SYNTHESES AND BIOLOGICAL EVALUATION OF ANALOGS OF LUTEINIZING HORMONE-RELEASING HORMONE (LH-RH) MODIFIED IN POSITION 2, 3, 4 OR 5

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SUMMARY. Syntheses by the conventional methods as well as the chemical, physical and biological properties are described of the following analogs of the LH-releasing hormone (LH-RH): [Leu^3]-LH-RH, [Phe^3]-LH-RH, [Trp^2][His^3]-LH-RH, Des-Trp^3-LH-RH, Des-His^2-[Phe^5]-LH-RH, [Ala^4] LH-RH, [Phe^5]-LH-RH and [Ala^4][Phe^5]-LH-RH. In vivo assays showed that [Leu^3]-LH-RH did not release LH in doses as high as 5 - 25 μg, having less than 0.0008% of LH-RH activity, while [Phe^3]-LH-RH had 0.43% of the LH-RH activity of natural LH-RH. The LH-RH activities of [Trp^2][His^3]-LH-RH, Des-Trp^3-LH-RH and Des-His^2-[Phe^5]-LH-RH were extremely low. On the other hand, [Ala^4]-LH-RH, [Phe^5]-LH-RH and [Ala^4][Phe^5]-LH-RH had significant LH-RH activity. The structure-activity relationship of LH-RH is discussed on the basis of these findings.

In previous communications (1, 2, 3, 4) we have described the syntheses and biological activities of LH-RH analogs which were modified in positions 1, 2 and 8, in order to evaluate the role of pGlu, His and Arg residues for the activity of LH-RH, based on the decapeptide sequence, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (5, 6).

Baba et al. (7) and Schally et al. (8) treated LH-RH with various chemical reagents, and their observation suggested that His, Trp, Ser and Tyr residues in LH-RH molecule are probably important for biological

activity. In order to derive more information with regard to the role of His, Trp, Ser and Tyr residues for biological activity of LH-RH, we prepared [Leu^3]-LH-RH, [Phe^3]-LH-RH, [Trp^2][His^3]-LH-RH, Des-Trp^3-LH-RH, Des-His^2-[Phe^5]-LH-RH, [Ala^4]-LH-RH, [Phe^5]-LH-RH and [Ala^4][Phe^5]-LH-RH. This paper describes the syntheses and LH-RH activities of these analogs.

The syntheses of [Phe^3]-LH-RH (9), [Ala^4]-LH-RH (10) and [Phe^5]-LH-RH (9, 11) by different methods have also been reported by others.

Synthesis

The syntheses of these analogs were achieved by following our method established for the preparation of LH-RH (2).

[Leu^3]-LH-RH, [Phe^3]-LH-RH and [Trp^2][His^3]-LH-RH were prepared by the stepwise method from H-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (2). Carbobenzoxyleucine N-hydroxysuccinimido ester (Z-Leu-OSu) (12), carbobenzoxyphenylalanine 2,4,5-trichlorophenyl ester (Z-Phe-OTCP)(13), carbobenzoxytryptophan 2,4,5-trichlorophenyl ester (Z-Trp-OTCP) (13), carbobenzoxyhistidine azide (Z-His-N_3) (14) and carbobenzoxypyroglutamic acid N-hydroxysuccinimido ester (Z-pGlu-OSu) (2) were used as acylating agents for introduction of the respective amino acid residues. Des-Trp^3-LH-RH was prepared by the interaction of Z-pGlu-OSu with H-His-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 which is the intermediate on the synthesis of [Trp^2][His^3]-LH-RH.

In the syntheses of [Ala^4]-LH-RH, [Phe^5]-LH-RH and [Ala^4][Phe^5]-LH-RH, the azides derived from Z-Ala-Tyr-hydrazide (15), Z-Ser-Phe-hydrazide [m.p. 221-222°, $[\alpha]_D^{33}$ -3.48° (c, 2.2, DMF), Rf^I 0.67, Anal. Found: N, 14.24%] and Z-Ala-Phe-hydrazide (16) were employed as

acylating agents, respectively, for the preparation of the protected hepta-peptide amide intermediates.

Des-His^2-[Phe^5]-LH-RH was prepared by the chain elongation of H-Ser-Phe-Gly-Leu-Arg-Pro-Gly-NH_2 which is the intermediate for the synthesis of [Phe^5]-LH-RH.

The purity of the intermediates was examined by elementary analysis and thin-layer chromatography in two solvent systems. The final products of these syntheses were purified by column chromatography on CM-Sephadex using ammonium acetate buffer (pH 6.5) as described before (2). Finally the products, dissolved in 1M AcOH, were desalted by gel filtration on Bio-Gel P-2. All the samples of synthetic analogs of LH-RH were homogeneous chromatographically. For the detection on thin-layer chromatography, Pauly, Sakaguchi, chlorine-tolidine and ninhydrin reagents were used. Their acid hydrolysates contained the constituent amino acids in theoretical ratios. Table I indicates chemical and physical properties of the analogs prepared in the present investigation. Their detailed syntheses and purification will be reported elsewhere.

Hormonal activity

The LH-RH activity of the analogs were determined in vivo by the stimulation of release of LH in ovariectomized rats pretreated with estrogen and progesterone (1, 17, 18), followed by radioimmunoassay for LH according to the method of Niswender et al. (19). As reported previously (1), the responses to the synthetic peptides were examined at 2 dose levels and 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

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

[Leu^3]-LH-RH : pGlu-His-Leu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -62.1° (c 1.3, 1M AcOH) ; Rf^I 0.18, Rf^{II} 0.59.[a)]

Anal. Calcd. for $C_{50}H_{76}N_{16}O_{13}\cdot 2CH_3COOH\cdot 5H_2O$ (1319.4) :
C, 49.16; H, 7.18; N, 16.99.

Found : C, 48.78; H, 7.50; N, 17.08.

Amino acid composition of acid hydrolysate[b)] : $Glu_{1.08}His_{0.97}Leu_{1.98}Ser_{0.89}Tyr_{1.00}Gly_{2.06}Arg_{0.96}Pro_{0.94}$ (90%).[c)]

[Phe^3]-LH-RH : pGlu-His-Phe-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -57.9° (c 1.3, 1M AcOH) ; Rf^I 0.21, Rf^{II} 0.68.

Anal. Calcd. for $C_{53}H_{74}N_{16}O_{13}\cdot 2CH_3COOH\cdot 5H_2O$ (1353.6) :
C, 50.58; H, 6.85; N, 16.56.

Found : C, 50.64; H, 6.85; N, 16.94.

Amino acid composition of acid hydrolysate : $Glu_{0.99}His_{0.97}Phe_{1.02}Ser_{0.91}Tyr_{1.00}Gly_{2.05}Leu_{1.00}Arg_{1.03}Pro_{0.93}$ (86%).

[Trp^2][His^3]-LH-RH : pGlu-Trp-His-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -51.8° (c 0.9, 1M AcOH) ; Rf^I 0.38, Rf^{II} 0.60.

Anal. Calcd. for $C_{55}H_{75}N_{17}O_{13}\cdot 2CH_3COOH\cdot 7H_2O$ (1427.5) :
C, 49.64; H, 6.78; N, 16.68.

Found : C, 49.24; H, 6.73; N, 17.06.

Amino acid composition of acid hydrolysate : $Glu_{0.95}His_{0,98}Ser_{0.82}Tyr_{1.03}Gly_{2.00}Leu_{1.02}Arg_{1.05}Pro_{0.98}$ (83%) ; (Tyr/Trp=0.96)[d)]

Des-Trp^3-LH-RH : pGlu-His-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{31}$ -57.9° (c 1.4, 1M AcOH) ; Rf^I 0.21, Rf^{II} 0.58.

Anal. Calcd. for $C_{44}H_{65}N_{15}O_{12}\cdot 2CH_3COOH\cdot 5H_2O$ (1206.3) :
C, 47.79; H, 6.94; N, 17.42.

Found : C, 47.51; H, 7.32; N, 17.42.

Amino acid composition of acid hydrolysate : $Glu_{1.00}His_{1.01}Ser_{0.92}Tyr_{0.99}Gly_{2.07}Leu_{0.98}Arg_{1.00}Pro_{0.94}$ (88%).

Des-His^2-[Phe^5]-LH-RH : pGlu-Trp-Ser-Phe-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{28}$ -44.2° (c 1.0, 1M AcOH) ; Rf^I 0.28, Rf^{II} 0.71.

Table I (Cont.)

Anal. Calcd. for $C_{49}H_{68}N_{14}O_{11} \cdot CH_3COOH \cdot 6H_2O$ (1197.3):
C, 51.16; H, 7.07; N, 16.38.

Found: C, 51.40; H, 6.67; N, 16.16.

Amino acid composition of acid hydrolysate: $Glu_{0.95}Ser_{0.89}Phe_{1.02}Gly_{2.01}Leu_{1.02}Arg_{1.01}Pro_{1.00}$ (83%).[e)]

[Ala^4]-LH-RH: pGlu-His-Trp-Ala-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{27}$ -48.8° (c 1.1, 1M AcOH); Rf^I 0.17, Rf^{II} 0.56.

Anal. Calcd. for $C_{55}H_{75}N_{17}O_{12} \cdot 2CH_3COOH \cdot 7H_2O$ (1412.5):
C, 50.17; H, 6.92; N, 16.86.

Found: C, 50.16; H, 6.62; N, 16.64.

Amino acid composition of acid hydrolysate: $Glu_{0.96}His_{0.99}Ala_{1.00}Tyr_{1.01}Gly_{2.09}Leu_{0.99}Arg_{1.01}Pro_{0.95}$ (86%); (Tyr/Trp=0.98).

[Phe^5]-LH-RH: pGlu-His-Trp-Ser-Phe-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{23}$ -54.0° (c 1.0, 1M AcOH); Rf^I 0.24, Rf^{II} 0.62.

Anal. Calcd. for $C_{55}H_{75}N_{17}O_{12} \cdot 2CH_3COOH \cdot 5H_2O$ (1376.5):
C, 51.48; H, 6.81; N, 17.30.

Found: C, 51.14; H, 6.61; N, 18.09.

Amino acid composition of acid hydrolysate: $Glu_{0.95}His_{0.98}Ser_{0.91}Phe_{1.02}Gly_{2.02}Leu_{1.03}Arg_{0.98}Pro_{1.02}$ (86%).[d)]

[Ala^4][Phe^5]-LH-RH: pGlu-His-Trp-Ala-Phe-Gly-Leu-Arg-Pro-Gly-NH_2

$[\alpha]_D^{28}$ -49.9° (c 1.0, 1M AcOH); Rf^I 0.21, Rf^{II} 0.68.

Anal. Calcd. for $C_{55}H_{75}N_{17}O_{11} \cdot 2CH_3COOH \cdot 5H_2O$ (1360.5)
C, 52.08; H, 6.89; N, 17.50.

Found: C, 52.36; H, 6.71; N, 17.58.

Amino acid composition of acid hydrolysate: $Glu_{0.95}His_{0.99}Ala_{1.05}Phe_{1.06}Gly_{1.96}Leu_{0.98}Arg_{1.04}Pro_{0.98}$ (85%).[d)]

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 with 6N HCl at 110° for 24 hr in a sealed tube.
c) Figure is average recovery of amino acids based on formula weight.
d) Estimation was carried out by the method of Bencze and Schmid (Anal. Chem., 29, 1193 (1957)).
e) Trp was not determined.

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	—	8.8 ± 0.4	—
Natural LH-RH	0.5	33.0 ± 2.0	0.01
	2.5	97.0 ± 0	0.01
[Leu^3]-LH-RH	5,000	5.7 ± 0.4	NS
	25,000	6.1 ± 0.9	NS
[Ala^4][Phe^5]-LH-RH	100	66.0 ± 5.0	0.01
	500	114.0 ± 25.0	0.01
Saline	—	10.5 ± 1.6	—
Natural LH-RH	0.5	21.8 ± 1.5	0.05
	2.5	80.9 ± 27.1	0.01
[Phe^3]-LH-RH	1,000	100.0 ± 6.0	0.01
	5,000	154.0 ± 8.0	0.01
[Trp^2][His^3]-LH-RH	1,000	6.3 ± 0.4	NS
	5,000	20.3 ± 3.2	0.05
Saline	—	5.6 ± 0.6	—
Natural LH-RH	0.5	13.0 ± 1.6	0.01
	2.5	63.3 ± 10.9	0.01
Des-Trp^3-LH-RH	5,000	42.9 ± 2.0	0.01
	25,000	103.3 ± 32.7	0.05
Des-His^2-[Phe^5]-LH-RH	5,000	17.4 ± 0.7	0.01
	25,000	54.7 ± 1.4	0.01
Saline	—	7.3 ± 0.7	—
Natural LH-RH	0.5	14.8 ± 1.7	0.05
	2.5	50.2 ± 5.3	0.01
[Ala^4]-LH-RH	5	13.3 ± 1.8	0.05
	25	45.5 ± 5.5	0.01
[Phe^5]-LH-RH	1	16.6 ± 5.2	NS
	5	43.7 ± 9.5	0.05

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%
[Leu^3]-LH-RH	< 0.0008% (0.0001 - 0.002%)
[Phe^3]-LH-RH	0.43% (0.12 - 59.8%)
[Trp^2][His^3]-LH-RH	0.0093% (0.0012 - 46.2%)
Des-Trp^3-LH-RH	0.0026% (0.00058 - 36.1%)
Des-His^2-[Phe^5]-LH-RH	0.007% (0.002 - 0.017%)
[Ala^4]-LH-RH	8.6% (5.4 - 13.5%)
[Phe^5]-LH-RH	44.3% (19.1 - 97%)
[Ala^4][Phe^5]-LH-RH	1.01% (0.23 - 15.8%)

LH-RH potencies of the synthetic analogs with 95% confidence limits (20) are summarized in Table III.

Discussion

A variety of homogeneous LH-RH analogs with substitution or deletion of amino acid in position 2, 3, 4 or 5 were prepared according to the method of Yanaihara et al. (2). These analogs were assayed quantitatively in vivo for LH-RH activity. [Leu^3]-LH-RH in which Trp residue in position 3 of LH-RH molecule is replaced by Leu was inactive in doses as high as 5 - 25 ug. This finding strongly suggests the importance of Trp residue for the functional effect of LH-RH. It also supports the results of inactivation of LH-RH by

2-hydroxy-5-nitrobenzyl bromide or by performic acid, which are known to have high selectivity for a reaction with Trp residue (7). However, it can not be completely excluded that Trp is essential for binding to the pituitary receptors. On the other hand, [Phe^3]-LH-RH showed 0.43% of the activity of pure natural LH-RH. The low but definite activity of this analog suggests that aromatic properties of the benzene structure in position 3 may in part substitute for the indole residue of Trp. Interestingly, our observations coincide with the finding of Hofmann et al. (21) that the replacement of Trp residue by Phe in Gln^5-β-corticotropin$_{1-20}$ results in a marked decrease of in vivo adrenocorticotropic activity. They suggested that the indole portion of Trp residue is not essential for the physiological activity of β-corticotropin, but may contribute to the binding of the hormone to its receptor. Kimura et al. (9) reported before that [Phe^3]-LH-RH and [Phe^5]-LH-RH raised serum LH levels in rats, but the results of their quantitative assays were not described.

[Trp^2][His^3]-LH-RH and Des-Trp^3-LH-RH had only 0.009% and 0.0026%, respectively, of the activity of pure natural LH-RH. Des-His^2-[Phe^5]-LH-RH was inactive in doses of 5 - 25 µg. This was expected from the poor activity of Des-His^2-LH-RH (22). The extremely low potency of [Trp^2][His^3]-LH-RH, Des-Trp^3-LH-RH and Des-His^2-[Phe^5]-LH-RH suggests that conformational changes of LH-RH have a marked effect on the LH-RH activity and that Trp-His transposition or deletion of His or Trp residue may result in the shift or elimination of vital active or binding sites of the LH-RH molecule.

[Ala^4]-LH-RH, [Phe^5]-LH-RH and [Ala^4][Phe^5]-LH-RH were found to possess significant LH-RH activities, which were 8.6%, 44.3% and 1.01%, respectively. [Ala^4][Phe^5]-LH-RH did not lose its biological activity in

spite of the fact that acylation of LH-RH in pyridine leads to considerable inactivation (7). The LH-RH activities of [Ala^4]-LH-RH (10) and [Phe^5]-LH-RH (11), prepared by different methods, were in excellent agreement with our results. These findings virtually eliminate the hydroxyl groups of both Ser and Tyr residues as essential for the function of LH-RH.

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