Effect of N-Methyl Substitution of the Peptide Bonds in Luteinizing Hormone-Releasing Hormone Agonists†,‡

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Each peptide bond in leuprolide (1), deslorelin (13), and nafarelin (24) was separately substituted with *N*-methyl. The synthesized compounds were tested for in vitro receptor binding, LH release, and stability against chymotrypsin and intestinal degradation. The NMe-Ser[4] (30), NMe-Leu[7] (33), and Sar[10] (35) analogues of nafarelin had pD_2 values 2-, 20-, 9-fold higher than their respective parent. All the other *N*-methyl agonists were less active. For the first time, conversion of LHRH agonists to antagonists was observed as a result of *N*-methyl substitution in the peptide backbone. [NMe-Phe[2],DLeu[6],Pro[9]NHEt]LHRH (4), [NMe-1Nal[3],DLeu[6],Pro[9]NHEt]LHRH (6), [NMe-His[2],DTrp[6],Pro[9]NHEt]LHRH (14), [NMe-Phe[2],DNal[6]]LHRH (27), and [D2Nal[6],NMe-Arg[8]]LHRH (34) exhibited antagonist responses. Substitutions of NMe-1Nal[3], NMe-Ser[4], or NMe-Tyr[5] in leuprolide rendered the 3-4 peptide bond in these compounds completely stable to chymotrypsin. Examination of the three-dimensional structure of leuprolide when bound to the active site of chymotrypsin, reveals the NH's of residues 3 and 5 are involved in hydrogen bond interactions with the enzyme. N-Methylation at these positions is not only disrupting the hydrogen bond interactions, but is also sterically preventing the substrate from fitting in the enzyme's active site. All the compounds in the leuprolide series were also tested against intestinal degradation using an in vitro rat jejunum sac assay. In this model the pattern of stabilization was similar, but not identical, to that against chymotrypsin. The pharmacokinetics of all the analogues in the leuprolide series and of several others in the deslorelin and nafarelin series were determined. The clearance values of all the three NMe-Tyr[5] analogues 8, 20, and 31 were lower than their respective parents. These slower clearances suggest lower rates of metabolism.

Introduction

Several agonists of luteinizing hormone-releasing hormone (LHRH), pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2, are currently used in the treatment of prostate cancer, endometriosis, precocious puberty, and other indications which are testosterone or estrogen dependent.[1-4] All of them are administered either subcutaneously, nasally, or as depot.[5,6] As an initial step toward the development of an orally active agonist, we tried to stabilize the leuprolide[4] (1) molecule against enzymatic degradation. We previously reported the stabilization of several LHRH agonists against chymotrypsin and intestinal degradation by structural modification of positions 1, 2, and 3.[7,8] We also demonstrated that substitution of NMe-Ser[4] in leuprolide (1) stabilized the peptide bond Trp[3]-Ser[4] against enzymatic digestion.[7,8] Stimulated by these findings we pursued the effect of *N*-methyl substitution at each position of three LHRH agonists: leuprolide (1), deslorelin[9,10] (13), and nafarelin[11,12] (24). The synthesized analogues were tested in vitro for LHRH receptor binding, LH release, and stability to chymotrypsin and intestinal degradation. We also measured the in vivo pharmacokinetics of the peptides.

Peptide Synthesis

All the peptides were synthesized using solid phase peptide synthesis (SPPS) techniques.[13] The appropriate Boc-protected amino acids, Boc-Pro-Merrifield resin for leuprolide and deslorelin analogues, and Boc-Gly-4-methylbenzhydrylamine resin for nafarelin analogues were used. The synthesis protocol, cleavage of the peptide from resin, removal of the protecting groups, workup, and HPLC purification were analogous to those extensively described in our recent publication.[8,14] No difficulties were encountered in coupling the Boc-*N*-methyl amino acids to the peptide resin.[8] The same activator and the same coupling time, which were applied for regular Boc-amino acids, were used for the Boc-*N*-methyl amino acids.[8] All the peptides were characterized by analytical HPLC, FAB mass spectrometry (FABMS), and amino acid analysis (AAA). Boc-NMe-Ser(OBzl) was synthesized according to D. H. Rich et al.[15] Boc-NMe-His(Tos) was synthesized by N-methylation of Boc-His(Tos).[16] Analogous syntheses were used for Boc-NMe-1-Nal and Boc-NMe-D-2Nal.

Bioassays

Peptides were tested in vitro for rat pituitary LHRH receptor binding and for LH release from cultured rat pituitary cells.[14] The binding affinities are reported as pK_I. The LH release potencies for agonists are reported as pD_2, those for antagonist as pA_2 (for definitions of pK_I, pD_2, and pA_2 see footnotes of Table I). The stability of peptides against chymotrypsin degradation was deter-

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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: D2Nal, D-3-(2-naphthyl)alanine; 1Nal, 3-(1-naphthyl)alanine; NMe-1Nal, N-α-methyl-3-(1-naphthyl)alanine; HPLC, high-pressure liquid chromatography; LH, luteinizing hormone; sc, subcutaneous; FABMS, fast atom bombardment mass spectrum; AAA, amino acid analysis.

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mined using a reported assay.[8] To determine in vitro intestinal stability of peptides we used the rat jejunum sac model, which we previously described.[8] To measure pharmacokinetics, the compounds were administered to rats by iv bolus. The serum levels of the compounds, were determined by a RIA using an antibody to an LHRH analogue which recognizes the C-terminal residues Leu-Arg-ProNHEt or Leu-Arg-Pro-GlyNH$_2$.[17] 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 expressed in units of mL/min per kg, and calculated as the dose divided by the area under the curve of blood concentration of compound as a function of time.

Results and Discussion

Effect of *N*-Methyl Substitution on in Vitro Potency. In our previous publication we reported[8] several LHRH agonists which derived from NAc-Sar[1], NMe-Phe[2], 1Nal[3], and NMe-Ser[4] substitutions in leuprolide (1), deslorelin (13), and nafarelin (24). To complete the studies of positions 2 and 3 we substituted NMe-His[2] and NMe-1Nal[3] in the three parent agonists. For completeness and comparison purposes we have included in Table I some of the compounds from our previous studies.[8]

Leuprolide Series (1–12). N-Methylation of each residue separately in leuprolide caused significant losses in in vitro potency (Table I). Losses in binding affinity ranging from 10- to 295-fold were observed in this series with NMe-DLeu[6] (9) and NMe-His[2] (2) compounds at the extremes. Likewise, 10- to 1860-fold reductions in pD_2 values were obtained, with the NMe-DLeu[6] (9) and NMe-Arg[8] (11) analogues being most and least potent, respectively. In two cases, the NMe-Phe[2] (4) and NMe-1Nal[3] (6), the biological responses changed to antagonists, having very modest pA_2 values (7.53 and 6.59, respectively). This is the first time that an agonist was converted to an antagonist just by N-methylation of position 2 or 3. We previously reported[14] in the (4–9) reduced size LHRH analogues a similar switch of biological response from agonist to antagonist effected by varying the size or the shape of the substituent at position 3 or 6.

Deslorelin Series (13–23). The analogous *N*-methyl substitutions, described above, were performed on deslorelin. In the receptor binding assay the affinities were again lower, ranging from 6-, for NMe-Leu[7] (21), to 140-fold for NMe-His[2] (14). The pD_2 values were decreased from 4-, for the NMe-Leu[7] (21), to 1000-fold for the NMe-1Nal[3] (18) compounds. In this series, unlike the previous leuprolide series, only the NMe-His[2] analogue (14) was an antagonist. These findings suggest that there is a feedback between position 6 and 2 which is capable of influencing the agonist to antagonist conversion as a result of N-methylation at position 2.

Nafarelin Series (24–35). This series, unlike the previous two, is a decapeptide rather than a nonapeptide. The analogous *N*-methyl backbone substituents reduced the receptor binding affinities ranging from 3- to 2884-fold, with the NMe-Tyr[5] (31) and the NMe-Arg[8] (34) analogues at the extremes. The pD_2 values for NMeSer[4] (30), NMe-Leu[7] (33), and Sar[10] (35) derivatives were, for the first time, higher by 2-, 20-, and 9-fold, respectively, than the parent. The pD_2 for the NMe-Tyr[5] analogue (31) was in the range of nafarelin. The NMe-Phe[2] (27) and NMe-Arg[8] (34) peptides were antagonists. Again, this is the first time that agonist to antagonist conversion was observed by substitution of *N*-methyl at position 8 of a decapeptide LHRH agonist.

A number of groups, applying theoretical methods to both agonists[18,19] and antagonists,[20] have indicated the presence of a type II′ β-turn extending from residues 5 to 8 in the bioactive conformation of LHRH analogues when bound to their receptor (Figure 1). Experimental NMR studies[21,22] have confirmed the presence of this β-turn in cyclic antagonists. The introduction of a *N*-methyl group in the peptide's backbone at position 5 would have the effect of interfering with the β-turn conformation and especially the hydrogen bond formed from the main chain NH of Tyr[5] to the C=O of residue Arg[8] (Figure 1). It is surprising therefore, that for the NMe-Tyr[5] agonists reported here (8, 20, and 31), the binding affinity and in vitro potency is quite close to that of the parent, loosing only 10-fold or less in the three agonist series. Similar studies have been performed with antagonists where N-methylation of Tyr[5] resulted in retention or even increase in binding affinity and in vitro potency.[23,24] This suggests that in these agonists, as well as in the antagonists, the β-turn in the bioactive conformation does not have a classic type II′ conformation.

In examining the conformation of the β-turn (Figure 1), N-methylation of the amide at position 8 should be even more disruptive. Position 8 is one of the central residues of the β-turn and N-methylation should greatly destabilize a β-turn and prevent hydrogen bond formation between the main chain NH of Arg[8] and the C=O of Tyr[5]. Indeed, the NMe-Arg[8] analogues (11 and 34) show greater losses, and are the worst compounds of the leuprolide and nafarelin series, with binding affinity reductions of 1800- and 2900-fold, respectively. However, the NMe-Arg[8] analogue in the deslorelin series (22) is surprisingly good, with reduction in binding affinity of only 30-fold relative to the parent, giving a compound that is better than LHRH (36). Thus, it is clear that the actual conformation of LHRH analogues in the receptor is not a simple type II′ β-turn and appears to vary from series to series based on the detailed interactions of the various side chains with the receptor.

Stability against Enzymatic Degradation. All the *N*-methyl analogues in the leuprolide series and several from the deslorelin and nafarelin series were tested for enzymatic stability. Two model systems were used: purified chymotrypsin ($t_{1/2}$) and the intestinal rat jejunum sac ($T_{1/2}$).[8] Leuprolide is highly labile to chymotrypsin under the testing conditions, with a half-life of 1 min (Table I and Figure 2). N-Methylation at positions 3, 4, and 5 effectively blocked chymotrypsin cleavage giving half-lives of over 60 min (5 and 1 versus 6, 7, and 8). The same modification at position 2, whether the residue is His or Phe, increased the half-life by 7- and 6-fold, respectively (1 and 3 versus 2 and 4). N-Methylation at the remaining positions 6, 7, 8, or 10 had no influence whatsoever (1 versus 9, 10, 11, and 12). Chymotrypsin has been shown to cleave the Trp[3]-Ser[4] bond in leuprolide.[8] Therefore, N-methylation of Ser[4] (7) was clearly expected to stabilize this peptide bond to cleavage, since it blocks the mechanism of action of the enzyme as we already reported.[7,8] More unexpected, though, was that N-methylation of the peptide bond preceding or following the 3-4 bond also stabilized it against cleavage by chymotrypsin, as dem-

Table I. In Vitro Biological Activity, Enzymatic Stability, and Pharmacokinetics of LHRH Agonists

*p*Glu-His-Trp-Ser-Tyr-DLeu-Leu-Arg-Pro-NHEt

1

compd	substitution	MH$^+$ [a]	t_R [b]	pK_I [c]	pD_2 [d]	pA_2 [e]	$t_{1/2}$ [f]	$T_{1/2}$ [g]	clearance [h]
1 (leuprolide)[i]				9.73 (±0.04)	10.69 (±0.04)		1.0	4.0 (2.5–9.5)	9.0 (±0.6)
2	NMeHis^2	1223	18.46	7.48 (±0.07)	8.22 (±0.05)		7.0	48.0 (34–78)	7.16 (±0.39)
3	Phe^2[i]			8.66 (±0.06)	9.81 (±0.05)			2.2 (1.1–4.9)	25.4 (±5.5)
4	NMePhe^2	1233	26.65	7.92 (±0.01)		7.53 (±0.00)	6.0	18 (15–21)	30.96 (±7.42)
5	1Nal^3[i]			10.03 (±0.16)	10.35 (±0.45)		>15.0	25.0 (15.5–65)	28.4 (±4.7)
6	NMe1Nal^3	1234	18.70	8.49 (±0.57)		6.59 (±0.00)	>60.0	44.0 (32–74)	14.45 (±2.9)
7	NMeSer^4[i]			8.85 (±0.09)	9.42 (±0.18)		>60.0	>90.0	16.9 (±0.8)
8	NMeTyr^5	1223	13.20	8.60 (±0.07)	9.57 (±0.18)		>60.0	42 (24–147)	8.2 (±0.5)
9	NMeDLeu^6	1223	35.18	8.42 (±0.08)	9.61 (±0.16)		1.0	54 (51–58)	21.0 (±1.3)
10	NMeLeu^7	1223	32.90	9.80 (±0.00)	10.45 (±0.05)		1.0	4.1 (2.0–9.1)	8.3 (±0.68)
11	NMeArg^8	1223	30.35	6.46 (±0.10)	7.00 (±0.10)		1.0	6.6 (6.2–6.9)	
12	Sar^{10}NH$_2$	1252	17.90	7.99 (±0.05)	8.73 (±0.15)		1.0	8.4 (7.1–10)	9.77 (±1.88)
13	DTrp^6 (deslorelin)			11.00 (±0.17)	11.33 (±0.14)				34.3 (±5.48)
14	NMeHis^{2}DTrp^6	1296	16.85	8.85 (±0.09)		8.26 (±0.26)			16.4 (±1.07)
15	Phe^{2}DTrp^6[i]			10.61 (±0.05)	10.81 (±0.13)			10.8 (6.8–26)	28.8 (±2.4)
16	NMePhe^{2}DTrp^6[i]			9.68 (±0.07)	8.40 (±0.15)		3.7	44.4 (28–133)	44.5 (±4.41)
17	1Nal^{3}DTrp^6	1293	18.40	10.71 (±0.12)	11.49 (±0.00)				
18	NMe1Nal^{3}DTrp^6	1307	33.40	9.00 (±0.19)	8.35 (±0.05)				15.14 (±1.08)
19	NMeSer^{4}DTrp^6[i]			10.11 (±0.06)	10.10 (±0.40)			39.1 (25–91)	19.9 (±1.2)
20	NMeTyr^{5}DTrp^6	1296	25.15	10.07 (±0.15)	10.55 (±0.45)				11.1 (±0.43)
21	DTrp^{6}NMeLeu^7	1296	34.38	10.24 (±0.27)	10.70 (±0.10)				19.5 (±0.14)
22	DTrp^{6}NMeArg^8	1296	17.90	9.51 (±0.01)	9.35 (±0.53)				25.0 (±2.4)
23	DTrp^{6}Sar^{10}NH$_2$	1325	20.15	9.50 (±0.02)	10.05 (±0.05)				4.32 (±0.16)
24	D2Nal^{6}Gly^{10}NH$_2$ (nafarelin)			11.01 (±0.28)	11.05 (±0.45)		0.5	28 (18–63)	5.50 (±0.60)
25	NMeHis^{2}D2Nal^{6}Gly^{10}NH$_2$	1336	26.13	8.85 (±0.12)	8.18 (±0.14)				4.13 (±0.19)
26	Phe^{2}D2Nal^{6}Gly^{10}NH$_2$	1332	42.55	10.13 (±0.04)	10.23 (±0.22)				
27	NMePhe^{2}D2Nal^{6}Gly^{10}NH$_2$	1346	30.93	9.53 (±0.09)		8.80 (±0.02)			
28	1Nal^{3}D2Nal^{6}Gly^{10}NH$_2$	1333	37.20	10.57 (±0.15)	11.55 (±0.18)				
29	NMe1Nal^{3}D2Nal^{6}Gly^{10}NH$_2$	1347	22.70	9.13 (±0.02)	8.70 (±0.30)				
30	NMeSer^{4}D2Nal^{6}Gly^{10}NH$_2$[i]			10.37 (±0.14)	11.30 (±0.20)		>60.0	47.0 (38–78)	27.55 (±2.25)
31	NMeTyr^{5}D2Nal^{6}Gly^{10}NH$_2$	1336	23.88	10.51 (±0.00)	10.95 (±0.15)		>60.0	33 (25–45)	3.40 (±0.34)
32	NMeD2Nal^{6}Gly^{10}NH$_2$	1336	24.88	8.57 (±0.19)	8.30 (±0.20)				
33	D2Nal^{6}NMeLeu^{7}Gly^{10}NH$_2$	1336	28.10	10.50 (±0.14)	12.35 (±0.05)				
34	D2Nal^{6}NMeArg^{8}Gly^{10}NH$_2$	1336	35.60	7.55 (±0.13)		7.95 (±0.38)			3.87 (±0.22)
35	D2Nal^{6}Sar^{10}NH$_2$	1336	25.45	10.49 (±0.03)	11.87 (±1.02)				3.55 (±0.21)
36	LHRH			8.90 (±0.05)	9.27 (±0.18)				

[a] Values determined by FABMS. [b] t_R = HPLC retention time in min. [c] pK_I = the negative logarithm of the 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] pA_2 = the negative logarithm of the concentration of antagonist that requires 2-fold higher concentration of agonist to release LH from cultured rat pituitary cells. [f] $t_{1/2}$ = chymotrypsin degradation half-life in min. [g] $T_{1/2}$ = the time, in min, required for the lumenal concentration of compound in the rat sac jejunal to decrease by 50%. [h] 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, units are mL/min per kg. [i] Compound reported in ref 8.

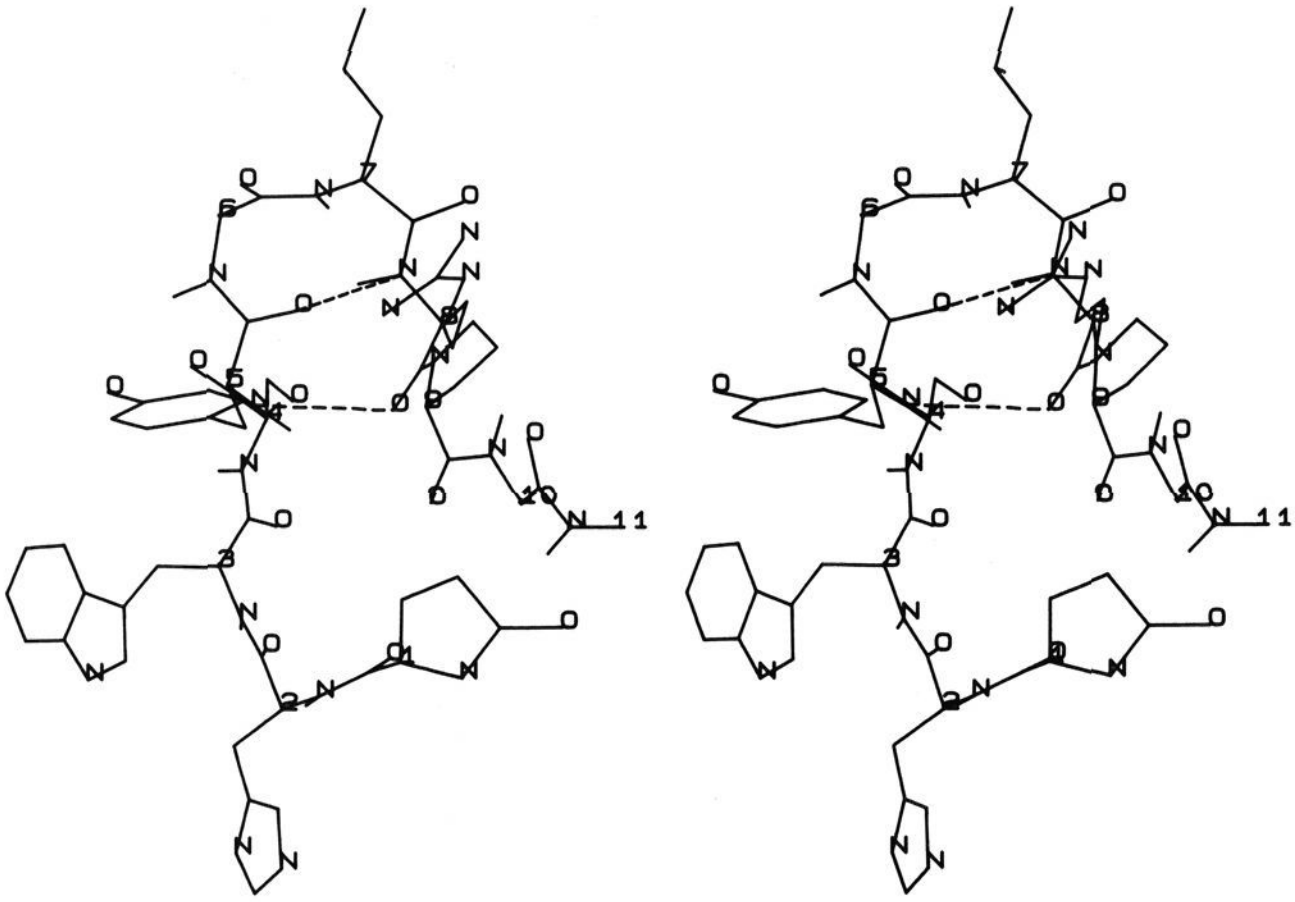

Figure 1. Stereo presentation of the three-dimensional structure of LHRH as calculated by Momany.[18,19] This structure depicts Momany's C conformation. The dotted lines show the possible hydrogen bonds that occur between the NH of Arg^8 and the C=O of Tyr^5 and between the NH of Tyr^5 and the C=O of Arg^8. Both of these hydrogen bonds and the main chain conformation would be disrupted by N-methylation at the 5 and especially at the 8 positions.

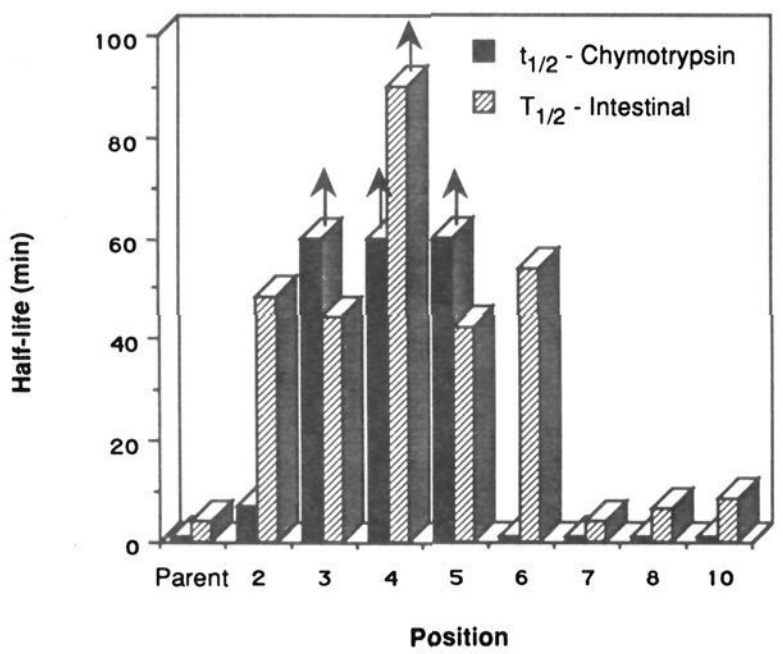

Figure 2. Bar graph of the digestion data for leuprolide (**1**) and its *N*-methyl analogues **2** and **6–12** in the chymotrypsin ($t_{1/2}$) and the in vitro rat intestinal jejunum ($T_{1/2}$) models. The arrows represent values greater than that shown. For the chymotrypsin digestion, N-methylation at positions 3, 4, and 5 stabilizes the molecule. For the intestinal model, N-methylation at positions 2 (with the His substitution), 3, 4, 5, and 6 stabilizes the peptide. Thus, the two patterns appear similar but are different in detail.

onstrated by the half-life values of compounds **6** and **8**. To understand the molecular basis for this protection, we examined the three-dimensional structure of chymotrypsin[25] and a model structure for the conformation of a substrate peptide bound in the active site[26] to see how it was affected when the peptide bonds prior to and following the scissile bond, i.e. positions 3 and 5, are N-methylated. Figure 3 shows the active site of chymotrypsin with leuprolide bound in the substrate binding site in position to be cleaved between residues 3 and 4. The side chain of Trp^3 sits in the primary specificity pocket. There are a number of hydrogen bonds between the substrate, leuprolide, and the enzyme. Both main chain NH's of residues 3 and 5 are involved in hydrogen bond interactions with the enzyme, with the main chain carbonyls of Ser 214 and Phe 41, respectively. Substitution of the amide hydrogen with *N*-methyl will disrupt the hydrogen bond, and steric hindrance of the larger methyl group forces a distortion in the conformation of the substrate on the enzyme. One would therefore expect that these compounds are significantly poorer substrates of chymotrypsin thereby stabilizing the molecules against cleavage between residues 3 and 4.

When the compounds were tested in the in vitro rat jejunum sac model, N-methylation at position 4 was the most stabilizing with a half-life of over 90 min relative to 4 min for the parent leuprolide (**7** versus **1**). *N*-Methyl substitutions at positions 2, 3, 5, and 6 gave intermediate increases in half-life ranging from 42 to 54 min (**2**, **6**, **8**, and **9**). Interestingly, unlike with chymotrypsin, the degree of stabilization was different for the two different residues at position 2, with NMe-Phe^2 much less stable than NMe-His^2, 18 versus 48 min, in the leuprolide series (**4** versus **2**). However, the NMe-Phe^2 substitution in the deslorelin (**16**) series had a half-life (44 min) longer than its parent, and similar to the NMe-His^2 in leuprolide (**2**). *N*-Methyl substitution at position 7, 8, or 10 had no significant influence on the half-life of the compounds. Overall, the pattern of stabilization in the intestinal model is rather similar to that found for chymotrypsin (Figure 2). However, an interesting difference is that the NMe-His^2 and NMe-DLeu^6 analogues (**2** and **9**) showed a large increase in stability in the intestinal model but were as labile as the parent leuprolide to chymotrypsin (Table I and Figure 2). This result suggests that chymotrypsin is not the major proteolytic enzyme in the rat intestinal model.

Pharmacokinetics. The clearance values for all the analogues in the leuprolide series, except compound **11** for which we do not have an antibody, were determined using our previous method.[17] The pharmacokinetics of several analogues in the deslorelin and nafarelin series

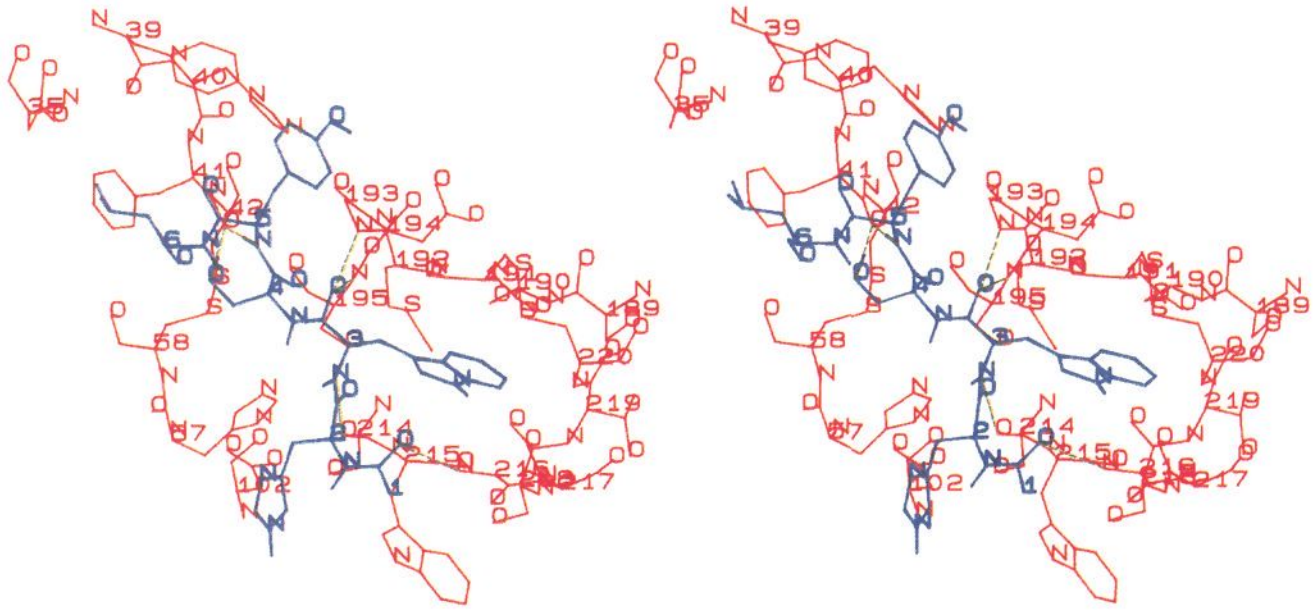

Figure 3. Three-dimensional structure of the active site region of chymotrypsin[25] (red) with leuprolide (blue) placed in the active site so that Trp[3] is in the primary specificity pocket and the 3-4 peptide bond is positioned for cleavage. It is clear that N-methylation of the Ser[4] of leuprolide would block cleavage of the Trp-Ser scissile bond. The main chain NHs of residues 3 and 5 of leuprolide form hydrogen bonds (green dashed lines) with the enzyme, to the main chain carbonyl oxygens of Ser 214 and Phe 41, respectively. Substitution of the amide hydrogens with methyls will block hydrogen bond formation and the increased bulk of the methyl groups sterically prevent the peptide from binding to the active site making these analogues poor substrates of chymotrypsin.

were also determined (Table I). The low value obtained for the clearance of nafarelin agrees with the previous report that this agonist extensively binds to plasma protein.[27] The pattern of clearance values in the three series does not reflect a clear correlation between in vitro enzymatic stabilization and in vivo clearance. This finding can be rationalized since clearance is constituted from three major components, distribution, metabolism, and elimination; the physicochemical properties of the compounds may have a significant effect on in vivo elimination.[17] In spite of this, the NMe-Tyr[5] substitution in the three series consistently gave lower clearance values, suggesting that metabolic stabilization of the 4-5 peptide bond, independently of the substituent at position 6 or 10, has a significant effect on the peptides' clearance rates. These findings agree with previous metabolism studies of leuprolide which indicated the presence of a (5–9) fragment as a metabolite.[28] Recently we have reported the effect on NMe-Tyr[5] substitution in LHRH antagonists on in vitro and in vivo potency.[23,24]

Conclusions

The structure–activity relationships of the receptor binding affinities and the in vitro LH release activities of the *N*-methyl analogues in the leuprolide, deslorelin, and nafarelin series were quite subtle and varied with the substitutions at positions 6 and 10. With the exception of compounds **30**, **33**, and **35**, which were more potent than nafarelin, all the N-methylated analogues were less active than the respective parents. In five cases (compounds **4**, **6**, **14**, **27**, and **34**) for the first time the agonist response was converted to antagonist upon N-methylation of the peptide bond. The agonist/antagonist switch was influenced by a combination of the site of N-methylation and the substituents at positions 6 and 10. Cleavage of leuprolide by chymotrypsin was blocked not only by the N-methylation of the 3-4 scissile bond, but also by N-methylation of the adjacent 2-3 or 4-5 peptide bonds. These findings were rationalized by examining the three-dimensional structure of the substrate bound to the enzyme's active site. The lower clearance values for the NMe-Tyr[5] analogues **8**, **20**, and **31** suggest slower rates of metabolism.

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 of 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 (except NMe-Tyr, NMe-Phe, NMe-His). That was confirmed by FABMS.

All the Boc-protected amino acids, Boc-Pro, Boc-Sar, Boc-Arg(Tos), Boc-Leu, Boc-NMe-Leu, Boc-DLeu, Boc-DTrp, Boc-D2Nal, Boc-Tyr(*O*-2-Br-Cbz), Boc-NMe-Tyr(*O*-2,6-Cl-Bzl), Boc-Ser(*O*-Bzl), Boc-Trp(*N*-ind-formyl), Boc-His(*N*-im-Cbz), Boc-Phe, Boc-NMe-Phe, and Cbz-*p*-Glu 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-Gly-4-methylbenzhydrylamine resin (with a substitution varying from 0.4 to 0.7 mmol/g) was obtained from Peninsula Laboratories, Inc., (Belmont, CA). Boc-NMe-Arg-(Tos) was purchased from Bachem Bioscience Inc. (Philadelphia, PA). 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 Procedure for the Synthesis of *N*-(*tert*-Butoxycarbonyl)-*N*-methyl-D-3-(2-naphthyl)alanine (37) and *N*-(*tert*-Butoxycarbonyl)-*N*-methyl-3-(1-naphthyl)alanine (38). Sodium hydride (60% dispersion in oil, 2.5 g, 63 mmol) was washed with anhydrous pentane (3 × 15 mL) to remove oil, and then suspended in dry THF (25 mL) and cooled, with stirring, to 0 °C in an ice-bath. A solution of the Boc-amino acid (5 g, 16 mmol) in THF (25 mL) was added via cannula, followed by the portionwise addition of methyl iodide (7.9 mL, 126 mmol) over 10 min. The reaction mixture was allowed to warm to room temperature and stirring was continued for 24 h. The reaction

was slowly added to a cold 1 N sodium hydrogen sulfate (80 mL) solution with rapid stirring. The resulting mixture was extracted with ethyl acetate (3 × 75 mL), washed with 1 N sodium thiosulfate (50 mL) and brine (50 mL), dried (Na_2SO_4), and concentrated in vacuo. The sticky crystals obtained were triturated with anhydrous hexane overnight, filtered, and dried to yield a white powder.

Compound **37** was isolated in 93% yield (4.90 g): mp 136–137 °C; $[\alpha]^{22}_D$: +62.2° (EtOH, c = 1); ^{1}H NMR ($CDCl_3$, 3:2 mixture of rotamers) δ 1.31, 1.38 (s, 9 H), 2.67, 2.78 (s, 3 H), 3.22, 3.35 (dd, 1 H, J = 14.35, 10.83 Hz), 3.48 (dd, 1 H, J = 14.35, 5.14 Hz), 4.75, 4.87 (dd, J = 10.83, 5.14 Hz), 7.30 (m, 1 H), 7.45 (m, 2 H), 7.65 (s, 1 H), 7.79 (m, 3 H); IR ($CDCl_3$) ν_{max} (cm^{-1}) 2975 (m), 1715 (s), 1685 (s), 1390 (m), 1370 (m), 1170 (m), 1155 (s), 1145 (s); MS (M + H)$^+$ 330. Anal. ($C_{19}H_{23}NO_4$) C, H, N.

Compound **38** was isolated in 92.6% yield (4.82 g): mp 42–46 °C; $[\alpha]^{22}_D$ −141.6° (EtOH, c = 1); ^{1}H NMR ($CDCl_3$, 3:2 mixture of rotamers) δ 1.05, 1.44 (s, 9 H), 2.50, 2.78 (s, 3 H), 3.35, 3.69 (dd, 1 H, J = 14.16, 6.84 Hz), 3.82, 3.93 (dd, 1 H, J = 14.16, 0.98 Hz), 4.64, 4.95 (dd, 1 H, J = 6.84, 0.98 Hz), 7.31 (m, 1 H), 7.39 (m, 1 H), 7.53 (m, 2 H), 7.77 (m, 1 H), 7.88 (m, 1H), 8.07 (m, 1H); IR (KBr) ν_{max} (cm^{-1}), 2980 (m), 1740 (s), 1700 (s) 1390 (m), 1365 (m), 1170 (s), 1155 (s). MS, (M + H)$^+$ 330. Anal. ($C_{19}H_{23}NO_4 \cdot {}^1/_4H_2O$) C, H, N.

General Synthesis and Purification of Peptides 2, 4, 6, 8–14, 17, 18, 20–23. All the peptides were synthesized using the solid phase peptide synthesis (SPPS) techniques[13] analogously to our previously reported syntheses for LHRH agonists.[8,14] The crude peptides were 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-mm 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 20–45% acetonitrile over 40 min. The UV detector was set at 254 nm. Preparative HPLC separation was accomplished with a C_{18} Dynamax column using analogous conditions to those previously reported.[8] The purity of the final compounds was over 95% on the basis of analytical HPLC, FABMS, and AAA.

Biological Assays. We previously reported the receptor binding and LH release assays.[14]

Rat Jejunum Sac Assay. This test was described in our recent publication.[8]

Pharmacokinetics Determination. Each compound was administered iv to castrate male rats at a dose of 100 μg/kg. Blood samples were drawn over a 6-h period, using EDTA as anticoagulant. The plasma concentration of each compound was determined by RIAs using a LHRH analogue antibody that recognizes the C-terminal residues Leu-Arg-Pro-NHEt or Leu-Arg-Pro-GlyNH_2. The antiserum and tracer used for leuprolide and deslorelin analogues were rabbit antiserum C-402 and [^{125}I-Tyr^5]leuprolide. The RIAs for nafarelin analogues and for Sar^{10}-substituted leuprolide and deslorelin analogues used rabbit antiserum 5328W and [^{125}I-Tyr^5,DLys^6]LHRH as reagents. Both rabbit antisera were obtained from Dr. P. Michael Conn of the University of Iowa. The area under the plasma concentration versus time curve was calculated via the trapezoidal rule. The whole body clearance was determined from the ratio of the dose divided by the area under the curve and is expressed in units of mL/min per kg.

Chymotrypsin Cleavage Assay. We previously reported our method for measuring the resistance of LHRH agonists to chymotrypsin.[9]

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