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Novel analogues of degarelix incorporating hydroxy-, methoxy- and pegylated-urea moieties at positions 3, 5, 6 and the N-terminus:

Part III†

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Abstract

Novel degarelix (Fe200486) analogues were screened for antagonism of GnRH-induced response (IC₅₀) 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 (IC₅₀ = 0.69 nM) and degarelix (IC₅₀ = 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, *p*-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) 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 B⁴ are three structurally closely related antagonists of GnRH with high therapeutic index and a common scaffold: Ac-D-2Nal¹-D-4Cpa²-D-3Pal³-Ser⁴-4Aph(X)⁵-D-4Aph(Y)⁶-Leu⁷-Ilys⁸-Pro⁹-D-Ala¹⁰-NH₂; in degarelix, X = *l*-hydroorotyl (Hor) and Y = carbamoyl (Cbm); in acyline, X = Y = acetyl (Ac) and in azaline

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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. Early¹ 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 4Aph⁵ by *L*-hydroroctic acid in conjunction with carbamoylation of *D*-4Aph⁶ 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-ethyleneglycol moieties (PEG)¹¹ or of hydrophobic side chains using fatty acid functionalities¹² respectively, may modulate solubility and bio-availability. For example, the introduction of a *p*-methoxyureidophenyl moiety in the non-peptide 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 study¹⁰ that demonstrated that the non aromatic substitution of *D*-3Pal in 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 (NaCNBH₃) 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 described¹⁴ 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 carbamoylation¹ 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 methods¹⁶⁻²⁰ reported to assemble ureas on solid support.

In the present study, we synthesized three urea donors as 4-nitrophenyl-*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-ethoxy-ethoxy)-ethylamine **Ic** was prepared from 2-(2-ethoxy-ethoxy)-ethanol (carbitol) by stepwise transformation (OH→OTos→N₃→NH₂) as described in the literature.²¹ The benzylprotection 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*^α-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, 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 IC₅₀, 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, ten days after castration, rats (5 or more per group) were injected 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 pre-dose 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 radioimmunoassay²⁵ (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 antioviulatory 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-diamino propionic acid to 2, 4-diamino butyric 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 4Aph⁵ with hydroxy- (**7**) and methoxy- (**8**) ureas as well as congeneric polyethylene glycols (**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 4Aph⁵ with polyethylene 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 as well as 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*-4Aph⁶. 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 physico-chemical 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 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 4Aph⁵ or *D*-4Aph⁶ (see **8** and **16**, respectively) was not as favorable *in vivo* as the *L*-hydroxytyrosyl at 4Aph⁵ and a carbamoyl group at *D*-4Aph⁶ 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 mono-substitutions. With **23**, we examined 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 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-Arg²⁹ and Nal-Glu²⁵ 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 para-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 4Aph⁵ did not materialize, suggesting a relatively important contribution of the methoxy-carbamoyl group on the para-amino function of 4Aph⁵ to histamine release. The observation that **9** and **14**, both bearing a PEG group on 4Aph⁵, were essentially inactive in that assay is encouraging (Table 2).

Further, the double substitution (short PEG units) on 4Aph⁵ and *D*-4Aph⁶ (**24**) and increasing the length of PEG group on *D*-4Aph⁶ (**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 4Aph⁵ and *D*-4Aph⁶ 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 **29-30** 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 intra molecular hydrogen bonding. All three analogues were short acting with IC₅₀s between 1.5 to 3.0 nanomolar.

Conclusion

All analogues described here have antagonist potency (IC₅₀) 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 RT > than 33 min (**12, 14, 19, 24, 26-28**) are significantly shorter acting than degarelix (RT 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-L-4Aph(L-Hor)¹ were synthesized according to published procedures. 2-(2-ethoxy-ethoxy)-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 resin³⁵ 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.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, H₂O, 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 C₁₈ (0.21 × 15 cm), buffer A: 0.1% TFA in H₂O, buffer B: 0.1% TFA in 60% ACN/40% H₂O, 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%; ¹H NMR (CDCl₃) δ 3.85 (s, 3H, CH₃), 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%; ¹H NMR (CDCl₃) δ 4.97 (s, 2H, CH₂), 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 t₁ = 5.2 min, purity = 97%; ¹H NMR (CDCl₃) δ 1.28 (t, 3H, CH₃), 3.40-3.72 (m, 10H, CH₂'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 methodology¹⁴ 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 1-2 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 4Aph⁵, 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 μ mol, 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 μ mol, 10.2%).

Analogues **8-9** 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 like 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 4Aph⁵ 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*-4Aph⁶ 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 μ mol, 20.07%).

Analogues **16-17** 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-(C₂H₄O)₃-C₂H₄-NH-CO-NH-OCH₃)⁵]degarelix (**10**)

This analogue was synthesized using the same protocol used for **7** except that to the deprotected orthogonal amino group of 4Aph⁵, 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 μ 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 -4Aph⁶ 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-OCH₃)⁵, D -4Aph(CO-NH-OCH₃)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 4Aph⁵ and D -4Aph⁶ 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 μ mol, 15.59%).

Analogues **29** and **31** were obtained using this procedure and D -Gln substitution at position 3 and Orn(IGly) substitution² 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 -Dab³/4Aph⁵/ D -4Aph⁶ 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 like 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-OCH₃)⁵, D -4Aph(CO-NH-OCH₃)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 4Aph⁵ 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 sodium cyanoborohydride (NaCNBH₃, 350 mg) to the reaction mixture, and that mixture was shaken for an additional half hour. 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-4Aph⁵ 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 4Aph⁵ and D -4Aph⁶ derivatization of these orthogonal amino function 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 $\mu\text{g}/\text{rat}$ in 50 μL of aqueous buffer. Blood was sampled from the tail tip (300 μL) at the given times. Plasma LH was determined by 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 μg 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 μL per well in white 96 well culture plates. The cells were incubated at 37 $^{\circ}\text{C}$ under 5% CO_2 overnight for assay the next day.

IC₅₀ 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 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$ 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 IC₅₀, 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 IC₅₀ value was derived by non-linear 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 IC₅₀ from at least two independent experiments is reported for each compound.

Antiovolatory assay (AOA)

The AOA was carried out as described by Corbin and Beattie⁴³ 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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Abbreviations

Ac, acetyl
 ACN, acetonitrile
 4Aph, 4-aminophenylalanine
 AOA, antioviulatory 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*^α-isopropyllysine
 LH, luteinizing hormone
 MBHA, *p*-methylbenzhydramine
 2Nal, 3-(2-naphthyl)-alanine
 NMP, *N*-methylpyrrolidinone
 3Pal, 3-(3-pyridyl)-alanine
 PEG, polyethyleneglycol
 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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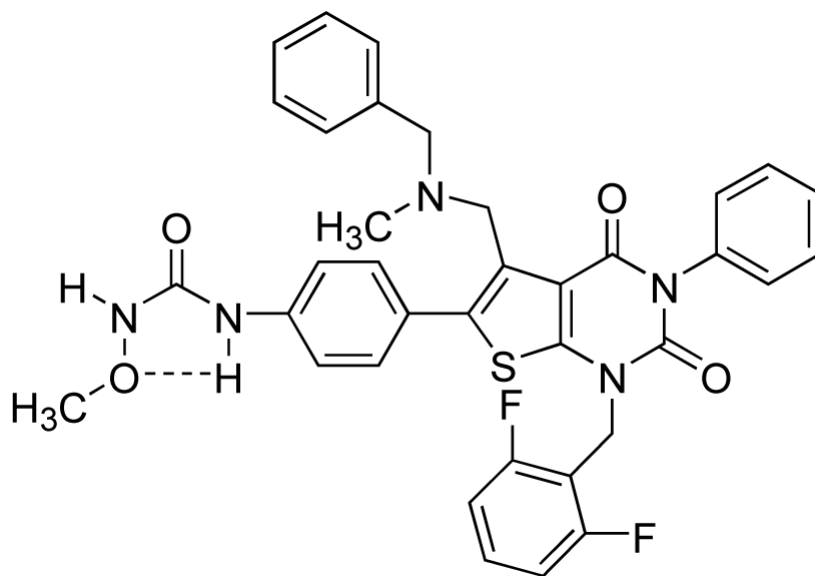
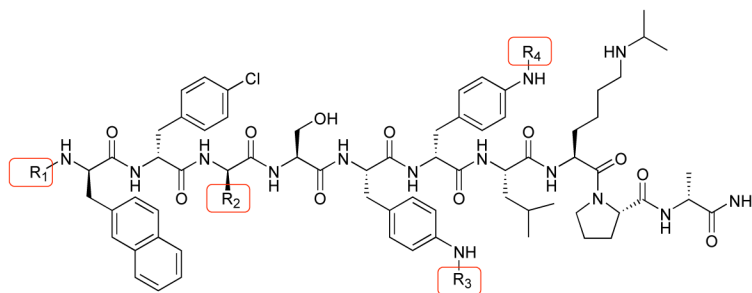
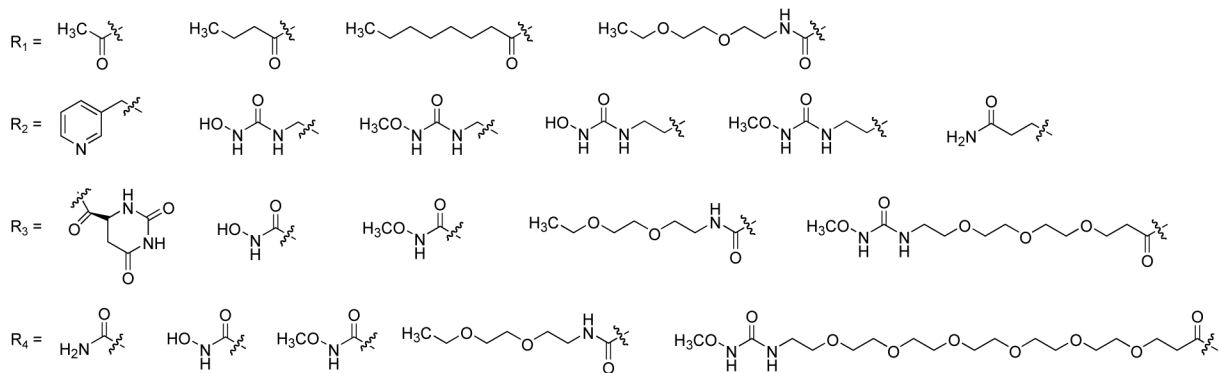


Figure 1.
Chemical structure of a non-peptide GnRH antagonist.¹³

(a)



(b)

**Figure 2.**

a) Amino acid scaffold upon which acyline, azaline B and degarelix are built; b) Structures of the substitutions incorporated at R₁ to R₄.

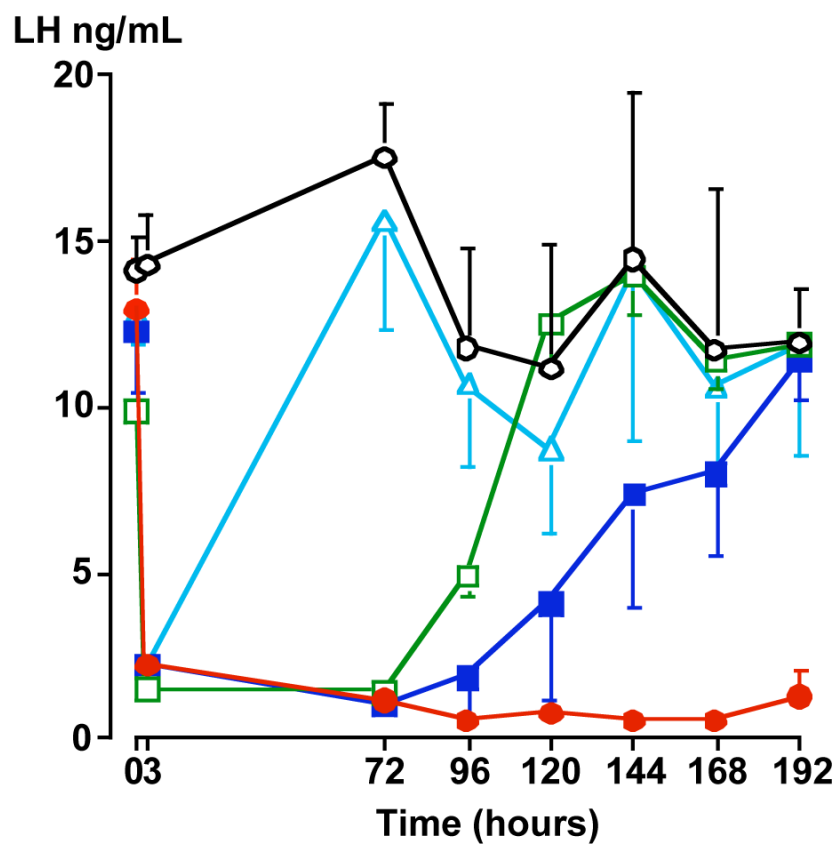
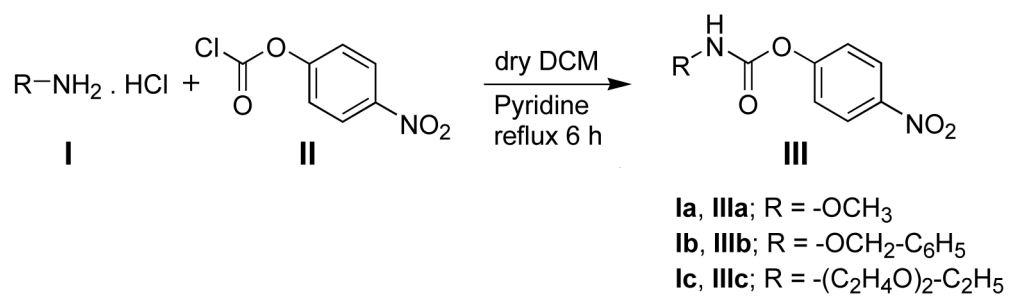


Figure 3. Inhibition of LH secretion after sc administration of analogues (**1**, **8**, **9** and **25**). Total dose was 50 μ g in 50 μ L 5% mannitol containing 0.1% DMSO. Blood samples were collected at the times shown on the abscissa. Results are mean plasma LH levels ($n = 5-8$ rats) \pm SEM. The SEM, where not appearing, are encompassed within the size of the symbols in the graph. Vehicle ; degarelix (**1**) \bullet ; **8** \square ; **9** \blacksquare ; **25** \triangle .

**Scheme 1.**

Synthesis of 4-nitrophenyl-*N*-substituted-carbamates as urea donors for SPPS.

Table 1

Physicochemical and biological characterization of GnRH antagonists

Compound	Purity	t _R ^c (min)	MS ^d (M + H) ⁺	pIC ₅₀ ^e Avg. ± SEM	IC ₅₀ ^f (nM)	Duration of action ^g
$\text{p-}[\text{D}-2\text{Nal}^1, \text{D}-4\text{Cpa}^2, \text{D}-3\text{Pal}^3, 4\text{Aph}(\text{C}-\text{Hor})^5, \text{D}-4\text{Aph}(\text{Cbm})^6, \text{Ily}^8, \text{D}-\text{Ala}^{10}] \text{GnRH (degarelix)}$	96	23.8	1631.8	9.24 ± 0.16	0.58	Very long
$\text{p-}[\text{D}-2\text{Nal}^1, \text{D}-4\text{Cpa}^2, \text{D}-3\text{Pal}^3, 4\text{Aph}(\text{Ac})^5, \text{D}-4\text{Aph}(\text{Ac})^6, \text{Ily}^8, \text{D}-\text{Ala}^{10}] \text{GnRH (acviline)}$	98	28.2	1532.7	9.16 ± 0.06	0.69	Long
$\text{Dab}(\text{CO}-\text{NH}-\text{OH})^2 \text{degarelix}$	97	21.7	1628.7	8.46 ± 0.01	3.46	Short
$\text{Dab}(\text{CO}-\text{NH}-\text{OH})^2 \text{degarelix}$	99	21.3	1642.8	8.57 ± 0.12	2.68	Short
$\text{Dap}(\text{CO}-\text{NH}-\text{OCH}_3)^3 \text{degarelix}$	95	23.3	1642.8	8.50 ± 0.05	3.16	Short
$\text{Dap}(\text{CO}-\text{NH}-\text{OCH}_3)^3 \text{degarelix}$	98	22.8	1656.8	8.77 ± 0.02	1.69	Short
$\text{Dab}(\text{CO}-\text{NH}-\text{OCH}_3)^3 \text{degarelix}$	99	24.4	1550.7	9.07 ± 0.19	0.86	Short
$\text{p}[\text{CO}-\text{NH}-\text{OCH}_3]^3 \text{degarelix}$	99	26.3	1564.7	9.22 ± 0.03	0.60	Long
$\text{p}[\text{CO}-\text{NH}-\text{OCH}_3]^3 \text{degarelix}$	98	29.1	1650.8	8.50 ± 0.02	3.16	Long*
$\text{p}[\text{CO}-\text{NH}-\text{OCH}_3]^3 \text{degarelix}$	98	26.1	1767.9	8.97 ± 0.13	1.06	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{CH}_2(\text{CH}_2)_2-\text{CO}-\text{degarelix}$	98	30.1	1592.8	8.83 ± 0.31	1.46	Long*
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{CH}_2(\text{CH}_2)_6-\text{CO}-\text{degarelix}$	97	40.4	1648.8	8.38 ± 0.14	4.17	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{C}_2\text{H}_4-\text{C}_2\text{H}_4-\text{NH}-\text{CO}-\text{degarelix}$	96	30.6	1681.8	8.53 ± 0.47	2.98	Long*
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{C}_2\text{H}_4-\text{C}_2\text{H}_4-\text{NH}-\text{CO}-\text{degarelix}$	95	33.3	1767.9	8.95 ± 0.04	1.12	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{C}_2\text{H}_4-\text{C}_2\text{H}_4-\text{NH}-\text{CO}-\text{degarelix}$	97	23.7	1647.7	9.09 ± 0.12	0.80	Long*
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{degarelix}$	98	26.0	1661.8	9.29 ± 0.12	0.51	Long*
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{degarelix}$	98	28.3	1747.8	9.06 ± 0.11	0.88	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{degarelix}$	98	29.7	1689.8	8.67 ± 0.26	2.13	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{CH}_2(\text{CH}_2)_2-\text{CO}-\text{degarelix}$	98	39.6	1745.9	8.02 ± 0.18	9.47	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{CH}_2(\text{CH}_2)_6-\text{CO}-\text{degarelix}$	95	29.9	1778.8	8.79 ± 0.25	1.61	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{C}_2\text{H}_4-\text{C}_2\text{H}_4-\text{NH}-\text{CO}-\text{degarelix}$	95	32.1	1864.9	8.34 ± 0.37	4.55	ND
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{C}_2\text{H}_4-\text{C}_2\text{H}_4-\text{NH}-\text{CO}-\text{degarelix}$	95	28.5	1594.8	9.04 ± 0.01	0.91	Long*
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{degarelix}$	99	28.9	1608.8	9.15 ± 0.05	0.70	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{degarelix}$	96	33.5	1767.0	8.69 ± 0.31	2.04	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{degarelix}$	98	28.5	1929.9	8.78 ± 0.24	1.67	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{degarelix}$	99	32.3	1622.8	8.86 ± 0.35	1.37	Short
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{degarelix}$	99	42.5	1678.8	8.30 ± 0.34	4.99	Inactive
$\text{Ac}-[4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3]-\text{degarelix}$	95	37.6	1883.9	8.59 ± 0.22	2.55	Short
$\text{Gln}^2, 4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^3, \text{D}-4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^6 \text{degarelix}$	99	27.6	1574.7	8.53 ± 0.24	2.98	Short
$\text{Dab}(\text{CO}-\text{NH}-\text{OCH}_3)^3, 4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^5, \text{D}-4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^6 \text{degarelix}$	98	28.9	1619.6	8.83 ± 0.04	1.49	Short
$\text{p}[\text{CO}-\text{NH}-\text{OCH}_3]^3, \text{D}-4\text{Aph}(\text{CO}-\text{NH}-\text{OCH}_3)^6, \text{Orn}(\text{Gly})^8 \text{degarelix}$	99	30.9	1637.8	8.82 ± 0.20	1.53	Short

^a purity determined by HPLC using buffer system A; TEAP, pH 2.30, buffer system B, 60% CH₃CN/40% A under gradient conditions (30% to 80% B over 50 min), at flow rate of 0.2 mL/min ac C18 column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm.

^b 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. 10 mM sodium phosphate (85:15, H₂O:CH₃CN), pH 2.50, on a Agilent μSilI bare fused-silica capillary (75 μm i.d. × 40 cm length). Detection at 214 nm.

^c retention time determined by HPLC using buffer system A; TEAP, pH 2.30, buffer system B, 60% CH₃CN/40% A under gradient conditions (30% to 80% B over 50 min), at flow rate of 0.2 mL/min ac C18 column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm.

^d molecular weight determined by HPLC using buffer system A; TEAP, pH 2.30, buffer system B, 60% CH₃CN/40% A under gradient conditions (30% to 80% B over 50 min), at flow rate of 0.2 mL/min ac C18 column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm.

^e pIC₅₀ 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. 10 mM sodium phosphate (85:15, H₂O:CH₃CN), pH 2.50, on a Agilent μSilI bare fused-silica capillary (75 μm i.d. × 40 cm length). Detection at 214 nm.

^f IC₅₀ 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. 10 mM sodium phosphate (85:15, H₂O:CH₃CN), pH 2.50, on a Agilent μSilI bare fused-silica capillary (75 μm i.d. × 40 cm length). Detection at 214 nm.

^g duration of action determined by HPLC using buffer system A; TEAP, pH 2.30, buffer system B, 60% CH₃CN/40% A under gradient conditions (30% to 80% B over 50 min), at flow rate of 0.2 mL/min ac C18 column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm.

^h ND = not determined.

ⁱ * = short duration of action (less than 24 h).

^dMass 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.

^eThe 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.

^gCastrated male rat assay. Duration of action: very long = over 80% inhibition of LH release for more than 120 h;

Table 2

Results from two rat peritoneal cell histamine release assays and from the antiovolulatory 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

^aEC₅₀s 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.

^bOne 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

^cAOA, antiovolulatory assay.⁴³ Peptide (2 μg/200 μL) was administered sc.