Journal of Medicinal Chemistry

Volume 17, Number 6 June 1974

Analogs of the Luteinizing Hormone-Releasing Hormone to Study Conformational Aspects of the Aromatic Amino Acid Moieties and Inhibition†

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[Ile^2]-LH-RH (<Glu-Ile-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), [Leu^{2,3}]-LH-RH (<Glu-Leu-Leu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), and [Tyr^3,Trp^5]-LH-RH (<Gly-His-Tyr-Ser-Trp-Gly-Leu-Arg-Pro-Gly-NH_2) have been synthesized by solid-phase procedures. These three analogs support further study of the possible parallel planarity of the bicyclic aromatic Trp and the monocyclic aromatic Tyr side chain moieties and conformation at the N terminus of LH-RH. [Ile^2]-LH-RH appeared 0.03% as active and [Tyr^3,Trp^5]-LH-RH appeared 0.003% as active as LH-RH to release both LH and FSH *in vitro;* neither analog showed inhibition. [Leu^{2,3}]-LH-RH did not release (<0.000003%) either LH or FSH and unequivocally inhibited the LH-RH-induced release of LH and FSH at the high dosage of 300,000-fold. As one guideline, an effective inhibitor may be sought which is designed from the structure of LH-RH and which does not exhibit even weak agonist activity. The data on these three analogs appear to support this guideline.

Following the determination of the sequence of the hypothalamic luteinizing hormone-releasing hormone (LH-RH) as <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, from both porcine[1,2] and ovine[3,4] tissues, many synthetic analogs have been assayed in connection with studies on structure–activity relationships and inhibitors.

The design of potential inhibitors of LH-RH has recognized the observations that [Gly^2]-LH-RH and [des-His^2]-LH-RH have inhibitor properties *in vitro.*[5,6] Also new inhibitors of LH-RH could embody Pro-$NHCH_2CH_3$ as a carboxyl terminus to reduce probable enzymatic degradation[7] and the conformational directing property of a D-Ala substituent in the 6 position.[6]

Several investigators have suggested that the "active site" of LH-RH is located in the N-terminal region or that the <Glu and the His residues have important roles in the hormonal activity.[8] From assay data on a series of analogs containing a linear chain in place of the <Glu of LH-RH, it was concluded that the "minimum necessary part" in the <Glu residue for biological activity is the -CO-NHCHCO- moiety.[8] Although replacement of His in the 2 position of LH-RH by the aromatic amino acids Trp[6] and Phe[6,9,10] led to the retention of considerable LH-releasing activity, replacement by nonaromatic amino acids caused a greater loss of activity.[5,6] The high activity (40%) of [Trp^2]-LH-RH indicates that aromatic properties in the 2 position may be more essential to the hormonal activity than other properties of the imidazole nucleus. However, a contribution from the peptide backbone may be significant for hormonal activity. Modifications in the 3 position (Trp) of LH-RH have produced either very weakly active compounds or weak antagonists.

We have proposed,[11] from inspection of a model of LH-RH, that the planar indole and benzenoid moieties of the Trp and Tyr could be in relatively parallel positions, because of possible π–π bond interactions, and that such parallel planarity, in the conformation of the decapeptide, may impart some structural specificity for hormonal activity to this molecule.

We now report the synthesis of and the bioassay data for three new decapeptide analogs of LH-RH which were designed to study specifically the significance of the side chains of His, Trp, and Tyr, the aromatic amino acids in LH-RH.

Experimental Section

Synthesis. The decapeptide analogs were prepared by the solid-phase method[12] on a Beckman 990 peptide synthesizer with the Merrifield resin. α-Amino functions were protected exclusively by the Boc group. Other protecting groups used were Bzl (Ser, Tyr), 2,6-dichlorobenzyl (Tyr), Z (<Glu), Tos (His, Arg), and nitro (Arg). The protected amino acid derivatives were coupled in methylene chloride, DMF (Trp), or in a mixture of these two solvents (<Glu) in a 2.5-fold excess for 4–12 hr with DCI in methylene chloride. The coupling reactions were monitored by the semiquantitative ninhydrin method[13] and by amino acid analysis. Removal of the Boc group was by a solution of 50% TFA in methylene chloride. After incorporation of Trp, 1,2-ethanedithiol was added (5%) to protect the indole nucleus.[14] The TFA salt of the peptide resin ester was neutralized with 10% Et_3N in methylene chloride.[15] The completed protected peptide was cleaved from the resin support with NH_3–CH_3OH (3 days at room temperature) and then completely deblocked with anhydrous (CoF_3), liquid HF in the presence of anisole (10%) for 1 hr at 0°.[16] The crude LH-RH analog was extracted with 10% aqueous acetic acid. The

†Hypothalamic Hormones. 55.

Table I, Chemical and Physical Properties of the Synthetic LH-RH Analogs

[Ile^2]-LH-RH (<Glu-Ile-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)
Amino acid analysis[a] (125°)[b] Glu 1.11, Ile 1.12, Trp 0.58, Ser 0.71, Tyr 0.89, Gly 2 × 0.98, Leu 0.97, Arg 0.96, Pro 0.99, NH_3 2.16; tlc data[c] R_f^{I} 0.78, R_f^{II} 0.74; $[\alpha]^{25}D^{d}$ −35.71° (*c* 1.029, CH_3OH)

[Leu^{2,3}]-LH-RH (<Glu-Leu-Leu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)
Amino acid analysis[a] (110°) Glu 0.87, Leu 3 × 1.04, Ser 0.99, Tyr 1.00, Gly 2 × 1.00, Arg 0.88, Pro 1.13, NH_3 1.56; tlc data[c] R_f^{I} 0.74, R_f^{II} 0.64, R_f^{III} 0.65; $[\alpha]^{25}D^{d}$ −43.77° (*c* 1.019, CH_3OH)

[Tyr^3,Trp^5]-LH-RH (<Glu-His-Tyr-Ser-Trp-Gly-Leu-Arg-Pro-Gly-NH_2)
Amino acid analysis[a] (110°) Glu 1.04, His 1.03, Tyr 0.87, Ser 0.94, Trp 0.95, Gly 2 × 1.05, Leu 1.10, Arg 0.97, Pro 0.94, NH_3 1.07; tlc data[c] R_f^{I} 0.65, R_f^{II} 0.43, R_f^{III} 0.57; $[\alpha]^{25}D^{d}$ −42.26° (*c* 0.876, CH_3OH)

[a]The peptide was hydrolyzed, *in vacuo*, in 6 *N* HCl containing 4% thioglycolic acid overnight at the temperatures specified and analyzed on a Beckman 119 amino acid analyzer set up for one-column methodology. [b]Hydrolysis of [Ile^2]-LH-RH at 110°, overnight, resulted in incomplete hydrolysis of the Ile residue. The ratios then observed were Ile, 0.62; Ser, 0.93; Leu, 1.00. [c]Tlc systems (silica gel): I, *n*-BuOH–CH_3CO_2H–ethyl acetate–H_2O (1:1:1:1); II, EtOH–H_2O (7:3); III, $CHCl_3$–CH_3OH–NH_4OH (60:45:20). The spots were detected by chlorotolidine (positive) and ninhydrin (negative) reagents. [d]Optical rotations were measured on a Perkin-Elmer 141 digital readout polarimeter.

Table II, *In Vitro* Agonist and Antagonist Activity of the Analogs

	Dose,[d] ng/ml of medium		LH			FSH		
Peptide analog	Peptide	LH-RH	Δ ng/ml of medium	S.E.M.	*p* value *vs.* 1	Δ ng/ml of medium	S.E.M.	*p* value *vs.* 1
[Ile^2]-LH-RH[a]	1,000		564	±45	ns	11,152	±2159	ns
	1,000	0.3	572	±31	ns	9,170	±1078	ns
		0.3	590	±27		9,230	±1043	
[Leu^{2,3}]-LH-RH[b]	100,000		49	±23	ns	640	±264	ns
	100	0.3	177	±32	ns	2,111	±308	<0.01
	1,000	0.3	207	±57	ns	2,165	±374	<0.01
	10,000	0.3	238	±55	ns	2,171	±249	<0.01
	100,000	0.3	83	±20	0.05	28	±336	<0.001
		0.3	200	±49		4,083	±401	
[Tyr^3,Trp^5]-LH-RH[c]	10,000		656	±8	ns	10,008	±1356	ns
	100,000	0.3	671	±23	ns	8,691	±1320	0.02
		0.3	635	±25		13,294	±1026	

[a]Incubation for 6 hr, Δ value = mean (8) P_2 minus I_3, I_4, I_5. [b]Incubation for 6 hr, Δ value = mean (4) P_2 minus I_3, I_4 (agonist); P_2 minus I_5, I_6 (antagonist). [c]Incubation for 5 hr, Δ value = mean (6) P_2 minus I_3, I_4, I_5. [d]Intermediate dose levels have been omitted for brevity.

Table III, *In Vitro* Dosage of LH-RH and LH-RH Analogs and Their Relative Degrees of LH and FSH Activity

	Dosage, μg/ml of medium		Rel act. (% LH-RH act.)	
Peptide	LH	FSH	LH	FSH
LH-RH	3×10^{-4}	3×10^{-4}	100	100
[Ileu^2]-LH-RH	1	1	0.03	0.03
[Tyr^3,Trp^5]-LH-RH	1×10	1×10^{1}	0.003	0.003
[Leu^2,Leu^3]-LH-RH	1×10^{2}	1×10^{2}	NA[a]	NA[a]

[a]NA = not active or <0.000003%.

lyophilized product was passed over a Bio-gel P2 column (110 × 2.5 cm) with 1.3% aqueous acetic acid. Peptide peaks were located by uv absorption at 256, 260, or 280 nm. The main peak was partitioned over Sephadex G25 (100 × 1.5 cm, 20–80 μ) using the system 0.1% acetic acid–1-butanol–pyridine (11:5:3), and the peptide peaks were located by the Folin–Lowry method[15] at 660 or 700 nm.

Purity of the purified peptides was checked by amino acid analyses and tlc on silica gel; the data including optical rotations are in Table I.

Biological Assays. All hormonal activities were obtained from *in vitro* studies using pituitaries of 20-day-old female Sprague–Dawley rats (Charles River Laboratory). To determine the LH and FSH agonist and antagonist activity, two pituitaries were incubated at 37° in 1 ml of lactated Ringer's solution (Travenol Laboratories) in 10-ml Teflon beakers in a Dubnoff shaker. Pituitaries were incubated for a total of 6 hr. Medium was removed each hour for RIA[17] for LH and FSH and fresh medium was added. After two preincubation periods (P_1, P_2), the LH-RH analog was added to the incubation medium (I_3, I_4, I_5, I_6) with and without LH-RH. When both peptides were added together the LH-RH analog was always added to the incubation medium 5 min before LH-RH. The RIA reagents for FSH were distributed by NIAMD, NIH. Dr. G. Niswender supplied the antiovine LH serum No. 15 for the rat LH assay while Dr. L. E. Reichert supplied an ovine LH preparation for labeling and the LH rat reference preparation. The values for these assays are calculated in terms of ng of the following standards: LH-LER-1240-2 (0.60 NIH-LH-SI units/mg) and FSH (2.1 × NIH-FSH-SI units/mg).

Results and Discussion

The activity data in Table II for the three analogs concern the assays of agonist and antagonist activities. The approximate agonist activities, expressed in percentages obtained from the *in vitro* doses of the analogs that have the same degree of LH and FSH agonist activity as 0.3 ng LH-RH, are in Table III.

The low activity of [Tyr^3,Trp^5]-LH-RH is particularly noteworthy and provides more support for our hypothesis[11] of parallel planarity for the Trp-Tyr residues in LH-RH. The bicyclic indole nucleus at position 3 may be essential for maximal orbital overlap with the monocyclic aromatic moiety of Tyr. Peptides with the reversed sequence for Tyr and Trp would not be expected to have any effective overlap in the conformation of the decapeptide and, therefore, only very weak activities would be anticipated. The low activities observed for replacement of

Trp with other monocyclic aromatic amino acids such as Phe,[18,19] D-Trp,[6] and His, as in [Trp^2,His^3]-LH-RH,[18] may be rationalized along this guideline. Although the actual function of the Trp-Tyr grouping in LH-RH at the receptor level is not known, possible functions may be proposed. A π–π interaction may be a significant cause of the molecule having its "active conformation;" in this case, the 1,3 relationship of Trp and Tyr may consist of a "conformational component" similar to the effect of replacing Gly in the 6 position of LH-RH with D-Ala.[6] Such an interaction of Trp and Tyr may selectively influence the conformation of the N-terminal portion of LH-RH and thereby influence the "active site" as well as binding to the receptor.

The reversed sequence of Tyr and Trp in this analog is comparable with the tetrapeptide, <Glu-Tyr-Arg-Trp-NH_2, which releases LH at μg levels.[20] In this small peptide, however, the peptide backbone conformation may require such a reversed sequence for maximal overlap of the aromatic moieties. Furthermore, the guanidino group in the 3 position of the tetrapeptide may then be in a position comparable with that in LH-RH when referred to the position of the 1,3-aromatic unit.

The observation of low (0.03%), but significant, activity for [Ile^2]-LH-RH, in which His has been replaced by Ile, is worthy of comment. Relatively high activity has previously been observed when His was replaced by nonaromatic amino acid residues; *e.g.*, [Met^2]-LH-RH is 1% active *in vitro*.[6] These results, taken together with the reported low activities of other analogs, such as [3-methyl-His^2]-LH-RH,[9,21,22] [1-methyl-His^2]-LH-RH,[22] and [Phe^2]-LH-RH,[6,9,10] indicate that the imidazole moiety does not contribute directly to the "active site" of LH-RH. The cause of inhibition by [des-His^2]-LH-RH and [Gly^2]-LH-RH[5,6] may then be considered to result from a conformational change of the N-terminal portion, involving the <Glu residue and the peptide backbone in the 2 position, caused by removal of the His residue or its replacement by a more "flexible" amino acid residue. A similar model could also explain the observation of weaker inhibition produced from certain replacements for Trp in the 3 position;[6] a conformational change affecting the <Glu residue, the peptide backbone in the 2 position, and the residue in the 3 position would then be considered. Since this change involves a larger proportion of the molecule, it would be expected to have a greater effect on conformation than that for the 2-substituted analogs and to produce a weaker inhibitor.

The replacement of both His and Trp by Leu, as in [Leu^{2,3}]-LH-RH, may result in the loss of several internal conformational stabilizing factors and, therefore, lead to a molecule with a distorted N-terminal segment, which has a low affinity for the LH-RH receptor. However, [Leu^{2,3}]-LH-RH did show inhibition of LH-RH-induced release of LH and FSH in contrast to [Ile^2]-LH-RH which did not do so (Table III). As recorded in Table III, an active dose level of [Tyr^3,Trp^5]-LH-RH slightly inhibited the FSH but not the LH release of LH-RH; however, this effect was not dose dependent. Of these three analogs, only [Leu^{2,3}]-LH-RH showed inhibition and no release and may underscore one guideline that effective antagonists might not have even weak agonist activity.

Acknowledgment, Appreciation is expressed to Dr. Marvin Karten and for the support of Contract NIH NICHD 72-2713 of the National Institutes of Health and Public Health Service Research Grant No. CA-14200-01 from the National Cancer Institute, and for grants from the Rockefeller Foundation and the Robert A. Welch Foundation. We are grateful to Drs. R. W. Bates, Albert Parlow, G. Niswender, and L. E. Reichert for their RIA preparations and procedures.

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