Novel Analogues of Degarelix Incorporating Hydroxy-, Methoxy-, and Pegylated-Urea Moieties at Positions 3, 5, 6 and the N-Terminus. Part III[1,2]

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Novel degarelix (Fe200486) analogues were screened for antagonism of GnRH-induced response (IC_{50}) in a reporter gene assay. Inhibition of luteinizing hormone release over time was measured in the castrated male rat. N^{ω}-Hydroxy- and N^{ω}-methoxy-carbamoylation of Dab and Dap at position 3 (**3**−**6**), and N^{ω}-hydroxy-,N^{ω}-methoxy-carbamoylation and pegylation of 4Aph at positions 5 and 6 (**7**−**10**, **15**−**17**, **22**−**25**) were carried out. Modulation of hydrophobicity was achieved using different acylating groups at the N-terminus (**11**−**14**, **18**−**21**, **26**−**28**). Analogues **8**, **15**−**17**, **22**, and **23** were equipotent to acyline (IC_{50} = 0.69 nM) and degarelix (IC_{50} = 0.58 nM) in vitro. Analogues **7**, **17**, and **23** were shorter acting than acyline, when **9**, **11**, **13**, **15**, **16**, and **22** were longer acting. Only **9** and **14** were inactive at releasing histamine. No analogue exhibited a duration of action comparable to that of degarelix. Analogues with shorter and longer retention times on HPLC (a measure of hydrophilicity) than degarelix were identified.

Introduction

Gonadotropin-releasing hormone (GnRH,[a] *p*-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) plays a major role in the modulation of reproductive functions.[5] GnRH antagonists are now recognized as potential drugs in addition to superagonists 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, premenstrual syndrome, and induction of ovulation.[6−9] If GnRH antagonists are to be used successfully in humans, they need to have a high therapeutic index (relative potency at inhibiting gonadotropin secretion over relative potency at stimulating histamine release) for safety reasons and long duration of action for economical imperative.

Degarelix (**1**, Fe200486)[1], acyline (**2**),[3] and azaline B[4] are three structurally closely related antagonists of GnRH with high therapeutic index and a common scaffold: Ac-D-2Nal[1]-D-4Cpa[2]-D-3Pal[3]-Ser[4]-4Aph(X)[5]-D-4Aph(Y)[6]-Leu[7]-Ilys[8]-Pro[9]-D-Ala[10]-NH_2; in degarelix, X = L-hydroorotyl (Hor) and Y = carbamoyl (Cbm); in acyline, X = Y = acetyl (Ac) and in azaline B, X = Y = 5′-(3′-amino-1*H*-1′,2′,4′-triazolyl) (Atz). Whereas X and Y vary considerably in the structure without significant influence on the antagonistic potencies of the derived antagonist in vitro, introduction of X = L-Hor and Y = Cbm in degarelix resulted in an unexpected very long duration of action precluding the need to rely on slow-release formulations. On the other hand, acyline and azaline B could not be formulated to achieve inhibition of gonadotropins for more than four weeks due to their tendency to form poorly soluble gels at high concentration in aqueous buffers, thus limiting bioavailability. The development of GnRH antagonists whose effects would last for more than a month after a single injection in animal models or in human, with or without formulation, remains a challenging goal. Early[1] introduction of some urea and carbamoyl functionalities was optimized to yield analogues with dramatically improved duration of action, which ultimately led to the selection of degarelix for clinical investigations. Since the acylation of 4Aph[5] by L-hydroorotic acid in conjunction with carbamoylation of D-4Aph[6] in degarelix versus acetylation of 4Aph[5,6] in acyline resulted in a dramatic increase in duration of action, we wondered if alternate substitutions exist that would lead to even more potent and long acting analogues easy to formulate for acute or slow release and that could be readily synthesized. Increasing the number of hydrogen bonding sites by introducing urea functionalities[1,10] and poly(ethylene glycol) moieties (PEG)[11] or of hydrophobic side chains using fatty acid functionalities,[12] respectively, may modulate solubility and bioavailability. For example, the introduction of a *p*-methoxyureidophenyl moiety in the nonpeptide GnRH antagonist (TAK-013, Figure 1)[13] forms an intramolecular hydrogen bond between the aniline NH and the methoxy oxygen atom, resulting in an increase in lipophilicity, membrane permeability, and consequently the oral absorption in monkeys. We present here the biological properties of a series of degarelix analogues containing the hydroxy-, methoxy-, and pegylated-urea functionalities on the side-chains of selected amino acids at positions 3, 5, 6 and the N-terminus of the acyline/azaline B/degarelix scaffold shown in Figure 2.

The present study of the effect of substitutions at position 3 of degarelix (**1**) is an extension of an earlier study[10] that demonstrated that the nonaromatic substitution of D-3Pal in

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[a] Abbreviations: Ac, acetyl; ACN, acetonitrile; 4Aph, 4-aminophenylalanine; AOA, antiovulatory assay; Atz, 5′-(3′-amino-1*H*-1′,2′,4′-triazolyl); Boc, *tert*-butoxycarbonyl; Bzl, benzyl; Cbm, carbamoyl; 4Cpa, 4-chlorophenylalanine; CZE, capillary zone electrophoresis; Dab, 2,4-diaminobutyric acid; Dap, 2,3-diaminopropionic acid; DCM, dichloromethane; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, *N*,*N*′-diisopropylethylamine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; GnRH, gonadotropin-releasing hormone; HOBt, 1-hydroxybenzotriazole; L-Hor, L-hydroorotyl; IGly, N^{α}-isopropylglycine; ILys, N^{ϵ}-isopropyllysine; LH, luteinizing hormone; MBHA, *p*-methylbenzhydrylamine; 2Nal, 3-(2-naphthyl)alanine; NMP, *N*-methylpyrrolidinone; 3Pal, 3-(3-pyridyl)alanine; PEG, poly(ethylene glycol); PyBrOP, bromo-tris-pyrrolidino-phosphonium hexafluorophosphate; RP-HPLC, reversed phase high performance liquid chromatography; RGA, Reporter Gene Assay; RT, room temperature; sc, subcutaneous; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

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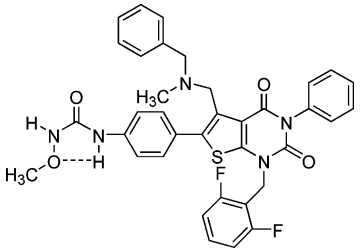


Figure 1. Chemical structure of a nonpeptide GnRH antagonist.[13]

position 3 by D-glutamine 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 short acting. This suggested that very minor substitutions at position 3 have an unexpected impact on duration of action with minimal effect on antagonist binding affinity. An additional study whereby the side chain amino function of 2,4-diaminobutyric acid (Dab) and 2,3-diaminopropionic acids (Dap) at position 3 were reductively alkylated with 2-pyridinecarboxaldehyde, 2-quinolinecarboxaldehyde, and imidazolcarboxaldehyde in the presence of sodium cyanoborohydride ($NaCNBH_3$) resulted in degarelix analogues with reduced antagonist potency and short duration of action.[2] This suggested that both a positive charge and a bulky heterocycle at position 3 are incompatible with high antagonist potency.

Significant optimization of substitutions at positions 5 and 6 was achieved with the introduction of 4Aph(L-Hor)[5] and D-4Aph(Cbm)[6] in **1** versus 4Aph(Ac)[5] and D-4Aph(Ac)[6] in acyline resulting in increased water solubility, decreased tendency to form gels, and considerable extension of duration of action. There is no mechanistic explanation for this property unique to degarelix and a few very closely related analogues all containing 4Aph(L-Hor) at position 5.[1] Will the introduction of hydroxy-carbamoyl, methoxy-carbamoyl, and pegylated moieties at the N-terminus and at the 4-amino function of 4Aph at positions 5 and 6 result in improved properties? Because these analogues had increased water solubility compared to that of acyline and degarelix, we tested the hypothesis that the optimal balance between hydrophilicity and hydrophobicity found in degarelix could be reached by increasing hydrophobicity of the newly developed analogues by acylation of the N-terminus with butyric and octanoic acids as well as a PEG derivative.

Results and Discussion

Synthesis, Purification, and Chemical Characterization (Table 1). All of the analogues shown in Table 1 were synthesized manually on a *p*-methylbenzhydrylamine resin (MBHA-resin, *tert*-butoxycarbonyl (Boc) strategy) using the solid-phase peptide synthesis (SPPS) methodology and protocols previously described[14] or shown below. In most cases, analogues were obtained by acylation/carbamoylation at positions 3, 5, 6 and the N-terminus on the partially deprotected peptido-resin obtained after the removal of the orthogonal protecting group (for example, after the removal of the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group of Boc-4Aph(Fmoc) at position 5, Boc-D-4Aph(Fmoc) at position 6, Boc-D-Dab/D-Dap(Fmoc) at position 3, and after the removal of the Boc group with 60% TFA in DCM of D-2Nal at the N-terminus). The reference compounds degarelix (**1**) and acyline (**2**) were synthesized using the published protocols. The carbamoylation[1] at position 6 was carried out by the reaction of *tert*-butyl isocyanate in dimethylformamide (DMF) with the free 4-amino function of D-4-aminophenylalanine on the otherwise fully protected resin-bound peptides. However, this methodology cannot be used for introducing hydroxy-carbamoyl, methoxy-carbamoyl, or pegylated carbamoyl functions in the GnRH antagonists reported in Table 1 due to the limited commercial availability of the isocyanates. Our strategy for the synthesis of GnRH antagonists (Table 1) with a ureido group at the amino function was analogous with the method reported by Kim et al.[15] for the solid-phase synthesis of oligoureas. In this approach, the urea donors were synthesized separately using solution-phase synthesis (Scheme 1) and then reacted with the free amino function of the resin-bound peptide in the presence of *N*,*N*′-diisopropylethylamine (DIPEA) in DMF. The reaction was performed on the resin after completion of the peptide prior to HF cleavage and deprotection (see Experimental Section). We found this method to be advantageous in terms of purity and yield of the final peptide over several other methods[16−20] reported to assemble ureas on solid support.

In the present study, we synthesized three urea donors as 4-nirophenyl-*N*-substituted carbamates (**IIIa**−**c**, Scheme 1) that can be conveniently used for the derivatization of amino function in solid-phase peptide synthesis (SPPS) or solid-phase organic synthesis. The synthesis of 4-nitrophenyl-N-substituted carbamates involved the reaction of 4-nitrophenylchloroformate with the commercially available amines (**Ia**,**b**) or with pegylated amine (**Ic**) in the presence of pyridine. The amines **Ia**,**b** are commercially available, and 2-(2-ethoxyethoxy)ethylamine **Ic** was prepared from 2-(2-ethoxyethoxy)ethanol (carbitol) by stepwise transformation (OH→OTos→N_3→NH_2) as described in the literature.[21] The benzyl protection on the hydroxyl group of **IIIb** was designed to avoid any side reactions during coupling of the amino acids on the resin. The debenzylation takes place during HF cleavage of the resin avoiding an additional deprotection step.

The introduction of the *N*-methyl group in **23** was achieved during the elongation of the peptide chain on the partially assembled peptide resin using the published procedure of Kaljuste et al.[22]

The protected peptido-resins were cleaved and deprotected in anhydrous HF 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 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 (Tables 1 and 2, and Figure 3). Analogues were tested in vitro for their antagonism at the GnRH receptor in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a stably integrated luciferase reporter gene.[1,2,23] 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%.

Since 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 castrated male rat assay,[24,25] the most efficacious analogues are those with the longest duration of action. In fact, we used this assay for screening purposes that led to the discovery of **1**. In short, 10 days after castration, rats (5 or more per group) were injected

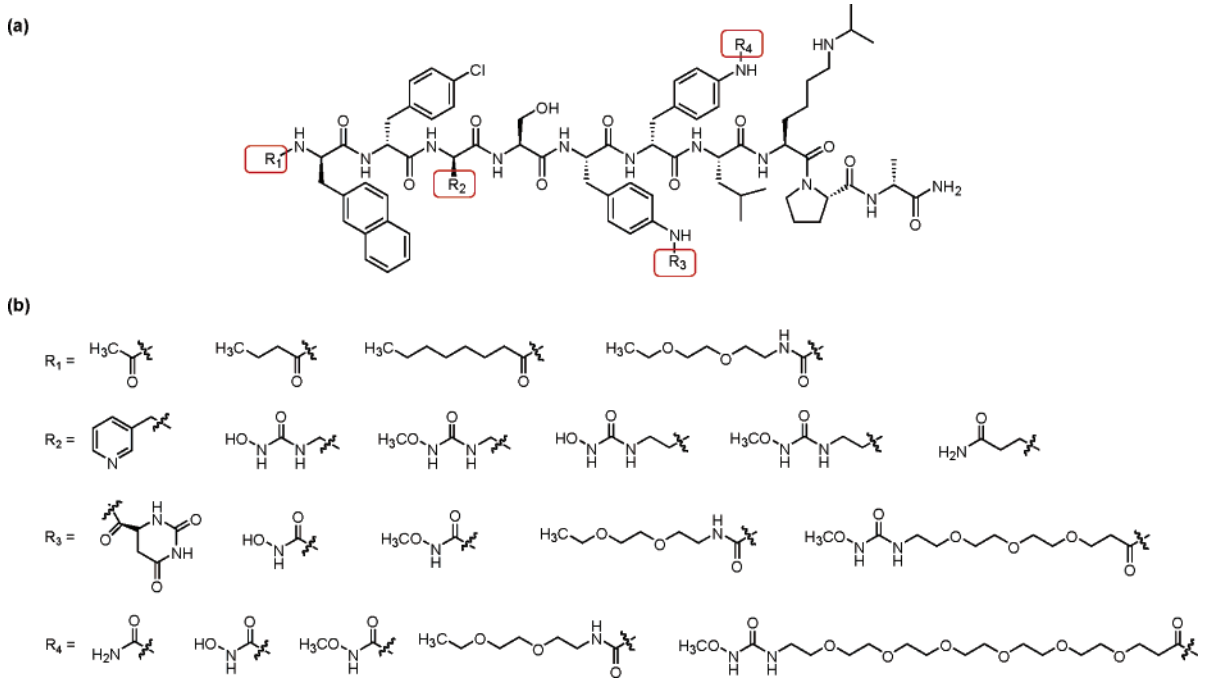


Figure 2. (a) Amino acid scaffold upon which acyline, azaline B, and degarelix are built. (b) Structures of the substitutions incorporated at R_1 to R_4.

on a Friday with 5% mannitol (50 μL), degarelix and acyline (50 μg/50 μL 5% mannitol) as an internal standard, and the novel analogues (50 μg/50 μL 5% mannitol). Blood sampling was performed predose and then at 3 h, 72 h, 96 h, 120 h, 144 h, 168 h, and occasionally at 192 h post-sc administration (bloods were not collected over weekends). The effects of the test compounds on the gonadotropic axis were determined by measurement of plasma LH levels using a radioimmunoassay[25] (Figure 3).

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 and less than 120 h, an analogue that is long acting will inhibit LH (>80%) secretion for at least 72 h, and an analogue that is short acting will inhibit LH (>80%) secretion at 3 h but not at 72 h. This is illustrated in Figure 3 where we describe the time course of activity of four selected analogues [**1** (very long acting), **25** (short acting), **8** (long acting), and **9** (long* acting)].

The antiovulatory assay (AOA) and histamine release assay (Table 2) are two other in vivo assays used to estimate the relative efficiency of the GnRH antagonists.

Structure−Activity Relationships. The overall rationale for the synthesis of the analogues described in Table 1 is presented in our introduction and consisted predominantly in understanding the structural basis for long duration of action and the development of a strategy leading to water soluble GnRH antagonists that can be formulated for acute or slow release. The structures of different substitutions incorporated in degarelix at positions 3, 5, 6 and the N-terminus resulting in differences in duration of action are presented in Figure 2. Analogues **3**−**6** closely resemble members of a series of position 3-substituted degarelix analogues found to be long acting.[10] The short duration of action of these analogues can be attributed in part to their lower affinity (ca. 5-fold) for the GnRH receptor as compared to that of degarelix and the highly hydrophilic character of the substituted ureas. Interestingly, extension of the side chains from 2,3-diaminopropionic acid to 2,4-diaminobutyric acid (Dap to Dab) had no significant effect on either antagonist potency or duration of action.

The next series (**7**−**10**) illustrates the effect of elaborate substitutions of the 4-amino function of 4Aph[5] with hydroxy- (**7**) and methoxy- (**8**) ureas as well as congeneric poly(ethylene glycol)s (**9** and **10**) on antagonist potency and duration of action. In this case, we see a clear correlation between hydrophobicity measured by retention time of **7**−**9** on HPLC and duration of action although inconsistent with the very long duration of action of **1** the retention time of which is similar to that of **7**. Despite the fact that **7** and **8** have affinities comparable to that of degarelix, neither is very long acting.

Analogues **11**−**14** are substituted at the N-terminus and at the 4-amino function of 4Aph[5] with poly(ethylene glycol) (Figure 2). In this series, **11** and **13** inhibit LH release (>80%) for 96 h but not for 120 h. It is noteworthy to suggest that the short duration of action of many of these analogues may result from chemical instability, rapid elimination, and formation of a depot at the site of injection. There is ample description of diminished bioavailability with increased concentration of GnRH antagonists upon sc administration.[26]

In the next series of analogues (**15**−**17**), we evaluated the effect of hydroxylation (**15**), methoxylation (**16**), and pegylation (**17**) of the carbamoyl function at the 4-amino function of D-4Aph.[6] Unexpectedly, addition of a hydroxyl or methoxyl group on the carbamoyl functionality resulted in loss of duration of action despite the fact that degarelix and **15** have equal retention time on HPLC and antagonistic potencies suggesting very similar physicochemical properties.

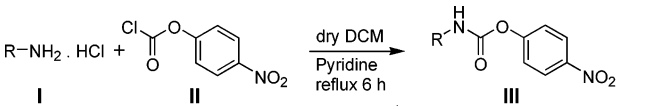
Analogues **18**−**20** are different from **16** with respect to the substitution at the N-terminus. In addition to the methoxy-carbamoyl function at position 6 as in **16**, the N-terminus was either acylated (**18** and **19**) or pegylated (**20**). It was hypothesized that if methoxy-carbamoylation at position 6 as in **16** resulted in a peptide with shorter duration of action than degarelix, it might be due to the decreased ability of this analogue to gel as compared to degarelix (an observation

Table 1. Physicochemical and Biological Characterization of GnRH Antagonists

no.	compound	purity HPLC[a]	purity CZE[b]	t_R[c] (min)	MS[d] (M + H)$^+$ calcd	MS[d] (M + H)$^+$ obsd	pIC$_{50}$,[e] avg ± SEM	IC$_{50}$[f] (nM)	duration of action[g]
1	[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	23.8	1631.8	1631.9	9.24 ± 0.16	0.58	very long
2	[Ac-D-2Nal^1,D-4Cpa^2,D-3Pal^3, 4Aph(Ac)^5,D-4Aph(Ac)^6,Ilys^8,D-Ala^{10}]-GnRH (acyline)	98	98	28.2	1532.7	1532.7	9.16 ± 0.06	0.69	long
3	[D-Dap(CO−NH−OH)^3]degarelix	97	98	21.7	1628.7	1628.7	8.46 ± 0.01	3.46	short
4	[D-Dab(CO−NH−OH)^3]degarelix	99	99	21.3	1642.8	1642.8	8.57 ± 0.12	2.68	short
5	[D-Dap(CO−NH−OCH_3)^3]degarelix	95	99	23.3	1642.8	1642.7	8.50 ± 0.02	3.16	short
6	[D-Dab(CO−NH−OCH_3)^3]degarelix	98	98	22.8	1656.8	1656.8	8.77 ± 0.05	1.69	short
7	[4Aph(CO−NH−OH)^5]degarelix	99	98	24.4	1550.7	1550.7	9.07 ± 0.19	0.86	short
8	[4Aph(CO−NH−OCH_3)^5]degarelix	99	99	26.3	1564.7	1564.7	9.22 ± 0.03	0.60	long
9	[4Aph(CO−NH−$(C_2H_4O)_2$−C_2H_5)^5]degarelix	98	98	29.1	1650.8	1650.8	8.50 ± 0.62	3.16	long*
10	[4Aph(CO−$(C_2H_4O)_3$−C_2H_4−NH−CO−NH−OCH_3)^5]degarelix	98	97	26.1	1767.9	1767.9	8.97 ± 0.13	1.06	short
11	des-Ac-[4Aph(CO−NH−OCH_3)^5]−CH_3−$(CH_2)_2$−CO-degarelix	98	97	30.1	1592.8	1592.7	8.83 ± 0.31	1.46	long*
12	des-Ac-[4Aph(CO−NH−OCH_3)^5]−CH_3−$(CH_2)_6$−CO-degarelix	97	98	40.4	1648.8	1648.6	8.38 ± 0.14	4.17	short
13	des-Ac-[4Aph(CO−NH−OCH_3)^5]-C_2H_5-$(C_2H_4O)_2$−NH−CO-degarelix	96	96	30.6	1681.8	1681.9	8.53 ± 0.47	2.98	long*
14	des-Ac-[4Aph(CO−NH−$(C_2H_4O)_2$−C_2H_5)^5]−C_2H_5−$(C_2H_4O)_2$−NH−CO-degarelix	95	95	33.3	1767.9	1767.9	8.95 ± 0.04	1.12	short
15	[D-4Aph(CO−NH−OH)^6]degarelix	97	99	23.7	1647.7	1647.7	9.09 ± 0.12	0.80	long*
16	[D-4Aph(CO−NH−OCH_3)^6]degarelix	98	99	26.0	1661.8	1661.8	9.29 ± 0.12	0.51	long*
17	[D-4Aph(CO−NH−$(C_2H_4O)_2$−C_2H_5)^6]degarelix	98	98	28.3	1747.8	1747.9	9.06 ± 0.11	0.88	short
18	des-Ac-[D-4Aph(CO−NH−OCH_3)^6]−CH_3−$(CH_2)_2$−CO-degarelix	98	98	29.7	1689.8	1689.9	8.67 ± 0.26	2.13	short
19	des-Ac-[D-4Aph(CO−NH−OCH_3)^6]−CH_3−$(CH_2)_6$−CO-degarelix	98	98	39.6	1745.9	1745.8	8.02 ± 0.18	9.47	short
20	des-Ac-[D-4Aph(CO−NH−OCH_3)^6]−C_2H_5−$(C_2H_4O)_2$−NH−CO-degarelix	95	99	29.9	1778.8	1778.7	8.79 ± 0.25	1.61	short
21	des-Ac-[D-4Aph(CO−NH−$(C_2H_4O)_2$−C_2H_5)^6]−C_2H_5−$(C_2H_4O)_2$−NH−CO-degarelix	95	99	32.1	1864.9	1864.9	8.34 ± 0.37	4.55	nd
22	[4Aph(CO−NH−OCH_3)^5,D-4Aph(CO−NH−OCH_3)^6]degarelix	95	96	28.5	1594.8	1594.8	9.04 ± 0.01	0.91	long*
23	[N^{α}-Me-4Aph(CO−NH−OCH_3)^5,D-4Aph(CO−NH−OCH_3)^6]degarelix	99	98	28.9	1608.8	1608.8	9.15 ± 0.05	0.70	short
24	[4Aph(CO−NH−$(C_2H_4O)_2$-C_2H_5)^5,D-4Aph(CO−NH−$(C_2H_4O)_2$−C_2H_5)^6]-degarelix	96	99	33.5	1767.0	1767.1	8.69 ± 0.31	2.04	short
25	[4Aph(CO−NH−OCH_3)^5,D-4Aph(CO−$(C_2H_4O)_6$−C_2H_4−NH−CO−NH−OCH_3)^6]degarelix	98	98	28.5	1929.9	1929.8	8.78 ± 0.24	1.67	short
26	des-Ac-[4Aph(CO−NH−OCH_3)^5,D-4Aph(CO−NH−OCH_3)^6]−CH_3-$(CH_2)_2$−CO-degarelix	99	99	32.3	1622.8	1622.9	8.86 ± 0.35	1.37	short
27	des-Ac-[4Aph(CO−NH−OCH_3)^5,D-4Aph(CO−NH−OCH_3)^6]−CH_3-$(CH_2)_6$−CO-degarelix	99	99	42.5	1678.8	1678.9	8.30 ± 0.34	4.99	inactive
28	des-Ac-[4Aph(CO−NH−$(C_2H_4O)_2$−C_2H_5)^5,D-4Aph(CO−NH−$(C_2H_4O)_2$−C_2H_5)^6]-C_2H_5-$(C_2H_4O)_2$−NH−CO-degarelix	95	97	37.6	1883.9	1883.9	8.59 ± 0.22	2.55	short
29	[D-Gln^3, 4Aph(CO−NH−OCH_3)^5,D-4Aph(CO−NH−OCH_3)^6]-degarelix	99	99	27.6	1574.7	1574.7	8.53 ± 0.24	2.98	short
30	[D-Dab(CO−NH−OCH_3)^3, 4Aph(CO−NH−OCH_3)^5,D-4Aph(CO−NH−OCH_3)^6]-degarelix	98	98	28.9	1619.6	1619.8	8.83 ± 0.04	1.49	short
31	[4Aph(CO−NH−OCH_3)^5,D-4Aph(CO−NH−OCH_3)^6,Orn(IGly)^8]-degarelix	99	99	30.9	1637.8	1637.7	8.82 ± 0.20	1.53	short

[a] Percentage purity determined by HPLC using buffer system A; TEAP, pH 2.30, buffer system B, 60% CH_3CN/40% A under gradient conditions (30% to 80% B over 50 min), at flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 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. × 40 cm length). Detection at 214 nm. [c] Retention times under gradient conditions (30% to 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 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; short = over 80% inhibition of LH release at 3 h but not at 72 h; nd = not determined.

Scheme 1. Synthesis of 4-Nitrophenyl-*N*-substituted-carbamates as Urea Donors for SPPS



I II III

Ia, **IIIa**; R = -OCH_3
Ib, **IIIb**; R = -OCH_2-C_6H_5
Ic, **IIIc**; R = -$(C_2H_4O)_2$-C_2H_5

difficult to quantitate). Therefore, to restore some gelling property to **16**, we introduced longer acyl groups than acetyl at the N-terminus to yield **18** and **19**. These analogues are clearly more hydrophobic and show a short duration of action. We concluded that **18** and **19** did not diffuse rapidly enough from the site of injection to sustain a bioactive concentration of the peptide in the blood stream. While pegylation at the N-terminus, as in **20**, did not improve duration of action, additional pegylation at position 6 (**21**) resulted in loss of antagonist potency suggesting that we may have some steric interference with the receptor.

With **22**−**25**, we explored the effect of the combined introduction of methoxy-carbamoyl and PEG functionalities at positions 5 and 6. Clearly, the methoxy-carbamoyl functionality at 4Aph^5 or D-4Aph^6 (see **8** and **16**, respectively) was not as favorable in vivo as the L-hydroorotyl at 4Aph^5 and a carbamoyl group at D-4Aph^6 found in degarelix (**1**). In **22**, these two substitutions did not result in any increase in duration of action or in vitro potency as compared to the monosubstitutions. With **23**, we examiined whether methylation of the backbone nitrogen at position 5 would yield an analogue with increased solubility in aqueous buffer as originally observed by Haviv et al.[27] It was hypothesized that a methyl group at that position would prevent the formation of stable beta sheets known to be sparingly soluble. We had shown however, that such substitution also

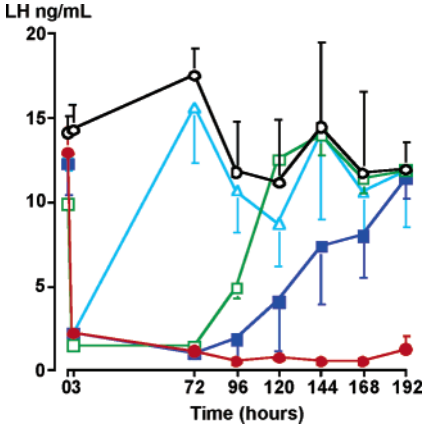


Figure 3. Inhibition of LH secretion after sc administration of analogues (**1**, **8**, **9**, and **25**). Total dose was 50 μg in 50 μL 5% mannitol containing 0.1% DMSO. Blood samples were collected at the times shown on the abscissa. Results are mean plasma LH levels ($n = 5-8$ rats) ± SEM. The SEM, where not appearing, are encompassed within the size of the symbols in the graph. Vehicle ○; degarelix (**1**) ●; **8** □; **9** ■; **25** △.

Table 2. Results from Two Rat Peritoneal Cell Histamine Release Assays and from the Antiovulatory Assay

compound	EC_{50}[a] (μg/mL) histamine ELISAs[b]	AOA[c] rats ovulating/total
Nal-Arg[29]	0.434	0/8
Nal-Glu[25]	3.05; 2.43	0/8
1 (degarelix)	>1000; >1000	0/8
2 (acyline)	461.4; >1000	0/8
7	117.8	1/8
9	910.3; 608.3	0/8
13	50.6; 89.6	0/8
14	>1000; 965.3	0/8
22	46.0	0/8
23	43.1	0/8

[a] EC_{50}s were determined with GraphPad Prism using a sigmoidal dose−response curve fit with the constraints that the bottom of the curve was a constant equal to 0% histamine released and the top, a constant equal to 100% histamine released. [b] One incubation with mast cells was performed for each compound, and the supernatants were collected and frozen. The supernatants were subsequently assayed once or twice in separate histamine ELISAs as indicated.[30] [c] AOA, antiovulatory assay.[43] Peptide (2 μg/200 μL) was administered sc.

increased the propensity of these analogues to release histamine.[28] Since histamine release could be traced to the presence of a hydrophobic N-terminus and basic residues at position 5, 6, and 8 (best illustrated for Nal-Arg[29] and Nal-Glu[25] in Table 2), we investigated whether it was still true after the introduction of neutral functionalities such as a hydroxy-carbamoyl (**7**), methoxy-carbamoyl (**22**, **23**), and PEG (**9**, **13**, and **14**) at positions 5, 6 (on the *p*-amino function of 4-aminophenylalanine) and at the N-terminus. Interestingly, the expectation that **23** would be more potent than **22** at releasing histamine as a result of the introduction of the N^{α}-methylation of 4Aph[5] did not materialize, suggesting a relatively important contribution of the methoxy-carbamoyl group on the *p*-amino function of 4Aph[5] to histamine release. The observation that **9** and **14**, both bearing a PEG group on 4Aph,[5] were essentially inactive in that assay is encouraging (Table 2).

Further, the double substitution (short PEG units) on 4Aph[5] and D-4Aph[6] (**24**) and increasing the length of PEG group on D-4Aph[6] (**25**) resulted in insignificant loss of antagonist potency and significant loss of duration of action. Compounds **26**−**28** had the favored methoxy-urea or PEG functionalities at 4Aph[5] and D-4Aph[6] and their N-terminal was either acylated with butanoic acid (**26**) or octanoic acid (**27**) and pegylated (**28**). The short duration of action of **26** and **28** and the inactivity of **27**, despite significant antagonist potency, was different from that of the same analogue **22** acetylated at the N-terminus. We conclude that the acetylated N-terminus of degarelix (**1**) is important to maximize its in vivo efficacy.

Analogues **29** and **30** were synthesized and tested to gain an appreciation of possible side chain interactions between positions 3, 5, and 6 (**29**, **30**) and positions 5, 6, and 8 (**31**) that would result in increased biological and structural stability as the result of intramolecular hydrogen bonding. All three analogues were short acting with IC_{50}s between 1.5 and 3.0 nM.

Conclusion

All analogues described here have antagonist potency (IC_{50}) in a reporter gene assay lower than 5 nm, suggesting that the observed variation in duration of action is strictly dependent on a slow release from the sc site of injection, binding to plasma proteins, plasma clearance, and/or enzymatic stability. In this series, analogues with t_R > than 33 min (**12**, **14**, **19**, **24**, **26**−**28**) are significantly shorter acting than degarelix (t_R ca. 23.8 min). The observations that **9**, **11**, **13**, **15**, **16**, and **22** were longer acting than acyline and azaline B and that they did not form gels as readily (general observation), suggest that they may be good candidates for successful incorporation in a delivery system that would prolong their activity. Taking into consideration the ability of these analogues to release histamine,[30] **9** would meet the minimum requirements for further pre-clinical investigation.

Experimental Section

Starting Materials. Most amino acid derivatives were obtained from Reanal Finechemical Co. (Budapest, Hungary), including Boc-D-Ala, Boc-D-Gln(Xan), Boc-Leu, Boc-Pro, and Boc-Ser(Bzl). Fmoc-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid and Fmoc-12-amino-4,7,10-trioxadodecanoic acid were purchased from Neosystem Groupe SNPE (Strasbourg, France). Boc-Orn(Fmoc) was obtained from Bachem Inc. (Torrance, CA). Boc-D-4Cpa, Boc-D-2Nal, and Boc-D-3Pal were provided by the Contraceptive Development Branch, Center for Population Research, NICHD. Boc-L- and Boc-D-4Aph(Fmoc) were synthesized according to a published procedure.[4] Boc-D-Dab(Fmoc) and Boc-D-Dap(Fmoc) were prepared according to a published procedure.[31] The L-isomer of hydroorotic acid was prepared using a published procedure.[32] IGly(Z),[33] Boc-ILys(Z),[34] and Boc-4Aph(L-Hor)[1] were synthesized according to published procedures. 2-(2-ethoxyethoxy)ethylamine was prepared as described in the literature.[21] Methoxylamine hydrochloride, *O*-benzylhydroxylamine hydrochloride, and *tert*-butyl isocyanate were purchased from Aldrich Chemical Co. (Milwaukee, WI). The MBHA resin[35] 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−Crafts step.[36] All solvents were reagent grade or better.

General Synthesis of 4-Nitrophenyl-N-substituted-carbamates. In a typical reaction, amine hydrochloride (**Ia**−**c**, Scheme 1, 100 mmol) was suspended in dry dichloromethane (DCM) (200 mL) and pyridine (7.9 g, 100 mmol). 4-Nitrophenylchloroformate (20.16 g, 100 mmol) dissolved in DCM (100 mL) was added dropwise while stirring at room temperature (RT) for 45 min. After the addition was completed, the reaction mixture was refluxed for 6 h and then cooled to RT, diluted with DCM (200 mL), washed sequentially with 1 N HCl, H_2O, 1 M sodium bicarbonate solution, water, and brine. The DCM layer was dried over sodium sulfate and evaporated under vacuum. The crude product was purified by flash chromatography using a mixture of ethyl acetate/hexane. **IIIa**: Yield = 86%; light yellow crystals; mp 128−130 °C; ESI-MS $(M + H)^+$ Calcd = 213.16, Found = 213.20; HPLC assay: column C_{18} (0.21 × 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; retention time t_R = 9.3 min, purity = 98%; ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H, CH_3), 7.38 (d, 2H, ArH's), 7.80 (br s, 1H, NH), 8.29 (d, 2H, ArH's).

IIIb: Yield = 84%; light yellow crystals; mp 95−97 °C; ESI-MS $(M + H)^+$ Calcd = 289.20, Found = 289.20; HPLC assay as for **IIIa**, retention time t_R = 13.2 min, purity = 97%; ^{1}H NMR ($CDCl_3$) δ 4.97 (s, 2H, CH_2), 7.35 (d, 2H, Ar's), 7.42 (br s, 5H, ArH's), 7.75 (br s, 1H, NH), 8.40 (d, 2H, ArH's).

IIIc: Yield = 78%; yellowish oil; ESI-MS $(M + H)^+$ Calcd = 299.29, Found = 299.30; HPLC assay as for **IIIa**: retention time t_R = 5.2 min, purity = 97%; ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H, CH_3), 3.40−3.72 (m, 10H, CH_2's), 5.93 (br s, 1H, NH), 7.32 (d, 2H, ArH's), 8.25 (d, 2H, ArH's).

Peptide Synthesis. All peptides were synthesized manually by SPPS methodology[14] using previously described *tert*-butoxycarbonyl (Boc) strategy on MBHA resin (approximately 1 g of starting resin per peptide). Trifluoroacetic acid (TFA) treatment (60% TFA in DCM) 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. The couplings were mediated with DIC (*N*,*N*′-diisopropylcarbodiimide) or TBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) in NMP (*N*-methylpyrrolidinone). 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.[1,3]

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.

Synthesis of [D-Dap(CO−NH−OH)^3]degarelix (3). First, we synthesized [Boc-D-4Aph(Fmoc)-Leu-ILys(Z)-Pro-D-Ala]-MBHA resin on 1 g of resin. The individual amino acids were incorporated in a sequential manner utilizing either DIC or TBTU mediated activation of the carboxyl group. The completion of couplings was qualitatively determined by ninhydrin test as described by Kaiser et al.[37] The N^α-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^α-Boc protected, resin-bound pentapeptide was treated with 30% piperidine in NMP (20 min) to selectively liberate the 4-amino function of D-4Aph which was carbamoylated using *tert*-butyl isocyanate (2 mmol) in dry dimethylformamide (DMF, 5 mL) at RT for 12 h. The completion of the reaction was monitored with the ninhydrin test. After the removal of N^α-Boc from D-4Aph, the synthesis was continued with the coupling of Boc-4Aph(L-Hor). After the removal of N^α-Boc from 4Aph,[5] chain elongation with the four N-terminal amino acids and acetylation gave the fully protected [Ac-D-2Nal-D-4Cpa-D-Dap(Fmoc)-Ser(Bzl)-4Aph(L-Hor)-D-4Aph(Cbm)-Leu-ILys(Z)-Pro-D-Ala]MBHA resin. 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 reacted with an excess of urea donor **IIIb** (Scheme 1) (1.65 mmol) in the presence of diisopropylethylamine (DIPEA, 2.31 mmol) in DMF (5 mL) at RT for 4 h. The progress of the reaction was monitored by ninhydrin test. HF treatment (anhydrous) of the completed peptido-resin at 0−5 °C in the presence of anisole (10% v/v) for 60 min yielded the desired crude analogue. The crude peptide was purified by preparative RP-HPLC.[38] The peptide was dissolved in 0.25 M triethylammonium phosphate (200 mL), pH 2.25 (TEAP 2.25) and loaded onto the cartridge as 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 by 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 **3** after purification was 48 mg (29.49 μmol, 8.94%).

Analogues **4**−**6** were obtained in comparable yields using this procedure and derivatization of orthogonal amino group of D-Dap/D-Dab with the appropriate urea donors **IIIa** or **IIIb** (Scheme 1) on the resin.

Synthesis of [4Aph(CO−NH−OH)^5]degarelix (7). The fully protected [Ac-D-2Nal-D-4Cpa-D-3Pal-Ser(Bzl)-4Aph(Fmoc)-D-4Aph(Cbm)-Leu-ILys(Z)-Pro-D-Ala]MBHA resin was first synthesized manually on MBHA resin (1 g, 0.33 mmol/g substitution). The Fmoc side chain protecting group of 4Aph was then removed with 30% piperidine in NMP (20 min). The reaction of the exposed amino group of 4Aph with excess of urea donor **IIIb** (Scheme 1), workup, and purification of the completed peptide was performed as described above. Yield of **7** after purification was 52 mg (33.55 μmol, 10.2%).

Analogues **8** and **9** were obtained in comparable yields using the same procedure and derivatization of orthogonal amino group of 4Aph with the appropriate urea donors **IIIa** or **IIIc** (Scheme 1) on the resin. Analogues **11**−**14** were synthesized the same way as **7** except the deblocked N-terminus of the resin-bound decapeptide [Boc-D-2Nal-D-4Cpa-D-3Pal-Ser(Bzl)-4Aph(Fmoc)-D-4Aph(Cbm)-Leu-ILys(Z)-Pro-D-Ala] was coupled with the appropriate capping groups such as butyric acid (in **11**), octanoic acid (in **12**), and urea donor **IIIb** (in **13** and **14**) instead of acetic anhydride. The coupling of butyric acid or octanoic acid to the N-terminus was mediated by DIC/HOBt in NMP. **IIIb** was reacted with the N-terminus in the presence of DIPEA in DMF at RT for 4 h. Then, the Fmoc group of 4Aph[5] was removed and reacted with **IIIa** (in **11**−**13**) and with **IIIc** (in **14**).

Synthesis of [D-4Aph(CO−NH−OH)^6]degarelix (15). The fully protected [Ac-D-2Nal-D-4Cpa-D-3Pal-Ser(Bzl)-4Aph(L-Hor)-D-4Aph(Fmoc)-Leu-ILys(Z)-Pro-D-Ala]MBHA resin was first synthesized manually on MBHA resin (1 g, 0.33 mmol/g substitution). The Fmoc side chain protecting group of D-4Aph[6] was then removed with 30% piperidine in NMP (20 min). The reaction of the exposed D-4Aph amino group with an excess of urea donor **IIIb** and the workup and purification of the completed peptide were performed as described above. Yield of **15** after purification was 109 mg (66.19 μmol, 20.07%).

Analogues **16** and **17** were obtained in comparable yields using this procedure and derivatization of orthogonal amino group of D-4Aph with the appropriate urea donors (**IIIa** or **IIIc**) on the resin. Analogues **18**−**21** were synthesized the same way as **11**−**14** starting with [Boc-D-2Nal-D-4Cpa-D-3Pal-Ser(Bzl)-4Aph(L-Hor)-D-4Aph-(Fmoc)-Leu-ILys(Z)-Pro-D-Ala]MBHA resin.

Synthesis of [4Aph(CO−(C_2H_4O)$_3$-C_2H_4-NH−CO−NH−OCH_3)^5]degarelix (10). This analogue was synthesized using the same protocol used for **7** except that to the deprotected orthogonal amino group of 4Aph,[5] Fmoc-12-amino-4,7,10-trioxadodecanoic acid (1 mmol) was coupled with DIC (1 mmol) and HOBt (1 mmol) in NMP. After removal of the Fmoc side chain protection of 12-amino-4,7,10-trioxadodecanoic acid with 30% piperidine in NMP (20 min), the freed amino group was reacted with an excess of urea donor **IIIa** (1.65 mmol) in the presence of DIPEA (2.31 mmol) in DMF (5 mL) at RT for 4 h. The completed resin-bound peptide was then cleaved from the resin and purified by preparative RP-HPLC as described above. Yield of **10** after purification was 52 mg (29.43 mol, 8.92%).

In the synthesis of analogue **25**, Fmoc-21-amino-4,7,10,13,16,-19-hexaoxaheneicosanoic acid was coupled to the orthogonal amino function of D-4Aph[6] during the chain elongation on the resin. The urea donor **IIIa** at position 5 and 6 was then introduced at the end of the synthesis prior to HF cleavage.

Synthesis of [4Aph(CO−NH−OCH_3)^5,D-4Aph(CO−NH−OCH_3)^6]degarelix (22). Analogue **22** was derived from the fully protected [Ac-D-2Nal-D-4Cpa-D-3Pal-Ser(Bzl)-4Aph(Fmoc)-D-4Aph(Fmoc)-Leu-ILys(Z)-Pro-D-Ala]MBHA-resin (1 g; 0.33 mmol/substitution). The 4-amino function of 4Aph[5] and D-4Aph[6] were freed with 30% piperidine in NMP and then reacted with excess of urea donor **IIIa** (3.30 mmol) in the presence of DIPEA

(4.62 mmol) in DMF (10 mL) at RT for 4 h. The completion of the reaction was monitored by ninhydrin test. The completed resin-bound peptide was then cleaved and purified by preparative RP-HPLC using the same conditions described for analogue **3**. Yield of **22** after purification was 82 mg (51.45 mol, 15.59%).

Analogues **29** and **31** were obtained using this procedure and D-Gln substitution at position 3 and Orn(IGly) substitution[2] at position 8, respectively. Analogue **24** was prepared using urea donor **IIIc**. In the synthesis of analogue **30**, D-Dab(Fmoc) was introduced at position 3 during the chain elongation on resin. The Fmoc protection of D-Dab[3]/4Aph[5]/D-4Aph[6] was removed with 30% piperidine in NMP and then the freed amino group was reacted with the excess of urea donor **IIIa** (4.95 mmol) in the presence of DIPEA (6.93 mmol) in DMF (10 mL) at RT for 4 h.

Analogues **26**−**28** were synthesized the same way as **22** except the deblocked N-terminus of the resin-bound decapeptide [Boc-D-2Nal-D-4Cpa-D-3Pal-Ser(Bzl)-4Aph(Fmoc)-D-4Aph(Fmoc)-Leu-ILys(Z)-Pro-D-Ala] was coupled with the appropriate capping groups such as butyric acid (in **26**), octanoic acid (in **27**), and urea donor **IIIc** (in **28**) instead of acetic anhydride.

Synthesis of [N^{α}-Me-4Aph(CO−NH−OCH_3)[5],D-4Aph(CO−NH−OCH_3)[6]]degarelix (23). We used the methodology of Kaljuste et al.[39] for the introduction of a methyl group at the N^{α}-amino function of 4Aph[5] on the resin. In short, the hexapeptide [Boc-4Aph-(Fmoc)-D-4Aph(Fmoc)-Leu-ILys(Z)-Pro-D-Ala]MBHA-resin was built on the MBHA resin (1 g; 0.33 mmol/substitution). The N^{α}-Boc group of 4Aph was freed with 60% TFA in DCM, and the neutralized amino function was alkylated by shaking the resin with 4 equiv of Dod-Cl (4,4′-dimethoxybenzhydryl chloride)[40] in the presence of 4 equiv of DIPEA in DCM for 1 h. The resin was sequentially washed with DCM, triethylamine solution (10% in DCM), methanol, and DCM. A formalin solution (37%, 8 mL) and 0.15 mL of acetic acid in NMP (15 mL) were added to the resin, the mixture was shaken for 5 min followed by the addition of $NaCNBH_3$ (350 mg) to the reaction mixture, and that mixture was shaken for an additional 0.5 h. The resin was washed with DCM, and for completeness of the reaction, the reductive methylation was repeated. The removal of the Dod group with TFA (60% in DCM) provided the corresponding N^{α}-methylated hexapeptide on the resin. Then Boc-Ser(Bzl) (1 mmol) was coupled to N^{α}-methyl-4Aph[5] using bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBrop) (1 mmol)[41] and DIPEA (3 mmol) in NMP. Further chain elongation with the three N-terminal amino acids, acetylation, Fmoc-deprotection of 4Aph[5], and D-4Aph[6] derivatization of these orthogonal amino functions with **IIIa** gave the resin-bound peptide precursor. The workup and purification of the completed peptide was performed as described above. Yield of **23** was 22 mg (13.68 μmol, 4.15%).

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 L) 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. 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 alpha 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 g streptomycin per mL medium), and L-glutamine (2 mM). The cells were harvested and plated at 5×10^4 cells per well in a volume of 80 L 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, were 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 measured on a Molecular Devices Analyst after a 10 min incubation at RT in the absence of direct light.

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. The geometric mean of the IC_{50} from at least two independent experiments is reported for each compound.

Antiovulatory Assay (AOA). The AOA was carried out as described by Corbin and Beattie[43] using an aqueous vehicle; cycling rats (250−300 g at the time of the assay) were injected sc with the peptides dissolved in saline or 5% DMSO in saline (200 μL) at noon on proestrus. Results are expressed in terms of the dosage in micrograms/rat (rats ovulating/total number of treated rats).

Histamine release assay was carried out according to published protocols.[30,44] Results are expressed in terms of the concentration of peptide necessary for 50% histamine release by mast cells.

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References

(1) Jiang, G.; Stalewski, J.; Galyean, R.; Dykert, J.; Schteingart, C.; Broqua, P.; Aebi, A.; Aubert, M. L.; Semple, G.; Robson, P.; Akinsanya, K.; Haigh, R.; Riviere, P.; Trojnar, J.; Junien, J. L.; Rivier, J. E. GnRH antagonists: A new generation of long acting analogues incorporating urea functions at positions 5 and 6. *J. Med. Chem.* **2001**, *44*, 453−467.

(2) Samant, M. P.; Gulyas, J.; Hong, D. J.; Croston, G.; Rivier, C.; Rivier, J. Iterative approach to the discovery of novel degarelix analogues: substitutions at positions 3, 7 and 8. Part II. *J. Med. Chem.* **2005**, *48*, 4851−4860.

(3) Rivier, J. E.; Jiang, G.; Porter, J.; Hoeger, C.; Craig, A. G.; Corrigan, A. Z.; Vale, W. W.; Rivier, C. L. GnRH antagonists: novel members of the azaline B family. *J. Med. Chem.* **1995**, *38*, 2649−2662.

(4) Theobald, P.; Porter, J.; Rivier, C.; Corrigan, A.; Perrin, M.; Vale, W.; Rivier, J. Novel gonadotropin releasing hormone antagonist: Peptides incorporating modified N^{ω}-cyanoguanidino moieties. *J. Med. Chem.* **1991**, *34*, 2395−2402.

(5) Matsuo, H.; Baba, Y.; Nair, R. M.; Arimura, A.; Schally, A. V. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 1334−1339.

(6) Lunenfeld, B.; Insler, V. *GnRH Analogues. The State of the Art 1993*; The Parthenon Publishing Group: Carnforth, Lancaster, 1993; pp 1−156.

(7) Lunenfeld, B.; Insler, V. *GnRH Analogues. The State of the Art 1996*; The Parthenon Publishing Group: Carnforth, Lancaster, 1996; pp 1−190.
(8) Lunenfeld, B.; Insler, V. Future trends in infertility treatment: challenges ahead. *Fertil. Steril.* **1997**, *68*, 977−980.
(9) Lunenfeld, B. *GnRH Analogues: The State of the Art at the Millennium*; The Parthenon Publishing Group: New York, 1999; pp 1−136.
(10) Jiang, G.; Stalewski, J.; Galyean, R.; C., S.; Broqua, P.; Aebi, A.; Aubert, M. L.; Semple, G.; Akinsanya, K.; Haigh, R.; Riviere, P.; Trojnar, J.; Junien, J. L.; Rivier, J. E. Novel, water soluble and long acting GnRH antagonists. *Peptides for the New Millennium; Proceedings of the Sixteenth American Peptide Symposium*; Kluwer Academic Publishers: Minneapolis, MN, 2000; pp 658−659.
(11) Greenwald, R. B.; Choe, Y. H.; McGuire, J.; Conover, C. D. Effective drug delivery by PEGylated drug conjugates. *Adv. Drug Delivery Rev.* **2003**, *55*, 217−250.
(12) Kurtzhals, P.; Havelund, S.; Jonassen, I.; Kiehr, B.; Larsen, U. D.; Ribel, U.; Markussen, J. Albumin binding of insulins acylated with fatty acids: characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect in vivo. *Biochem. J.* **1995**, *312* (Pt 3), 725−731.
(13) Sasaki, S.; Cho, N.; Nara, Y.; Harada, M.; Endo, S.; Suzuki, N.; Furuya, S.; Fujino, M. Discovery of a Thieno[2,3-d]pyrimidine-2,4-dione Bearing a p-Methoxyureidophenyl Moiety at the 6-Position: A Highly Potent and Orally Bioavailable Non-Peptide Antagonist for the Human Luteinizing Hormone-Releasing Hormone Receptor. *J. Med. Chem.* **2003**, *46*, 113−124.
(14) Stewart, J. M.; Young, J. D. Solid-Phase Peptide Synthesis. *Solid-Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984; p 176.
(15) Kim, J.-M.; Bi, Y.; Paikoff, S. J.; Schultz, P. G. The solid-phase synthesis of oligoureas. *Tetrahedron Lett.* **1996**, *37*, 5305−5308.
(16) Wang, G. T.; Chen, Y.; Wang, S.; Sciotti, R.; Sowin, T. Solid-phase synthesis of ureas of secondary amines via carbamoyl chloride. *Tetrahedron Lett.* **1997**, *38*, 1895−1898.
(17) Hutchins, S. M.; Chapman, K. T. A strategy for urea linked diamine libraries. *Tetrahedron Lett.* **1995**, *36*, 2583−2586.
(18) Zheng, C.; Combs, A. P. A general method for the solid-phase synthesis of unsymmetrical tri- and tetrasubstituted ureas. *J. Comb. Chem.* **2002**, *4*, 38−43.
(19) Acharya, A. N.; Ostresh, J. M.; Houghten, R. A. Solid-phase synthesis of substituted imidazoline-tethered 2,3-diketopiperazines, cyclic ureas, and cyclic thioureas. *J. Comb. Chem.* **2001**, *3*, 612−623.
(20) Burgess, K.; Ibarzo, J.; Linthicum, D. S.; Russell, D. H.; Shin, H.; Shitangkoon, A.; Totani, R.; Zhang, A. J. Solid-phase syntheses of oligoureas. *J. Am. Chem. Soc.* **1997**, *119*, 1556−1564.
(21) Neumayer, D. A.; Belot, J. A.; Feezel, R. L.; Reedy, C.; Stern, C. L.; Marks, T. J.; Liable-Sands, L. M.; Rheingold, A. L. Approaches to Alkaline Earth Metal-Organic Chemical Vapor Deposition Precursors. Synthesis and Characterization of Barium Fluoro-beta-ketoiminate Complexes Having Appended Polyether "Lariats". *Inorg. Chem.* **1998**, *37*, 5625−5633.
(22) Kaljuste, K.; Undén, A. New method for the synthesis of *N*-methyl amino acids containing peptides by reductive methylation of amino groups on the solid phase. *Int. J. Pept. Protein Res.* **1993**, *42*, 118−124.
(23) Samant, M. P.; Hong, D. J.; Croston, G.; Rivier, C.; Rivier, J. Novel gonadotropin-releasing hormone antagonists with substitutions at position 5. *Biopolymers* **2005**, *80*, 386-391.
(24) Beattie, C. W.; Corbin, A.; Foell, T. J.; Garsky, V.; McKinley, W. A.; Rees, R. W. A.; Sarantakis, D.; Yardley, J. P. Luteinizing hormone-releasing hormone. Antiovulation activity of analogues substituted in position 2 and 6. *J. Med. Chem.* **1975**, *18*, 1247.
(25) Rivier, J.; Porter, J.; Rivier, C.; Perrin, M.; Corrigan, A. Z.; Hook, W. A.; Siraganian, R. P.; Vale, W. W. New effective gonadotropin releasing hormone antagonists with minimal potency for histamine release in vitro. *J. Med. Chem.* **1986**, *29*, 1846−1851.
(26) Hutchinson, J.; Liao, S.; Smith, J.; Phillips, A. Clinical pharmacology of azaline B. In *Gynecological Endocrinology; The 5th International Symposium on GnRH Analogues in Cancer and Human Reproduction*; Parthenon Publishing: Geneva, Switzerland, 1999.
(27) Haviv, F.; Fitzpatrick, T. D.; Nichols, C. J.; Swenson, R. E.; Mort, N. A.; Bush, E. N.; Diaz, G.; Nguyen, A. T.; Holst, M. R.; Cybulski, V. A.; Leal, J. A.; Bammert, G.; Rhutasel, N. S.; Dodge, P. W.; Johnson, E. S.; Cannon, J. B.; Knittle, J.; Greer, J. The effect of NMeTyr^5 substitution in luteinizing hormone-releasing hormone antagonists. *J. Med. Chem.* **1993**, *36*, 928−933.
(28) Rivier, J.; Porter, J.; Hoeger, C.; Theobald, P.; Craig, A. G.; Dykert, J.; Corrigan, A.; Perrin, M.; Hook, W. A.; Siraganian, R. P.; Vale, W.; Rivier, C. Gonadotropin releasing hormone antagonists with N^{ω}-triazolyl-ornithine, -lysine or, -para-aminophenylalanine residues at positions 5 and 6. *J. Med. Chem.* **1992**, *35*, 4270−4278.
(29) Schmidt, F.; Sundaram, K.; Thau, R. B.; Bardin, C. W. [Ac-D-Nal-(2)^1, 4FD-Phe^2, D-Trp^3, D-Arg^6]LHRH, a potent antagonist of LHRH, produces transient edema and behavioral changes in rats. *Contraception* **1984**, *29*, 283−289.
(30) Karten, M. J.; Hook, W. A.; Siraganian, R. P.; Coy, D. H.; Folkers, K.; Rivier, J. E.; Roeske, R. W. In vitro histamine release with LHRH analogs. *LHRH and Its Analogs, Contraceptive and Therapeutic Applications, Part 2*; MTP Press: Ltd.: Lancaster, Boston, The Hague, 1987; pp 179−190.
(31) Stanfield, C. F.; Felix, A. M.; Danho, W. The preparation of N^{α}-t-BOC-2,3-diaminopropionic acid (DPR) and of N^{α}-t-BOC-2,4-diaminobutyric acid (DBR) derivatives suitable for solid-phase peptide synthesis. *Org. Prep. Proc. Int.* **1990**, *22*, 597−603.
(32) Miller, C. S.; Gordon, J. T.; Engelhardt, E. L. Dihydroörotic acid. *J. Am. Chem. Soc.* **1953**, *75*, 6086−6087.
(33) Mazaleyrat, J.-P.; Rage, I.; Mouna, A. M.; Savrda, J.; Wakselman, M. Peptoid mimics of a C_2-symmetric inhibitor of the HIV-1 protease. *Biorg. Med. Chem. Lett.* **1994**, *4*, 1281−1284.
(34) Ljungqvist, A.; Feng, D.-M.; Tang, P.-F. L.; Kubota, M.; Okamoto, T.; Zhang, Y.; Bowers, C. Y.; Hook, W. A.; Folkers, K. Design, synthesis and bioassays of antagonists of LHRH which have high antiovulatory activity and release negligible histamine. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 849−856.
(35) Stewart, J.; Pèna, C.; Matsueda, G. R.; Haris, K. Some improvements in the solid-phase synthesis of large peptides. *The 14th European Peptide Symposium*; Editions de l'Université de Bruxelles, Bruxelles (Belgique): Wépion, Belgium, 1976; pp 285−290.
(36) Rivier, J.; Vale, W.; Burgus, R.; Ling, N.; Amoss, M.; Blackwell, R.; Guillemin, R. Synthetic luteinizing hormone releasing factor analogues Series of short chain amide LRF homologues converging to the amino terminus. *J. Med. Chem.* **1973**, *16*, 545−549.
(37) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595−598.
(38) Miller, C.; Rivier, J. Peptide chemistry: Development of high-performance liquid chromatography and capillary zone electrophoresis. *Biopolymers Pept. Sci.* **1996**, *40*, 265−317.
(39) Kaljuste, K.; Undén, A. A new general solid-phase method for the synthesis of backbone-to-backbone cyclized peptides. *Int. J. Peptide Protein Res.* **1994**, *43*, 505−511.
(40) Hanson, R. W.; Law, H. D. Substituted diphenylmethyl protecting groups in peptide synthesis. *Journal of The Chemical Society*; The Chaucer Press: Ltd.: London, 1965; pp 7285−7297.
(41) Coste, J.; Frérot, E.; Jouin, P. Coupling *N*-methylated amino acids using PyBroP1 and PyCloP halogenophosphonium salts: mechanism and fields of application. *J. Org. Chem.* **1994**, *59*, 2437−2446.
(42) Kay, T. W.; Chedrese, P. J.; Jameson, J. L. Gonadotropin-releasing hormone causes transcriptional stimulation followed by desensitization of the glycoprotein hormone alpha promoter in transfected alpha T3 gonadotrope cells. *Endocrinology* **1994**, *134*, 568−573.
(43) Corbin, A.; Beattie, C. W. Inhibition of the pre-ovulatory proestrous gonadotropin surge, ovulation and pregnancy with a peptide analogue of luteinizing hormone releasing hormone. *Endocr. Res. Commun.* **1975**, *2*, 1−23.
(44) Hook, W. A.; Karten, M.; Siraganian, R. P. Histamine release by structural analogs of LHRH. *Federation Proc.* **1985**, *44*, 1323.

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