analogues that would release histamine. Last but not least, our conclusion from an earlier publication also suggested that greater hydrophilicity most often translated into shorter duration of action.[11]

Over the past 10 years, analogues **1**−**6** have been tested extensively in humans[1,2,4] and are described here for comparison purposes. Their pA_2 values range from 8.6 (acyline) to 9.3 (Nal-Glu and ganirelix). Cetrorelix, azaline B, and acyline are long acting by our definition, ganirelix has an intermediate duration of action, and Nal-Glu and abarelix have a short and very short duration of action, respectively. The ED_{50} of these analogues to release histamine was calculated from data shown in Table 1 and varies from 0.5 μg/mL for Nal-Glu, 1.3 μg/mL for cetrorelix, 11 μg/mL for ganirelix, 19 μg/mL for azaline B, to 66 μg/mL for acyline and 170 μg/mL for FE200486. In that assay, GnRH itself has an ED_{50} greater than 100 μg/mL (17% and 51% release at 100 and 300 μg/mL, repsectively), thus suggesting this concentration of 100 μg/mL as a lower acceptable limit for safety.

Our search for favorable substitutions concentrated on residue 5 of acyline and was first limited to the introduction of a carbamoyl moiety on the methylated amino function of Nα'-Me-aminoglycine (**7**), on the amino function of Dap (**8**) and Dab (**9**), and to the introduction of the substituted carbamoyl moieties [EtCbm (**10**) and MeOPhCbm (**11**)] on the amino function of Dap. All of these substitutions in acyline resulted in very short (**7**) or short (**8**−**11**) acting analogues. Similarly, acylation of the β-amino function of [Dap^5]-acyline with L-dihydroorotic acid, which offers multiple options for hydrogen bonding (Figure 2), yielded the very short acting analogue **12**.

On the basis of the observation that azaline B congeners are longer acting than the corresponding azaline analogues (azaline B has a 4-amino-phenylalanine at positions 5 and 6 as compared to lysines at positions 5 and 6 for azaline analogues[8]), we introduced several amido-, ureido-, and cyclic ureido-containing substitutions (see Figure 2A for structures) on the amino-function of 4-aminophenylalanine (Aph) at position 5. Of the analogues that were tested in this series (**13**−**20**), one was long acting (**17**), four had intermediate durations of action (**13**, **14**, **16**, and **18**), one was short acting (**15**), and one was very short acting (**19**). All had high affinity in a reporter gene assay, suggesting that duration of action is strictly dependent on unique properties of distribution from the sc site of injection, binding to plasma proteins, plasma clearance, and/or enzymatic stability. The very long duration of action obtained for **20** was particularly rewarding as it suggested that more efficient analogues than acyline could be designed. Efficiency is a measure of degree of inhibition times the duration of the inhibition for a given dose of an antagonist. Because all analogues (except for those that are very short acting) inhibit LH secretion maximally to about the same level, the most efficient analogues are those with the longest duration of action. Although **20** was more soluble than acyline in aqueous buffers, we synthesized the corresponding [N$^{\alpha}$-methyl-Aph^5(Hor)] analogue **21** with the expectation that it would be even more soluble in aqueous solution while wishing that it retained a very long duration of action, which it did not. These results strongly suggest that the ability of GnRH antagonists to form β sheets can be modulated and ultimately optimized. The fact that **22** is not as long acting as **20** as the result of a change in chirality of the hydroorotyl substituent suggests important interactions of this class of peptides at a locus or loci other than the receptor since both analogues have the same affinity within statistical error. Another argument in favor of yet to be discovered stereochemically dependent interactions between GnRH antagonists and components of the subcutaneous milieu or between antagonist molecules themselves derives from the observation that **23** with an additional methylene group is also shorter acting than **20** although it should be noted that there are great differences between the electronic and steric properties of a benzylamide (as in **23**) and an anilinamide (as in **20**).

With the identification of the favorable [Aph(Hor)] substitution at position 5, we investigated the effect of that substitution at position 6. Whereas [DAph^6(Hor)]-acyline (**24**) is shorter acting than acyline (**6**) and therefore than [Aph^5(Hor)]acyline (**20**), [DAph^6(Cbm)]-acyline (**25**) is as long acting as acyline. This suggests that, in addition to a different chirality of the aminophenylalanine residues at positions 5 (L) and 6 (D), quite different SAR apply to the choice of optimal substitutions. The introduction of [DAmf^6(Hor)], as in **26**, also resulted in some loss of efficiency despite high affinity for the GnRH receptor.

In the next series of analogues (**27**−**29**) we mimicked acyline in that the acylating groups of the two Aph at positions 5 and 6 were identical (Cbm, MeCbm, and EtCbm, respectively). It is noteworthy that duration of action decreased as the substitutions became more hydrophobic and bulky: **27** is long acting, **28** has an intermediate duration of action, while **29** is short acting. This observation may suggest the existence of a limited inverse correlation between duration of action and the increasing lipophilicity of the substituted urea from hydrogen to methyl and ethyl or different susceptibilities to degradation or elimination. It is unlikely that the steric hindrance brought about by the two methyl

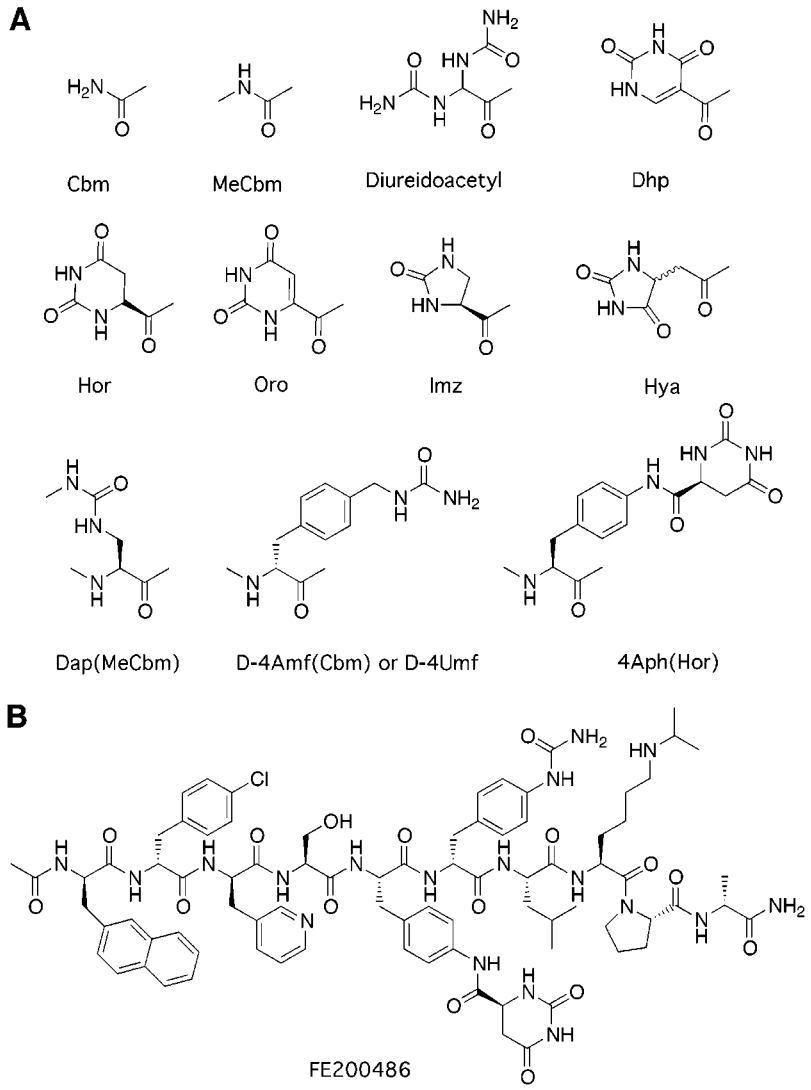

Figure 2. (A) Structures of substitutions of the 4-amino function in 4-aminophenylalanine and 4-aminomethylphenylalanine at positions 5 and 6. (B) Structure of FE200486 (**31**).

and ethyl groups in **28** and **29**, respectively, is the major factor influencing duration of action since the L-hydroorotyl substitutions which are bulkier than the two ethyl groups in **29** yield **30**, which is long acting.

We have shown that **20** was very long acting and that this property resulted from the substitution at position 5 of Aph(Ac) in acyline by Aph(Hor) since a number of other acylations at Aph^5 yielded analogues that were not as long acting. Substitutions at position 6, such as those introduced in **24**−**26**, also seemed to modulate the duration of action of these analogues, although not favorably since **24** and **26** are shorter acting than acyline. We have shown that substitution of (Hor) in **20** by (DHor) in **22** resulted in shortened duration of action from very long to long. The possibility of an interaction between the side chains of residues 5 and 6 and any substitution at position 6 other than Aph(Ac) remained that would have a favorable or deleterious effect on duration of action. To systematically test this possibility, the effect of homologation and carbamoylation at the 6 position, either separately or in combination, was investigated in both the Aph^5(Hor) (**31**, **33**, **35**) and the Aph^5(DHor) (**32**, **34**, **36**) series. Only **31** (see Figure 2B for structure) and **35** were found to be very long acting, suggesting that the Aph(Hor) substitution is definitely more advantageous than the Aph(DHor) and that the $DAph^6$ and $DAmf^6$ substitutions are compatible when carbamoylated (as in **31** and **35**, respectively) but not when acetylated (as in **33**). Additionally, methylation of the carbamoyl group at position 6 in **35** to yield **37** was also compatible with very long duration of action.

We recognized that past SAR studies in the GnRH agonist series suggested that most D-amino acids at position 6 would influence potency in vivo favorably to about the same extent,[23−25] and that in the antagonists series the nature of this D-amino acid substitution may have a significant effect on duration of action.[11,26] It was therefore important to validate these early observations in conjunction with the very favorable Aph(Hor) substitution at position 5. Analogues **38** ($DCit^6$), **39** ($DPal^6$), **40** ($DNal^6$), and **41** [$DAph^6$(DHor)] illustrate such study and confirm a certain unpredictability of the compatibility of a particular substitution at position 6 with respect to substitutions at position 5.

Whereas substitutions such as [Aph^5(Hor)-$DAph^6$(Ac)] in **20**, [Aph^5(Hor)-$DAph^6$(Cbm)] in **32**, [Aph^5(Hor)-$DPal^6$] in **39**, and [Aph^5(Hor)-$DAph^6$(DHor)] in **41** yield analogues that are all very long acting, very similar substitutions such as [Aph^5(Hor)-$DAph^6$(Hor)] in **30** and

Table 2. Solubility Data and Propensity To Gel for Selected GnRH Antagonists

	FE200486 in water	**35·Ac**[a] in water	abarelix in water	cetrorelix in water	FE200486 in 5% mannitol	**35·Ac**[a] in 5% mannitol	abarelix in 5% mannitol	cetrorelix in 5% mannitol	azaline B in 5% mannitol
highest concn attempted (mg/mL)	50	50	50	50	50	50	50	50	50
highest concn attained (mg/mL)	50	50	50	30	50	50	50	30	50
pH	5.0	5.2	5.0	4.1	4.9	5.2	4.8	4.8	4.8
initial appearance	clear, slightly viscous	clear, free-flowing	clear, free-flowing	clear, very viscous	clear, free-flowing	clear, free-flowing	clear, free-flowing	opalescent, viscous	opalescent, free-flowing
gel formation after 24 h ambient T.	none, signs of turbidity	no gel	complete	clear, more viscous	none, signs of turbidity	no gel	complete	almost complete	complete
gel formation after 1 week ambient T.	none, solution turbid with some sediment	no gel	complete	complete	none, solution turbid with some sediment	no gel	complete	complete	complete

[a] FE200486 and **35·Ac** are the acetate salt forms of **31** and **35**, respectively.

[Aph^5(Hor)-DCit^6] in **38** yield analogues that have shorter duration of action (long acting), while the [Aph^5-(Hor)-DNal^6] substitution in **40** is clearly unfavorable in the castrated male rat assay.

The observation made earlier that compatibility of different acylating agents of the Aph^5 and DAph^6 and more particularly of Hor is important was vindicated in that the [Aph^5(Hor)-DAph^6(DHor)] substitution in **41** is more favorable than the corresponding [Aph^5(Hor)-DAph^6(Hor)] substitution in **30**. The structural basis for such a pharmacological effect will elude us for as long as a good model of the environment experienced by these molecules after sc administration other than their receptor, for which preliminary information is available,[27−29] is not better defined.

In an earlier publication, it was shown that the [Aph^5-(P)-DAph^6(Q)] substitutions at positions 5 and 6 were clearly advantageous over the corresponding [Lys^5(P)-DLys^6(Q)] substitutions used by others in terms of duration of action.[8] This was further confirmed here with **42** [Lys^5(Hor)-DLys^6(Hor)], which is very short acting as compared to **31** [Aph^5(Hor)-DAph^6(Hor)], which is long acting.

One further attempt at identifying favorable substitutions led us to modify the C-terminus of some of our longest acting analogues with the introduction of an ethylamide of proline at position 9 (**43** and **44**) or reduction of the C-terminus alanine or D-alanine to the corresponding alcohol. Whereas the Pro^9-NHEt substitution was not favorable in that analogues **43** and **44** are not as long acting as their parents **31** and **35**, the reduced analogues **45**−**48** retained very long duration of action in all cases but that of **48**, where the C-terminus is an L-alaninol as compared to the D-alaninol of the corresponding **47**.

We studied the ability to dissolve and to form gels upon standing in water and 5% mannitol of cetrorelix·acetate, **31**, as the acetate salt (**31·Ac**, FE200486) and **35** as the acetate salt (**35·Ac**) and compared it to that of the acetate salts of abarelix, cetrorelix, and azaline B in 5% mannitol (Table 2). Whereas this simple assay may lend itself to some subjectivity, there were some very clearly observable differences in the behavior of the different analogues. All analogues shown in Table 2 were in the acetate form and were handled similarly. Dissolution was achieved with gentle swirling using the same protocol, avoiding vortexing and the formation of foam. All peptides, with the exception of cetrorelix in 5% mannitol dissolved within minutes at a concentration of 50 mg/mL. The pH of the resulting solutions varied from 4.8 to 5.2. The initial appearance of the solutions is described in Table 2 and ranged from clear and free flowing to viscous and opalescent. Upon standing at room temperature for 24 h, however, these solutions behaved very differently in that some formed gels and other remained unchanged. By our definition, a gel had formed when the tube in which the original solution had been made could be turned upside down without a change of shape of the content of the tube. While FE200486 remained in solution, yet showed some signs of precipitation in water and 5% mannitol, solutions of **35·Ac** remained unchanged over several weeks. All other peptides had formed gels within 24 h, as shown in Table 2. We concluded from these experiments that FE200486 and **35·Ac** were more soluble than abarelix, cetrorelix, and azaline B in aqueous media.

As mentioned earlier, we had evidence that increased solubility may have its own liabilities in that the introduction of an NMe at position 5 resulting in increased solubility and the prevention of gel formation also consistently conferred on the analogues a significantly increased ability to release histamine. We further confirmed these early observations since N-methylation of **20** at position 5 yielded **21** which is considerably shorter acting than **20** and is also significantly more potent at releasing histamine. It was therefore important to demonstrate that the very long acting and water soluble analogues (**20**, **31**, **35**, **37**, **39**, **41**) did not suffer from the same limitation and did not release histamine at doses lower than those found, for example, to release histamine in GnRH superagonists used extensively in the clinic. [DTrp^6,Pro^9-NHEt]GnRH, for example, was tested in the same assay as the antagonists reported here, and the percents of histamine release at given doses were as follows: 0% (3 μg/mL), 6% (10 μg/mL), 28% (30 μg/mL), 56% (100 μg/mL), 65% (300 μg/mL).

We had found azaline B to release little histamine compared to several other GnRH antagonists. In the assay described here (Table 1), azaline B releases histamine at concentrations equal to or greater than 10 μg/mL (93% release of histamine at 30 μg/mL). Acyline is somewhat less potent than azaline B (43% release of histamine at 30 μg/mL). Other known GnRH antagonists (Nal-Glu, cetrorelix, and ganirelix) were significantly more potent than azaline B as shown here and earlier.[8] The very long acting analogues (**20**, **31**, **35**, **37**,

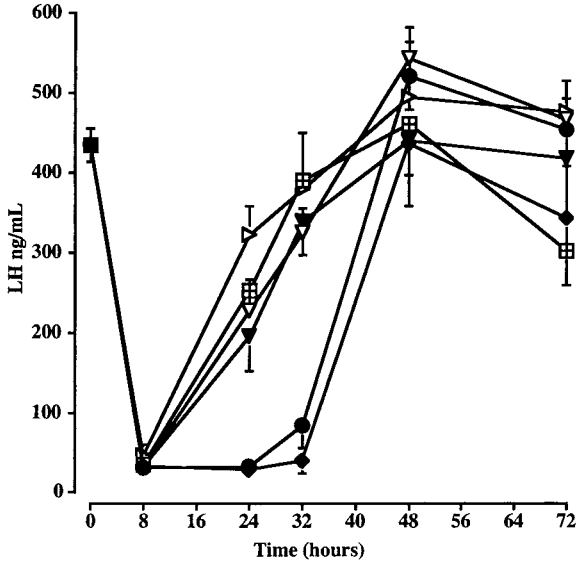

Figure 3. Inhibition of LH secretion after iv administration of analogues (**5**, **6**, **31**, **33**, **35**, **37**). Total dose was 100 μg in 100 μL of 40 mM phosphate buffer containing 0.1% and 0.6% DMSO. Blood samples were collected at the time shown on the abscissa. Azaline B (**5**) ■; acyline (**6**) ●; FE200486 (**31**) cross-hatched box; Compound (**33**) ▽; Compound (**35**) ▼; Compound (**37**) sideways triangle. Results are mean plasma LH levels (n = 5 rats) ± SEM.

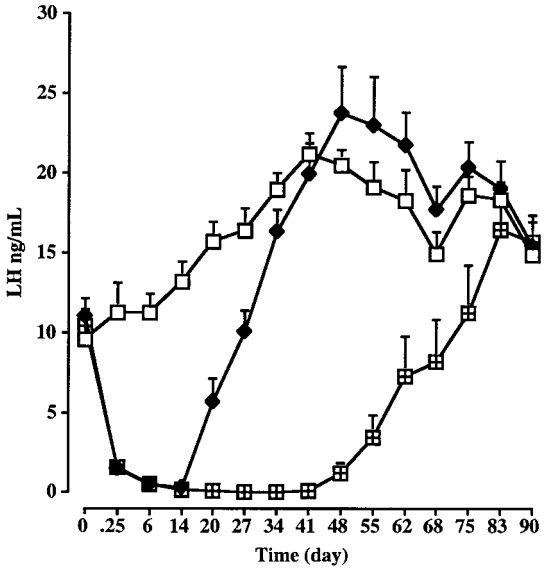

Figure 4. Effects on plasma LH levels (ng/mL) of FE200486 and azaline B injected sc at 2 mg/kg in 5% mannitol (20 μL/rat). Vehicle □; FE200486 (**31**) cross-hatched box; azaline B (**5**) ◆. Results are mean plasma LH levels (n = 6−7 rats) ± SEM.

39, **41**) distinguish themselves in that they release histamine at doses only equal to or exceeding 100 μg/mL similarly to [DTrp^6,Pro^9-NHEt]GnRH. Additionally, even at doses of 300 μg/mL, the very long acting analogues tested and the agonist [DTrp^6,Pro^9-NHEt]-GnRH or even GnRH itself did not release histamine maximally whereas **21** released histamine maximally at 100 μg/mL.

Analogues **20**, **31**, **35**, **37**, **39**, **41**, and **45**−**47** were found to be very long acting in our screening assay in the rat. Duration of action, however, is an integrated response that takes into consideration a number of factors including affinity to the receptor, chemical stability in vivo, resistance to elimination, and rate of diffusion from the site of administration or compartmentalization, among others. We attempted to identify which of these parameters may have been prevalent in conferring long duration of action. In vitro data were obtained that show no correlation between duration of action and affinity since all analogues had about the same high affinity for the GnRH receptor. We then investigated the effect of intravenous (iv) administration of several peptides (**5**, **6**, **31**, **33**, **35**, and **37**) (Figure 3). Interestingly, the time courses of LH inhibition for azaline B (**5**) and acyline (**6**) at the dose tested (100 μg/rat in 100 μL of 40 mM phosphate buffer containing 0.1% BSA and 0.6% DMSO) were longer than for the urea containing analogues **31**, **33**, **35**, and **37**, in contradiction to their duration of action after sc injection. These results strongly suggest that the very long duration of action of **31**, **35**, and **37** after sc administration as compared to long duration of action for **33**, acyline, and azaline B is the result of the peptide's bioavailability at, and distribution from, the injection site rather than one of stability in the circulation.

In other words, all analogues were not necessarily equally stable or excreted at about the same rate, and the very long duration of action of some of the analogues was most likely the result of a slow release of the peptide from the injection site. We had shown that duration of action of several GnRH antagonists, but not all, was concentration dependent in addition to dose dependent.[8] Whereas binding to plasma proteins was suggested to play an important role in the differences in pharmacokinetics of GnRH and its potent agonist Nafarelin ([DNal^6]GnRH),[30] no such evidence was found for GnRH antagonists.

To demonstrate the validity of our original hypothesis that GnRH antagonists could be made more soluble by increasing the number of H-bonding sites and at the same time have extended duration of action precluding the need of a slow release formulation to achieve gonadotropin inhibition for more than a month, we injected azaline B and FE200486 sc (2 mg/kg in 20 μL 5% mannitol) and observed complete inhibition of LH release for 14 and 41 days, respectively (Figure 4). These results with azaline B whereby a 10-fold increase in dose resulted in a 3-fold increase in duration of action (see Figure 1 for comparison) were unexpected and disappointing. This observation was later confirmed by Hutchison et al.[31] whereby they showed that increasing the concentration of azaline B beyond a certain point dramatically affected bioavailability in humans. This was explained by the tendency of azaline B to form gels beyond a certain concentration, gels that will not effectively reach the circulation at a rate that is sufficient to maintain LH inhibition. On the other hand, the same increase in dose of FE200486 resulted in a 7-fold increase in duration of action (Figures 4 and 5).

The question remained as to whether the profound and long lasting inhibition of LH seen using a single injection of FE200486 would translate into similar inhibition of testosterone in the normal rat. Data are shown in Figure 5. Seven days after administration of ganirelix and abarelix (2 mg/kg in 20 μL 5% mannitol, sc) in intact rats, plasma testosterone had almost

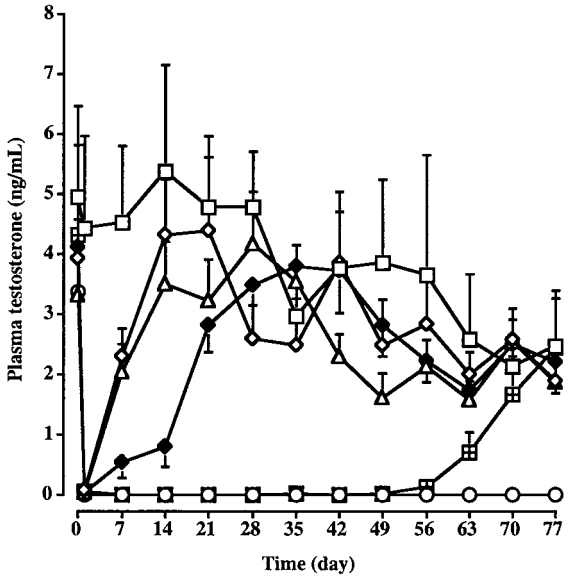

Figure 5. Effects on plasma testosterone levels in the intact rat of FE 200486, ganirelix, abarelix, and azaline B injected at 2 mg/kg s.c., in 5% mannitol, 20 μL/rat, comparison with surgical castration. Vehicle □; ganirelix (**3**) ◇; abarelix (**4**) △; castrated ○; azaline B (**5**) ◆; FE200486 (**31**) cross-hatched box. Results are mean plasma testosterone levels ($n = 8$) ± SEM.

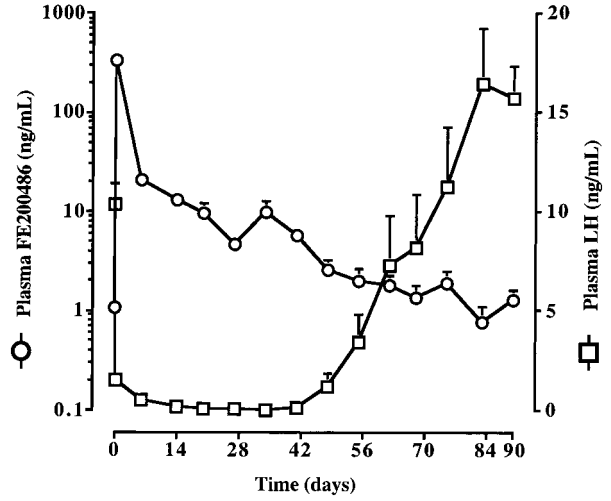

Figure 6. Relationship between pharmacokinetics and pharmacodynamics of FE200486 (2 mg/kg, 5% mannitol, 20 μL/animal). Plasma LH and FE200486 levels were measured by radioimmunoassay.

returned to baseline levels, demonstrating the need for these compounds to be formulated as slow-release preparations. Under the same conditions, azaline B, which suppressed LH in castrated male rats for 2 weeks, also decreased testosterone levels in the intact rat for 2 weeks although some rats had already escaped from castrate levels by day 7. By comparison FE200486, at the same dose (Figure 5), maintained testosterone at castrate levels for 49 days, with one animal out of eight escaping by day 56. Testosterone levels then gradually increased to return to baseline levels at day 77. Inhibition of LH correlated well with plasma levels of the analogue (Figure 6). In rats, after single acute bolus sc administration (2.0 mg/kg in 20 μL 5% mannitol), FE200486 is rapidly absorbed giving rise to plasma levels reaching a C_{max} of 330 ng/mL at 6 h (Figure 6). Thereafter, plasmatic peptide concentrations decrease gradually over time to reach about 1 ng/mL at day 90. Parallel analysis of plasmatic LH levels indicate that LH release remains completely inhibited up to day 41 and starts to increase gradually thereafter. Plasmatic levels of FE200486 are 6 and 3 ng/mL at day 41 and 48, respectively. All together the above data suggest that FE200486 administered in an aqueous buffer (5% mannitol) is able to maintain a maximal inhibition of LH release at plasmatic concentrations of 5 ng/mL or above, corresponding to molar concentrations ranging from approximately 3 to 10 nM, which is consistent with the affinity and activity of FE200486 at the GnRH receptor.

As expected, rats sacrificed on day 45 had considerably reduced prostate, seminal vesicle, and testis weights. Of all analogues tested, **31**, **35**, **37**, **39**, and **41** were the least potent at releasing histamine from rat peritoneal mast cells at the maximal dose of 300 μg/mL. FE200486 was also less potent than **1**−**6** in inducing a subcutaneous anaphylactoid response in the rat[32] (data to be reported elsewhere).

Conclusion

As the search for clinical candidates of GnRH antagonists continues, we recognized at the initiation of this study that there were two possible approaches to identifying analogues capable of inhibiting reproductive functions for 30 days or more after a single administration. The first was to identify a highly water soluble analogue with the highest possible affinity for the GnRH receptor and design a formulation that would yield a slow release of the molecule at a concentration sufficient to antagonize the effect of endogenous GnRH over the desired period. This is the approach followed by Molineaux et al. who selected abarelix (**4**) which they formulated in a proprietary injectable (abarelix-depot) and found to have great clinical promise.[12,33] An alternative possibility was to build the extended duration of action directly into the peptide, thus eliminating the need for a slow-release formulation. We found that the introduction of some urea and carbamoyl functionalities could be optimized to yield analogues with dramatically improved duration of action after subcutaneous administration but not after iv administration. The fact that the pA_2 of **31** ($pA_2 = 8.8$ for the trifluoroacetate salt or 9.1 for FE200486, the corresponding acetate salt) is essentially equal and within experimental error to that of other GnRH antagonists reported here, such as Nal-Glu ($pA_2 = 9.3$), ganirelix ($pA_2 = 9.3$), or abarelix ($pA_2 = 9.1$), suggests that the affinity of the analogues for the GnRH receptor is not responsible for the observed increase in duration of action of **31** over that of **1**−**6**. Therefore it would be important to show that the urea functionality stabilizes a favorable secondary structure(s) less amenable to forming gels than is the case for azaline B, for example, or complementarily that the urea functionality prevents the formation of β-structures shown to be responsible for gel formation.[22] We provide data (Figure 6) suggesting that such structure(s) resulting from the addition of urea functionalities favor(s) a slow diffusion from the subcutaneous sites of administration.

Altogether the chemistry of ureas offers great versatility and ease of introduction on solid supports as demonstrated here. The discovery of very long acting

GnRH antagonists, such as FE200486, suggests that the introduction of urea functions in the design of peptide analogues may be a generally applicable strategy to modulate diffusion from injection site and therefore bioavailability in peptide drug design while retaining high therapeutic ratio.

Experimental Section

Instruments. Optical rotations were measured on a Perkin-Elmer model 341 polarimeter in a 1 dm microcell at 25 °C at the concentration indicated (w/v %). Thin-layer chromatography (TLC) was performed in a solvent-vapor-saturated chamber on Merck silica gel 60 F-254 plates using the following solvent systems: (A) *n*-BuOH:AcOH:H_2O = 4:1:1; (B) ACN:AcOH:MeOH = 4:1:1; (C) CH_3Cl:MeOH:AcOH = 95:5:3. The plates were visualized by UV, I_2, and ninhydrin spray. Melting points (uncorrected) were determined in open capillaries on a Thomas-Hoover Unimelt apparatus. ^1H and ^{13}C NMR spectra (500 or 125.77 MHz) were recorded on a Bruker-500 instrument by NuMega Resonance Labs. Chemical shifts (δ) are expressed in parts per million relative to internal standard tetramethylsilane (TMS), and coupling constants (J) are given in hertz. Elemental analysis (C, H, N) was performed by NuMega Resonance Labs, and the results were within 0.4% of the theoretical values. Mass spectra were recorded on a Finnigan LCQ mass spectrometer with ESI source.

Analytical RP-HPLC was performed on a Waters 600 multi solvent delivery system with a Waters 486 tunable absorbance UV detector and a Waters 746 data module. HPLC analyses of the novel protected amino acids were carried out using a Vydac C_{18} column (0.46 × 25 cm, 5 μm particle size, 300 Å pore size) at a flow rate of 2.0 mL/min. Solvents A and B were 0.1% TFA in H_2O and 0.1% TFA in 60% ACN/40% H_2O, respectively. A linear gradient of 20% to 90% buffer B over 35 min was applied to all the novel amino acids with UV detection 0.2 AUFS at 214 nm. Retention times (t_R) are given in min. Preparative RP-HPLC was accomplished using a Vydac C_{18} preparative cartridge (5 × 30 cm, 15−20 μm particle size, 300 Å pore size) at a flow rate of 100 mL/min, on a Waters Prep LC 2000 preparative chromatograph system with a Waters 486 tunable absorbance UV detector and a Servogor 120 strip chart recorder. Buffer B was 60% ACN in aqueous buffer in all cases.

Starting Materials. Most amino acid derivatives were obtained from Bachem (Torrance, CA), including Boc-D-Ala, Boc-Arg(Tos), Boc-D-Asn, Boc-D-Nal, Boc-D-Cit, Boc-D-Cpa, Boc-D-Gln, Boc-ILys(Cbz), Boc-Leu, Boc-L- and Boc-D-Lys(Fmoc), Boc-D-Pal, Boc-Pro, Boc-Ser(Bzl), Boc-D-Trp, and Boc-Tyr(2-BrCbz). Boc-L- and Boc-D-Aph(Fmoc) were synthesized from Boc-L- and Boc-D-4-nitro-Phe obtained from Bachem (Torrance, CA) according to the published procedure.[10] Boc-L- and Boc-D-Amf(Fmoc) were synthesized from L- and D-Phe following the method of Rivier et al.[11] Boc-D/L-Agl(Me,Fmoc) was synthesized using the procedures described by Jiang et al.[34] Boc-L- and Boc-D-Dap(Fmoc) and Boc-L- and Boc-D-Dab(Fmoc) were synthesized according to the published procedure.[35] The L- and D-isomers of N^{α}-Cbz-2-imidazolidone carboxylic acid, for the introduction of L- and D-Imz moieties, were prepared according to a published procedure.[36] Both L- and D-isomers of hydroorotic acid were prepared using a published procedure.[37]

The methyl benzhydrylamine (MBHA) resin with substitutions varying from 0.5 to 0.8 mequiv/g was obtained from Bachem (Torrance, CA). All solvents were reagent grade or better. N^{α}-Methylation of **21** was achieved by direct methylation on the α-nitrogen on the resin using the protocols of Kaljuste et al.[17]

***N*$^{\alpha}$-(*tert*-Butyloxycarbonyl)-*p*-*tert*-butylureido-D-phenylalanine: [Boc-D-Aph(*t*BuCbm)-OH].** To a cold and stirred solution of N^{α}-Boc-*p*-amino-D-phenylalanine (28.0 g, 100 mmol) and DIEA (26.1 mL, 150 mmol) in dry DMF (250 mL) was added *tert*-butyl isocyanate (34.3 mL, 300 mmol). The reaction mixture was stirred at room temperature (rt) for 3 days, and then rotary evaporated at 30 °C to dryness. The residual syrup was washed with ethyl acetate (EtOAc, 2 × 100 mL) and then mixed with 500 mL of EtOAc and 300 mL of H_2O. The pH was adjusted to 3.0 with 3 N HCl, and the separated aqueous layer was extracted with EtOAc (2 × 150 mL). The combined EtOAc extracts were washed with water and brine, dried (Na_2SO_4), and concentrated in vacuo, yielding the product as white crystals (34.7 g, 91% yield): mp 200 °C (dec); $[\alpha]^{25}_D = -21.4°$ (c = 1.0, MeOH); TLC R_f 0.92 (A), 0.92 (B), 0.11 (C). HPLC analysis: t_R = 21.08 min, purity >99%; ^1H NMR (CD_3OD) δ 1.35 (s, 9H), 1.39 (s, 9H), 2.86 (d d, 1H, J = 13.9, J = 8.8), 3.08 (d d, 1H, J = 13.9, J = 5.0), 4.30 (d d, 1H, J = 8.6, J = 5.1), 7.10 (d, 2H, J = 8.3), 7.22 (d, 2H, J = 8.4); ^{13}C NMR (CD_3OD) δ 28.8, 29.8, 38.1, 51.2, 56.5, 80.6, 120.1, 130.8, 132.2, 139.9, 157.5, 157.9, 175.6; MS (ESI) m/z 379.9 $(M + H)^+$, 759.0 $(2M + H)^+$. Anal. ($C_{19}H_{29}N_3O_5$) C, H, N.

***N*$^{\alpha}$-(*tert*-Butyloxycarbonyl)-*p*-ureido-D-phenylalanine: [Boc-DUph-OH or Boc-DAph(Cbm)-OH].** A mixture of N^{α}-Boc-*p*-amino-D-phenylalanine (28.0 g, 100 mmol) and trimethylsilyl isocyanate (40.6 mL, 300 mmol) in dry DMF (100 mL) was stirred at room temperature for 3 days. After the same workup as that of Boc-DAph(*t*BuCbm)-OH, the product was obtained as white crystals (27.6 g, 85% yield): mp 157 °C (dec); $[\alpha]^{25}_D = -24.1°$ (c = 1.0, MeOH); TLC R_f 0.78 (A), 0.83 (B). HPLC analysis: t_R = 9.04 min, purity >98%; ^1H NMR (CD_3OD) δ 1.38 (s, 9H), 2.87 (d d, 1H, J = 13.9, J = 8.8), 3.09 (d d, 1H, J = 13.9, J = 5.1), 4.30 (d d, 1H, J = 8.7, J = 5.1), 7.13 (d, 2H, J = 8.4), 7.28 (d, 2H, J = 8.3); ^{13}C NMR (CD_3OD) δ 28.8, 38.1, 56.5, 80.7, 120.7, 130.8, 132.9, 139.4, 157.9, 159.7, 175.6; MS (ESI) m/z 345.9 $(M + Na)^+$, 646.9 $(2M + H)^+$, 668.7 $(2M + Na)^+$. Anal. ($C_{15}H_{21}N_3O_5$) C, H, N.

***N*$^{\alpha}$-(Fluorenylmethyloxycarbonyl)-*p*-ureido-D-phenylalanine: [Fmoc-DUph-OH or Fmoc-DAph(Cbm)-OH].** Same procedure as that of Boc-DUph-OH except for 0.60 M concentration in dry DMF and different starting material, giving the product as white solid (85% yield): mp 163 °C (dec); $[\alpha]^{25}_D = -13.2°$ (c = 1.0, MeOH); TLC R_f 0.84 (A), 0.89 (B). HPLC analysis: t_R = 21.61 min, purity >98%; ^1H NMR (DMSO-d_6) δ 2.78 (d d, 1H, J = 13.7, J = 10.6), 2.98 (d d, 1H, J = 13.8, J = 4.3), 4.11 (m, 1H), 4.19 (m, 3H), 5.78 (s, 1H), 7.11 (d, 2H, J = 8.5), 7.30 (m, 4H), 7.41 (m, 2H), 7.64 (d, 1H, J = 7.6), 7.66 (d, 1H, J = 7.6), 7.69 (d, 1H, J = 8.7), 7.88 (d, 2H, J = 7.5), 8.43 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 35.8, 46.5, 55.7, 65.6, 117.6, 120.1, 125.2, 125.3, 127.1, 127.6, 129.2, 130.3, 138.9, 140.7, 143.7, 155.9, 156.0, 173.4; MS (ESI) m/z 446.0 $(M + H)^+$, 468.2 $(M + Na)^+$, 891.0 $(2M + H)^+$, 912.9 $(2M + Na)^+$. Anal. ($C_{25}H_{23}N_3O_5 \cdot {}^1/_2H_2O$) C, H, N.

***N*$^{\alpha}$-(Fluorenylmethyloxycarbonyl)-*p*-ureido-L-phenylalanine: [Fmoc-LUph-OH or Fmoc-LAph(Cbm)-OH].** Same procedure as that of Fmoc-DUph-OH with different starting material, affording the product as a white solid (86% yield): mp 180 °C (dec); $[\alpha]^{25}_D = +12.8°$, (c = 1.0, MeOH); TLC R_f 0.84 (A), 0.89 (B). TLC R_f 0.84 (A), 0.89 (B). HPLC: t_R = 21.62 min, purity >98%; ^1H NMR (DMSO-d_6) δ 2.80 (d d, 1H, J = 13.7, J = 10.4), 3.00 (d d, 1H, J = 13.8, J = 4.4), 4.13 (m, 1H), 4.21 (m, 3H), 5.80 (s, 1H), 7.12 (d, 2H, J = 8.1), 7.31 (m, 4H), 7.41 (m, 2H), 7.64 (d, 1H, J = 7.6), 7.66 (d, 1H, J = 7.6), 7.69 (d, 1H, J = 8.7), 7.88 (d, 2H, J = 7.5), 8.45 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 35.9, 46.6, 55.8, 65.6, 117.7, 120.1, 125.2, 125.3, 127.1, 127.6, 129.3, 130.4, 139.0, 140.7, 143.8, 155.9, 156.0, 173.5; MS (ESI) m/z 445.9 $(M + H)^+$, 468.0 $(M + Na)^+$, 890.7 $(2M + H)^+$, 912.5 $(2M + Na)^+$. Anal. ($C_{25}H_{23}N_3O_5 \cdot {}^1/_2H_2O$) C, H, N.

***N*$^{\alpha}$-(*tert*-Butyloxycarbonyl)-*p*-ureidomethyl-D-phenylalanine: [Boc-DUmf-OH or Boc-DAmf(Cbm)-OH].** A mixture of N^{α}-Boc-*p*-aminomethyl-D-phenylalanine (5.89 g, 20 mmol) and potassium cyanate (4.86 g, 60 mmol) in water (200 mL) was stirred at room temperature for 3 days. After EtOAc extraction and evaporation, the product was obtained as a white foam (5.91 g, 87% yield): $[\alpha]^{25}_D = -15.3°$ (c = 1.0, MeOH); TLC R_f 0.70 (A), 0.74 (B). HPLC analysis: t_R = 9.52 min, purity >98%; ^1H NMR (DMSO-d_6) δ 1.33 (s, 9H), 2.81 (d d, 1H, J = 13.7, J = 10.2), 2.98 (d d, 1H, J = 13.7, J = 4.5), 4.06 (m, 1H), 4.14 (d, 2H, J = 5.9), 5.53 (s, 1H), 6.39 (t, 1H, J = 5.9), 7.04 (d, 1H, J = 8.2), 7.16 (AA′BB′ system, 4H), ca. 12.5 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 28.2, 39.2, 42.6, 55.3,

78.1, 126.9, 129.0, 136.3, 138.9, 155.5, 158.8, 173.7; MS (ESI) *m*/*z* 337.9 $(M + H)^+$, 360.1 $(M + Na)^+$, 675 $(2M + H)^+$. Anal. ($C_{16}H_{23}N_3O_5$) C, H, N.

***N*$^\alpha$-(*tert*-Butyloxycarbonyl)-*N*^{4}-(*tert*-butylcarbamoyl)-D-4-aminomethylphenylalanine: [Boc-DAmf(tBuCbm)-OH].** To a cold and stirred suspension of *N*$^\alpha$-Boc-*p*-aminomethyl-D-phenylalanine (35.3 g, 0.12 mol) in a mixture of THF (100 mL) and H_2O (400 mL) was added *tert*-butyl isocyanate (17.1 mL, 0.15 mol) dropwise while keeping the pH at 9.5 with 2 N Na_2CO_3 solution. The reaction mixture was stirred at room temperature for 2 h and evaporated. After acidification, EtOAc extraction, and evaporation, the desired product was obtained as a white solid (39.5 g, 84% yield): mp 140−142 °C; $[\alpha]^{25}_D = -13.8°$ (*c* = 1, MeOH); TLC R_f 0.92 (A), 0.91 (B), 0.10 (C); HPLC analysis: t_R = 19.42 min, purity >98%; ^{1}H NMR (DMSO-d_6) δ 1.23 (s, 9H), 1.33 (s, 9H), 2.80 (d d, 1H, *J* = 13.7, *J* = 10.3), 2.97 (d d, 1H, *J* = 13.7, *J* = 4.5), 4.05 (m, 1H), 4.12 (d, 2H, *J* = 5.8), 5.69 (s, 1H), 6.02 (t, 1H, *J* = 5.8), 7.06 (d, 1H, *J* = 8.2), 7.15 (AA′BB′ system, 4H), ca. 12.6 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 28.2, 29.3, 36.0, 42.3, 49.1, 55.2, 78.1, 126.9, 129.0, 136.2,139.0, 155.5, 157.3, 173.6; MS (ESI) *m*/*z* 394.2 $(M + H)^+$, 416.3 $(M + Na)^+$, 787.2 $(2M + H)^+$, 809.0 $(2M + Na)^+$. Anal. ($C_{20}H_{31}N_3O_5$) C, H, N.

***N*$^\alpha$-Boc-*N*^{4}-(L-Hydroorotyl)-L-4-aminophenylalanine: [Boc-Aph(Hor)-OH].** To a stirred solution of L-hydroorotic acid (23.4 g, 148 mmol) and *N*-hydroxysuccinimide (18.7 g, 163 mmol) in dry DMF (600 mL) was added DIC (25.0 mL, 163 mmol) with external ice−water cooling. The reaction mixture was stirred at room temperature overnight. The precipitate (diisopropylurea) was filtered off, and the filtrate was evaporated. The oily residue was washed with diethyl ether (100 mL) and dissolved in dry DMF (300 mL). To this solution was added a solution of *N*$^\alpha$-Boc-L-4-aminophenylalanine (40.0 g, 143 mmol) in dry DMF (300 mL), followed by DIEA (28.4 mL, 163 mmol). The reaction mixture was stirred at room temperature for 24 h, and the solvent was evaporated. The residue was mixed with water (750 mL), and the pH of the resulting suspension was adjusted to 9.0 with saturated sodium carbonate solution. The precipitate of remaining diisopropylurea was filtered off, and the filtrate was washed with EtOAc. The aqueous layer was acidified to pH 2.5 with 6 N HCl, and the resulting precipitate was collected by filtration. The product was obtained as a slightly yellowish solid (53.66 g, 89% yield): mp >270 °C; $[\alpha]^{25}_D = +63.0°$ (*c* = 1.0, 1% $NaHCO_3$); TLC R_f 0.66 (A), 0.81 (B); HPLC analysis: t_R = 8.75 min, purity >99%; ^{1}H NMR (DMSO-d_6) δ 1.32 (s, 9H), 2.55 (d d, 1H, *J* = 16.6, *J* = 2.5), 2.77 (d d, 1H, *J* = 13.7, *J* = 10.2), 2.95 (m, 2H), 4.04 (m, 1H), 4.15 (m, 1H), 7.06 (d, 1H, *J* = 8.3), 7.18 (d, 1H, *J* = 8.2), 7.46 (d, 1H, *J* = 8.4), 7.66 (s, 1H), 10.06 (s, 1H), 10.09 (s, 1H), 12.5 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 28.2, 33.2, 36.0, 50.4, 55.3, 78.1, 119.2, 129.5, 133.4, 136.9, 153.9, 155.5, 169.2, 169.3, 173.7; MS *m*/*z* 443.2 $(M + Na)^+$. Anal. ($C_{19}H_{24}N_4O_7 \cdot {}^1/_2H_2O$) C, H, N.

The resin-bound peptides incorporating the Fmoc protected amino functions were synthesized manually by SPPS methodology[38] with use of previously described protocols on the methyl benzhydrylamine (MBHA) resin (approximately 1 g of starting resin per peptide) using *tert*-butyloxycarbonyl groups for *N*$^\alpha$-amino protection. TFA treatment was extended to two times 15 min. Coupling time was 90−120 min followed by acetylation (excess acetic anhydride in CH_2Cl_2 for 15 min). A 2- to 3-fold excess of 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**−**6** were synthesized and purified as previously described.[8] Other analogues were derived from the fully protected [Boc-DAph/DAmf(N$^\omega$-Fmoc)-Leu-Lys(N$^\epsilon$-isopropyl,N$^\epsilon$-Cbz)-Pro-DAla]-MBHA resin obtained manually using established solid-phase peptide synthesis (SPPS) techniques[39] and the N$^\alpha$-Boc strategy with the following side chain protecting groups: Aph(Fmoc), Amf(Fmoc), Lys(N$^\epsilon$-isopropyl, N$^\epsilon$-Cbz). The individual amino acids were incorporated in a sequential manner utilizing either DIC or PyBOP 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.[40] The *tert*-butyloxycarbonyl (Boc) group was removed after each coupling cycle by treatment of the growing peptide-resin with 50% TFA in DCM in the presence of 1% *m*-cresol. This protected, resin-bound pentapeptide 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-DAph/DAmf(N$^\omega$-Fmoc)-Leu-Lys-(N$^\epsilon$-isopropyl,N$^\epsilon$-Cbz)-Pro-DAla]. Acylation of the free N$^\omega$-amino functions with a variety of isocyanates or ureido-carboxylic acids (i.e., Hor and Imz) was then carried out using 10-fold excess of isocyanates in dry DMF or 10-fold excess of carboxylic acids in the presence of DIC and HOBt in dry DMF. After removal of *N*$^\alpha$-Boc on DAph[6] or DAmf,[6] chain elongation with Boc-Agl(Me)/Dap/Dab/Aph/Amf(*N*$^\omega$-Fmoc) at position 5, and the four *N*-terminal amino acids and acetylation gave the fully protected resin-bound peptide precursor. The ω-amino function of position 5 was then freed with 20% piperidine in DMF, and reacted with isocyanates or ureido-carboxylic acids using the same protocol as described above to afford the desired functionality in the final resin-bound peptide. HF treatment (anhydrous) at 0−5 °C in the presence of anisole (10% v/v) yielded the desired crude analogue after elimination of HF under vacuum, diethyl ether wash, extraction with 0.1% TFA in 25% ACN/H_2O or 10% acetic acid, and lyophilization.

[Ac-DNal^1,DCpa^2,DPal^3,Aph(Hor)^5,DAph(Cbm)^6,ILys^8,DAla^{10}] GnRH (31). Analogue **31** was synthesized via DIC/HOBt mediated coupling reaction of L-hydroorotic acid and the partially deprotected resin-bound peptide precursor, [Ac-DNal-DCpa-DPal-Ser(Bzl)-Aph(Fmoc)-DAph(*t*BuCbm)-Leu-Lys(isopropyl,Cbz)-Pro-DAla]MBHA-resin. The precursor was assembled manually on 0.52 g of 0.54 mmol/g substituted MBHA resin. The Aph(Fmoc) was then deprotected with 20% piperidine in DMF (2 × 15 min). Coupling reaction proceeded with a mixture of L-hydroorotic acid (0.44 g, 2.8 mmol), DIC (0.36 g, 2.8 mmol), and HOBt (0.38 g, 2.8 mmol) in dry DMF (10 mL). The mixture was agitated at room temperature for 2 h, and a ninhydrin test (NT) indicated a completed reaction. The completed peptide-resin (1.0 g) was then cleaved and deprotected in anhydrous HF in the presence of anisole (90 min, 0−5 °C), washed with diethyl ether after HF removal, and extracted with 0.1% TFA in 25% ACN/H_2O. After lyophilization, the crude (0.46 g) was purified by preparative RP-HPLC in TEAP 2.3/ACN. Gradient was 38% B isocratic for 10 min followed by a 60 min linear gradient to 58% B. Desalting of TEAP-purified fractions was achieved with 0.1% TFA/ACN (45% B isocratic for 10 min followed by a 60 min linear gradient to 65% B). Yield of **31** was 113 mg (59 μmol, 21%).

Analogues **7**−**26** and **32**−**41** were obtained by use of this general procedure in comparable yields.

[Ac-DNal^1,DCpa^2,DPal^3,Aph(Cbm)^5,DAph(Cbm)^6,-ILys^8,DAla^{10}] GnRH (27). One of the syntheses of analogue **27** involved reaction of *tert*-butyl isocyanate and the partially deprotected resin-bound peptide precursor, [Ac-DNal-DCpa-DPal-Ser(Bzl)-Aph(Fmoc)-DAph(Fmoc)-Leu-Lys(isopropyl,Cbz)-Pro-DAla]MBHA-resin. The precursor was assembled manually on 0.26 g of 0.54 mmol/g substituted MBHA resin. The Aph-(Fmoc) and DAph(Fmoc) were then deprotected with 20% piperidine in DMF (2 × 15 min). Carbamoylation proceeded with *tert*-butyl isocyanate (0.28 g, 2.8 mmol) in dry DMF (5 mL). The mixture was agitated at room temperature for 14 h, and NT indicated a completed reaction. The completed peptide-resin (0.79 g) was then cleaved and deprotected in anhydrous HF in the presence of anisole (90 min, 0−5 °C), washed with diethyl ether after HF removal, and extracted with 0.1% TFA in 25% ACN/H_2O. After lyophilization, the crude peptide (0.28 g) was purified by preparative RP-HPLC in TEAP 2.3/ACN.

Gradient was 42% B isocratic for 10 min followed by a 60 min linear gradient to 62% B. Desalting of TEAP-purified fractions was achieved with 0.1% TFA/ACN (35% B isocratic for 10 min followed by a 60 min linear gradient to 75% B). Yield of **27** was 54 mg (30 μmol, 21%).

Analogues **28**−**30** and **42** were obtained as TFA salts in comparable yields using this procedure.

[Ac-D-Nal[1],D-Cpa[2],D-Pal[3],Aph(Hor)[5],D-Aph(Cbm)[6],-ILys[8],D-Ala-ol[10]] GnRH (47). Condensation of the (1−9)-peptide fragment Ac-D-Nal-D-Cpa-D-Pal-Ser-Aph(Hor)-D-Aph-(Cbm)-Leu-Lys(isopropyl)-Pro-OH with D-alaninol yielded **47**. The fragment was assembled manually on 2.0 g of 0.60 mmol/g substituted Boc-Pro-Merrifield resin that was prepared in-house using the classical method. The completed peptide-resin (3.8 g) was then cleaved and deprotected in anhydrous HF in the presence of anisole (90 min, 0−5 °C), washed with diethyl ether after HF removal, and extracted with 0.1% TFA in 25% ACN/H_2O. After lyophilization, the crude (1.6 g) was purified by preparative RP-HPLC in 0.1% TFA buffer system to yield 887 mg (0.48 mmol, 40%) of the (1−9)-peptide fragment.

The coupling reaction of D-alaninol (as a reagent and a base) with the fragment was mediated with PyBOP in dry DMF. PyBOP (0.27 g, 0.52 mmol) was added to a solution of the above fragment (203 mg, 0.11 mmol) and D-alaninol (0.20 g, 2.6 mmol) in 4 mL of dry DMF, and the mixture was stirred at room temperature for 30 min. Analytical HPLC analysis in a TEAP 6.5 buffer system showed a clean and complete reaction. The mixture was diluted with 2% acetic acid and then directly loaded into a preparative C_{18} cartridge for purification using TEAP 6.5. Gradient was 45% B isocratic for 10 min followed by a 60 min linear gradient to 65% B. Desalting of TEAP-purified fractions was achieved with 0.1% TFA/ACN (35% B isocratic for 10 min followed by a 40 min linear gradient to 95% B). Yield of **47** was 112 mg (59 μmol, 53%).

Analogues **43**−**46** and **48** were obtained as TFA salts in comparable yields using this procedure and the appropriate protected nonapeptides. Condensation of Ac-D-Nal-D-Cpa-D-Pal-Ser-Aph(Hor)-D-Aph(Cbm)-Leu-Lys(isopropyl)-Pro-OH with ethylamine hydrochloride was mediated by PyBOP to yield **43**. Condensation of Ac-D-Nal-D-Cpa-D-Pal-Ser-Aph(Hor)-D-Amf(Cbm)-Leu-Lys(isopropyl)-Pro-OH with ethylamine hydrochloride was mediated by isobutyl chloroformate and DIEA to yield **44**.

Peptide Purification. Peptides were subsequently purified by preparative 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 the cartridge described earlier. The peptide was eluted using a flow rate of 100 mL/min with a mixture of A (TEAP 2.25) and B (60% ACN, 40% A) and an appropriate gradient (90 min) such that retention time was ca. 45 min. The collected fractions were screened by analytical RP-HPLC under isocratic conditions, 0.1% TFA/H_2O at a flow rate of 2.0 mL/min (Vydac C_{18} column, 5 μm, 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/ACN gradient: 20% B (10 min) followed by a 20 min gradient to 90% B (where B contains 60 or 80% ACN depending on the hydrophobicity of the analogue). See references 15 and 41 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[15] allowed easy separation of the methylated analogue.

Peptide Characterization (Table 1). Purity of the peptides was assessed using RP-HPLC and CZE under conditions reported in the legend of Table 1. Composition of the analogues was confirmed by mass spectrometric analysis.

Biological Testing. Castrated Male Rat Assays. Male Sprague−Dawley rats (180−200 g at beginning of experiments, $n = 5$−8) were castrated under ether anesthesia 10 days prior to the start of the experiment. The peptides were either dissolved in 40 mM phosphate buffer containing 0.1% and 0.6% DMSO (Figures 1 and 3) or in 5% mannitol (Figures 4−6) and injected either sc (Figures 1, 4, 5, and 6) or iv in rats bearing an indwelling catheter in the jugular vein (Figure 3). Blood was sampled from the tail tip (300 μL) at the given times. Plasma LH was determined by RIA using reagents provided by the National Pituitary and Hormone Distribution Program of the NIDDK (Bethesda, MD) with the exception of the second antiserum. NIDDK anti-rat LH S11 serum was used. Values reported in Figures 1 and 3 are expressed in terms of the RP-1 reference standard and values reported in Figure 4 in terms of the RP-3 reference standard. For each experiment, all plasma samples (vehicle control and tested peptides) were measured in the same RIA. Plasma testosterone levels were determined by radioimmunoassay using kits purchased from Diagnostic Systems Laboratories (Webster, TX).

Cell Culture. Human embryonic kidney cells (HEK293 cells), genetically modified to express a cloned human GnRH receptor (Larry Jameson, Northwestern University, IL), and a luciferase reporter gene under the control of LH α subunit promoter[42] were cultured in phenol red free DMEM containing 10% (v/v) FBS, G418 (0.4 mg/mL), penicillin/streptomycin solution (100 units penicillin and 100 μg streptomycin per mL medium), and L-glutamine (2 mM). The cells harvested from one confluent 75 cm^2 flask were resuspended in 33 mL of DMEM, and 100 μL of the cell suspension was added to each well of three white 96-well culture plates (supplied by Canberra Packard). The cells were incubated at 37 °C under 5% CO_2 for a further 24 h before assay.

IC_{50} Determination Using the Reporter Gene Assay. Each compound was assayed in duplicate at six different concentrations. Thus, compounds in 1% DMSO (5 μL), or 1% DMSO (5 μL) alone (GnRH controls and blanks), were added to the HEK293 cells followed by gentle mixing and incubation for an additional 10 min at 37 °C under 5% CO_2. Following this, GnRH (5 μL) was added to a final concentration of 3 nM. At the same time, blanks and controls (3 nM GnRH final concentration) were established, and a GnRH standard curve was set up in duplicate ranging from 30 nM to 0.03 nM final concentrations. Following a further gentle mixing, the plates were incubated for 5 h at 37 °C under 5% CO_2, after which 100 μL of LucLite was added to each well. The plates were sealed with Packard Topseal film and gently mixed for a final time before luminescence as counts per second (cps) was measured in a Packard TopCount after a 10 min preincubation at room temperature in the dark.

To derive the IC_{50}, the test compound cps values (minus blank cps) were expressed as a percentage of the control cps values (minus blank cps). The percentage values were plotted against the log of the concentration used, and a curve fitted to the data. An IC_{50} value was derived by nonlinear regression to a four-parameter logistic equation [sigmoidal dose−response (variable slope)], using the GraphPad Prism (version 2.01) curve fitting software package.

pA_2 Determination Using the Reporter Gene Assay. To generate the data required for a pA_2 calculation, a seven-concentration GnRH standard curve was produced in duplicate (10 μM to 10 pM final concentration) in the absence or presence of four to five increasing concentrations of test compound. Thus, the diluted compound in 1% DMSO (5 μL) was added to the HEK293 cells in the 96-well culture plates. For the GnRH standard curve in the absence of compound, 1% DMSO (5 μL) was added. After gentle mixing, the cells were incubated for 10 min at 37 °C under 5% CO_2 before adding GnRH in 1% DMSO (5 μL) at the required concentrations. The plates were then processed exactly as described above.

An EC_{50} value was derived for GnRH without compound and with each concentration of compound, by nonlinear regression to a four-parameter logistic equation [sigmoidal dose−response (variable slope), using the GraphPad Prism curve fitting software package]. The EC_{50} figures were then divided by the EC_{50} for GnRH alone. The log of the EC_{50} − 1 (log dr − 1 (log [X])) was then plotted against the log concentration of compound (log [Y]). A linear regression analysis was then carried out with the pA_2 value being that at the *X*-intercept.

Histamine Release Assay. The assay for histamine release by rat mast cells has been reported previously[19] and was performed at CEREP (Celle L'Evescault, France). Briefly, mast cells were isolated from the abdominal cavity of Sprague−Dawley rats and suspended in heparinized BSSA (balanced salt solution with bovine albumin), pH 7.2, and purified on a Percoll gradient. Mast cells were incubated at 37 °C for 2 min

with the compounds or their vehicles and centrifuged. The amount of histamine liberated in the supernatants was measured using a spectrofluorometric assay after derivatization with *o*-phthalaldehyde.[19] The mast cell pellets were lysed, and the amount of intracellular histamine was measured. The histamine releasing activity of the compound was calculated as the percentage of total histamine (released + retained in mast cells) released into the supernatant after correction for spontaneously released histamine, measured as released histamine in assays with the vehicle alone.

Abbreviations. IUPAC rules are used for nomenclature except for the following: Ac = acetyl; ACN = acetonitrile; Agl (Me) = $N^{\alpha'}$-methyl-aminoglycine; Amf = 4-aminomethylphenylalanine; Aph = 4-aminophenylalanine; Aph(Atz) = 4-(*N*-5′-(3′-amino-1*H*-1′,2′,4′-triazolyl)phenylalanine; Atz = 5′-(3′-amino-1*H*-1′,2′,4′-triazolyl); Cbm = carbamoyl; Cbz = carbobenzoxy; Cit = citrulline; Cpa = 4-chlorophenylalanine; Dab = α,γ-diaminobutyric acid; Dap = α,β-diaminopropionic acid; DCM = dichloromethane; DGlu(AA) = 4-(*p*-methoxybenzoyl)-D-2-aminobutyric acid; Dhp = 2,4-dihydroxy-pyrimidine-5-carbonyl or uracil-5-carbonyl; DIC = *N*,*N*′-diisopropylcarbodiimide; diCbm = dicarbamoyl; DIEA = *N*,*N*′-diisopropylethylamine; DMF = dimethylformamide; EtCbm = ethylcarbamoyl; GnRH = gonadotropin-releasing hormone; Har(Et_2) = N^G,$N^{G'}$-diethyl-homoarginine; HF = hydrofluoric acid; Fpa = 4-fluorophenylalanine; HOBt = 1-hydroxybenzotriazole; Hor = L-hydroorotyl; Hya = hydantoin-5-acetyl; ILys = N^{ϵ}-Isopropyl lysine; Imz = L-2-imidazolidone-4-carbonyl; LH = luteinizing hormone; MeCbm = methylcarbamoyl; MeOPheCbm = 4-methoxyphenylcarbamoyl; Nal = 3-(2-naphthyl)-alanine; Oro = orotyl; Pal = 3-(3-pyridyl)-alanine; PyBOP = benzotriazole-1-yloxy-tris-pyrrolidino-phosphonium hexafluorophosphate; RGA = reporter gene assay; sc = subcutaneous; TFA = trifluoroacetic acid; TolylCbm = *p*-tolylcarbamoyl; Umf = 4-ureidomethylphenylalanine; Uph = 4-ureido-phenylalanine.

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