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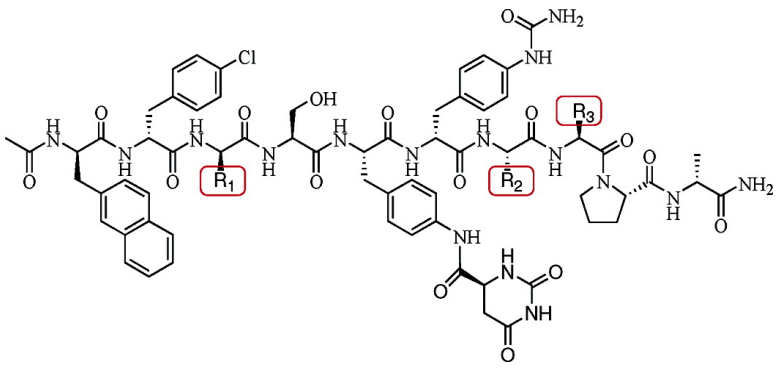

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Iterative Approach to the Discovery of Novel Degarelix Analogues: Substitutions at Positions 3, 7, and 8. Part II

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Degarelix (FE200486, Ac-D-2Nal[1]-D-4Cpa[2]-D-3Pal[3]-Ser[4]-4Aph(L-Hor)[5]-D-4Aph(Cbm)[6]-Leu[7]-ILys[8]-Pro[9]-D-Ala[10]-NH_2) is a potent and very long acting antagonist of gonadotropin-releasing hormone (GnRH) after subcutaneous administration in mammals including humans. Analogues of degarelix were synthesized, characterized, and screened for the antagonism of GnRH-induced response in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor. The duration of action was also determined in the castrated male rat assay to measure the extent (efficacy and duration of action) of inhibition of luteinizing hormone (LH) release. Structurally, this series of analogues has novel substitutions at positions 3, 7, and 8 and N^{α}-methylation at positions 6, 7, and 8 in the structure of degarelix. These substitutions were designed to probe the spatial limitations of the receptor's cavity and to map the steric and ionic boundaries. Some functional groups were introduced that were hypothesized to influence the phamacokinetic properties of the analogues such as bioavailability, solubility, intra- or intermolecular hydrogen bond forming capacity, and ability to bind carrier proteins. Substitutions at positions 3 ([N^{β}-(2-pyridyl-methyl)D-Dap[3]]degarelix, IC_{50} = 2.71 nM) (**5**), 7 ([Pra[7]]-degarelix, IC_{50} = 2.11 nM) (**16**), and 8 ([N^{δ}-(IGly)Orn[8]]degarelix, IC_{50} = 1.38 nM) (**20**) and *N*-methylation ([N^{α}-methyl-Leu[7]]degarelix, IC_{50} = 1.47 nM) (**32**) yielded analogues that were equipotent to degarelix (**2**) in vitro (IC_{50} = 1.64 nM) but shorter acting in vivo. Out of the 33 novel analogues tested for the duration of action in this series, two analogues ([N^{ϵ}-cyclohexyl-Lys[8]]degarelix, IC_{50} = 1.50 nM) (**23**) and ([N^{β}-(IβAla)Dap[8]]degarelix, IC_{50} = 1.98 nM) (**26**) had antagonist potencies and duration of action similar to that of azaline B {inhibited LH (>80%) release for >72 h after sc injection to castrated male rats at a standard dose of 50 μg/rat in 5% mannitol}. Under similar conditions analogues ([N^{γ}-(IGly)Dab[8]]degarelix, IC_{50} = 1.56 nM) (**21**) and ([IOrn[8]]degarelix, IC_{50} = 1.72 nM) (**18**) had a longer duration of action {inhibited LH (>96 h) release} than azaline B; however they were shorter acting than degarelix. Hydrophilicity of these analogues, a potential measure of their ability to be formulated for sustained release, was determined using RP-HPLC at neutral pH yielding analogues with shorter as well as longer retention times. No correlation was found between retention times and antagonist potency or duration of action.

Introduction

Gonadotropin-releasing hormone (GnRH) antagonists are used in the protocols for ovulation induction and are recognized as potential drugs for the management of sex steroid-dependent pathophysiologies, such as hormone-responsive prostate cancers and, in females, the management or treatment of breast and gynecological cancers, endometriosis, precocious puberty, uterine myoma, ovarian hyperandrogenism, and premenstrual syndrome.[1−4]

Most of these disorders can be treated with long-acting preparations of the superagonists, which desensitize the gonadotrophs after approximately two weeks of treatment. An optimized 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.

The present study of the effect of substitutions at position 3 of degarelix is an extension of an earlier study,[5] which demonstrated that the nonaromatic substitution of D3Pal by D-glutamine in Ac-D-2Nal[1]-D-4Cpa[2]-D-3Pal[3]-Ser[4]-4Aph(L-Hor)[5]-D-4Aph(Ac)[6]-Leu[7]-ILys[8]-Pro[9]-D-Ala[10]-NH_2 was compatible with high potency as well as very long duration of action (i.e., >80% inhibition of LH in the castrated male rat after sc administration of 50 μg in 5% mannitol for >96 h), whereas the equivalent D-asparagine-containing analogue was comparatively shorter acting. This suggested that very minor modifications at position 3 might possibly have an unexpected impact on duration of action with minimal effect on pA_2 (the x intercept in a Schild analysis of receptor antagonism, the concentration of antagonist in log terms, which right-shifts agonist dose response curves 2-fold).

Substitutions at position 7 explore the effect of the

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introduction of functional groups such as a primary amide (in asparagine), imidazole (in histidine), triple bonds (in propargylglycine), phenol (in tyrosine), and sulfhydryl (in cysteine) on the antagonist potency and the duration of action in inhibition of luteinizing hormone (LH) release. Early studies[6] suggested that a hydrophobic moiety would be preferable, yet the parent analogues were structurally quite different from degarelix, and the question remained whether side chains prone to hydrogen bonding could be tolerated in degarelix for intramolecular stabilization.

Additionally, we investigated the effect of novel substitutions at position 8 of degarelix. It is well documented that substitution of arginine by isopropyllysine at position 8 in GnRH antagonists yields potent analogues in antiovulatory and GnRH receptor assays with low potency to release histamine.[7] It was therefore hypothesized that other closely related structures to that of ILys might be even more favorable. Most substitutions are based on acylation of the side chains of dibasic residues of α,*β*-diaminopropionic acid (Dap) and α,*γ*-diaminobutyric acid (Dab), as well as ornithine, reminiscent to our work on betidamino acids (acylated α,α-diaminoacetic acids or amino-glycines).[8]

Finally, the key residues encompassing a *β*-turn[9] at positions 6, 7, and 8 of degarelix were N^{α}-methylated to confer enzymatic stability,[10] increasing solubility,[11] and to induce or disrupt sterical constraints with possible effects on activities.

Results and Discussion

Synthesis, Purification, and Chemical Characterization. Analogues were synthesized by the solid-phase peptide synthesis (SPPS) methodology on a *p*-methylbenzhydrylamine resin (MBHA-resin, *tert*-butyloxycarbonyl (Boc) strategy) using protocols previously described[12] or shown below. In most cases, analogues were obtained by the introduction of each of the side chain substituents at positions 3, 5, 6, and 8 on the partially deprotected peptido-resin obtained after the removal of the 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group with 30% piperidine in *N*-methylpyrrolidinone (NMP) (for example, after the introduction of Boc-D-Dab/D-Dap(Fmoc) at position 3, Boc-D-4Aph-(Fmoc) at position 5, Boc-4Aph(Fmoc) at position 6, and Boc-Dab/Dap/Orn/Lys(Fmoc) at position 8).

The reference compounds azaline B (**1**) and degarelix (**2**) were synthesized using published protocols.[13,14] The synthesis of all other analogues deserves further comment.

In all of the analogues, the side chain substitutions at position 5 and 6 were carried out during the elongation of the peptide chain on the resin. The carbamoylation[14] at position 6 was carried out by the reaction of *tert*-butyl isocyanate in dimethylformamide (DMF) with the free *ω*-amino function of D-*p*-aminophenylalanine (D-4Aph) on the otherwise fully protected resin-bound peptides. Coupling of L-hydroorotic acid (L-Hor) to the resin-bound deprotected *ω*-amino group of L-4Aph at position 5 was mediated by *N*,*N*′-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) in NMP.

The synthesis of analogues **3**−**10** involved the reductive alkylation of the deprotected side chain amino group of D-Dap/D-Dab at position 3 on the fully assembled peptide resin, [Ac-D-2Nal-D-4Cpa-D-Dap/Dab(Fmoc)-Ser-(Bzl)-4Aph(L-Hor)-D-4Aph(Cbm)-Leu-ILys(N^{ϵ}-Cbz)-Pro-D-Ala]MBHA-resin. A number of reductive alkylation protocols on solid phase have been reported in the literature.[15−18] Hocart et al.[15,16] have described the reductive alkylation of the side chain of D-Lys with various aldehydes and ketones in the presence of sodium cyanoborohydride ($NaCNBH_3$) at position 6 of the GnRH antagonist [Ac-D-2Nal^1-D-Phe^2-D-Phe^3-Ser^4-Tyr^5-D-Lys^6-Phe^7-Leu^8-Pro^9-D-Ala^{10}-NH_2] in an attempt to reduce the histamine-releasing activity. In our hands, the reductive alkylation of the free amino group on the resin with an aromatic aldehyde in the presence of $NaCNBH_3$ gave the mixture of mono- and dialkylated products. The formation of the dialkylated product was observed even when the reaction time was shortened to 20 min. The N^{β},N^{β}- or N^{γ},N^{γ}-dialkylated peptides **3** and **4** were prepared by exhaustive reductive alkylation (2 × 3 h) of the deprotected amino group of D-Dap and D-Dab, respectively, with an excess of 2-pyridinecarboxaldehyde and $NaCNBH_3$ in DMF on the resin (see Experimental Section).

In the synthesis of analogues **5**−**10**, we used the stepwise or indirect reductive alkylation[19] involving the preformation of the intermediate imine on the resin-bound peptide followed by reduction in a separate step. The synthesis of analogues **5**−**10** involved the reaction of an excess of aromatic aldehydes [2-pyridinecarboxaldehyde (in **5** and **6**), 2-quinolinecarboxaldehyde (in **7** and **8**), and 2-imidazolecarboxaldehyde (in **9** and **10**)] in DMF with the unprotected orthogonal amino function of amino acids (D-Dap/D-Dab) on the otherwise fully protected and assembled peptide resins resulting in aldamines. A significant reaction time (4 h) was required, and in the second step, the reduction of the aldimine was carried out directly in DMF with an excess of $NaCNBH_3$ to give the desired secondary amine. The formation of the secondary amine on the resin was qualitatively determined by the chloranil test.[20]

Most of the unusual amino acids substituted at positions 7 and 8 of degarelix were obtained commercially or synthesized as described in the literature. However, the synthesis of two new amino acids N^{α}-Boc-Lys(N^{ϵ}-cyclohexyl, N^{ϵ}-Cbz) and I*β*Ala(N^{α}-Cbz) substituted at position 8 of degarelix deserves further comment. The N^{α}-Boc-Lys(N^{ϵ}-cyclohexyl, N^{ϵ}-Cbz) was prepared via reductive alkylation of N^{α}-Boc-Lys(N^{ϵ}-Cbz) with cyclohexanone under Pd/C−H_2 conditions, followed by secondary amine protection using Cbz-Cl. The synthesis of I*β*Ala(N^{α}-Cbz) involved direct nucleophilic displacement reaction of 3-bromopropionic acid with isopropylamine to give isopropyl-*β*Ala (I*β*Ala) followed by Cbz protection (see Experimental Section).

Analogues **11**−**18** and **23** were synthesized by introducing different amino acids at position 7 or 8 during the elongation of the peptide chain and using the strategy reported for the synthesis of degarelix.[14]

The synthesis of analogues **19**−**22**, **24**−**26**, and **30** was accomplished by DIC and HOBt-mediated coupling reaction of the resin-bound deprotected orthogonal amino group at position 8 with Gly(N^{α}-isopropyl, N^{α}-Cbz) (in **19**−**22**), Gly(N^{α}-cyclohexyl, N^{α}-Cbz) (in **24** and **25**), *β*Ala(N^{α}-isopropyl, N^{α}-Cbz) (in **26**), and L-Hor (in **30**). Further elongation of the peptide chain, HF cleav-

Table 1. Physicochemical and Biological Characterization of GnRH Antagonists

		purity			MS[d] (M + H)$^+$		pIC$_{50}$[e]		
no.	compound	HPLC[a]	CZE[b]	t_R[c] (min)	calcd	obsd	avg ± SEM	IC$_{50}$[f] (nM)	duration of action[g]
1	[Ac-D-2Nal[1],D-4Cpa[2],D-3Pal[3],4Aph(Atz)[5], D-4Aph(Atz)[6],Ilys[8],D-Ala[10]] GnRH **azaline B**	99	99	27.4	1612.8	1612.7	8.9 ± 0.07	1.4	long
2	[Ac-D-2Nal[1],D-4Cpa[2],D-3Pal[3],4Aph(L-Hor)[5], D-4Aph(Cbm)[6],Ilys[8],D-Ala[10]]GnRH **degarelix**	96	98	27.9	1631.8	1631.9	8.8 ± 0.03	1.64	very long
3	[N^{β}, N^{β}-di-(2-pyridyl-methyl)D-Dap[3]]degarelix	97	99	33.4	1751.8	1751.6	8.3 ± 0.01	5.31	short
4	[N^{γ}, N^{γ}-di-(2-pyridyl-methyl)D-Dab[3]]degarelix	89	93	33.5	1765.8	1765.8	7.6 ± 0.01	22.58	short
5	[N^{β}-(2-pyridyl-methyl)D-Dap[3]]degarelix	95	98	29.5	1660.8	1660.8	8.6 ± 0.05	2.71	short
6	[N^{γ}-(2-pyridyl-methyl)D-Dab[3]]degarelix	95	97	28.8	1674.8	1674.8	8.4 ± 0.18	3.79	short
7	[N^{β}-(2-quinolyl-methyl)D-Dap[3]]degarelix	94	96	35.1	1710.8	1710.6	8.4 ± 0.07	4.25	short
8	[N^{γ}-(2-quinolyl-methyl)D-Dab[3]]degarelix	95	95	34.8	1724.8	1724.6	8.3 ± 0.02	5.52	short
9	[N^{β}-(2-imidazolyl-methyl)D-Dap[3]]degarelix	95	96	27.6	1649.8	1649.6	8.5 ± 0.06	3.50	intermediate
10	[N^{γ}-(2-imidazolyl-methyl)D-Dab[3]]degarelix	98	97	26.7	1663.8	1663.8	7.9 ± 0.03	13.47	short
11	[Asn[7]]degarelix	97	97	22.5	1632.7	1632.6	8.0 ± 0.03	9.18	short
12	[N^{γ}-methyl-Asn[7]]degarelix	97	98	23.2	1646.7	1646.8	7.6 ± 0.01	25.79	short
13	[N^{γ}, N^{γ}-dimethyl-Asn[7]]degarelix	98	98	24.2	1660.7	1660.7	7.3 ± 0.08	48.91	short
14	[His[7]]degarelix	95	99	22.7	1655.7	1655.7	8.0 ± 0.10	10.64	short
15	[Tyr[7]]degarelix	95	99	25.1	1681.7	1681.7	8.5 ± 0.01	2.85	intermediate
16	[Pra[7]]degarelix	96	97	24.9	1613.7	1613.7	8.7 ± 0.03	2.11	short
17	[Cys[7]]degarelix	97	96	25.3	1621.7	1621.7	6.7 ± 0.01	178.19	short
18	[IOrn[8]]degarelix	99	99	28.1	1617.7	1617.7	8.8 ± 0.14	1.72	long*
19	[N^{ϵ}-(IGly)Lys[8]]degarelix	99	98	30.6	1688.8	1688.7	8.7 ± 0.11	1.83	short
20	[N^{δ}-(IGly)Orn[8]]degarelix	98	99	30.1	1674.8	1674.7	8.9 ± 0.12	1.38	intermediate
21	[N^{γ}-(IGly)Dab[8]]degarelix	97	98	30.3	1660.7	1660.7	8.8 ± 0.09	1.56	long*
22	[N^{β}-(IGly)Dap[8]]degarelix	95	98	30.6	1646.7	1646.8	8.8 ± 0.02	1.54	intermediate
23	[N^{ϵ}-cyclohexyl-Lys[8]]degarelix	98	97	31.7	1671.8	1671.7	8.8 ± 0.08	1.50	long
24	[N^{γ}-(N^{α}-cyclohexyl-Gly)Dab[8]]degarelix	98	98	35.8	1700.8	1700.7	8.8 ± 0.15	1.77	short
25	[N^{β}-(N^{α}-cyclohexyl-Gly)Dap[8]]degarelix	99	97	36.0	1686.8	1686.7	8.7 ± 0.14	2.01	short
26	[N^{β}-(IβAla)Dap[8]]degarelix	96	98	28.3	1660.7	1660.7	8.7 ± 0.25	1.98	long
27	[N^{β}-(IβAla)-N^{β}-methyl-Dap[8]]degarelix	98	98	29.0	1674.8	1674.7	8.7 ± 0.19	1.97	intermediate
28	[N^{β}-(ICbm)Dap[8]]degarelix	98	97	31.7	1632.7	1632.7	8.8 ± 0.16	1.62	short
29	[N^{γ}-(ICbm)Dab[8]]degarelix	99	98	31.8	1646.7	1646.7	8.6 ± 0.06	2.24	short
30	[N^{δ}-(L-Hor)Orn[8]]degarelix	98	98	27.2	1715.7	1715.7	8.8 ± 0.16	1.77	short
31	[N^{α}-methyl-D-4Aph(Cbm)[6]]degarelix	97	98	30.2	1645.8	1645.6	8.7 ± 0.15	1.85	short
32	[N^{α}-methyl-Leu[7]]degarelix	98	99	30.3	1645.8	1645.7	8.8 ± 0.11	1.47	short
33	[N^{α}-methyl-Ilys[8]]degarelix	99	96	29.1	1645.8	1645.8	7.3 ± 0.01	52.24	short

[a] Percentage purity determined by HPLC using buffer system A (TEAP, pH 2.30) and buffer system B (60% CH_3CN/40% A) under gradient conditions (30%−80% B over 50 min) at a flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 cm × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm. [b] Percentage purity determined by capillary zone electrophoresis (CZE) using a Beckman P/ACE System 2050 controlled by an IBM Personal system/2 model 50Z; field strength of 15 kV at 30 °C; buffer, 100 mM sodium phosphate (85:15, H_2O/CH_3CN), pH 2.50, on a Agilent μSil bare fused-silica capillary (75 μm i.d. x 40 cm length). Detection at 214 nm. [c] Retention times under gradient conditions (30%−80% B over 50 min); buffer system A, TEAP, pH 7.0; buffer system B, 60% CH_3CN/40% A. [d] Mass spectra (MALDI-MS) were measured on an ABI−Voyager DESTR instrument using saturated solution of α-cyano-4-hydroxycinnamic acid in 0.3% trifluoroacetic acid and 50% acetonitrile as matrix. The calculated $[M + H]^+$ of the monoisotope is compared with the observed $[M + H]^+$ monoisotopic mass. [e] The pIC$_{50}$ is the negative log of the IC$_{50}$ in molar, as determined in the GnRH reporter gene assay. [f] IC$_{50}$ is the concentration of antagonist required to repress the GnRH induced response by 50% in the reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a GnRH-responsive stably integrated luciferase reporter gene. [g] Castrated male rat assay. Duration of action: very long = over 80% inhibition of LH release for more than 120 h; long* = over 80% inhibition of LH release at 96 h but not at 120 h; long = over 80% inhibition of LH release at 72 h but not at 96 h; intermediate = partial inhibition of LH release at 72 h; short = over 80% inhibition of LH release at 3 h but not at 72 h.

age, and purification afforded the desired analogues. The synthesis of **28** and **29** involved the reaction of isopropyl isocyanate with the unprotected orthogonal amino function of Dap (in **28**) or Dab (in **29**) at position 8 on the otherwise fully protected and assembled peptide resins.

The introduction of the N^{β}-methyl group in **27** and N^{α}-methyl group in analogues **31**−**33** was achieved during the elongation of the peptide chain on the partially assembled peptide resin using the published procedure of Kaljuste et al.[21]

The protected peptido-resins were cleaved and deprotected in anhydrous HF (1.5 h at 0−5 °C) in the presence of a scavenger (anisole). The crude peptides were purified by reversed-phase HPLC (RP-HPLC) in two steps and isolated as their trifluoroacetate (TFA) salts. The analytical techniques used for the characterization of the analogues included RP-HPLC with two different solvent systems (acidic and neutral) and capillary zone electrophoresis (CZE). With very few exceptions, all of the analogues were greater than 98% pure. Mass spectrometric analysis supported the identity of the intended structures, Table 1.

Biological Characterization. Analogues were tested in vitro for their antagonism on the GnRH receptor in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a stably integrated luciferase reporter gene.[14,22] The antagonism of the GnRH agonist-induced response by each analogue was determined at several concentrations and reported as IC$_{50}$, the concentration required to suppress the response in the reporter gene assay by 50%, Figure 1.

Since there is strong evidence showing that most of the GnRH antagonists (except for those that are very short acting) inhibit LH secretion maximally to about the same level in the highly reproducible in vivo

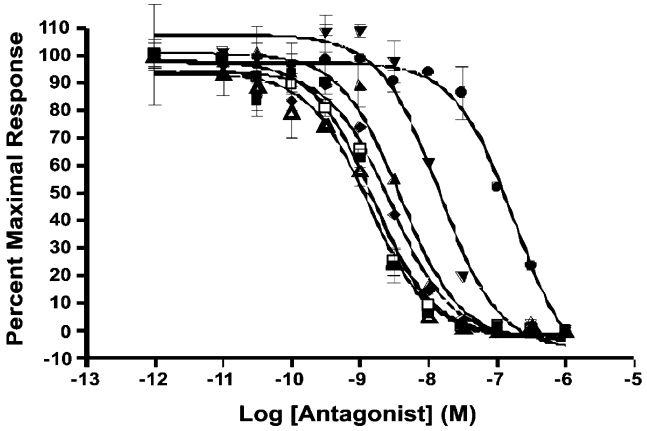

Figure 1. Antagonism of GnRH by selected antagonists in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a GnRH-responsive stably integrated luciferase reporter gene: (■) degarelix **2** , (▲) **9**, (▼) **10**, (◆) **15**, (●) **17**, (□) **19**, and (△) **26**.

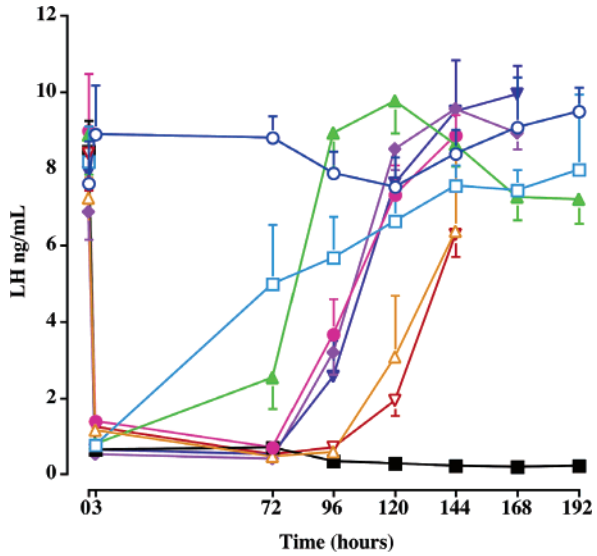

Figure 2. Inhibition of LH secretion after sc administration of analogues. Total dose was 50 μg in 50 μL of 5% mannitol containing 0.1% DMSO. Blood samples were collected at the times shown on the abscissa. Results are mean plasma LH levels (n = 5−8 rats) ± SEM. The SEM, where not appearing, are encompassed within the size of the symbols in the graph: (○) vehicle; (●) azaline B (**1**); (■) degarelix (**2**); (▲) **9**; (□) **16**; (△) **18**; (▽) **21**; (▼) **23**; (◆) **26**.

castrated male rat assay,[23,24] the most efficient analogues are those with the longest duration of action, suggesting the use of this assay for screening purposes as validated by the discovery of degarelix (FE200486).[14] In short, 10 days after castration, rats (five or more per group) were injected sc on a Friday with the excipient (50 μL of 5% mannitol), degarelix, and azaline B (50 μg/50 μL of 5% mannitol) as an internal standard, or the novel analogues (50 μg/50 μL of 5% mannitol). Blood sampling was performed predose, then 3, 72, 96, 120, 144, 168, and occasionally 192 h post-sc administration. The effects of the test compounds on the gonadotropic axis were determined by measurement of plasma LH levels by radioimmunoassay[24] (Figure 2).

By our definition, an analogue is very long acting when 50 μg in 5% mannitol in a volume of 50 μL injected sc results in an inhibition of LH (>80%) that lasts for more than 120 h. Under the same conditions, an analogue that is long* acting (see Table 1) will inhibit LH (>80%) secretion for at least 96 h, an analogue that is long acting will inhibit LH (>80%) secretion for at least 72 h, an analogue that is intermediate acting will inhibit LH secretion only partially at 72 h, and an analogue that is short acting will inhibit LH (>80%) secretion at 3 h but not at 72 h.

The overall rationale for the synthesis of the different analogues of degarelix was presented in our introduction and consists of probing the steric boundaries, ionic properties, and hydrogen bond forming capacity of the pharmacophores at positions 3, 7, and 8, known to be critical yet not necessarily optimized in the analogues reported so far.[6,25] Additionally, some functional groups were introduced that were hypothesized to influence the phamacokinetic properties of the analogues through altered solubility, stability, and ability to bind to carrier proteins. There is precedence for favorable substitutions at position 3; it includes glutamine and carbamoylation of Dap and Dab.[5] Another favorable substitution at position 3 includes the aminotriazole functionality.[13] Yet there is no available data on the effect of alkylation and dialkylation of an ω-NH_2 at this position. To mimic the functionality of the favored D-3Pal[3] found in azaline B,[26] acyline,[27] degarelix[14] and many other analogues, we carried out reductive alkylation on D-Dap/D-Dab to generate a variety of amino acids that can provide a combined modulation of steric effects, basicity, aromaticity, and hydrophobicity at position 3. The reductive alkylation with 2-pyridinecarboxaldehyde under two different experimental conditions resulted in the formation of the mono- and dialkylated products (**5**−**6** and **3**−**4**, respectively). Comparison of the antagonist potency data indicated that increasing the length of the side chain from Dap (in **3** and **5**) to Dab (in **4** and **6**) resulted in ca. 2−4-fold loss of potency (compare IC_{50}'s of **4** = 22.58 nM versus **3** = 5.31 nM and of **6** = 3.79 nM versus **5** = 2.71 nM). All four analogues are short acting. A similar set of derivatives (**7**−**10**) was significantly less potent in vitro, likely due to increased steric hindrance (**7** and **8**) and charge distribution (**9** and **10**). Interestingly, **9** (with intermediate duration of action) showed some extended duration of action over **10** (with short duration of action).

Several different amino acids have been introduced at position 7 of GnRH agonists and antagonists in the past.[6] Interestingly, leucine, found in mammalian GnRH, is not a conserved amino acid in many other species: tryptophan is found in salmon GnRH,[28] dogfish GnRH,[29] chicken II GnRH,[30] lamprey I GnRH,[31] and lamprey III GnRH,[32] phenylalanine is found in tunicate GnRH-1[33] and tunicate GnRH-3,[34] histidine is found in tunicate GnRH-2,[33] and tyrosine is found in tunicate GnRH-5 and -6.[34]

In the present study, we have introduced amino acids such as Asn (in **11**), Asn(Me) (in **12**), $Asn(Me)_2$ (in **13**), Pra (in **16**), or Cys (in **17**), along with His (in **14**) and Tyr (in **15**) found in GnRHs of other species (see above). It is noteworthy that all of these diverse substitutions with the exception of [Tyr[7]] and [Pra[7]] yielded analogues (**15** and **16**, respectively) with increased IC_{50} and significantly shorter duration of action than degarelix.

The rationale for the introduction of Asn, Asn(Me) and $Asn(Me)_2$ was to increase the potential for hydrogen bond formation. While Pra in **16** could induce an intra- or intermolecular $\pi-\pi$ interaction with aromatic groups in degarelix or the receptor, respectively, the sulfhydryl of cysteine in **17** could be used for further derivatization at position 7. While **16** was the most potent analogue in vitro of this series (IC_{50} = 2.11 nM), the antagonist potency of **17** was almost 100 times less (IC_{50} = 178.2 nM). The poor affinity of **17** was confirmatory of an earlier study (unpublished results) whereby a partial cysteine scan of degarelix had shown loss of activity when introduced at positions 1, 2, and 3. Overall, our observations are complementary to those of Millar et al.[35] and Hocart et al.[36] who observed a limited preference for hydrophobic residues at position 7.

Isopropyl-ornithine (IOrn) and isopropyl-lysine (ILys) were first introduced in GnRH antagonists at position 8 by Ljungqvist et al.[7] to yield [Ac-D-2Nal^1,D-4Cpa^2,D-3Pal^3,D-Lys(Nic)^6,IOrn/ILys^8,D-Ala^{10}]GnRH. They found that the IOrn-containing peptide was less potent than the ILys-containing one, both in an antiovulatory assay and in a histamine release assay. Whereas these two substitutions, that is, IOrn^8 in **18** and ILys^8 in degarelix (**2**) have nonsignificant effect on antagonist potency (IC_{50}'s = 1.72 and 1.64 nM, respectively), the former is not as long acting as degarelix. However, analogue **18** is longer acting than azaline B, Figure 2. It is rather intriguing that such a subtle modification (shortening the chain length by an additional methylene) in **18** and **2** may have an effect on their ability to release histamine (by analogy with Ljungqvist's observation, see above), and to influence those parameters responsible for the duration of action (present investigation). This is further documented in another series whereby the side chains of Lys, Orn, Dab, and Dap are each extended by an isopropyl-glycine residue to yield analogues **19**−**22**. The antagonistic potency for all of these analogues **19**−**22** is equal to that of degarelix; however, the duration of action is extended from short (for **19**) to intermediate (for **20**) to long* (for **21**) to revert back to intermediate (for **22**), as if an optimal chain length had been reached in **21** somewhat independently from the actual hydrophobicity of the peptide as measured by the retention time on HPLC at physiological pH (retention time, t_R = 30.6, 30.1, 30.3, and 30.6 min for **19**−**22**, respectively). In this series, **21** had a longer duration of action than azaline B and inhibited LH (>80%) release for >96 h while still being shorter acting than degarelix.

To further probe the steric environment of the pharmacophore at residue 8, we introduced the cyclohexyl substituent instead of the isopropyl group in **23**. Whereas the antagonistic potency of **23** is identical to that of degarelix, the duration of action was significantly altered. Analogue **23** has duration of action similar to that of azaline B, but it is about half as long acting as degarelix. Interestingly, **23** may be significantly more hydrophobic than degarelix (measured by the retention time on HPLC, t_R for **23** = 31.7 min versus 27.9 min for degarelix). Short duration of action may be due to several factors among which one should consider the rapid release of the peptide from the injection site exhausting all bioactive material or, alternatively, insufficient release (due to gel formation) after an initial burst resulting in the retention of a fraction of the material at the site of injection. Although degarelix levels were measured and shown to be above 1.6 ng/mL for the observed duration of gonadotropin inhibition in ovariectomized rhesus monkeys, similar studies were not carried out on shorter acting analogues thus precluding us from any conclusion.[37] Additional analogues (**24** and **25**) with the cyclohexyl substitution and otherwise analogous to **21** and **22** are significantly shorter acting. In **26** and **27**, we have two analogues differing by one methyl group on the β-nitrogen of Dap^8. Whereas both analogues are potent (IC_{50} ca. 2.0 nM each), the N^{β}-methylated **27** is shorter acting than its corresponding nonmethylated analogue **26**. Another subtle structural difference such as that found in **21** (with (IGly)-Dab^8) as compared to **26** (with (IβAla)Dab^8) with the same composition yield compounds with different and difficult to understand in vivo activities; while analogue **21** is active for >96 h, analogue **26** is shorter acting by approximately 24 h. Does this reflect yet to be identified inter- or intramolecular interactions that significantly alter one or several physicochemical parameters such as enzymatic resistance, solubility, ability to gel, or clearance rates, among others? On the other hand, two analogues with the same composition but with a quite different side chain configuration at position 8 such as **20** (N^{δ}-(IGly)Orn^8) and **27** (N^{β}-(IβAla)-N^{β}-methyl-Dap^8) both have an intermediate duration of action. NMR studies in progress will try to address such observations that may be critical for a rational design of optimized structures.

In an attempt to further increase the number of inter- or intramolecular hydrogen bonding opportunities, three additional analogues, **28** (with N^{β}-(ICbm)Dap^8) and **29** (with N^{γ}-(ICbm)Dab^8), which contain an alkylated carbamoyl function, and **30** with an L-hydroorotyl group (N^{δ}-(L-Hor)Orn^8) at position 8, were synthesized; all showed antagonist potency similar to that of degarelix in vitro and short duration of action in vivo.

In our previous paper describing the design of degarelix (FE200486), we investigated the effect of N^{α}-methylation at position 5, which was deleterious (loss of duration of action). Here we scanned positions 6, 7, and 8 with the introduction of an N^{α}-methyl group in analogues **31**−**33**. Whereas it was well documented that N^{α}-substitution at position 7 is well tolerated in both agonists and antagonists of GnRH,[38,39] N^{α}-methylation of ILys in azaline B was reported to be detrimental to biological activity in a rat antiovulatory assay.[27] As shown here, such a substitution resulted in a shortened duration of action when introduced at position 6 of degarelix and reduced antagonistic potency significantly when ILys (IC_{50} = 52.24 nM) was substituted at position 8.

Conclusions

As we probe closely related structures of degarelix, a promising drug candidate that can be administered sc to achieve long duration of action (>30 days in human),[40] we modulated the steric boundaries, ionic properties, and hydrogen bond forming capacity of the pharmacophore at positions 3, 7, and 8 of degarelix known to be critical yet not necessarily optimized in

analogues reported so far.[14,22,27,41] To generate degarelix analogues mono-N-alkylated at position 3, we developed an indirect two-step reductive alkylation protocol of the orthogonal amino group of D-Dap/D-Dab residues in otherwise protected resin-bound peptides with various aldehydes thereby generating a wide range of basic amino acids having unique aromatic, steric, and electronic characteristics. Concomitantly, we probed the effect of novel substitutions to influence solubility characteristics favorable for slow release formulations comparable to those used for GnRH agonists, which may depend primarily on the hydrophilic character of the analogue. Interestingly, some early generation analogues of degarelix[14] including azaline B[11] and acyline[27] had a significant tendency to gel upon standing in aqueous buffers such as 5% mannitol at concentrations as low as 2.5 mg/mL. Very few of the analogues (**10**, **16**, and **23**) reported here show such tendency.

The highlight of this work is the observation that very minor modifications at positions 3, 7, or 8 of degarelix aimed at modifying the steric properties, the hydrogen bonding potential, or the ability to form $\pi-\pi$ interactions resulted in all cases in loss of duration of action with retention in most cases of antagonistic potency thus paving the way for promising structural studies.

Experimental Section

Abbreviations. IUPAC rules are used for nomenclature except for the following: Ac, acetyl; ACN, acetonitrile; 4Aph, 4-aminophenylalanine; Atz, 5′-(3′-amino-1*H*-1′,2′,4′-triazolyl); Boc, *tert*-butyloxycarbonyl; 2Br−Cbz, 2-bromocarbobenzoxy; Bzl, benzyl; Cbm, carbamoyl; Cbz, carbobenzoxy; 4Cpa, 4-chlorophenylalanine; CZE, capillary zone electrophoresis; Dab, α,γ-diaminobutyric acid; Dap, α,β-diaminopropionic acid; DCM, dichloromethane; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, *N*,*N*′-diisopropylethylamine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; GnRH, gonadotropin-releasing hormone; HF, hydrofluoric acid; HOBt, 1-hydroxybenzotriazole; Hor, L-hydroorotyl; Iβ Ala, N^{α}-isopropyl-β alanine; ICbm, isopropylcarbamoyl; IGly, N^{α}-isopropylglycine; ILys, N^{ϵ}-isopropyllysine; IOrn, N^{δ}-isopropylornithine; LH, luteinizing hormone; MBHA, *p*-methylbenzhydrylamine; $NaCNBH_3$, sodium cyanoborohydride; 2Nal, 3-(2-naphthyl)-alanine; NMP, *N*-methylpyrrolidinone; 3Pal, 3-(3-pyridyl)-alanine; Pra, propargylglycine; PyBrOP, bromo-tris-pyrrolidino-phosphonium hexafluorophosphate; RP-HPLC, reversed phase high-performance liquid chromatography; RGA, reporter gene assay; sc, subcutaneous; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

Instruments. The optical rotation was measured on a Perkin-Elmer model 241 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) EtOAc/hexane = 1:1, (B) $CHCl_3$/MeOH = 9:1, (C) *n*-butanol/AcOH/H_2O = 4:1:2, (D) $CHCl_3$/MeOH/AcOH = 95:5:3. The plates were visualized by UV, I_2, and ninhydrin spray. ^1H NMR spectra were recorded on a Varian Mercury 200 MHz spectrometer with $CDCl_3$ as a solvent. Chemical shifts (δ) are expressed in parts per million relative to internal standard tetramethylsilane (TMS). Mass spectra of the amino acids were recorded on a Bruker Esquire 3000 plus instrument using nitrogen/helium gas ESI-MS.

Analytical RP-HPLC was run on a system using two Waters 501 HPLC pumps, a Schimadzu SPD-6A UV detector, Rheodyne model 7125 injector, Omniscribe (Houston Instrument) chart recorder, and a Vydac C_{18} column (0.46 cm × 25 cm, 5 μm particle size, 300 Å pore size). Preparative RP-HPLC was accomplished on a Vydac C_{18} preparative cartridge (5 cm × 30 cm, 15−20 μm particle size, 300 Å pore size) on a Waters Prep LC 4000 preparative chromatograph system with a Waters 486 tunable absorbance UV detector and Omniscribe (Houston Instrument) chart recorder. Matrix-assisted laser desorption−ionization mass spectroscopy (MALDI-MS) of the peptide analogues was measured on an ABI-Perseptive DE STR instrument. The instrument employs a nitrogen laser (337 nm) at a repetition rate of 20 Hz. The applied accelerating voltage was 20 kV. Spectra were recorded in delayed extraction mode (300 ns delay). All spectra were recorded in the positive reflector mode. Spectra were sums of 100 laser shots. Matrix α-cyano-4-hydroxycinnamic acid was prepared as saturated solutions in 0.3% trifluoroacetic acid in 50% acetonitrile. The observed monoisotopic $(M + H)^+$ values of each peptide corresponded with the calculated $(M + H)^+$ values. The capillary zone electrophoresis (CZE) analysis of peptides was performed on a Beckman P/ACE System 2050 controlled by an IBM Personal system/2 model 50Z, field strength of 15 kV at 30 °C using buffer, 100 mM sodium phosphate (85:15, H_2O/ACN), pH 2.50, on a Agilent μSil bare fused-silica capillary (75 μm i.d. x 40 cm length).

Starting Materials. Most amino acid derivatives were obtained from Reanal Finechemical Co. (Budapest, Hungary), including Boc-D-Ala, Boc-Asn(Xan), Boc-Cys(4-MeOBzl), Boc-His(Dnp), Boc-Leu, Boc-Lys(Cbz), Boc-Lys(Fmoc), Boc-Orn-(Fmoc), Boc-Pro, Boc-Ser(Bzl), and Boc-Tyr(2Br-Cbz). Boc-Pra was purchased from Neosystem Groupe SNPE (France). Fmoc-Dap(Boc) was obtained from Bachem Inc. (Torrance, CA). Boc-D-4Cpa, Boc-D-2Nal, and Boc-D-3Pal 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. Boc-L- and Boc-D-4Aph(Fmoc) were synthesized according to the published procedure.[13] Boc-L- and Boc-D-Dab-(Fmoc) and Boc-L- and Boc-D-Dap(Fmoc) were synthesized according to the published procedure.[42] The L-isomer of hydroorotic acid was prepared using a published procedure.[43] Boc-Asn(Me) and Boc-Asn$(Me)_2$ were prepared according to the procedure reported earlier.[44−46] The benzylchloroformate (Cbz-Cl), 3-bromopropionic acid, cyclohexanone, 2-imidazolecarboxaldehyde, isopropylamine, isopropyl isocyanate, 10% Pd/C, 2-pyridinecarboxaldehyde, 2-quinolinecarboxaldehyde, *tert*-butyl isocyanate, and sodium cyanoborohydride were purchased from Aldrich Chemical Co. (Milwaukee, WI). N^{α}-cyclohexyl-Gly(N^{α}-Cbz),[47] IGly(N^{α}-Cbz),[48] Boc-Ilys(N^{ϵ}-Cbz)[6] and Boc-IOrn(N^{δ}-Cbz)[6] were synthesized according to the published procedure. The methylbenzhydrylamine (MBHA) resin[49] with substitution 0.33 mequiv/g was obtained according to the published procedure of Rivier et al. using *p*-toluoyl chloride in lieu of benzoyl chloride in the Friedel and Crafts step.[50] All solvents were reagent grade or better.

Synthesis of Novel Amino Acids. L-N^{α}-Boc-Lys(N^{ϵ}-cyclohexyl, N^{ϵ}-Cbz). L-N^{α}-Boc-Lys(N^{ϵ}-Cbz) (15.20 g, 40 mmol), cyclohexanone (7.85 g, 80 mmol), MeOH (100 mL), and molecular sieves (6.0 g, 4 Å) were added in a 500 mL Parr hydrogenation vessel. The mixture was purged with N_2 for 10 min, and then Pd/C 10% (600 mg) was added. The reductive alkylation under H_2 at 40 psi was monitored by HPLC for 26 h. After the filtration of the catalyst and molecular sieves and the evaporation of the solvent in a vacuum, the desired intermediate l-N^{α}-Boc-Lys(N^{ϵ}-cyclohexyl) was obtained as an oil. The L-N^{α}-Boc-Lys(N^{ϵ}-cyclohexyl) was cooled to 0 °C and then Cbz-protected using benzyl chloroformate (Cbz-Cl; 8.6 mL, 60 mmol) in a mixture of THF/H_2O (1:1, 200 mL) maintained at pH 9.5 by the use of an autotitrator delivering 0.5 N NaOH. The mixture was then stirred at room-temperature overnight. The mixture was concentrated under vacuum to remove THF and then extracted with petroleum ether (3 × 100 mL) to remove excess Cbz-Cl. The pH of the aqueous layer was brought to 2.5 with saturated sodium hydrogen sulfate and then extracted with EtOAc (3 × 150 mL). The combined organic layers were washed with a saturated sodium chloride solution (2 × 100 mL) and dried over anhydrous sodium sulfate. After evaporation of EtOAc under vacuum L-N^{α}-Boc-Lys(N^{ϵ}-cyclohexyl, N^{ϵ}-Cbz) was obtained as a foam. Yield =

11.47 g (24.80 mmol, 62%); $[\alpha]^{25}_D = -5.5°$ ($c = 1$, MeOH); ESI-MS $(M + H)^+ = 463.20$; $(M + Na^+) = 485.20$. HPLC assay: column (C_{18} 0.21 cm × 15 cm); buffer A, 0.1% TFA in H_2O; buffer B, 0.1% TFA in 60% ACN/40% H_2O; gradient condition, 40−90% buffer B over 50 min at a flow rate of 0.2 mL/min; UV detection, 0.1 AUFS at 214 nM; $t_R = 44.3$ min; purity = 98%. ^{1}H NMR ($CDCl_3$) δ 1.15−1.40 (m, 15H, CH_3s + CH_2s-cyclohexyl), 1.45−1.56 (m, 4H, $^{\gamma}CH_2$-Lys + $^{\delta}CH_2$-Lys), 1.58−1.92 (m, 6H, $^{\beta}CH_2$-Lys + CH_2s-C−cyclohexyl), 3.10 (t, 2H, $^{\delta}CH_2$-Lys), 3.68 (m, 1H, CH−cyclohexyl), 4.28 (m, 1H, $^{\alpha}$CH-Lys), 5.12 (s, 2H, CH_2−Cbz), 6.19 (br, 1H, NH), 7.32 (br s, 5 H, ArHs-Cbz), 9.30 (br, 1H, COOH).

βAla(*N*$^{\alpha}$-isopropyl, *N*$^{\alpha}$-Cbz). Isopropylamine (11.90 g, 200 mmol) in absolute ethanol (100 mL) was added dropwise to the stirred solution of 3-bromopropionic acid (15.3 g, 100 mmol) in absolute ethanol (100 mL). After the addition, the mixture was slowly heated and refluxed for 2 h. The reaction mixture was cooled to room temperature, and then NaOH (250 mmol) in H_2O (100 mL) was added. The excess of isopropylamine and ethanol was distilled off in a vacuum. The residue was dissolved in a mixture of THF/H_2O (1:1, 200 mL) and Cbz-protected using the procedure similar to that used for L-*N*$^{\alpha}$-Boc-Lys(*N*$^{\epsilon}$-cyclohexyl, *N*$^{\epsilon}$-Cbz) to yield the desired βAla(*N*$^{\alpha}$-isopropyl, *N*$^{\alpha}$-Cbz) as a thick oil. Yield = 19.90 g (75 mmol, 75%); ESI-MS $(M + H)^+ = 266.10$, $(M + Na^+) = 288.10$. HPLC assay: column (C_{18} 0.21 cm × 15 cm); buffer A, 0.1% TFA in H_2O; buffer B, 0.1% TFA in 60% ACN/40% H_2O; gradient condition, 40−90% buffer B over 50 min at a flow rate of 0.2 mL/min; UV detection, 0.1 AUFS at 214 nM; $t_R = 13.6$ min; purity = 99%. ^{1}H NMR ($CDCl_3$) δ 1.15 (d, 6H, CH_3s), 2.64 (t, 2H, CH_2−CO), 3.44 (t, 2H, CH_2−N), 4.30 (br, 1H, CH−$(CH_3)_2$), 5.15 (s, 2H, CH_2−Cbz), 7.32 (br, 5H, ArHs-Cbz), 11.20 (br s, 1H, COOH).

Peptide Synthesis. All of the peptides were synthesized manually by solid-phase peptide synthesis (SPPS) methodology[12] using previously described *tert*-butyloxycarbonyl (Boc) strategy on methylbenzhydrylamine (MBHA) resin (approximately 1 g of starting resin per peptide). Trifluoroacetic acid (TFA) treatment was used for Boc removal for 20 min. Two- to three-fold excess of protected amino acid based on the original substitution of the resin was used for coupling for 90−120 min. *N*-terminal acetylation was performed by using excess acetic anhydride in DCM for 15 min. Compounds **1** and **2** were synthesized and purified as previously described.[13,14]

Synthesis of [IOrn^8]degarelix (18). Analogue **18** was derived from the fully protected [Boc-D-4Aph(*N*$^{\omega}$-Fmoc)-Leu-IOrn(*N*$^{\delta}$-Cbz)-Pro-D-Ala]-MBHA resin (1 g; 0.33 mmol/ substitution). The individual amino acids were incorporated in a sequential manner utilizing either DIC (*N*,*N*′-diisopropylcarbodiimide) or TBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-hexamethyluronium tetrafluoroborate) 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.[51] The *N*$^{\alpha}$-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% *m*-cresol for 20 min. The *N*$^{\alpha}$-Boc-protected, resin-bound pentapeptide was treated with 30% piperidine in NMP (20 min) to selectively liberate the ω-amino function of D-4Aph. Carbamoylation of the free *N*$^{\omega}$-amino function of D-4Aph was then carried out using *tert*-butyl isocyanate (2 mmol) in dry DMF (5 mL) at room temperature for 12 h. The completion of reaction was monitored by ninhydrin test. After the removal of *N*$^{\alpha}$-Boc from D-4Aph, the synthesis on the resin was continued with the coupling of Boc-4Aph(*N*$^{\omega}$-Fmoc). The ω-amino function of 4Aph was freed with 30% piperidine in *N*-methylpyrrolidone (NMP) and then L-hydroorotic acid (1 mmol) was coupled with DIC (1 mmol) and HOBt (1 mmol) in NMP. The mixture was agitated at room temperature for 2 h and a ninhydrin test indicated the completed reaction. After the removal of *N*$^{\alpha}$-Boc from 4Aph^5 at position 5, chain elongation with the four *N*-terminal amino acids and acetylation gave the fully protected resin-bound peptide precursor. 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 40% acetonitrile (ACN)/H_2O and lyophilization. The crude peptide (0.45 g) was purified by preparative RP-HPLC.[52] The peptide was 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 (35% B for 10 min followed by a 90 min linear gradient to 65% B). The collected fractions were screened by analytical RP-HPLC under isocratic conditions, with a mixture of A (0.1% TFA) and B (60% ACN, 40% A) at a flow rate of 1.0 mL/min. Appropriate fractions were then combined (diluted 1:2 with water) and desalted on preparative HPLC with a mixture of A (0.1% TFA) and B (60% ACN, 40% A) using a gradient: 35% B (10 min) followed by a 40 min gradient to 75% B. Yield of **18** after purification was 80 mg (47.88 μmol, 14.52%).

Analogues **11**−**17** and **23** were obtained in comparable yields using this general procedure with the appropriate amino acids introduced at position 7 or 8 during the chain elongation step of the resin-bound peptide. The dinitrophenyl (Dnp) protection of the imidazole of histidine in **14** was removed with thiophenol/NMP at the end of the synthesis and prior to HF cleavage.

Synthesis of [*N*$^{\beta}$, *N*$^{\beta}$-di-(2-pyridyl-methyl)D-Dap^3]degarelix (3). The fully protected [Ac-D-2Nal-D-4Cpa-D-Dap-(Fmoc)-Ser(Bzl)-4Aph(L-Hor)-D-4Aph(Cbm)-Leu-ILys(*N*$^{\epsilon}$-Cbz)-Pro-D-Ala]MBHA resin was synthesized manually on methylbenzhydrylamine resin (1 g, 0.33 mmol/g substitution). The Fmoc side chain protecting group of D-Dap was then removed with 30% piperidine in NMP (20 min). The exposed D-Dap amino group was reductively alkylated with 2-pyridinecarboxaldehyde (16.5 mmol) in the presence of $NaCNBH_3$ (3.3 mmol) in DMF (25 mL) at room temperature for 3 h. The cycle was repeated with fresh reagents for an additional 3 h. The progress of the reaction was monitored by chloranil test[20] (used for detection of secondary amine on resin) and ninhydrin test.[51] The completed resin-bound peptide (1.45 g) was then cleaved, washed, extracted, and purified by preparative RP-HPLC using the same conditions described for analogue **18.** Yield of **3** after purification was 68 mg (38.80 μmol, 11.77%).

Analogue **4** was obtained in comparable yield using this procedure and D-Dab at position 3.

Synthesis of [*N*$^{\beta}$-(2-pyridyl-methyl)D-Dap^3]degarelix (5). This analogue was synthesized the same way as analogue **3** except the deprotected amino group of the side chain of D-Dap was first reacted with the excess of 2-pyridinecarboxaldehyde (20 mmol) in DMF (10 mL) at room temperature for 4 h. All of the solvents and reagents were drained, and the resin was washed sequentially with methanol (2 × 25 mL) and dichloromethane (2 × 25 mL). The resin bound peptide aldimine was agitated for 30 min with solid $NaCNBH_3$ (3.3 mmol) in DMF (15 mL). The reduction step was repeated with fresh reagents for an additional 30 min. The progress of the reaction was monitored by chloranil test.[20] The workup and purification of the completed peptide was performed as described above. Yield of **5** after purification was 42 mg (25.30 μmol, 7.66%).

Analogues **6**−**10** were obtained in comparable yields using this procedure; different aldehydes (2-quinolinecarboxaldehyde or 2-imidazolecarboxaldehyde) and D-Dab were substituted at position 3.

Synthesis of [*N*$^{\epsilon}$-(IGly)Lys^8]degarelix (19). First, we synthesized [Boc-Lys(*N*$^{\epsilon}$-Fmoc)-Pro-D-Ala]-MBHA resin on 1 g of methylbenzhydrylamine resin with 0.33 mmol/g substitution. This protected, resin-bound peptide was treated with 30% piperidine in NMP (20 min) to free the *N*$^{\epsilon}$-amino group of lysine and then IGly(*N*$^{\alpha}$-Cbz) (1 mmol) was coupled by using DIC (1 mmol) and HOBt (1 mmol) in NMP. After the removal of *N*$^{\alpha}$-Boc from Lys, the chain elongation with the seven *N*-terminal amino acids and acetylation gave the completed peptide resin (1.60 g). The workup and purification of the

completed peptide was performed as described above. Yield of **19** after purification was 120 mg (71.80 μmol, 21.78%).

Analogues **20**−**22**, **24**−**26**, and **30** were obtained in comparable yields using this procedure and different amino acid derivatives: N^{α}-isopropyl-Gly(N^{α}-Cbz); N^{α}-cyclohexyl-Gly(N^{α}-Cbz); N^{α}-isopropyl-βAla(N^{α}-Cbz); L-Hor coupled on the orthogonal amino group of Orn/Dap/Dab at position 8 during the chain elongation of the peptide resin.

Synthesis of [N^{β}-(ICbm)Dap^{8}]degarelix (28). This analogue was synthesized the same way as analogue **19** except the deprotected amino group of the side chain of Dap introduced at position 8 was reacted with isopropyl isocyanate (2 mmol) in dry DMF (5 mL) on the resin. The mixture was agitated at room temperature for 12 h, and the ninhydrin test indicated a complete reaction. After the removal of N^{α}-Boc from Dap, chain elongation with the seven *N*-terminal amino acids and acetylation was carried out on the resin. The completed peptide resin (1.65 g) was then cleaved and purified by preparative RP-HPLC as described above. Yield of **28** after purification was 110 mg (67.40 μmol, 20.41%).

Analogue **29** was obtained in comparable yields using this procedure, and Dab was substituted at position 8.

N^{α}-Methylation on the Resin. A general procedure for the introduction of methyl group at the N^{α}-amino function of a nascent peptide chain on the resin is described in the literature.[53] We have used this methodology for the synthesis of some of the analogues (**31**−**33**) presented here. In short, the analogue was built on the MBHA resin up to and including the amino acid that would ultimately contain the N^{α}-methyl group. Removal of the N^{α}-Boc group (60% TFA in DCM, 20 min) provided the unprotected N^{α}-amino function. Alkylation of this primary amino group with 4,4′-dimethoxybenzhydryl chloride[54] 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 NMP (3:7) 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. The coupling of *N*-protected amino acid to a *N*-methyl amino acid on the resin is difficult and usually gives low to moderate yield of products, which are often contaminated with unwanted diastereomers. We used bromotris-pyrrolidino-phosphonium hexafluorophosphate[55] (PyBrop)/DIPEA mediated coupling in NMP to get the desired analogues.

Analogue **27** was synthesized by introducing N^{α}-Fmoc-Dap-(Boc) at position 3 during the elongation of the peptide chain followed by N^{β}-methylation of Dap as described above on the resin. βAla(N^{α}-isopropyl, N^{α}-Cbz) was then coupled to N^{β}-methyl of Dap using PyBrop/DIPEA in NMP. After the removal of Fmoc group from N^{α} of Dap, chain elongation was carried out as described for **28**.

Peptide Characterization. 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 the beginning of experiments, $n = 5-8$) were castrated under ether anesthesia 10 days prior to the start of the experiment. The peptides (500 μg) were dissolved in a bacteriostatic water containing 5% mannitol and 0.6% DMSO. The rats were injected sc with a total dose of 50 μg/rat in 50 μL of aqueous buffer. Blood was sampled from the tail tip (300 μL) at the given times. Plasma LH was determined by radioimmunoassay (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. 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[56] were cultured in phenol red free DMEM containing 10% (v/v) FBS, G418 (0.4 mg/mL), penicillin/streptomycin solution (100 U of penicillin and 100 μg of streptomycin per mL of medium), and L-glutamine (2 mM). The cells were harvested and plated at 5×10^4 cells per well in a volume of 80 μL per well in white 96 well culture plates. The cells were incubated at 37 °C under 5% CO_2 overnight for assay the next day.

IC_{50} Determination Using the Reporter Gene Assay. Each compound was assayed in duplicate at 11 descending concentrations in half-log increments. Compounds in 1% DMSO (10 μL) or 1% DMSO alone as a control was added to the hGnRH receptor expressing HEK293 cells followed by gentle mixing and incubation for an additional 10 min at 37 °C under 5% CO_2. Following this, GnRH (10 μL) was added to a final concentration of 1 nM. Plates were then incubated for a minimum of 5 h at 37 °C under 5% CO_2, after which 100 μL of luciferase substrate mix was added to each well. Plates were sealed with Packard Topseal film, and luminescence was measured on a Molecular Devices Analyst after a 10 min incubation at room temperature in the absence of direct light.

To derive the IC_{50}, the test compound counts per second (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 was fitted to the data. An IC_{50} value was derived by nonlinear regression to a 4-parameter logistic equation [sigmoidal dose−response (variable slope)], using the GraphPad Prism (version 2.01) curve fitting software package. The geometric mean of the IC_{50} from at least two independent experiments is reported for each compound.

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