The Effect of NMeTyr[5] Substitution in Luteinizing Hormone-Releasing Hormone Antagonists[†,‡]

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Received August 5, 1992

Introduction

In humans, as well as in mammals, agonists of luteinizing hormone-releasing hormone (LHRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2), following acute administration, bind to the LHRH receptor in the pituitary gonadotrophs inducing the synthesis and release of gonadotropins. Upon chronic administration, the receptor is down-regulated, resulting in suppression of gonadotropins. This property of suppressing gonadotropin levels has enabled the use of LHRH agonists in the treatment of a variety of endocrine-based disease conditions such as prostate cancer, endometriosis, and precocious puberty.[1-4] However, the initial surge in gonadotropins and therefore sex steroids, following treatment with LHRH agonists, raises some concern in clinical therapy. Since a LHRH antagonist is expected to suppress gonadotropins from the onset and be devoid of a possible initial clinical flare, many attempts have been made over the past 20 years to design a potent and safe antagonist. The discovery of an antagonist suitable for therapeutic use was hampered first by low potency and then by low safety, caused by the property of some LHRH antagonists to degranulate mast cells and release histamine.[5,6] In the last 5 years, several antagonists with low histamine release properties were discovered and taken for clinical studies.[6] To date no known antagonist has proven suitable for therapeutic use.

As part of our earlier efforts to design novel LHRH analogues, we stabilized leuprolide (pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-ProNHEt), a LHRH agonist, against chymotrypsin and intestinal degradation by substituting NMeSer at position 4.[7] Further studies demonstrated that substitution of NMeTyr[5] in leuprolide also stabilized the peptide against enzymatic degradation and improved the pharmacokinetic profile of the compound over the parent by lowering the clearance rate.[8,9] These findings were particularly interesting and consistent with a previous report indicating that the 5-9 fragment of leuprolide was the major metabolite.[10] Consequently, we applied this new information about LHRH agonists to the design of new antagonists. Generally, most of the LHRH antagonists contain five D-amino acids at positions 1, 2, 3, 6, and 10, versus only one D-amino acid at 6 for the agonists. For this reason, the antagonists are expected to be metabolically more stable than the agonists. Nevertheless, recent metabolism studies with RS-26306, a LHRH antagonist in clinical studies, reported the 5-10 fragment as one of the metabolites.[11] Encouraged by our previous finding with the LHRH agonists, we decided to substitute NMeTyr[5] in the structures of several known LHRH antagonists to study their effect on in vitro LHRH receptor binding, inhibition of LH release from rat pituitary cells, release of histamine from rat peritoneal mast cells, and stability against enzymatic degradation. All the compounds were also tested in vivo for suppression of LH in castrate male rats.

Chemical Synthesis

All the peptides were synthesized by solid-phase peptide synthesis techniques (SPPS)[12] starting from the C-terminus with Boc-D-Ala attached to 4-methylbenzhydrylamine resin and sequentially coupling the commercially available Boc-amino acids.[13,14] The synthesis protocol, cleavage of the peptide from the resin, removal of the protecting groups, workup, and HPLC purification were analogous to those described for LHRH agonists in our recent publications.[9,14] The major change was in the coupling time of Boc-D3Pal, which was extended to 6 h. No difficulties were encountered in forming the *N*-methyl peptide bond using the routine coupling conditions. All the peptides were characterized by analytical HPLC, FABMS, and AAA.

Biological Testing

Antagonists were tested in vitro for LHRH receptor binding[13] and for LH release from cultured rat pituitary cells.[13] The binding affinities are reported as pK_I. The LH inhibition potencies for antagonists are reported as pA_2 (for definitions of pK_I and pA_2, see footnote of Table I). For initial characterization of the safety profile, the antagonists were tested for histamine release from rat peritoneal mast cells.[5,15] To evaluate stability of the peptides against enzymatic degradation, compounds were separately tested with chymotrypsin and with an intestinal preparation using the rat jejunal sac model.[14]

All antagonists were tested for LH suppression in castrate male rats following sc administration of a 30 μg/kg dose, dissolved in 1:4 propylene glycol/saline solution, to groups of three animals. Serial blood samples were collected for 24 h after dosing. Plasmas were separated by centrifugation and frozen at -20 °C until assayed. LH levels were measured by radioimmunoassay.[13,16] All the compounds were evaluated as the TFA salts unless otherwise indicated.

Results and Discussion

Effect of NMeTyr[5] on in Vitro Activity. NalGlu (7) is a well-known representative of the third-generation antagonists.[17] This antagonist has been tested in humans and was found to be effective in suppressing gonadotropins and sex steroids; however it suffers from side effects. While

[†] Part of this work was presented as a poster at the 74th Annual Meeting of the Endocrine Society, San Antonio, TX, June 24-27, 1992.

[‡] Abbreviations: The abbreviations for the amino acids are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, 138, 9-37). The symbols represent the L-isomer except when indicated otherwise. Additional abbreviations: D2Nal, D-3-(2-naphthyl)alanine; D3Bal, D-3-(3-benzothienyl)alanine; D4ClPhe, D-3-(4-chlorophenyl)alanine; D3Pal, D-3-(3-pyridyl)alanine; NMeTyr, *N*-methyltyrosine; DLys(Nic), D-lysine(*N*-ε-nicotinyl); Lys(Isp), lysine(*N*-ε-isopropyl); DGlu(AA), 4-(*p*-methoxybenzoyl)-D-2-aminobutyric acid; DHCA, dicyclohexylamine; IND, investigational new drug application; HPLC, high-pressure liquid chromatography; LH, luteinizing hormone; sc, subcutaneous; FABMS, fast atom bombardment mass spectrum; AAA, amino acid analysis.

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0022-2623/93/1836-0928$04.00/0

Table I. In vitro Functional Properties and Solubilities of LHRH Antagonists

NAc-A[1]-D4ClPhe-A[3]-Ser-A[5]-A[6]-Leu-A[8]-Pro-DAlaNH$_2$

compd	substitution	t_R[a]	MH$^+$ [b]	pK_I[c]	pA_2[d]	ED$_{50}$[e]	solubility[f]
1	D4ClPhe[1],D3Bal[3],Tyr[5],DLys[6],Arg[8] Org-30850[g]			10.48 (±0.12)	11.19 (±0.05)	0.62 (±0.38)	<0.1
2	D4ClPhe[1],D3Bal[3],NMeTyr[5],DLys[6],Arg[8]	24.32	1454	10.63 (±0.16)	11.30 (±0.12)	<0.5	>25.0
3	D2Nal[1],D3Pal[3],Tyr[5],DCit[6],Arg[8] SB-75[h]			9.93 (±0.07)	10.45 (±0.05)	1.11 (±0.29)	<1.0
4	D2Nal[1],D3Pal[3],NMeTyr[5],DCit[6],Arg[8]	17.90	1444	10.87 (±0.34)	10.80 (±0.10)	1.65 (±0.37)	12.0
5	D2Nal[1],D3Pal[3],Lys(Nic)[5],DLys(Nic)[6],Lys(Isp)[8] antide[i]			10.21 (±0.24)	10.63 (±0.10)	261 (±39)	1.1
6	D2Nal[1],D3Pal[3],NMeTyr[5],DLys(Nic)[6],Lys(Isp)[8] A-75998	17.62	1535	10.50 (±0.20)	11.23 (±0.14)	10.0 (±3.6)	>20.0
7	D2Nal[1],D3Pal[3],Arg[5],DGlu(AA)[6],Arg[8] NalGlu[j]			10.28 (±0.22)	11.06 (±0.20)	1.11 (±0.12)	

[a] Analytical HPLC retention time in minutes. HPLC conditions are described in the Experimental Section. [b] Values determined by FABMS. [c] The negative logarithm of the concentration of antagonist that inhibits 50% of the binding of ^{125}I-labeled leuprolide to the rat pituitary LHRH receptor. [d] The negative logarithm of the concentration of antagonist that requires 2-fold higher concentration of agonist to release LH from cultured rat pituitary cells. [e] Effective dose of antagonist that gives 50% of maximal release of histamine from rat peritoneal mast cells. Units are microgram per milliliter. [f] The solubility was determined in 5% dextrose, pH 4.5; units are milligram per milliliter. The solubilities of compounds 1 and 2 were measured as the TFA salts, whereas those of compounds 3–6 were measured as the acetate salts. [g] Reference 19. [h] References 20, 21. [i] References 22, 23. [j] Reference 17.

it does not cause any systemic anaphylactic responses, it causes some local skin reaction.[18] We chose NalGlu as the standard in our studies (Table I).

The first antagonist selected for structural modification was Org-30850 (1).[19] This antagonist had pK_I and pA_2 values similar to NalGlu (1 versus 7). Its ED$_{50}$ for histamine release (HR) was about 2-fold lower than the standard. Substitution of NMeTyr^5 in Org-30850 did not affect either the pK_I or the pA_2 values (1 versus 2) and had only a slight effect on HR. The results for compound 2, our first antagonist containing NMeTyr^5, are interesting because they differ from our previous findings with NMeTyr^5 agonists,[9] where 5–10-fold losses in activity were observed as a result of NMeTyr^5 substitutions.

The second target was SB-75 (3), another antagonist which currently is in clinical studies and reported to have a long duration of action in the rat.[20,21] Its NMeTyr^5 analogue (4) showed a 5-fold increase in receptor binding affinity and a 2-fold increase in inhibition of LH in vitro (4 versus 3). In this case, the NMeTyr^5 substitution had virtually the same HR ED$_{50}$ as the parent compound.

The third target was antide (5), another antagonist from the third generation, which is known for its high ED$_{50}$ in HR[22] and long duration of action in the monkey.[23] Antide (5), unlike Org-30850 (2) and SB-75 (3), contains Lys(Isp)^8 and Lys(Nic)^5 instead of Arg^8 and Tyr^5, both residues are believed to be partly responsible for causing release of histamine. Substitution of NMeTyr^5 in antide yielded A-75998 (6). This new antagonist had pK_I and pA_2 values 2- and 4-fold, respectively, higher than antide (6 versus 5). The HR ED$_{50}$ for A-75998 (6) was 10 μg/mL, which is 26-fold lower than antide (5), but 10-fold higher than NalGlu (7).

It is noteworthy that, as is indicated above, in our laboratory NalGlu (7) was more potent than SB-75 (3) in the receptor binding and LH inhibition assays (Table I). These results differ from those described in a recent publication by Rivier et al.[24] wherein they found SB-75 to be more potent than NalGlu in inhibiting LH in vitro by 1.56–1.0 with a range of 1.00–2.45. They also reported for NalGlu and antide, in the receptor binding assay, K_Ds of 0.67 (0.43–1.0) and 0.35 (0.25–0.47) nM, equivalent to pK_I values of 9.2 and 9.46, respectively. These values are over 10-fold lower than ours. The reason for the differences between the data is unclear. However, our pK_I values are consistent with the pA_2 values and have low SEM. The HR ED$_{50}$ values for NalGlu, antide, and A-75998, reported by them,[24] are within 2-fold of ours and thus are consistent.

Antagonists 1–7 were tested against chymotrypsin and intestinal degradation using the rat jejunal sac model, which we previously used to evaluate metabolic stability of LHRH agonists.[14] Antagonists 1–7 were completely stable against enzymatic degradation in both tests. These results indicate that the in vitro assays, which were appropriate for studying metabolic stability of agonists, are unsuitable for antagonists. However, since recent metabolism studies in the rat with the antagonist RS-26306 indicated the presence of (5–10) fragment as one of the metabolites,[11] it is reasonable to speculate that NMeTyr^5 substitution in LHRH antagonists would slow the metabolism rate. Detailed metabolism studies of A-75998 are in progress.

Effect of NMeTyr^5 on in Vivo LH Suppression. A dose of 30 μg/kg of NMeTyr^5-Org-30850 (2) and its parent (1) were administered sc to castrate rats. Both compounds (Figure 1A) were similar in suppressing LH during the first 12 h; after 24 h, compound 2 was 70% back to control, whereas compound 1 continued to suppress. In the same test the NMeTyr^5-SB-75 (4) and its parent (3) suppressed LH very effectively during the first 6 h; afterward the LH levels of antagonist 4 recovered faster than the parent (Figure 1B). The LH suppression of SB-75 (3) was in agreement with the results reported by Bokser et al.[21] For A-75998 (6) and antide (5), the LH suppression was similar during the first 6 h. From 6 through 24 h, the LH levels for antide recovered faster than for A-75998. Again our data for antide are consistent with those reported by Ljungqvist et al.[25] for the dose of 10 μg/rat. NalGlu (7) effectively suppressed LH during the first 8 h, and only 40% of the suppression was restored by 24 h. Rivier et al.[24] also tested NalGlu and antide in male castrated rats using a dose of 10 μg/rat, which is comparable to the 30 μg/kg used by us. With both antagonists, at the 10 μg/rat dose, they also observed LH suppression which lasted less

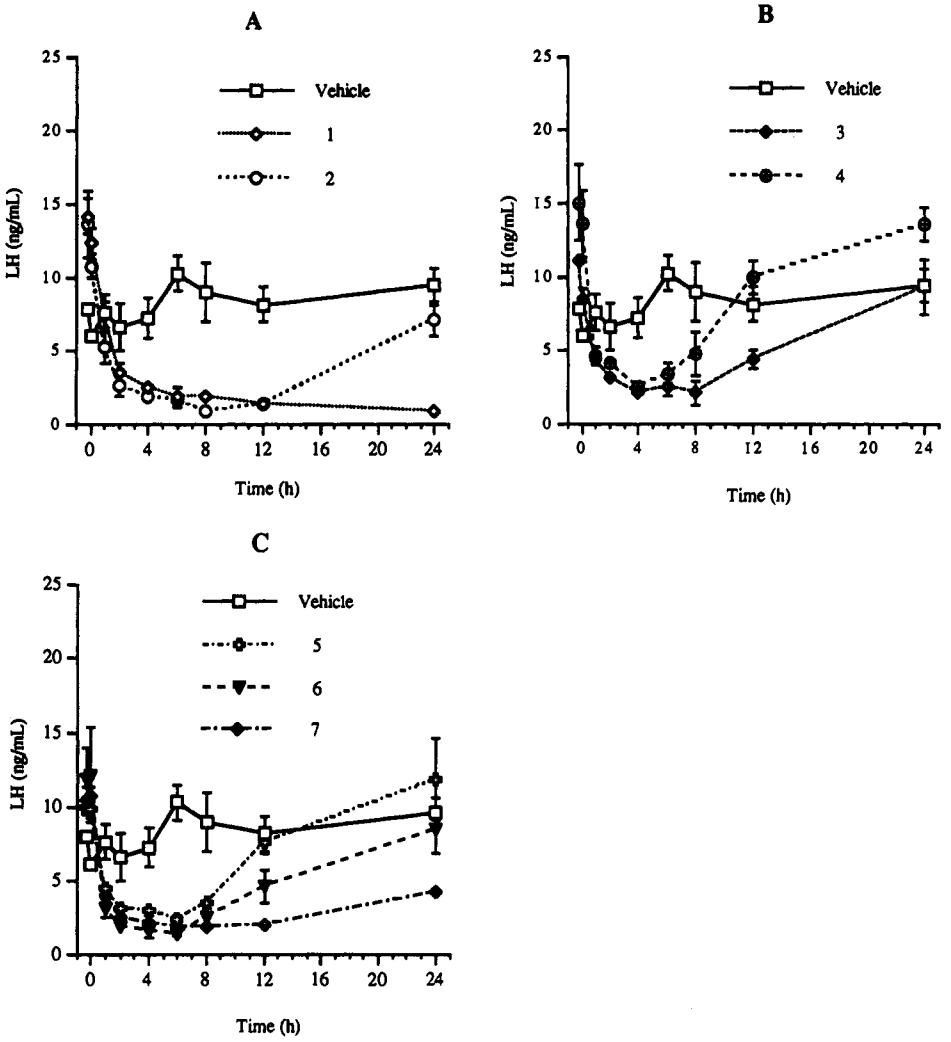

Figure 1. Plasma LH levels in nanograms per milliliter following the sc administration of 30 μg/kg of 1–7 antagonists to male castrate rats. A-75998 (6) was tested as the acetate salt; all the rest were the TFA salts.

than 24 h, but with antide only 40% of the suppression was restored by 24 h.

Effect of NMeTyr[5] on Water Solubility. During the HPLC purification of A-75998 (6) and the other antagonists, we noticed that all the compounds which contained NMeTyr[5] were very water soluble. To confirm this observation, we carried out comparative solubility studies of the peptides in 5% aqueous dextrose solutions at pH 4.5. The difference in solubility was striking. In every case, the solubility of the parent compounds was less than or equal to 1 mg/mL (Table I). However, the solubility of the NMeTyr[5] analogues was at least 12 mg/mL, and for A-75998 (6) was over 20 mg/mL (Table I). Initially, the reason for the increase in solubility was not apparent; however, when we considered the conformation of the molecule, the following explanation became evident. By substituting NMeTyr instead of Tyr or Lys(Nic) at position 5, the peptide backbone was restricted and distorted, thereby exposing these amphipathic peptides to better interact with the aqueous solvent resulting in an increase in water solubility. This interesting effect of increase in water solubility by the *N*-methyl, inserted in the peptide backbone, is novel and important. It is important because it demonstrates that it is possible to increase the water solubility of peptides without adding any hydrophilic groups. An additional importance of this finding is that it solves one of the major obstacles in the drug development of LHRH antagonists, since many of the known antagonists are limited in their in vivo efficacy by their low water solubility.

NMeTyr[5] and the Bioactive Conformation. Chemical modification studies on agonists,[26-28] theoretical calculations by Momany[29,30] on LHRH, and theoretical[31] and NMR[32,33] studies on conformationally constrained LHRH antagonists have indicated the presence of a type II′ β-turn extending from residues 5–8 in the bioactive conformation of the LHRH molecule when bound to its receptor (Figure 2). As a result of this, two hydrogen bonds are postulated to exist: between the C=O of 5 with the N-H of 8 and the N-H of 5 with the C=O of 8. It is therefore rather surprising that methylation of the N-H of residue 5, which prevents the formation of one of the above hydrogen bonds, did not reduce, but increased the binding affinities of antagonists 2, 4, and 6 (Table I). Recently we found that NMeTyr[5] substitution in three known LHRH agonists, leuprolide, deslorelin, and nafarelin, resulted in only modest reductions in the binding affinities by 4–10-fold.[9] These findings suggest: (1) the conformation of the residues 5–8 in the β-turn of the agonists differs from that of antagonists; (2) in the antagonists, the NMeTyr[5] restriction favors the bioactive conformation of the molecule; and (3) that probably no hydrogen bond occurs between the NH of Tyr[5] and CO of Arg[8] when the antagonists are bound to their receptor.

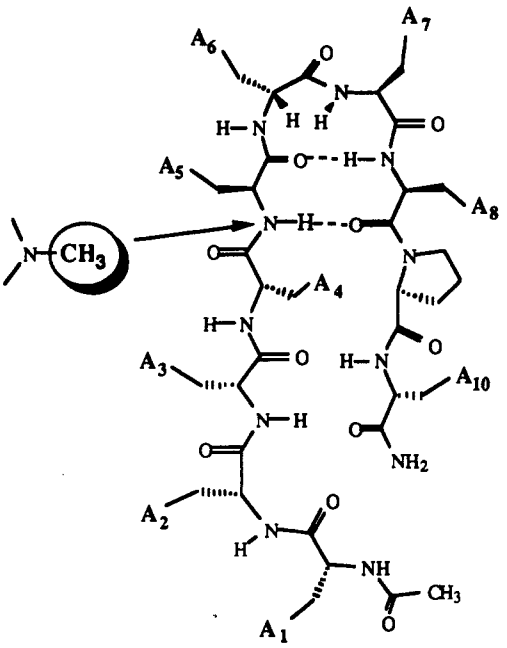

Figure 2. Schematic representation of the type II' β-turn in the conformation of LHRH antagonists.

Conclusions

A-75998 (6) appears to be a potent and safe LHRH antagonist. The uniqueness of its structure is the NMeTyr[5] moiety which bestows upon the molecule physicochemical and biological properties superior to antide (5). The NMeTyr[5] substitution seems to produce two major effects: (1) it introduces a backbone constraint that maintains or increases the in vitro intrinsic activity, which implies that it favors the bioactive conformation; and (2) it increases water solubility by enhancing solute–solvent interactions, thereby overcoming one of the major hurdles in the development of LHRH antagonists for clinical studies. Preliminary reports of additional in vivo tests with A-75998 have recently been presented.[15,34] These studies demonstrated this antagonist is effective in producing sustained testosterone suppression in both rats[16,35] and monkeys[34] following chronic administration. A-75998 (6) has passed all the functional and toxicological tests required for IND submission and is currently in clinical studies.

Experimental Section. All the peptides were synthesized using a Milligen-Biosearch Model 9500 automated peptide synthesizer (Milligen-Biosearch, Division of Millipore, Burlington, MA). The HF reaction apparatus, Type 1B, was from Peninsula Laboratories, Inc., Belmont, CA. Peptide purification was performed with a Rainin/Gilson Ternary HPLC system. FABMS were run using a Finningan MAT, MAT90 double-focusing magnetic sector (BE) mass spectrometer, xenon FAB ionization, and (1:1) glycerol/thioglycerol matrix. Amino acid analyses (AAA) were performed on a Beckman Model 6300 amino acid analyzer, using Ninhydrin derivatization. The peptides were hydrolyzed with 6 N HCl containing 0.5% phenol at 150 °C for 2 h. The data handling system was PE Nelson ACCESS CHROM. For calibration, Beckman standards were used. The values for Ser were generally low because of partial decomposition. The content of Ala, Pro, Leu, Lys, and NMeTyr were within ±10%. We did not look for the presence of any unnatural amino acid. That was confirmed by FABMS.

The following Boc-protected amino acids: Boc-D2Nal, Boc-D4ClPhe, Boc-D3Pal, Boc-Ser(OBzl), Boc-Tyr(O-2Br-Cbz), Boc-NMeTyr(O-2,6-di-Cl-Bzl), Boc-Lys(*N*-ε-nicotinyl), Boc-DLys(*N*-ε-nicotinyl), Boc-Leu, Boc-Arg-(Tos), Boc-Lys(*N*-ε-isopropyl-*N*-ε-Cbz)·DCHA, Boc-Pro were purchased from Bachem Inc. (Torrance, CA). Boc-DCit was purchased from Bachem Bioscience Inc. (Philadelphia, PA). Boc-D3Bal was purchased from SyntheTech Inc. (Albany, OR). TFA was obtained from Kali-Chemie Co. Inc. (Greenwich, CT). Boc-Gly-4-methylbenzhydrylamine resin (with a substitution varying from 0.4 to 0.7 mmol/g) was obtained from Peninsula Laboratories, Inc. (Belmont, CA). All the solvents were purchased from Fisher Scientific Co. (Fair Lawn, NJ). HF gas cylinders were purchased from AGA Gas Inc., Cleveland, OH. All other chemicals were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI).

General Synthetic Method for the SPPS and Purification of Peptides 1–6. A typical semimacro scale synthesis for a peptide used 0.8 g of Boc-D-Ala-4-methylbenzhydrylamine resin (with a substitution varying from 0.4–0.7 mmol/g). Boc-amino acids with protecting groups as defined above were loaded in the synthesizer according to the peptide sequence starting from the C-terminus. In the last amino acid vessel acetic acid was placed for coupling to Boc-D2Nal using identical conditions as the preceding amino acid. The resin was washed at the beginning of the synthesis and after each step, twice with CH_2Cl_2 (wash A), and three times with (1:1) DMF/CH_2Cl_2 (wash B). Each wash was for 40 s. The removal of the Boc substituent from the resin was performed using the deblocking solution (18 mL), containing 45% TFA, 50% CH_2Cl_2, 2.5% anisole, and 2.5% dimethyl phosphite, for 20 min. Afterward, the resin was washed three times with wash A and B, followed by three washes with base wash (18 mL), containing 10% *N*,*N*'-diisopropylethylamine in CH_2Cl_2, followed by two washes with A and three washes with B. The coupling step was performed using a 3-fold excess of a 0.3 M solution of protected amino acid (based on the degree of substitution of the resin) in DMF, simultaneously mixed with a 0.3 M activator solution containing 1,3-diisopropylcarbodiimide in CH_2Cl_2. All the amino acids were coupled twice. The duration of the couplings was as follows: for Boc-Arg(Tos), Boc-Lys(*N*-ε-isopropyl-*N*-ε-Cbz), Boc-Leu, Boc-D-Lys(*N*-ε-Cbz), Boc-D-Lys(Nic), Boc-D-Cit, Boc-Tyr(O-2-Br-Cbz), Boc-NMeTyr(O-2,6-Cl-Bzl) 1 h; for Boc-Ser(O-Bzl), Boc-D3Bal, Boc-D4ClPhe, Boc-D2Nal, and acetic acid, 2 h. For Boc-D3Pal the coupling time was extended to 6 h. The peptide resin was then dried over P_2O_5 under vacuum overnight and placed into the Teflon vessel of an HF reaction apparatus containing a stirring bar. Anisole (1 mL) was added to it. The reaction tube was attached to the apparatus and cooled with liquid nitrogen. The whole system was evacuated, and anhydrous HF (dried over CoF_3 for 24 h) was condensed into the reaction vessel (about 10 mL). The liquid nitrogen bath was replaced with an ice–water bath, and the resin was stirred over 75 min. The excess of HF and anisole was removed in vacuo over 2 h at 0 °C. The reaction tube was removed from the apparatus, and the residue was triturated with ether to remove all traces of anisole. A 1:1:0.1 water/acetonitrile/acetic acid solution (30 mL) was added, and the mixture was stirred for 10 min and filtered. The filtrate was frozen with dry ice/acetone and lyophilized to give the crude product. The

product was purified by HPLC using a C_{18} reversed-phase column. Analytical HPLC separation was achieved with a C_{18} Dynamax column (0.46 × 25 cm), 300-Å pore size, 5-μm particle size fitted with a guard column of the same material (0.46 × 1.5 cm). The solvent system was 0.1% TFA in water/acetonitrile and the gradient was 25–60% acetonitrile over 35 min. The UV detector was set at 254 nM. Preparative HPLC separation was accomplished with a C_{18} Dynamax column (2.14 × 25 cm), 60-Å pore size, 8-μm particle size, with a guard column of the same material (2.14 × 5.0 cm). Sixty fractions (30 s each) were collected in the interval from 10 to 40 min. Each fraction was checked by analytical HPLC for purity. The clean fractions were combined and lyophilized to provide a homogeneous fluffy white powder. The purity of the final compounds was over 95% on the basis of analytical HPLC, FABMS, and AAA.

In Vitro Biological Assays. Antagonists were tested in the receptor binding and LH release assays which we previously described.[13] For these assays the compounds were dissolved in 10 mmol of Tris buffer pH 7.4. The histamine release assay from rat peritoneal mast cells followed a reported procedure.[5,15] Histamine was measured using a RIA kit purchased from AMAC Inc. (Westbrook, ME). For this assay, the compounds were dissolved in 5.6 mM glucose, 0.1% BSA in Dulbecco phosphate buffer/saline pH 7.4.

In Vivo LH Inhibition. Antagonists were administered sc at 30 μg/kg to groups of three rats. The vehicle consisted of 1:4 propylene glycol/normal saline. The dose was administered in a volume of 1 mL/kg body weight. Under ether anesthesia, timed blood samples were withdrawn and assayed for LH in triplicate using reagents purchased from the Research and Education Institute (Torrance, CA) and from Dr. P. Michael Conn (Department of Pharmacology, University of Iowa, Iowa City, IA).

Acknowledgment. We thank Drs. Marvin Karten and Jean Rivier for supplying us with a sample of NalGlu. We are also in debt to Dr. P. Michael Conn of the University of Iowa for providing the anti-rat-LH and LHRH antagonist antibody.

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