SOME ANALOGS OF LUTEINIZING HORMONE RELEASING HORMONE (LH-RH) HAVING INTENSE OVULATION-INDUCING ACTIVITY

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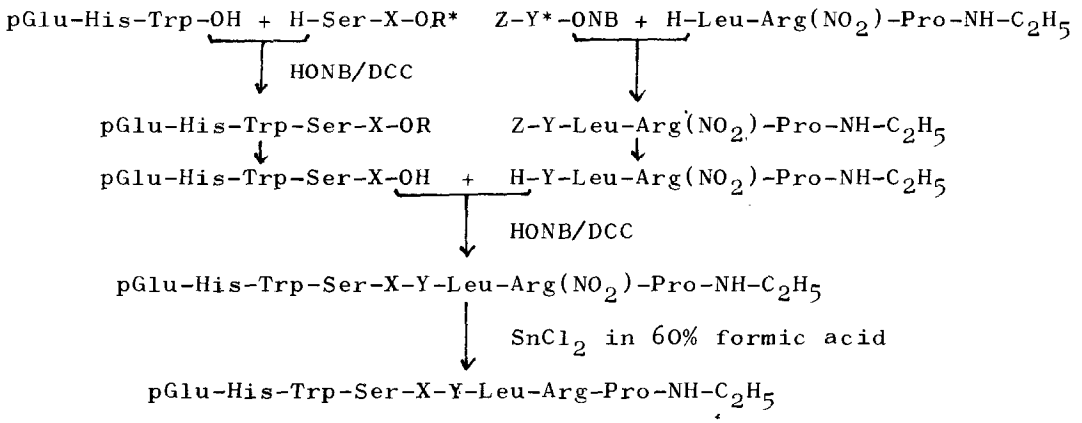
SUMMARY: Five new analogs of luteinizing hormone releasing hormone (LH-RH), des-Gly^{10}-[Ala^{6}]-LH-RH-ethylamide, des-Gly^{10}-[D-Ala^{6}]-LH-RH-ethylamide, des-Gly^{10}-[α-aminoisobutyric acid^{6}]-LH-RH-ethylamide, des-Gly^{10}-[Phe^{5}, D-Ala^{6}]-LH-RH-ethylamide and des-Gly^{10}-[Ile^{5}, D-Ala^{6}]-LH-RH-ethylamide were synthesized and evaluated for the ovulation-inducing activity in the rat, and it was found that the analogs, des-Gly^{10}-[D-Ala^{6}]-LH-RH-ethylamide and des-Gly^{10}-[Phe^{5}, D-Ala^{6}]-LH-RH-ethylamide, were 50 times or more active than the original molecule.

We have recently reported that replacement of the Gly-NH_2^{10} of the synthetic LH-RH by various alkylamines resulted in retention of the hormonal activity (1,2) and one of these alkylamine-substituted analog, des-Gly^{10}-LH-RH-ethylamide [I] was five times as potent as synthetic LH-RH in the *in vivo* assay (3), and also found that the analog I has considerably more prolonged action in the proestrous rat as compared with LH-RH (4). On the other hand, synthetic decapeptides having altered sequence of LH-RH have always led to a decreased hormonal activity in various degrees (5). With regard to the position 6 (Gly), Monahan et al. (6) reported that the synthetic analog [Ala^{6}]-LH-RH exhibited only 1% the activity of the natural hormone. Very recently, Monahan et al. (7) have reported that a newly synthesized [D-Ala^{6}]-LH-RH analog had exhibited a potency of 350-450% relative to LH-RH. These results were obtained *in*

vitro (rat pituitary primary cell cultures) and *in vivo* (estrogen treated ovariectomized rats). However, no data were given on the potencies of these compounds in the ovulation induction test. In our independent studies on structure-activity relations of LH-RH molecule, we have synthesized some analogs of LH-RH di- or tri-substituted in positions 5, 6 and 10, and found that [D-Ala^6]-analogs possessed very high activities when evaluated by the ovulation-induction index. In our view, studies of structure-activity relations of highly potent analogs of LH-RH should be very meaningful for an understanding of the sites on the LH-RH molecule which promote binding to the target receptor(s) and also in determining which structure features are necessary for the hormone to be resistant to *in vivo* inactivations. We believe that such studies are basic to the design of an inhibitor of LH-RH.

This paper describes the synthesis and the ovulation-inducing activity of five new analogs of LH-RH, des-Gly^{10}-[Ala^6]-LH-RH-ethylamide [II], des-Gly^{10}-[D-Ala^6]-LH-RH-ethylamide [III], des-Gly^{10}-[α-aminoisobutyric acid^6]-LH-RH-ethylamide [IV], des-Gly^{10}-[Phe^5, D-Ala^6]-LH-RH-ethylamide [V] and des-Gly^{10}-[Ile^5, D-Ala^6]-LH-RH-ethylamide [VI].

Synthesis of peptides: The syntheses of the analogs (except IV) have been achieved by a new route as shown in Fig. 1. For the preparation of N-terminal pentapeptides pGlu-His-Trp-Ser-X-OR [X-OR = Tyr-OMe, Phe-OMe and Ile-OBzl], a crystalline tripeptide pGlu-His-Trp-OH (8) was coupled with H-Ser-X-OR by the use of N-hydroxy-5-norbornene-2,3-dicarboximide (HONB)/dicyclohexylcarbodiimide (DCC) to minimize undesirable racemization during the coupling reaction, and the resulting pentapeptide esters were carefully subjected to saponification (or



[II] X=Tyr, Y=Ala; [III] X=Tyr, Y=D-Ala; [V] X=Phe, Y=D-Ala; [VI] X=Ile, Y=D-Ala.

* X-OR = Tyr-OMe, Phe-OMe or Ile-OBzl; Y = Ala or D-Ala.

Fig. 1. Syntheses of LH-RH analogs

hydrogenation) to give the corresponding free peptides. The other intermediates, Z-Y-Leu-Arg(NO_2)-Pro-NH-C_2H_5 [Y = Ala, D-Ala and α-aminoisobutyric acid (Aib)] were prepared by the coupling of H-Leu-Arg(NO_2)-Pro-NH-C_2H_5 (3) with the corresponding Z-amino acid via the HONB ester. The resulting intermediates were then treated with hydrogen bromide in acetic acid, followed by percolation through a column of Amberlite CG-410 (OH^-) to obtain the tetrapeptide-ethylamides. Couplings of N-terminal pentapeptides and C-terminal tetrapeptide-ethylamides were effected by the HONB/DCC method to give the crude protected nonapeptides which were purified by a column chromatography on Amberlite XAD-2 (gradient elution, 20% aqueous ethanol ⟶ 100% ethanol). The resulting peptides which have the nitro group as a protecting group were subjected to a reduction with stannous chloride in 60% formic acid for 120 min. at 80-85° [a modification of the method of Hayakawa et al. (9)]. The crude

peptides thus obtained were purified by a column chromatography on Amberlite XAD-2 (5% aqueous ethanol → 75% aqueous ethanol) and followed by a column chromatography on CMC (0.005 M NH_4OAc → 0.2 M NH_4OAc, pH 6.8) in a manner similar to that described for other LH-RH analogs (2).

The analog IV was also prepared by the conventional classical method. BOC-Ser-Tyr(Bzl)-Aib-Leu-Arg(NO_2)-Pro-NH-C_2H_5 was prepared by a stepwise method, starting from H-Aib-Leu-Arg(NO_2)-Pro-NH-C_2H_5 and using BOC-amino acid active esters. The BOC-group was removed from the intermediates by employing trifluoroacetic acid. The resulting free hexapeptide ethylamide was coupled with N-terminal tripeptide, pGlu-His-Trp-OH, by the HONB/DCC method to give the protected peptide which was treated with hydrogen fluoride to remove tha all protecting groups (10). The deblocked peptide was purified as the same manner described above.

The peptides thus obtained were all chromatographically pure in several solvent systems and gave the correct amino acid ratios and reasonable UV-spectra. The data for characterization of the key intermediates and the final products are listed in Table I and II, respectively.

Biological Results and Discussion: The ovulation inducing activities of these analogs were determined in diestrous rats (s.c., injection) by the method of Yamazaki and Nakayama (11). As indicated in Table III, analog III possessed an amazingly high potency of about 50-80 times the ovulation-inducing activity of LH-RH itself. This result suggested that the introduction of D-Ala at position 6 as well as ethylamine at the C-terminal position might led to a greater binding affinity of the peptide to the receptor(s) at the target organ, pituita-

Table I. Chemical and Physical Properties of Intermediates

Compound[a]	$[\alpha]_D$ (temp., conc., solvent)	Rf values of TLC[b]
Z-Ala-Leu-Arg(NO_2)-Pro-NH-C_2H_5	-70.4° (25°, 1.07, MeOH)	Rf^1=0.58, Rf^2=0.91
Z-D-Ala-Leu-Arg(NO_2)-Pro-NH-C_2H_5 (crystal, mp. 183-184°)	-49.2° (24°, 0.5, MeOH)	Rf^1=0.58, Rf^2=0.91
Z-Aib-Leu-Arg(NO_2)-Pro-NH-C_2H_5	-45.8° (24°, 0.55, DMF)	Rf^1=0.62, Rf^2=0.94
pGlu-His-Trp-Ser-Tyr-OH	+2.6° (27.5°, 0.55, AcOH)	Rf^3=0.67, Rf^4=0.34 Rf^5=0.46
pGlu-His-Trp-Ser-Phe-OH	-3.3° (26.5°, 0.55, AcOH)	Rf^3=0.69, Rf^4=0.37 Rf^5=0.51
pGlu-His-Trp-Ser-Ile-OH	-8.0° (26.5°, 0.10, AcOH)	Rf^3=0.69, Rf^4=0.39 Rf^5=0.54
BOC-Tyr(Bzl)-Aib-Leu-Arg(NO_2)-Pro-NH-C_2H_5 (crystal, mp. 127-129°)	-27.6° (24°, 0.54, DMF)	Rf^1=0.60
BOC-Ser-Tyr(Bzl)-Aib-Leu-Arg(NO_2)-Pro-NH-C_2H_5	-34.8° (25°, 0.44, DMF)	Rf^1=0.74

a) All compounds listed gave the correct analytical values (C, H, N).

b) Solvent system (Merck's precoated silica gel plate F 254): Rf^1=$CHCl_3$-MeOH-AcOH (9:1:0.5), Rf^2=AcOEt-Pyridine-AcOH-H_2O (60:20:6:10), Rf^3=n-BuOH-AcOEt-AcOH-H_2O (1:1:1:1), Rf^4= n-BuOH-AcOH-H_2O (4:1:1), Rf^5=n-BuOH-Pyridine-AcOH-H_2O (30:20:6:24).

ry, or a longer effective half-life of this analog *in vivo* than the original molecule, because the [Aib^6]-analog which might also be refractory toward an enzymic digestion shows a relatively high potency when compared with the [Ala^6]-analog.

Table II. Chemical and Physical Properties of LH-RH Analogs

Analog II: pGlu-His-Trp-Ser-Tyr-Ala-Leu-Arg-Pro-NH-C_2H_5
$[\alpha]_D^{23}$ -60.2° (C=0.5 in 5% AcOH)
TLC[a]: Rf^2=0.073, Rf^3=0.74, Rf^4=0.54, Rf^5=0.89
Amino acid analysis[b]: His 1.00; Arg 0.96; Trp 1.00; Ser 0.98; Glu 1.00; Pro 1.00; Ala 1.00; Leu 1.09; Tyr 1.02; Ethylamine 1.04 (87%)[c]

Analog III: pGlu-His-Trp-Ser-Tyr-(D)-Ala-Leu-Arg-Pro-NH-C_2H_5
$[\alpha]_D^{26}$ -41.0° (C=0.47 in 5% AcOH)
TLC: Rf^2=0.073, Rf^3=0.74, Rf^4=0.54, Rf^5=0.89
Amino acid analysis: His 0.96; Arg 1.03; Trp 0.90; Ser 0.92; Glu 1.03; Pro 1.00; Ala 1.02; Leu 1.05; Tyr 1.00; Ethylamine 1.08 (85%)

Analog IV: pGlu-His-Trp-Ser-Tyr-Aib-Leu-Arg-Pro-NH-C_2H_5
$[\alpha]_D^{27}$ -54.1° (C=0.31 in 5% AcOH)
TLC: Rf^2=0.099, Rf^3=0.72, Rf^4=0.80, Rf^5=0.90
Amino acid analysis: His 0.94; Arg 0.97; Trp 0.81; Ser 1.00; Glu 0.97; Pro 0.97; Aib 1.10; Leu 1.03; Tyr 1.03; Ethylamine 1.02 (81%)

Analog V: pGlu-His-Trp-Ser-Phe-(D)-Ala-Leu-Arg-Pro-NH-C_2H_5
$[\alpha]_D^{24}$ -38.49° (C=0.53 in 5% AcOH)
TLC: Rf^2=0.088, Rf^3=0.73, Rf^4=0.61, Rf^5=0.91
Amino acid analysis: His 0.96; Arg 1.04; Trp 0.92; Ser 0.96; Glu 0.99; Pro 1.08; Ala 1.00; Leu 1.00; Phe 1.00; Ethylamine 1.04 (85%)

Analog VI: pGlu-His-Trp-Ser-Ile-(D)-Ala-Leu-Arg-Pro-NH-C_2H_5
$[\alpha]_D^{24}$ -50.9° (C=0.47 in 5% AcOH)
TLC: Rf^2=0.084, Rf^3=0.71, Rf^4=0.62, Rf^5=0.90
Amino acid analysis: His 1.00; Arg 1.00; Trp 1.01; Ser 0.97; Glu 0.97; Pro 1.00; Ala 1.00; Ile[d] 0.98; Leu 0.99; Ethylamine 1.05 (86%)

a) Solvent systems used are listed in Table I. Rf^4 and Rf^5 are used Avicel precoated cellulose plate SF. b) Acid hydrolysate (5.7 N HCl, 105°, 30 hr., in the presence of thioglycolic acid) (13). c) Peptide content. d) Since a small peak of D-allo-isolucine was found (few %), recovery of Ile of this peptide indicates the racemization during the coupling reaction might be negligible.

Table III. Ovulation-Inducing Activity of LH-RH Analogs

Analog[a]	Ovulation-inducing activity in diestrous rat		In vitro	
	ED_{50}/100 g body weight[b]	Relative activity %	LH Release	FSH Release
LH-RH	215 ± 15 ng	100[c]	100[c]	100[c]
Analog I[d]	32.0 (24.8-41.3)[e]	672	300	280
Analog II	700.6 (600.2-793.6)	31	22	-
Analog III (lot-1)	2.6 (2.0-3.5)	8,269	180	300
(lot-2)	4.2 (3.7-5.1)	5,119		
Analog IV	14.5 (12.0-17.3)	1,483	470	300
Analog V (lot-1)	3.7 (2.2-4.7)	5,810	270	300
(lot-2)	4.4 (3.5-5.0)	4,886		
Analog VI	16.1 (12.0-20.2)	1,335	160	350
D-Ala^6-LH-RH[f]	7.6 (6.2-9.4)	2,829	570	475

a) The systematic names of these analogs are in Table II. b) The ED_{50} values were calculated from the data of five or six different dosages (5-10 rats each): in every case, a dose related ovulation was confirmed. c) Accepted to be 100. d) See reference 2. e) 95% confidence limits. f) This peptide was synthesized by the solid-phase method (M. Fujino and C. Kitada, unpublished): $[\alpha]_D^{24}$ -38.7° (C=0.52 in 5% AcOH), Rf^2=0.037, Rf^3=0.68, Rf^4=0.41, Rf^5=0.79 (solvent and plate, see Table I and II).

The surprizingly high activity of [Phe^5, $D\text{-}Ala^6$]-analog and relatively high potency of [Ile^5, $D\text{-}Ala^6$]-analog demonstrate that the aromatic ring system as well as the hydroxyl group of tyrosine residue of LH-RH (12) is not essential for the LH-release activity.

Previously (1,2), we reported good agreement between the *in vitro* and ovulating potency of a series of analogs in which $Gly\text{-}NH_2^{10}$ was replaced by various alkylamines. However, in the present work, the *in vitro* LH and FSH release potencies of the new highly active analogs are much lower than the potencies obtained by the ovulation test (Table III). At present we are unable to give a completely satisfactory explanation for these discordant results. Dose level studies in the diestrous rat (R. Rippel et al., unpublished) comparing LH-RH and analog III show a 50-80 fold increase in LH release by the analog which is in agreement with the ovulating activity.

Further detailed data for the biological properties including *in vitro* and *in vivo* LH- and FSH-release effects of these analogs will be reported elsewhere in the near future by White et al. and Yamazaki et al. (in preparation).

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References

1. Fujino, M., Kobayashi, S., Obayashi, M., Shinagawa, S., Fukuda, T., Kitada, C., Nakayama, R., Yamazaki, I., White, W. F., and Rippel, R. H., Biochem. Biophys. Res. Commun., 49, 863 (1972).

2. Fujino, M., Shinagawa, S., Obayashi, M., Kobayashi, S., Fukuda, T., Yamazaki, I., Nakayama, R., White, W. F., and Rippel, R. H., J. Med. Chem., 16, 1144 (1973).

3. Fujino, M., Shinagawa, S., Yamazaki, I., Kobayashi, S., Obayashi, M., Fukuda, T., Nakayama, R., White, W. F., and Rippel, R. H., Arch. Biochem. Biophys., 154, 488 (1973).

4. Rippel, R. H., Johnson, E. S., White, W. F., Fujino, M., Yamazaki, I., and Nakayama, R., Endocrinology, 93, 1449 (1973).

5. Fujino, M., Kobayashi, S., Obayashi, M., Fukuda, T., Shinagawa, S., Yamazaki, I., Nakayama, R., White, W. F., and Rippel, R. H., Biochem. Biophys. Res. Commun., 49, 698 (1972).

6. Monahan, M., Rivier, J., Vale, W., Ling, N., Grant, G., Amoss, M., Guillemin, R., Burgus, R., Nicolaides, E., and Ribstock, M., "Chemistry and Biology of Peptides" J. Meinhofer, ed., Ann Arbor Science Publishers, 1972, p.601.

7. Monahan, M., Amoss, M., Anderson, H., and Vale, W., Biochemistry, 12, 4616 (1973).

8. Fujino, M., Kobayashi, S., Fukuda, T., Obayashi, M., and Shinagawa, S., Proc. of 10th Symposium of Peptide Chemistry in Japan, J. Noguchi, ed., 1972, p. 7; Chem. Pharm. Bull. (Tokyo), in press.

9. Hayakawa, T., Fujiwara, Y., and Noguchi, J., Bull. Chem. Soc. Japan, 40, 1205 (1967).

10. Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., and Sugihara, H., Bull. Chem. Soc. Japan, 40, 2164 (1967).

11. Yamazaki, I., and Nakayama, R., Folia Endocrinologica Japonica, 49, 189 (1973); also see ref. 2.

12. Coy, D. H., Coy, E. J., and Schally, A. V., J. Med. Chem., 16, 83 (1973).

13. Matsubara, H., and Sasaki, K., Biochem. Biophys. Res. Commun., 35, 175 (1969).