Stabilization of the N-Terminal Residues of Luteinizing Hormone-Releasing Hormone Agonists and the Effect on Pharmacokinetics†,‡,§

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To stabilize leuprolide (1) against chymotrypsin and intestinal degradation several agonists of LHRH (2–12), modified at position 1, 2, or 3 and/or containing *N*-α-methyl at positions 1, 2, or 4, were synthesized by SPPS. These agonists were tested in vitro for (a) rat pituitary LHRH receptor binding, (b) LH release from rat pituitary cells, (c) stability against chymotrypsin, and (d) stability against rat intestinal degradation. The clearances of the compounds in the rat were determined using a RIA. Complete stabilization against chymotrypsin ($t_{1/2}$) and lumenal degradation ($T_{1/2}$) was achieved with substitution of NMe-Ser[4] in leuprolide; however, with an increase in clearance. Substitution with 1-Nal[3] increased both $t_{1/2}$ and $T_{1/2}$, while substitution with NAc-Sar[1] increased only $T_{1/2}$. [NAcSar[1],NMeSer[4],D-Trp[6],Pro[9]NHEt]LHRH (12), the doubly stabilized analogue, was tested in the rat by both iv and id administrations, and its bioavailabilities were measured. No significant improvement in id absorption over leuprolide was observed.

Introduction

Several agonists of LHRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2, are currently used for the treatment of prostate cancer, endometriosis, and other indications which are sex-hormone dependent.[1-5] Leuprolide (1), as well as other LHRH agonists, is administered either subcutaneously or as a depot.[6-9] As part of our interest in developing an orally active LHRH analogue, we tried to stabilize peptide 1 against metabolic degradation. We soon found that leuprolide is highly susceptible to degradation by the intestinal protease chymotrypsin. This observation led us to direct our chemical effort towards the design of analogues which are stable to chymotrypsin digestion. Furthermore, since an orally active compound must be absorbed through the intestinal wall, which is known to contain a large amount of endopeptidase activity, we examined the stability of our analogues in an in vitro rat intestinal sac model. We also measured their in vivo pharmacokinetics. Finally, we tried to determine whether stabilization of the peptide against metabolic degradation improves intestinal absorption.

Peptide Synthesis

Enzymatic studies with leuprolide (described below) indicated that chymotrypsin cleaves the Trp[3]-Ser[4] peptide bond. To stabilize this bond against enzymatic degradation we substituted Ser[4] with NMeSer[4]. Initially we synthesized [NMe-Ser[4],D-Leu[6],Pro[9]NHEt]LHRH (2) using conventional peptide solution chemistry. Though we obtained the desired product, the synthesis was tedious and time consuming, since each intermediate had to be purified and characterized. To accelerate our progress we looked for an alternative synthesis and were encouraged by a report[10] about the synthesis of [NMe-Leu[7]]LHRH which was carried out using the solid phase peptide synthesis (SPPS) technique.[11] Though this technique generally yields a less clean crude product, it appeared more attractive to us as being faster (since only the final product is purified and characterized). After several trials, we were able to optimize the SPPS protocol (described in

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§ 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: 1Nal, 3-(1-naphthyl)alanine: D-2Nal, D-3-(2-naphthyl)alanine; D-4ClPhe, D-3-(4-Cl-phenyl)alanine; Boc, *tert*-butoxycarbonyl; HPLC, high-pressure liquid chromatography; LH, luteinizing hormone; iv, intraveneous; id, intraduodenal; RIA, radioimmunoassay; SPPS, solid phase peptide synthesis; FABMS, fast atom bombardment mass spectroscopy; AAA, amino acid analysis.

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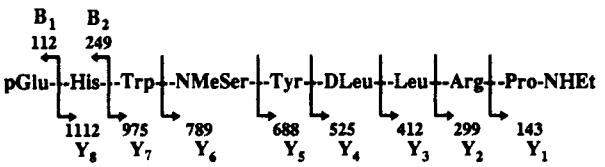

Figure 1. Fragment ions (m/z) observed in the FABMS of [NMe-Ser[4],D-Leu[6],Pro[9]NHEt]LHRH (2).

the Experimental Section) and reduce the amount of impurities. The same protocol was also used in the syntheses of compounds 2–12. Generally, the byproducts which we observed derived from (a) deletion of positions 1, 2, or 3; (b) ring opening of the p-Glu residue by ethylamine to give *N*-δ-ethylglutaminyl resulting in a byproduct with a shorter retention time by HPLC, and therefore was easily separable; and (c) only partial removal of the Bzl (on Ser) and the Tos (on Arg) groups resulting in byproducts with longer retention times by HPLC. The amount of deletion byproducts was reduced by increasing the coupling time for positions 1, 2, and 3. Similarly, by increasing the HF reaction time, almost complete removal of the protecting groups was achieved. However, it was difficult to find better conditions, in spite of a large number of trials, under which the ethylamine would not open the p-Glu. The amount of ring opening generally varied with the peptide structure. All the peptides were purified by HPLC and obtained as the TFA salts. They were characterized by analytical HPLC, FABMS, and AAA. Additionally, the peptide sequence of 2 was confirmed by analysis of the fragment ions (m/z), besides MH^+ at 1223, observed in the FABMS. Figure 1 illustrates the pattern of the fragment ions (from the Y and B series based on Roepstorff and Fohlman nomenclature[12]) which was observed and is consistent with structure 2.

Bioassays

Peptides were tested in vitro in the rat pituitary LHRH receptor binding assay and in the LH release from cultured rat pituitary cells assay.[13] The binding affinities are reported as pK_1. The LH release potencies for agonists are reported as pD_2 (for definitions of pK_1 and pD_2 see footnotes of Table I). The stability of peptides against chymotrypsin degradation was determined using an assay similar to the previously reported one.[14] To determine in vitro intestinal stability of peptides we developed a rat jejunum sac model, which is described in the Experimental Section. To measure pharmacokinetics, the compounds were administered to rats by iv bolus. The serum levels of the compounds, except compound 4, were determined by a RIA using an antibody to a LHRH analogue, which recognizes the C-terminal residues Leu-Arg-ProNHEt. Compound 4 was evaluated by a RIA using an antibody to [D-Lys[6]]LHRH.[15] The pharmacokinetics are reported as values of the whole body clearance, which is defined as the volume of plasma cleared of compound per unit time, and it is calculated as the dose divided by the area under the curve of blood concentration of compound as a function of time and is expressed in units of mL/min per kg.

Results and Discussion

Enzymatic Susceptibility of Leuprolide. Enzymatic digestion of leuprolide with trypsin (pH 8.0) or pepsin (under acidic conditions) at 37 °C did not cause any cleavage of the peptide. These results were somewhat puzzling since the peptide bond C-terminal to Arg[8] is theoretically cleavable by trypsin. The absence of cleavage was presumably due to the involvement of Pro[9] in the peptide bond. Similarly, pepsin, which is expected to cleave the peptide at both sides (N- and C-terminal) of Trp[3] and N-terminal of Leu[7], did not affect the peptide stability. It is possible that pepsin failed to cleave the peptide (under the attempted conditions) because the size of the molecule is too small for recognition. Incubation of leuprolide with chymotrypsin, which is known to be a very specific proteolytic enzyme, caused a clean cleavage of the bond Trp[3]-Ser[4] resulting in two fragments pGlu-His-Trp and Ser-Tyr-DLeu-Leu-Arg-ProNHEt. This cleavage was consistent and reproducible. Under the assay conditions the reaction was very fast and complete in 1 min (Table I) and produced no side products upon further incubation. The two fragments were identified by chromatographic comparison with authentic samples.

Stabilization of Trp[3]-Ser[4] Bond. To buttress the Trp[3]-Ser[4] bond in leuprolide against chymotrypsin degradation we synthesized the NMe-Ser[4] analogue 2 and tested its stability against this enzyme's digestion. Under conditions where leuprolide was rapidly degraded by chymotrypsin, the NMe-Ser[4] compound 2 was not cleaved for over 60 min (Table I). We then tested these two compounds for intestinal degradation using the rat jejunal sac model. While leuprolide had a 4-min half-life ($T_{1/2}$) in the intestinal model, the NMe-Ser[4] analogue 2 had a half-life increased over 22-fold (Table I). However, the NMe-Ser[4] analogue 2 showed about a 10-fold reduction in receptor binding affinity and in LH release, and its in vivo clearance was increased about 2-fold to 17 mL/min per kg. Substitution of D-Leu[6] with D-Trp[6] in compound 2 yielded the NMe-Ser[4],D-Trp[6] analogue 3 with improved potency (pD_2 10.10). While 3 is expected to be as resistant to chymotrypsin degradation as 2, it had a half-life of 39 min in the intestinal model and a similar 2-fold increased clearance in the rat (Table I). Further substitutions of positions 4 and 6 of LHRH with NMe-Ser[4] and D-2Nal[6], respectively, gave analogue 4 which was 4-fold more potent than leuprolide in LH release and had a 12-fold increase in half-life against lumenal degradation. From comparison of the $T_{1/2}$ and clearance values of 2, 3, and 4, it becomes evident that although an aromatic D-amino acid at position 6 enhances potency,[16,17] it seems to have a negative effect

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Table I. FABMS Data, in Vitro LHRH Receptor Binding Affinities, LH Release Activities, Stability against Chymotrypsin and Intestinal Degradation, and in Vivo Clearances of LHRH Agonists

compd	structure	t_R[a]	MH$^+$[b]	pK_I[c]	pD_2[d]	$t_{1/2}$[e]	$T_{1/2}$[f]	clearance[g]
1	[D-Leu^6,Pro^{9}NHEt]LHRH (leuprolide)	10.98	1209	9.73 (±0.04)	10.69 (±0.04)	1.0	4.0 (2.5–9.5)	9.0 (±0.6)
2	[NMe-Ser^4,D-Leu^6,Pro^{9}NHEt]LHRH	11.65	1223	8.85 (±0.09)	9.42 (±0.18)	>60.0	>90.0	16.9 (±0.8)
3	[NMe-Ser^4,D-Trp^6,Pro^{9}NHEt]LHRH	14.43	1296	10.11 (±0.06)	10.10 (±0.40)		39.1 (25–91)	19.9 (±1.2)
4	[NMe-Ser^4,D-2-Nal^6]LHRH	17.43	1336	10.37 (±0.06)	11.30 (±0.20)	>60.0	47.0 (37–78)	27.5 (±2.3)
5	[NAc-Sar^1,D-Leu^6,Pro^{9}NHEt]LHRH	11.97	1211	8.59 (±0.13)	9.45 (±0.01)	1.0	60.0 (39–125)	24.0 (±0.4)
6	[Phe^2,D-Leu^6,Pro^{9}NHEt]LHRH	18.10	1219	8.66 (±0.06)	9.81 (±0.05)		2.2 (1.1–4.9)	25.4 (±5.5)
7	[Phe^2,D-Trp^6,Pro^{9}NHEt]LHRH	23.62	1292	10.61 (±0.05)	10.81 (±0.13)		10.8 (6.8–26)	28.8 (±2.4)
8	[NMe-Phe^2,D-Trp^6,Pro^{9}NHEt]LHRH	20.63	1306	9.68 (±0.07)	8.40 (±0.15)	3.7	44.4 (28–133)	44.4 (±4.4)
9	[Tyr(OMe)^3,D-Leu^6,Pro^{9}NHEt]LHRH	10.52	1200	9.81 (±0.07)	8.76 (±0.00)		14.9 (12–19)	13.3 (±0.4)
10	[4ClPhe^3,D-Leu^6,Pro^{9}NHEt]LHRH	9.85	1204	8.03 (±0.51)	9.17 (±0.17)		4.4 (3.5–5.9)	7.6 (±0.01)
11	[1Nal^3,D-Leu^6,Pro^{9}NHEt]LHRH	13.82	1220	10.03 (±0.16)	10.35 (±0.45)	>15.0	25.0 (15.5–65)	28.4 (±4.7)
12	[NAc-Sar^1,NMeSer^4,D-Trp^6,Pro^{9}NHEt]LHRH	10.70	1298	9.42 (±0.11)	9.72 (±0.11)	>60.0	55.0 (36–122)	28.3 (±4.1)

[a] Analytical HPLC retention time in min. HPLC conditions are described in the Experimental Section. [b] Values determined by FABMS. [c] pK_I = the negative logarithm of equilibrium dissociation constant in the rat pituitary receptor binding assay. [d] pD_2 = the negative logarithm of the concentration of agonist that produces 50% of the maximum release of LH from cultured rat pituitary cells in response to the test compound. [e] $t_{1/2}$ = chymotrypsin degradation half-life in min. [f] $T_{1/2}$ = the time, in min, required for the lumenal concentration of compound in the rat jejunal sac to decrease by 50%. [g] Clearance = clearance of compound after iv administration in the rat, expressed as the dose divided by the area under the curve of the concentration of compound as a function of time, mL/min per kg.

on the clearance and the intestinal sac stability, particularly in the case of D-Trp^6.

Modification of Positions 1, 2, and 3. Once we achieved stabilization of the Trp^3-Ser^4 bond and maintained the potency of leuprolide, we directed our attention to residues 1, 2, and 3 in the molecule with the intent of identifying other substitutions which are chemically and metabolically more stable yet retain potency. Substitution of pGlu^1 in leuprolide with NAc-Sar^1 gave compound 5 which was 17-fold less potent than the parent and was degraded by chymotrypsin as fast as the parent, but its half-life in the jejunal test was 15-fold longer than that of leuprolide (Table I). These results suggest that chymotrypsin is either absent or present only in low concentration in our intestinal model. Substitution of His^2 with Phe^2 yielded compound 6 which was 7-fold less potent than the parent and with increased clearance. These results clearly indicate that Phe^2, although chemically more stable, is not advantageous over His^2 with respect to resistance against lumenal degradation or in vivo clearance. Substitution of D-Leu^6 in compound 6 with D-Trp^6 provided analogue 7 which was as potent as leuprolide and had about a 2-fold increase in the intestinal half-life. Previous reports[18,19] indicated that pyroglutamyl-aminopeptidase cleaves the bond pGlu^1-His^2. It is possible that the same enzyme, if it is present in the jejunal preparation, is cleaving the pGlu^1-Phe^2 bond in 6. To probe this hypothesis we synthesized the NMe-Phe^2,D-Trp^6 analogue 8 which was 200-fold less potent than leuprolide but was 4-fold more stable against chymotrypsin degradation and 11-fold more stable against intestinal degradation. It is possible that the increase in intestinal stability of compound 5 ($T_{1/2}$ 60 min), achieved upon substitution of NAcSar^1 in leuprolide, is also due to stabilization against the same pyroglutamyl-aminopeptidase (Table I). The in vivo clearances of both the Phe^2- (6) and NMe-Phe^2- (8) analogues were increased by 2.7- and 5.0-fold, respectively. We believe that these higher clearances were caused by the increase in the hydrophobicity of the peptides (Phe or NMe-Phe versus His) which may elicit increased hepatic clearance. These results are consistent with our previous report about the effect of physicochemical properties on pharmacokinetics of reduced size LHRH analogues.[15]

Substitution of Trp^3 in leuprolide with 4ClPhe^3 (10) caused a 33-fold loss in potency and did not improve stability. Substitution of the same position with Tyr(OMe)^3 (9) caused an 85-fold loss in potency but improved intestinal stability by 4-fold. Substitution of the same position with 1-Nal^3, a side chain which is known to be poorly accommodated in the chymotrypsin specificity pocket,[20] gave analogue 11, which was as potent as leuprolide in LH release and over 15-fold more stable against chymotrypsin degradation and 6-fold more stable in the jejunum model. However, the clearance of 11 was increased 3-fold. Next we substituted NAc-Sar^1 in 3 and obtained analogue 12, which was 10-fold less potent than

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leuprolide, but 14-fold more stable in the intestine and had a 3-fold increase in clearance.

The large differences in half-life values of peptides 5 and 8 against chymotrypsin ($t_{1/2}$) and intestinal ($T_{1/2}$) degradation (Table I) indicate that chymotryptic cleavage plays only a minor role in the jejunal sac model. Also, comparison of the half-lives ($t_{1/2}$, in the presence of chymotrypsin) of compounds 5, 8, 11, and 2 indicate a stability order of 2 > 11 > 8 > 5, which is in accordance with the proximity of the modified position (NMe-Ser^4 > 1-Nal^3 > NMe-Phe^2 > NAc-Sar^1) to the enzyme's cleavage site, the peptide bond between Trp^3-Ser^4. The closer the modification is to the cleavage site, the greater the stabilization.

Comparison of the pK_1 versus the pD_2 values of compounds 2–12 reveals three groups of agonists: the first consists of compounds 3, 7, 11, and 12 which have similar values for pK_1 and pD_2; the second includes compounds 2, 4-6, and 10 which have 2- to 10-fold increased pD_2 over pK_1 values; and the third contains compounds 8 and 9 which have 10-fold decreased pD_2 below pK_1 values, indicating that NMe-Phe^2 and Tyr(OMe)^3 substitutions are detrimental to the agonist activity. The basis for the pK_1 and pD_2 differences are not evident.

Stability and Intestinal Absorption. At this point we were interested in finding out how stabilization of the molecule would affect intraduodenal (id) absorption of leuprolide in vivo. For this purpose, we administered both leuprolide and analogue 12, [NAc-Sar^1,NMe-Ser^4,D-Trp^6,Pro^{9}NHEt]LHRH, separately to rats by both iv and id routes, measured the peptide levels in the serum and determined the absorption using the methods which we previously reported.[15] The bioavailability for leuprolide was 0.08% and for analogue 12 was 0.14%, clearly showing that the enzymatic stabilization did not improve intestinal absorption in vivo.

Conclusions

Up to 60- and 20-fold increases, respectively, in resistance to chymotrypsin and intestinal lumenal degradation were achieved upon separately substituting NMe-Ser^4, NMe-Phe^2, NAc-Sar^1, and 1-Nal^3 into several LHRH agonists. Several of these newly synthesized analogues showed potency in the range of leuprolide and other highly potent LHRH agonists. The doubly stabilized NAc-Sar^1,NMe-Ser^4,D-Trp^6 analogue 12 was only 10-fold lower in potency. Yet all of these compounds also showed increased clearance over leuprolide, with the exception of the 4Cl-Phe^3 analogue 10. All the synthesized compounds, again except for 10, were more hydrophobic than leuprolide suggesting that higher clearance values seem to be related to increased hydrophobicity of the molecule. The results also indicate that the low intestinal absorption of these LHRH analogues does not appear to be limited by chymotryptic or intestinal lumenal degradation.

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 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. If the peptide contained Trp, 0.5% phenol was replaced with 5% thioglycolic acid. The data handling system was PE Nelson ACCESS CHROM. For calibration, Beckman standards were used. The values for the Ser, His, and Trp were generally low because of partial decomposition. The values for Arg were high because of interference from the ethylamide residue and were corrected accordingly. The content of Glu, Phe, Tyr, Leu, Pro, Gly, and Sar were within ±10%. We did not look for the presence of any unnatural amino acid. That was confirmed by FABMS.

All the Boc-protected amino acids were purchased from Bachem Inc. (Torrance, CA). Boc-Pro-Merrifield resin (with a substitution varying from 0.4 to 0.7 mmol/g) was obtained from the same company. Boc-NMe-Ser(*O*-Bzl) was synthesized according to D. H. Rich et al.[21] 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). TFA was obtained from Kali-Chemie Co., Inc. (Greenwich, CT). All the solvents were purchased from Fisher Scientific Co. (Fairlawn, 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 1–3 and 5-12. A typical semimacro scale synthesis for peptide 2 used 0.8 g of Boc-Pro-Merrifield resin (0.64 mmol/g substitution). Boc-Arg(Tos), Boc-Leu, Boc-D-Leu, Boc-Tyr(*O*-2-Br-Cbz), Boc-NMe-Ser(*O*-Bzl), Boc-Trp(*N*-ind-Formyl), Boc-His(*N*-im-Cbz), and Cbz-pGlu were loaded in the synthesizer according to the peptide sequence starting from the C-terminus. 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. Afterwards, 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 Boc-Pro-O-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-Leu, Boc-D-Leu, and Boc-Tyr(*O*-2-Br-Cbz) it was 1 h; for Boc-NMe-Ser(*O*-Bzl) and Boc-His(*N*-im-Cbz) it was 2 h; for Boc-Trp(*N*-ind-Formyl) and Cbz-pGlu it was 3 h. The peptide resin was then dried, weighed, and transferred to a round-bottom flask (100 mL) containing a magnetic stirring bar. The flask was capped with a rubber septum (which was wired down) and immersed into a dry ice/acetone bath. Through a hypodermic needle a solution of freshly distilled ethylamine (15 mL) was added. The dry ice/acetone bath was removed, and the resin was stirred at room temperature for 40 h. Afterwards, the reaction vessel was cooled again with dry ice/acetone, and the rubber septum was removed. The cold bath was withdrawn, ethylamine was allowed to escape, and methanol (30 mL) was added. The resin was stirred for 10 min at room temperature and filtered. The filtrate was concentrated in vacuo, and the residue was triturated with water. The solid product was filtered and dried over P_2O_5 under vacuum overnight to yield the crude protected peptide. The ethylamine treatment also cleaved the formyl group from Trp^3 whenever that was present. The dry protected peptide was then 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 for 75 min. The excess of HF and anisole was removed

(21) Rich, D. H.; Dhaon, M. K.; Dunlap, B.; Miller, S. P. F. Synthesis and Antimitogenic Activities of Four Analogues of Cyclosporin A Modified in the 1-Position. *J. Med. Chem.* **1986**, *29*, 978–984.

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). The UV detector was set at 254 nm. 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.

[NMe-Ser⁴,D-2Nal⁶]LHRH (4). To synthesize compound 4 we used the same synthesis protocol described above, but substituted Boc-Gly-4-methylbenzhydrylamine resin (0.8 g, 0.48 mmol/g substitution) for Boc-Pro-Merrifield resin, added Boc-Pro as the first amino acid to be coupled, and substituted Boc-D-2Nal for Boc-D-Leu. The peptide resin was dried and treated with HF to cleave the peptide from the resin and to remove the protecting groups. After workup and lyophilization, 0.58 g of crude peptide 4 was obtained. This was purified by HPLC and characterized.

Biological Assays. We previously reported the receptor binding and LH release assays[13] and our method to measure pharmacokinetics of LHRH analogues.[15]

Rat Jejunum Sac Assay. The rat jejunum sac model was designed to determine intestinal stability in vitro. In this assay, the compounds were incubated at 37 °C in short closed rat jejunal segments in oxygenated pH 6.8 Ringer's buffer. At a specific time, each segment was removed from the incubation bath and its contents assayed for peptide using HPLC. Half-lives were estimated in duplicate by linear regression analysis of the logarithm of percent peptide remaining versus time, assuming minimal transport related losses of intact molecule.

Chymotrypsin Cleavage Assay. Solutions of peptides **1**, **2**, **4**, **5**, **8**, **11**, and **12** were prepared by dissolving 5.0 mg of each in 0.50 mL of MeOH. A 50-μL aliquot of each solution was placed in an incubator at 37 °C. The stock solution of buffered chymotrypsin was prepared as follows: a 30 mM sodium phosphate/100 mM sodium chloride solution was prepared and adjusted to pH 6.9 with 4 N HCl. This solution was equilibrated at 37 °C for 15 min. Chymotrypsin was then added to 5 μg/mL and allowed to equilibrate for another 5 min. To each peptide a 2450-μL aliquot of the buffered chymotrypsin solution was added, and the mixtures were allowed to incubate at 37 °C. Then, 100-μL liquots were removed from each sample at designated intervals (0.083, 0.5, 1, 2, 3, 4, 6, and 9 min for peptide **1** and 0.083, 1, 3, 6, 9, 15, 30, and 60 min for peptides **2**, **4**, **5**, **8**, **11**, and **12**) and quenched into 500 μL of (3:7) acetonitrile/water solution. The composition of each sample was analyzed by analytical HPLC. To each fraction was added a standard solution of analogue [NAc-D-2Nal¹,D-4ClPhe²,D-3Pal³,D-Cit⁶,D-Ala¹⁰]LHRH (**13**),[22] prepared as above without the chymotrypsin. A volume of 20 μL of each fraction was injected on a Dynamax C-18 (300 Å, 5 μm) analytical column eluted with a gradient from 25% to 45% acetonitrile (in water containing 0.1% TFA) over 30 min. The UV detector was set at 214 nm, and the quantitation was achieved by normalization against peptide **13** as internal standard.

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