SYNTHESES AND BIOLOGICAL ACTIVITIES OF ANALOGS OF LUTEINIZING HORMONE-RELEASING HORMONE (LH-RH) SUBSTITUTED IN POSITION 1 OR 2

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Received January 24, 1973

SUMMARY Syntheses are described of [Pro^1]-LH-RH, [Orotic acid^1]-LH-RH, [Glu^1]-LH-RH, [Ser^2]-LH-RH, [Leu^2]-LH-RH, [Gln^2]-LH-RH and [Phe^2]-LH-RH. The LH-releasing hormone (LH-RH) activity of each of these peptides was compared with that of natural LH-RH in vivo. [Glu^1]-LH-RH and [Phe^2]-LH-RH had significant LH-RH activity, while all the other analogs possessed extremely low activities. These findings are briefly discussed in the light of the structure-activity relationship for LH-RH.

After the primary structure of luteinizing hormone-releasing hormone (LH-RH) of porcine hypothalamus was shown to be pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (1, 2), various analogs of LH-RH, based on the decapeptide sequence, were synthesized in order to evaluate the relationship between the structure and the activity of this hormone.

Syntheses of [Gly^2]-LH-RH and Des-His^2-LH-RH were reported by Monahan et al. (3). Schally et al. (4) described in vivo quantitative assays of Des-pGlu^1-LH-RH, Des-pGlu^1-des-His^2-LH-RH and other peptides shorter than decapeptide. Chang et al. (5, 6, 7) and Fujino et al. (8) described the syntheses and hormonal activities of LH-RH analogs modified at the C-terminal portion. Yanaihara et al. (9) reported the syntheses of

LH-RH analogs modified in position 8 of LH-RH and the comparison of their biological activities with natural LH-RH. Synthesis of [Ala^4]-LH-RH was reported by Geiger et al. (10); this substance was also assayed for LH-RH activity.

This paper reports the syntheses of [Pro^1]-LH-RH, [Glu^1]-LH-RH, [Orotic acid^1]-LH-RH, [Ser^2]-LH-RH, [Leu^2]-LH-RH, [Gln^2]-LH-RH and [Phe^2]-LH-RH and their LH-RH activities.

Synthesis

These analogs were conveniently synthesized by a chain elongation starting from Des-pGlu^1-LH-RH or Des-pGlu^1-des-His^2-LH-RH according to the method of Yanaihara et al. (11). Carbobenzoxy (Z) group was used for α-amino protection and removal of the group was performed by hydrogenolysis. [Pro^1]-LH-RH was prepared by the coupling of Z-proline N-hydroxysuccinimido ester (Z-Pro-OSu) (12) with Des-pGlu^1-LH-RH, followed by hydrogenolysis. [Orotic acid^1]-LH-RH was produced by the interaction of orotic acid with Des-pGlu^1-LH-RH according to dicyclohexylcarbodiimide-N-hydroxysuccinimide procedure (13). For the synthesis of [Glu^1]-LH-RH, Z-Glu(OBu^t)-OSu (14) was used as acylating agent, and the resulting protected decapeptide amide was treated with trifluoroacetic acid to remove the tert-butyl group and then hydrogenated. Des-pGlu^1-des-His^2-LH-RH was employed as the starting material for the syntheses of [Ser^2]-LH-RH, [Leu^2]-LH-RH, [Gln^2]-LH-RH and [Phe^2]-LH-RH. Interaction of Z-Ser-N_3, derived from the corresponding hydrazide (15), with Des-pGlu^1-des-His^2-LH-RH gave protected nonapeptide amide which was hydrogenated. Coupling of the resulting material with Z-pGlu-OSu (11), followed by hydrogenolysis, gave [Ser^2]-LH-RH. Similarly [Leu^2]-LH-RH and [Phe^2]-LH-RH were prepared using Z-Leu-OSu (12) and Z-Phe-OTCP

(Z-phenylalanine 2,4,5-trichlorophenyl ester) (16), respectively, on preparations of the protected nonapeptide amide intermediates. [Gln^2]-LH-RH was produced by the interaction of Z-pGlu-Gln-N_3, derived from Z-pGlu-Gln-NHNH-Boc, with Des-$pGlu^1$-des-His^2-LH-RH, followed by hydrogenolysis. The final products of these syntheses were purified by column chromatography on CM-Sephadex using ammonium acetate buffer (pH 6.5). Finally the products dissolved in 1M AcOH were desalted by gel filtration on Bio-Gel P-2. The highly purified LH-RH analogs behaved as single component respectively on TLC in two solvent systems. Pauly, Sakaguchi, Ehrlich, chlorine-tolidine and ninhydrin reagents were used for detection on TLC. Their acid hydrolysates contained the constituent amino acids except tryptophan in theoretical ratios. The data in Table I demonstrate high purity of the LH-RH analogs prepared in this investigation.

Hormonal activities

The LH-RH activity of these analogs was determined in vivo by stimulation of release of LH in ovariectomized rats pretreated with estrogen and progesterone as reported previously (4, 17, 18). LH levels in the serum were measured by radioimmunoassay for rat LH, according to the method described by Niswender et al. (19). The responses to the synthetic peptides were examined at 2 dose levels. Serum LH levels resulting from the injection of samples were compared with those observed after administrations of saline and of 2 doses of pure natural LH-RH (18). The results are shown in Table II. The relative LH-RH potencies of synthetic analogs with 95% confidence limits (20) are listed in Table III.

Discussion

The present investigation is a continuation of our studies on the structure and function relationships of LH-RH. Homogeneous LH-RH analogs with

Table I CHEMICAL AND PHYSICAL PROPERTIES OF SYNTHETIC LH-RH ANALOGS

[Pro^1]-LH-RH : H-Pro-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -54.3° (c 1.3, 1M AcOH) ; Rf^I 0.25, Rf^{II} 0.60.[a)]

Anal. Calcd. for $C_{55}H_{76}N_{17}O_{12}\cdot 3CH_3COOH\cdot 6H_2O$ (1456.7) :
C, 50.29; H, 6.99; N, 16.35.

Found : C, 50.29; H, 6.79; N, 16.69.

Amino acid composition of acid hydrolysate[b)] : $Pro_{1.93}His_{1.01}Ser_{0.88}Tyr_{1.01}Gly_{2.03}Leu_{1.00}Arg_{1.03}$ (80%)[c)]; (Tyr/Trp=0.98[d)]).

[Orotic $acid^1$]-LH-RH : Orotyl-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -39.5° (c 0.74, 1M AcOH) ; Rf^I 0.25, Rf^{II} 0.68.

Anal. Calcd. for $C_{55}H_{72}N_{18}O_{14}\cdot 2CH_3COOH\cdot 5H_2O$ (1419.5) :
C, 49.92; H, 6.39; N, 17.76.

Found : C, 50.01; H, 6.28; N, 17.38.

UV λ_{max} : 289 mμ (ϵ 6.93 x 10^3), 283 mμ (ϵ 6.72 x 10^3), 320 mμ (ϵ 2.97 x 10^3).

Amino acid composition of acid hydrolysate : $His_{0.92}Ser_{0.82}Tyr_{0.96}Gly_{2.09}Leu_{1.00}Arg_{1.01}Pro_{1.02}$ (84%).[e)]

[Glu^1]-LH-RH : H-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -36.9° (c 2.0, 1M AcOH) ; Rf^I 0.21, Rf^{II} 0.60.

Anal. Calcd. for $C_{55}H_{77}N_{17}O_{14}\cdot 3CH_3COOH\cdot 6H_2O$ (1488.7) :
C, 49.22; H, 6.84; N, 16.00.

Found : C, 49.18; H, 6.68; N, 15.83.

Amino acid composition of acid hydrolysate : $Glu_{1.00}His_{0.98}Ser_{0.90}Tyr_{1.03}Gly_{2.11}Leu_{0.96}Arg_{1.02}Pro_{0.91}$ (83%) ; (Tyr/Trp=0.96).

[Ser^2]-LH-RH : pGlu-Ser-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -53.5° (c 0.9, 1M AcOH); Rf^I 0.23, Rf^{II} 0.60.

Anal. Calcd. for $C_{52}H_{73}N_{15}O_{14}\cdot CH_3COOH\cdot 10H_2O$ (1372.6) :
C, 47.25; H, 7.12; N, 15.30.

Found : C, 47.25; H, 6.89; N, 15.48.

Amino acid composition of acid hydrolysate : $Glu_{1.09}Ser_{1.78}Tyr_{0.95}Gly_{1.94}Leu_{0.93}Arg_{1.07}Pro_{1.01}$ (81%) ; (Tyr/Trp=1.02).

Table I (Cont.)

[Leu^2]-LH-RH : pGlu-Leu-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -43.4° (c 1.4, 1M AcOH) ; Rf^I 0.32, Rf^{II} 0.70.

Anal. Calcd. for $C_{55}H_{79}N_{15}O_{13} \cdot CH_3COOH \cdot 10H_2O$ (1398.5) :
C, 48.95; H, 7.42; N, 15.02.

Found : C, 48.65; H, 6.97; N, 14.84.

Amino acid composition of acid hydrolysate : $Glu_{0.95}Leu_{1.93}Ser_{0.89}Tyr_{1.02}Gly_{2.10}Arg_{1.03}Pro_{0.97}$ (82%) ; (Tyr/Trp=0.94).

[Gln^2]-LH-RH : pGlu-Gln-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -44.2° (c 1.2, 1M AcOH) ; Rf^I 0.23, Rf^{II} 0.62.

Anal. Calcd. for $C_{54}H_{76}N_{16}O_{14} \cdot CH_3COOH \cdot 6H_2O$ (1341.4) :
C, 50.13; H, 6.91; N, 16.71.

Found : C, 49.73; H, 6.56; N, 17.10.

Amino acid composition of acid hydrolysate : $Glu_{1.90}Ser_{0.92}Tyr_{1.03}Gly_{2.06}Leu_{1.01}Arg_{0.97}Pro_{1.03}$ (84%) ; (Tyr/Trp=0.95).

[Phe^2]-LH-RH : pGlu-Phe-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{28}$ -45.5° (c 0.71, 1M AcOH) ; Rf^I 0.35, Rf^{II} 0.70.

Anal. Calcd. for $C_{58}H_{77}N_{15}O_{13} \cdot CH_3COOH \cdot 7H_2O$ (1378.5) :
C, 52.28; H, 6.95; N, 15.24.

Found : C, 52.09; H, 6.85; N, 15.54.

Amino acid composition of acid hydrolysate : $Glu_{0.95}Phe_{1.00}$[e] $Ser_{0.91}Tyr_{1.02}Gly_{2.03}Leu_{1.00}Arg_{1.04}Pro_{0.96}$ (85%).

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a) Rf^I and Rf^{II} values refer to the solvent systems 1-BuOH-AcOH-H_2O (4 : 1 : 5) and 1-BuOH-pyridine-AcOH-H_2O (30 : 20 : 6 : 24), respectively
b) Acid hydrolysis was performed at 110° for 24 hr in the presence of phenol in a sealed tube.
c) Figure is average recovery of amino acids based on formula weight.
d) Estimated by the method of Bencze and Schmid (Anal. Chem., 29, 1193, (1957)).
e) Trp was not determined.

amino acid substitution in position 1 or 2 were prepared by a chain elongation of Des-pGlu^1-LH-RH or Des-pGlu^1-des-His^2-LH-RH, the stereohomogeneity of which has been established previously (11). These analogs were assayed in vivo for LH-RH activity. [Pro^1]-LH-RH and [Orotic $acid^1$]-LH-RH had

Table II

SERUM LH LEVELS AFTER INTRAVENOUS INJECTION OF LH-RH ANALOGS INTO OVARIECTOMIZED, ESTROGEN AND PROGESTERONE TREATED RATS

Sample	Dose ng/rat	Serum LH Level ng/ml ± S.E.	P Value*
Saline	---	10.0 ± 1.33	---
Natural LH-RH	0.5	27.5 ± 0.1	0.01
	2.5	69.2 ± 10.8	0.01
[Pro^1]-LH-RH	10,000	29.3 ± 0.3	0.01
	50,000	89.3 ± 7.8	0.01
[Ser^2]-LH-RH	5,000	52.5 ± 6.2	0.01
	25,000	131.3 ± 5.0	0.01
Saline	---	7.2 ± 1.4	---
Natural LH-RH	0.5	18.4 ± 1.5	0.01
	2.5	52.4 ± 6.5	0.01
[Glu^1]-LH-RH	50	55.6 ± 6.6	0.01
	250	102.7 ± 13.1	0.01
[Orotic $acid^1$]-LH-RH	10,000	21.4 ± 6.3	NS
	50,000	49.9 ± 13.0	0.05
[Leu^2]-LH-RH	200	19.7 ± 2.0	0.01
	1,000	40.6 ± 6.4	0.01
Saline	---	8.8 ± 0.4	---
Natural LH-RH	0.5	33.0 ± 2.0	0.01
	2.5	97.0 ± 0	0.01
[Gln^2]-LH-RH	5,000	42.5 ± 4.5	0.01
	25,000	90.0 ± 6.0	0.01
Saline	---	5.6 ± 0.6	---
Natural LH-RH	0.5	13.0 ± 1.6	0.01
	2.5	63.3 ± 10.9	0.01
[Phe^2]-LH-RH	10	14.5 ± 1.4	0.01
	50	31.8 ± 3.1	0.01

* Duncan's new multiple range test.

Table III

POTENCY ESTIMATES OF SYNTHETIC ANALOGS AGAINST NATURAL LH-RH

Sample	% LH-RH Activity with 95% Confidence Limits
Natural LH-RH	Accepted as 100%
[Pro^1]-LH-RH	0.0088% (0.0056 - 0.0154)
[Glu^1]-LH-RH	5.7% (2.66 - 23.8)
[Orotic $acid^1$]-LH-RH	0.005% (0.0019 - 0.013)
[Ser^2]-LH-RH	0.032% (0.02 - 0.06)
[Leu^2]-LH-RH	0.18% (0.086 - 0.35)
[Gln^2]-LH-RH	0.01% (0.0075 - 0.014)
[Phe^2]-LH-RH	1.4% (0.03 - 4.58)

only 0.0088% and 0.005%, respectively, of the activity of pure natural LH-RH, while [Glu^1]-LH-RH possessed higher activity (5.7%). These results indicate the importance of five membered ring lactam structure of the pGlu residue for the hormonal activity for release of LH. The extremely low activities of [Pro^1]-LH-RH and [Orotic $acid^1$]-LH-RH suggest that pGlu residue in position 1 is important for the function of LH-RH or for its binding to the pituitary receptor(s).

[Ser^2]-LH-RH, [Leu^2]-LH-RH and [Gln^2]-LH-RH had only very weak LH-RH activities, which were 0.032%, 0.18% and 0.01%, respectively. The very low LH-RH activity of these analogs implies that the His residue in position 2 of LH-RH molecule may be essential for biological function of this hormone. The report on low activity of [Gly^2]-LH-RH, prepared by solid phase synthesis (21), is in agreement with this view. On the other

hand, [Phe^2]-LH-RH was found to possess a significant activity, 1.4% of that of natural LH-RH. The low but definite activity of this compound suggests that not only the characteristic acid-base property of the imidazole in His residue, but aromatic properties of the structure (ring) in position 2 may be responsible for the LH-RH activity.

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