ACIDIC ANALOGS OF THE LUTEINIZING HORMONE-RELEASING HORMONE AND CONFORMATIONAL ASPECTS FOR ACTIVITY

by

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SUMMARY

[Gly^{1a}]-LHRH acid (<Glu-Gly-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH), [Gly^{2a}]-LHRH acid (<Glu-His-Gly-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH), and [Tyr^3, Trp^5]-LHRH acid (<Glu-His-Tyr-Ser-Trp-Gly-Leu-Arg-Pro-Gly-OH), were synthesized; they released LH with potencies of <0.0003, 0.0003, and 0.0003%, respectively, that of LHRH, but did not act as inhibitors up to a 30,000-fold relative dosage. Absence in these analogs of "conformational components" involving a hydrogen bond between the $<Glu^1$ and Ser^4 as proposed for LHRH and/or the proposed parallel planarity of the Trp-Tyr aromatic nuclei, and other effects including that of a C-terminal acid, could explain the observed data.

INTRODUCTION

The synthesis of analogs of the hypothalamic luteinizing hormone-releasing hormone (LHRH), <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, to elucidate structure-activity-conformation relationships and to seek inhibitors is a subject of increasing interest to many investigators. The sequence and conformational aspects of the decapeptide are being systematically investigated.

Most of the reported analogs of LHRH have possessed a <Glu moiety at the amino terminus and a Gly-NH_2 moiety at the carboxyl terminus; in particular, investigators have focused upon amides of the carboxyl terminus.

Certain naturally occurring and physiologically active peptides have a C-terminal acid function. Some of these peptides include angiotensin II,

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bradykinin, kallidin, β-MSH, ACTH, and particularly the tetradecapeptide, named somatostatin, isolated from the hypothalamus by Brazeau et al. (1) which apparently inhibits the release of somatotropin (growth hormone). In contrast to somatostatin, the thyrotropin releasing hormone (TRH) and LHRH have amide functionality at their C-terminus.

Sievertsson et al. (2) and Chang et al. (3) reported that the corresponding acid of LHRH has very low hormonal activity. Monahan et al. (4) reported that [Asp^2]-LHRH acted as a weak antagonist of the release of the luteinizing hormone (LH). The inactivity of <Glu-His-Trp-Ser-Tyr-Gly-OH was reported by Chang et al. (5).

The C-terminus of LHRH has been altered in other ways with the retention of considerable releasing activity. [$Gly^{10}NMe_2$]-LHRH of Coy et al. (6) and [Ala^{10}]-LHRH of Fujino et al. (7) were found to have significant releasing activity. Even [des-Gly^{10}]-LHRH had 10% of the releasing activity of LHRH (8). A series of nonapeptides based upon the [des-Gly^{10}]-LHRH alkylamide sequence was reported by Fujino et al. (9,10) to possess activities up to 500% (9,10).

In the vasopressin series, the conversion of the terminal amide to an acid has produced an inhibitor (11).

On the basis of such background information on both naturally occurring and synthetic peptide acids, one new decapeptide acid, two new undecapeptide acids and two fragments of LHRH as acids, have been synthesized and bioassayed. The two undecapeptide analogs of LHRH resulted from the intercalation of Gly into the backbone sequence of LHRH. Previously, intercalation has been an unexplored variation of the structure of LHRH.

EXPERIMENTAL

Synthesis. - The peptide analogs were synthesized by the solid phase method on a Beckman 990 Peptide Synthesizer with the Merrifield resin as described (12). Treatment of the completed protected resin ester with anhydrous, (CoF_3) liquid HF in the presence of anisole (10%) for 1 hr at 0° resulted in the cleavage of the peptide acid from the resin and the complete removal of all of the protecting groups (13). Other details of synthesis of the peptides and their purification were as described (12). Data on amino acid analyses, tlc and optical rotations for the analogs are in Table I.

Bioassays. - The analogs were tested, in vitro, for the release of LH and FSH, and for inhibition of the LHRH-induced release of LH and FSH, as reported (12).

RESULTS AND DISCUSSION

[Gly^{2a}]-LHRH acid had 0.0003% of the LH and FSH agonist activity of LHRH.

Table I

CHEMICAL AND PHYSICAL PROPERTIES OF THE SYNTHETIC LHRH ANALOGS

[Tyr^3, Trp^5]-LHRH acid (<Glu-His-Tyr-Ser-Trp-Gly-Leu-Arg-Pro-Gly-OH)

Amino Acid Analysis (110°)[a]: Glu, 1.06; His, 1.02; Tyr, 0.84; Ser, 0.90; Trp, 1.02; Gly, 2 x 1.06; Leu, 1.09; Arg, 0.98; Pro, 0.98.
Tlc Data[b]: R_f^{I} 0.62; R_f^{II} 0.53; R_f^{III} 0.51. $[\alpha]_D^{25}$ -44.50 (c = 0.91, CH_3OH).

[Gly^{1a}]-LHRH acid (<Glu-Gly-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH)

Amino Acid Analysis (130°)[a]: Glu, 0.91; Gly, 3 x 1.02; Trp, 0.65; Ser, 0.91; Tyr, 0.89; Leu, 1.13; Arg, 1.04; Pro, 1.03.
Tlc Data[b]: R_f^{II} 0.57; R_f^{IV} 0.58. $[\alpha]_D^{24}$ -47.44 (c = 0.93, 1% HOAc)

[Gly^{2a}]-LHRH acid (<Glu-His-Gly-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH)

Amino Acid Analysis (130°)[a]: Glu, 0.87; His, 0.82; Gly, 3 x 1.02; Trp, 0.68; Ser, 0.96; Tyr, 0.97; Leu, 0.87; Arg, 1.12; Pro, 1.09.
Tlc Data[b]: R_f^{II} 0.53; R_f^{III} 0.56; R_f^{IV} 0.59. $[\alpha]_D^{25c}$ -29.88° (c = 1.0, DMF).

a. The peptides were hydrolyzed, in vacuo, in 6 N HCl containing 4% thioglycollic acid, either overnight at 110°, or for 3 hr at 130°, and then analyzed on a Beckman 119 Amino Acid Analyzer set up for one-column methodology.

b. Tlc systems (silica gel): I, n-BuOH, CH_3CO_2H, ethyl acetate, H_2O (1:1:1:1); II, C_2H_5OH, H_2O (7:3); III, $CHCl_3$, CH_3OH, NH_4OH (60:45:20); IV, ethyl acetate, pyridine, CH_3COOH, H_2O (5:5:1:3). The spots were located by uv and iodine, chloro-tolidine (positive), and ninhydrin (negative) reagents.

c. Optical rotations were measured on a Perkin-Elmer 141 digital readout polarimeter.

[Tyr^3, Trp^5]-LHRH acid had 0.0003% of the LH agonist activity of LHRH, but only 0.00015% of the corresponding FSH agonist activity. Therefore, the LH/FSH releasing activity ratio of this analog, at a dosage of 100 μg, appears to be slightly greater than that observed for LHRH. [Gly^{1a}]-LHRH acid was less active since the LH and FSH agonist activity was <0.0003% that of LHRH.

These analogs, having two or more backbone and conformational changes, had only one-tenth of the releasing ability of LHRH-acid, which was 0.003% as potent as LHRH for releasing LH and FSH. <Glu-His-Trp-Ser-Tyr-Gly-OH and <Glu-His-Trp-OH were essentially inactive as agonists.

The lower releasing activity of [Tyr^3, Trp^5]-LHRH acid over that observed

for [Tyr^3, Trp^5]-LHRH, which was 0.003% as active as LHRH (12), reflects the difference in agonist activity resulting from single and multiple changes in the LHRH sequence.

[Gly^{2a}]- and [Gly^{1a}]-LHRH acids did not seem to inhibit the LHRH induced release of LH and FSH. However, the LHRH induced release of FSH, but not LH, was

TABLE II

IN VITRO AGONIST AND ANTAGONIST ACTIVITY OF LHRH ANALOGS OR DERIVATIVES[a]

Peptide Analog	Dose		LH			FSH		
	Peptide	LHRH						
	mμg/ml medium		△mμg/ml medium	SEM	p value vs LHRH	△mμg/ml medium	SEM	p value vs LHRH
[Tyr^3, Trp^5]-LHRH acid	-	0.3	635	± 25		13,294	±1026	
	10,000	-	27	± 10	<0.001	933	± 150	<0.001
	100,000	-	542	± 56	ns	7,067	± 540	<0.001
	10,000	0.3	603	± 50	ns	10,000	±1027	0.05
[Gly^{1a}]-LHRH acid	-	0.3	590	± 27		9,230	±1043	
	10,000	-	- 9	± 26	<0.001	1,238	± 493	<0.01
	100,000	-	69	± 26	<0.001	4,041	± 748	<0.001
	10,000	0.3	559	± 24	ns	8,443	± 437	ns
[Gly^{2a}]-LHRH acid	-	0.3	398	± 53		8,077	± 844	
	10,000	-	10	± 21	<0.001	949	± 627	<0.001
	100,000	-	599	± 64	0.05	10,639	±1147	ns
	10,000	0.3	478	± 53	ns	7,601	± 943	ns
LHRH acid	-	0.3	516	± 89		11,250	±3507	
	1,000	-	26	± 18	<0.001	283	± 589	<0.001
	10,000	-	504	±105	ns	16,150	± 666	ns
	1,000	0.3	583	± 53	ns	9,623	±2345	
<Glu-His-Trp-Ser-Tyr-Gly-OH	-	0.3	408	± 44		7,575	±1084	
	400,000	-	99	± 41	<0.001	2,069	± 888	<0.01
	400,000	0.3	547	± 33	ns	5,400	± 865	ns
<Glu-His-Trp-OH	-	0.3	400	± 38		15,922	± 859	
	200,000	-	5	± 13	<0.001	409	± 101	<0.001
	200,000	0.3	291	± 43	ns	15,161	± 929	ns

a. Incubation for 5 or 6 hr. Medium changed hourly (P_1, P_2, I_3, I_4, I_5, I_6). Experiments (#2,3) were performed by adding the LHRH analog or derivative to I_3-I_6 and LHRH to I_5 and I_6 or by adding LHRH analog or derivatives with and without LHRH to I_3-I_5 (#1,4,5,6). Values were obtained by subtraction of P_2 from I_3, I_4, I_5 and I_6 and each value recorded is the mean of 3-9.

slightly inhibited by a dosage of 1 μg and 10 μg, but not by 100 μg, of [Tyr^3, Trp^5]-LHRH acid.

The agonist and antagonist activities are recorded in Table II. In Table III, the LH and FSH agonist activities are expressed as percentages obtained from the in vitro effect of the analog and 0.3 ng of LHRH.

Table III

EQUIPOTENT AGONIST IN VITRO DOSAGE OF LHRH, LHRH ANALOGS AND THEIR RELATIVE DEGREES OF LH AND FSH ACTIVITY

Peptide	Dosage		Relative Activity	
	LH	FSH	LH	FSH
	μg/ml medium		% LHRH Activity	
1. LHRH	$3x10^{-4}$	$3x10^{-4}$	100	100
2. [Gly^{2a}]-LHRH acid	$1x10^{2}$	$1x10^{2}$	0.0003	0.0003
3. [Tyr^3, Trp^5]-LHRH acid	$1x10^{2}$	$2x10^{2}$	0.0003	0.00015
4. [Gly^{1a}]-LHRH acid	$1x10^{2}$	$1x10^{2}$	<0.0003	<0.0003
5. LHRH acid	$1x10^{1}$	$1x10^{1}$	0.003	0.003
6. <Glu-His-Trp-Ser-Tyr-Gly-OH	$1x10^{2}$	$1x10^{2}$	<0.0003	<0.0003
7. <Glu-His-Trp-OH	$1x10^{2}$	$1x10^{2}$	<0.0003	<0.0003

One interesting feature has emerged from this study. It is now clearly evident that the terminal glycinamide grouping is not necessary for LH-release. It is also evident that replacement of the terminal amide by a terminal carboxylic acid moiety produces a great loss of potency.

Inspection of a model of LHRH, with the His-Trp linkage in cis relationship, indicates that hydrogen bonding could occur either between the α-CO of <Glu and the OH of Ser, or between the α-NH in <Glu and the O of the OH in Ser. Although the OH of Ser in the 4-position cannot be regarded as essential for activity, since the in vitro releasing activities of [Ala^4]- and [Gly^4]-LHRH are 5-9% (7,14,15) and 1.5% (16), respectively, the OH is important for hormonal potency.

At the more hydrophobic receptor level, where the strength of a hydrogen bond may be maximized, the role of the OH of Ser^4 and <Glu^1 in a hydrogen bond relationship may constitute an important "conformational component" in LHRH. The driving force for the proposed cis-His-Trp peptide bond linkage could be the

formation of this internal hydrogen bond which could make this "cis-conformation" the more preferred.

Deslauriers et al. (17) reported that the C^{13}-nmr resonances of the His residue in TRH and LHRH were different, and this was interpreted to reflect some conformational differences for His in these two hypothalamic hormones. We consider that the differences in chemical shifts for the His carbon atoms in LHRH and in TRH may be a consequence of the diamagnetic anisotropy associated with the aromatic moieties of Trp and Tyr in LHRH which are absent in TRH. The conformation in which the His and the Trp-Tyr unit have a close proximity may require a cis-peptide linkage between the His and Trp residues.

The high potency of LHRH depends in part upon the availability of the "active conformation" of the hormone for its receptor site, and to "binding components" responsible for attracting and holding the hormone to its receptor. The most important "binding components" include the imidazole moiety of the His in the 2-position (12) and the protonated guanidino function of the Arg in the 8-position. The availability of the "active conformation" of long polypeptides depends, at least in part, on the existence of "conformational components", which are intramolecular interactions between certain moieties within the molecule which can cause a certain molecular conformation. Without these "conformational components", the molecule could be flexible, and could exist in a topographical spectrum. This flexibility would result in a low probability for existence of the "active conformation".

Another "conformational component" follows from our proposal that the bicyclic aromatic moiety of Trp and the monocyclic aromatic moiety of Tyr are in parallel planarity which is in addition to the hydrogen bonding interaction between the <Glu^1 and the Ser^4 residues. Recently, Monahan et al. (18) have speculated that the LHRH sequence, -Ser-Tyr-Gly-Leu-, may be involved with forming a β-type bend.

A cis-His-Trp peptide linkage in LHRH places the imidazole of His in the proximity, and on the same side of the molecule as the aromatic Trp and Tyr unit. This relationship contrasts with a trans-His-Trp linkage in which the His side chain and the Trp-Tyr unit are located on the opposite side of the backbone. This cis-conformation exposes the α-CO of His, the α-NH and α-CO of Trp, and the α-NH of Tyr for possible interaction with groupings of the receptor site and as such may be an essential part of the "active site". In support of these concepts, [des-His^2]-LHRH (19), which is a weak inhibitor of the release of LH probably does not have a cis-linkage between the <Glu and Trp residues. This biological result may support the concept that a cis-linkage between His^2 and Trp^3 in LHRH is necessary for high agonist activity.

The C-terminus of LHRH lies in the proximity of the so-called "active site" of LHRH (if there is a specific active site) which could be comprised of contributions from the 1 to 5 positions of LHRH. Consequently, the presence of a terminal carboxylic acid or its anion in place of a neutral amide moiety could perturbate the conformation arising from the <Glu-Ser interaction with a concomitant reduction in binding capability. Alternatively, the free carboxyl terminus may cause new binding to an additional site and lead to decreased activity. The extremely low potencies observed for LHRH acid, and [Gly^{1a}]-, [Gly^{2a}]-, and [Tyr^{3}, Trp^{5}]-LHRH acids may be rationalized by these concepts. The inactivities of <Glu-His-Trp-Ser-Tyr-Gly-OH and <Glu-His-Trp-OH may be substantially due to the absence of the Arg "binding component".

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