SYNTHESES AND BIOLOGICAL ACTIVITIES OF ANALOGS OF LUTEINIZING HORMONE RELEASING HORMONE (LH-RH)

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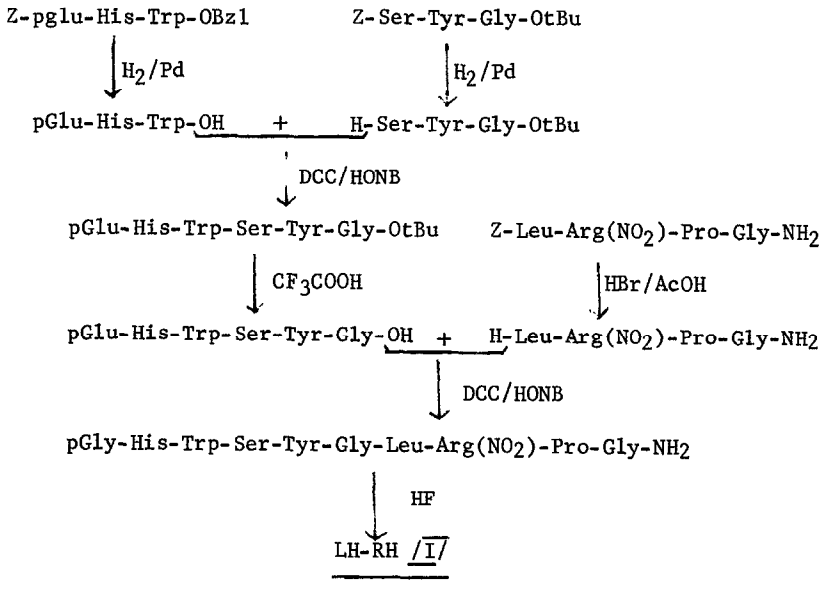
Summary: Twenty analogs of luteinizing hormone releasing hormone (LH-RH or pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), i.e., /-$^{O}\!\!\prec$Ser1/-, /-$^{O}\!\!\prec$Thr1/-, /Pro1/-, /Phe2/-, /3-Me-His2/-, /Lys2/-, /Arg2/-, /Ala4/-, /Thr4/-, /Gln5/-, /Cl-Tyr5/-, /di-Cl-Tyr7/-, /Gly7/-, /Ala7/-, /Val7/ /Ile7/-, /Nle7/-, /Lys8/-, /Orn8/-, and /Ala10/-LH-RH, were synthesized by the fragment condensation method and some biological properties of these decapeptide amides were studied. On the basis of in vitro and in vivo biological activities of these analogs, structure-activity relationships were discussed.

Since the amino acid sequence of porcine (1) and ovine (2) hypothalamic luteinizing hormone releasing hormone (LH-RH) has been demonstrated to be the decapeptide amide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 /1/, several syntheses of this releasing hormone by the solid phase method (3,4,5,6) and the solution method (7,8,9,10) have been reported.

Abbreviations: $^{O}\!\!\prec$Ser = 2-oxo-oxazolidine-4-carboxylic acid, $^{O}\!\!\prec$Thr = 2-oxo-5-methyl-oxazolidine-4-carboxylic acid, Cl-Tyr = o-Cl-tyrosine, di-Cl-Tyr = o,o'-Cl-tyrosine, 3-Me-His = $N^{3\text{-im}}$ methyl-histidine, Nle = norleucine, DCC = dicyclohexylcarbodiimide, HONB = N-hydroxy-5-norbornene-2, 3-dicarboximide.

We also reported a new method for the synthesis of the hormone by a solution method, in which d-isobornyloxycarbonyl (IBOC)-group (11,12) was used for protecting amino groups and for increasing the polarity of the protected peptide intermediates (13). We now wish to report the synthesis of decapeptide analogs of LH-RH by another improved method which was recently discovered in this laboratory and has the advantage of avoiding racemization during the coupling process. Detailed description of this method (DCC/HONB) including the synthesis of LH-RH will be given in a separate publication (14).

The key steps of our synthesis of the decapeptide amide can be outlined as follows:



In order to evaluate structure-activity relationships in the LH-RH molecule, we have synthesized twenty decapeptide amides (Tables I and II) essentially by the method outlined above. In the case of the first two analogs in the Tables, /-$^{O}\!\!=\!\!\langle$Ser/7-LH-RH and /-$^{O}\!\!=\!\!\langle$Thr/7-LH-RH a modification was used in which $^{O}\!\!=\!\!\langle$Ser-OSu and $^{O}\!\!=\!\!\langle$Thr-OSu were coupled with H-His-Trp-Ser-Tyr-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2(13), respectively, followed by treatment with HF (15).

TABLE I CHEMICAL AND PHYSICAL PROPERTIES OF LH-RH ANALOGS

Compound	tlc Values* Rf^1	Rf^2	Rf^3	$[\alpha]_D^{23}$ (in 5% AcOH)	Amino acid analysis**
LH-RH	0.32	0.06	0.61	-52.7°	
[$^0\tau_{Ser}{}^1$]-LH-RH	0.41	0.06	0.67	-33.4°	$His_{1.0}Arg_{1.0}Ser_{2.0}Pro_{1.1}Gly_{2.1}Leu_{1.1}Tyr_{1.0}Trp_{1.0}$
[$^0\tau_{Thr}{}^1$]-LH-RH	0.40	0.07	0.67	-38.2°	$His_{1.0}Arg_{1.0}Ser_{1.0}Thr_{1.0}Pro_{1.1}Gly_{2.0}Leu_{1.0}Tyr_{0.9}Trp_{1.0}$
[Pro^1]-LH-RH	0.14	0.04	0.65	-47.0°	$His_{0.9}Arg_{1.0}Ser_{1.0}Pro_{2.0}Gly_{2.0}Leu_{1.0}Tyr_{0.9}Trp_{1.0}$
[Phe^2]-LH-RH	0.22	0.32	0.79	-41.3°	$Arg_{0.9}Ser_{1.0}Glu_{1.0}Pro_{1.1}Gly_{2.0}Leu_{1.1}Phe_{1.0}Tyr_{0.9}Trp_{0.9}$
[3-Me-His^2]-LH-RH	0.19	0.03	0.64	-44.6°	$MeHis_{1.0}Arg_{1.0}Ser_{1.0}Glu_{1.0}Pro_{1.0}Gly_{2.0}Leu_{1.0}Tyr_{0.9}Trp_{1.0}$
[Lys^2]-LH-RH	0.25	0.05	0.65	-50.4°	$Arg_{1.0}Lys_{1.0}Ser_{0.9}Glu_{1.0}Pro_{1.1}Gly_{2.0}Leu_{1.0}Tyr_{0.8}Trp_{1.0}$
[Arg^2]-LH-RH	0.32	0.06	0.61	-51.2°	$Arg_{2.0}Ser_{0.9}Glu_{1.0}Pro_{1.0}Gly_{2.0}Leu_{1.1}Tyr_{0.9}Trp_{1.0}$
[Ala^4]-LH-RH	0.32	0.06	0.60	-52.6°	$His_{0.9}Arg_{1.0}Glu_{0.9}pro_{1.0}Gly_{2.0}Ala_{1.0}Leu_{1.0}Tyr_{1.0}Trp_{1.1}$
[Thr^4]-LH-RH	0.30	0.06	0.60	-56.6°	$His_{0.9}Arg_{0.9}Thr_{1.0}Glu_{1.0}Pro_{1.1}Gly_{2.0}Leu_{1.0}Tyr_{1.0}Trp_{1.0}$
[Gln^4]-LH-RH	0.25	0.04	0.58	-48.6°	$His_{1.0}Arg_{0.9}Glu_{1.[}Pro_{1.0}Gly_{1.[}Leu_{1.0}Tyr_{1.1}Trp_{0.9}$
[Cl-Tyr^5]-LH-RH	0.47	0.09	0.67	-46.8°	$His_{0.9}Arg_{1.0}Ser_{1.0}Glu_{1.0}Pro_{1.0}Gly_{2.0}Leu_{1.0}Cl\text{-}Tyr_{1.0}Trp_{0.9}$
[di-Cl-Tyr^5]-LH-RH	0.47	0.08	0.69	-57.0°	$His_{0.9}Arg_{1.0}Ser_{0.9}Glu_{0.9}Pro_{1.1}Gly_{2.0}Leu_{1.0}di\text{-}Cl\text{-}Tyr_{0.9}Trp_{0.9}$
[Gly^7]-LH-RH	0.17	0.04	0.50	-39.9°	$His_{1.1}Arg_{1.1}Ser_{0.9}Glu_{0.9}Pro_{0.9}Gly_{2.9}Tyr_{1.0}Trp_{1.0}$
[Ala^7]-LH-RH	0.18	0.01	0.51	-47.8°	$His_{0.9}Arg_{1.0}Ser_{1.0}Glu_{1.0}Pro_{1.1}Gly_{2.0}Ala_{1.1}Tyr_{1.0}Trp_{1.0}$
[Val^7]-LH-RH	0.23	0.03	0.55	-48.2°	$His_{1.1}Arg_{0.9}Ser_{0.9}Glu_{0.9}Pro_{1.0}Gly_{2.1}Val_{0.9}Tyr_{1.0}Trp_{1.0}$
[Ile^7]-LH-RH	0.27	0.06	0.59	-55.0°	$His_{1.2}Arg_{1.0}Ser_{1.0}Glu_{0.9}Pro_{0.9}Gly_{1.9}Ile_{1.1}Tyr_{1.0}Trp_{0.9}$
[Nle^7]-LH-RH	0.30	0.07	0.61	-46.2°	$His_{1.0}Arg_{1.0}Ser_{1.0}Glu_{1.0}Pro_{1.1}Gly_{2.1}Nle_{1.0}Tyr_{1.0}Trp_{1.0}$
[Lys^8]-LH-RH	0.24	0.06	0.58	-55.4°	$His_{1.0}Lys_{1.0}Ser_{0.9}Glu_{1.0}Pro_{1.0}Gly_{2.0}Leu_{1.0}Tyr_{1.0}Trp_{1.0}$
[Orn^8]-LH-RH	0.26	0.04	0.54	-61.0°	$His_{1.2}Orn_{1.0}Ser_{0.9}Glu_{0.9}Pro_{0.9}Gly_{1.9}Leu_{1.0}Tyr_{0.9}Trp_{1.0}$
[Ala^{10}]-LH-RH	0.34	0.07	0.59	-64.6°	$His_{1.0}Arg_{1.0}Ser_{0.9}Glu_{1.0}Pro_{1.0}Gly_{1.0}Ala_{1.0}Leu_{1.0}Tyr_{1.0}Trp_{1.0}$

* Rf^1, Rf^2 and Rf^3(on Woelm pre-coated silica gel F 254/366) values refers to the systems: n-BuOH:AcOH:EtOAc:H_2O(1:1:1:1); n-BuOH:AcOH:H_2O(4:1:1); n-BuOH:Pyridine:AcOH:H_2O(30:20:6:24),respectively.

** Acid hydrolysate(5.7N-HCl,110°,20hrs). Trp contents were caluclated from UV spectrum(in 0.1N-NaOH).

All the products were purified on an Amberlite XAD-II column and subsequently on a carboxymethylcellulose column in a similar way to the purification of LH-RH (14). The detailed account of the syntheses of these analogs will be reported in companion papers (16,17,18).

Criteria of the purity of the analogs were achieved by tlc on Woelm pre-coated silica gel F 254/366, using three solvent systems (50μg of each peptide was spotted) and also by amino acid analysis and UV analysis. All the peptides synthesized were chromatographically pure in all solvent systems and gave the correct amino acid contents and reasonable UV spectra. The data for characterization of the analogs are listed in Table I.

The hormonal evaluation in vivo of the analogs of LH-RH were performed by the method of Yamazaki and Nakayama (19). The ovulation inducing activities of the analogs were tested by using adult Sprague-Dawley rats and single subcutaneous injection at diestrus (at 14:40) and then the results were compared with the ED_{50} of synthetic LH-RH.

For more detailed evaluation, the analogs were incubated with hemisected male rat pituitaries and the media were assayed for LH, by the bioassay of Parlow (20) and/or the radioimmunoassay of Niswender et al (22) and for FSH by the bioassay of Steelman and Pohley (21) and/or the radioimmunoassay employing the NIAMD-Rat-FSH-Kit.

The data on the biological activities of the synthetic LH-RH analogs are summarized in Table II.

Discussion. Several publications have recently appeared describing studies of the activity-structure relationsip. On the basis of chemical modification of natural LH-RH, Baba et al (23) have concluded that His, Trp, Try and Arg in the LH-RH molecule are important for its hormonal activity and the hydroxy group of Ser is essential for the activity. The important nature of pyrrolidine structure of N-terminal for the hormonal activity was demonstrated by means of the treatment of natural LH-RH with pyrrolidone carboxylyl peptidase by Amoss et al (24) and

Schally et al (25). On the other hand, only a few instances have been recorded of the synthetic approach to the structure-activity relationship of the LH-RH analogs. Sakakibara et al (8) reported the synthesis and biological activity of /D-Phe2/-LH-RH and /D-Phe5/-LH-RH and found these analogs to have ca. 50% the LH-release activity of LH-RH itself. Monahan et al (26) described the synthesis of /D-Gly2/-LH-RH, which exhibits a low biological activity and some degree of inhibitory activity against LH-RH in its release of LH. Chang et al (27) reported recently the syntheses of /D-His8/-LH-RH, /D-Nva8/-LH-RH and /des-Arg8/-LH-RH, and showed the important nature of positive charge at position 8 for the hormonal action of LH-RH.

On the basis of our new data which is listed in Table II, it is concluded that: (a) the intramolecular cyclic carbonylamide at N-terminus is very important for release of LH and FSH, because /D-Pro1/-LH-RH has no activity, whereas /$^{0}\gamma$Ser1/-LH-RH has a significant potency for release of LH and FSH; (b) the imidazole ring at position 2 is not essential but appears to contribute to the higher level of the hormonal activity, and the basicity of this position has not so much contribution to the activity. Moreover it is interesting to note that the replacement of His by 3-Me-His at position 2 does not increase the activity but actually markedly decreases the specific activity; (c) the hydroxy-group of Ser at position 4 is not essential for the hormonal action; (d) the non-polar side chain of Leu at position 7 is also not essential but appears to contribute to the binding of the hormone to its receptor by means of its apolar character; (e) since replacement of Arg by Lys at position 8 results in only a small decrease in activity, basic character in this position appears to contribute to the hormonal action, as suggested by Chang et al (27); however the marked loss in activity caused by the insertion of Orn in this position suggests that chain length is also important.

TABLE II HORMONAL ACTIVITY OF LH-RH ANALOGS

Compound	Ovulation induce activity(ED_{50})	in vitro assays LH-release	in vitro assays FSH-release
LH-RH	165-230ng/100g b.w.*	100(%)	100(%)
[des-Gly^{10}, D-Ser^{1}]-LH-RH	3,000	5-25(P)*	6(SP)*
[des-Gly^{10}, D-Thr^{1}]-LH-RH	100,000 - 10,000	-	$\ll$ 5(SP)
[Pro^{1}]-LH-RH	Neg.*	< 0.1(RIA)*	< 0.1(SP)
[Phe^{2}]-LH-RH	100,000 - 10,000	4-7(RIA)	2(SP)
[Lys^{2}]-LH-RH	Neg.	< 0.1(RIA)	< 0.1(SP,RIA)
[Arg^{2}]-LH-RH	-	< 0.1(P,RIA)	< 0.1(SP,RIA)
[3-Me-His^{2}]-LH-RH	50,000	1(RIA)	1-2(SP,RIA)
[Ala^{4}]-LH-RH	10,000	3-6(RIA)	16(SP)
[Thr^{4}]-LH-RH	10,000	4(RIA)	17(SP)
[Gln^{4}]-LH-RH	10,000 - 1,000	8(RIA)	6(RIA)
[Cl-Tyr^{5}]-LH-RH	10,000 - 1,000	8(RIA)	5(SP)
[di-Cl-Tyr^{5}]-LH-RH	Neg.	< 1(P,RIA)	< 1(RIA)
[Gly^{7}]-LH-RH	-	3(P)	5(SP)
[Ala^{7}]-LH-RH	10,000	5-6(RIA)	3-5(SP)
[Val^{7}]-LH-RH	1,000	16(RIA)	20-35(SP)
[Ile^{7}]-LH-RH	600 - 400	45(RIA)	33(SP)
[Nle^{7}]-LH-RH	600	30(P)	22(SP)
[Lys^{8}]-LH-RH	2,000	11-28(P)	25(SP)
[Orn^{8}]-LH-RH	2,000	6-12(P)	5(SP)
[Ala^{10}]-LH-RH	10,000 - 1,000	$\sim$10(P)	-

* b.w.: body weight, P: Parlow's method, SP: Steelman-Pohley's method, RIA for LH: Niswender's method, RIA for FSH: NIAMD-Rat-Fsh-Kit, Neg.: Negative.

Our above-mentioned results, together with those reported by others (23,27), strongly suggest that none of the so-called "essential/functional amino acid" exists in the LH-RH molecule, but on the contrary it appears to us that all the amino acids may play important parts in the biological functions of the hormone.

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References

1. Matsuo, H., Baba, Y., Nair, R. M. G., Arimura, A. and Schally, A. V. Biochem. Biophys. Res. Commun., 43, 1334 (1971).
2. Burgus, R., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, J., Fellows, R., Blackwell, R., Vale, W. and Guillemin, R., Proc. Nat. Acad. Sci. (US), 69, 278 (1972).
3. Stievertson, H., Chang, J. K., Bogentoft, C., Currie, B. L., Folkers, K. and Bowers, C. Y., Biochem. Biophys. Res. Commun., 44, 1566 (1971).
4. Monahan, M., Rivier, J., Burgus, R., Amoss, M., Blackwell, R., Vale, W., Guillemin, R., C. R. Acad. Sci. (Paris), 273, 508 (1971).
5. Matsuo, H., Arimura, A., Nair, R. M. G. and Schally, A. V., Biochem. Biophys. Res. Commun., 45, 822 (1971).
6. Rivaille, D., Robinson, A., Kamen, M. and Milhand, G., Helv. Chim. Acta., 54, 2772 (1971).
7. Geiger, R., König, W., Wissmann, H., Geisen, K. and Enzmann, F., Biochem. Biophys. Res. Commun., 45, 767 (1971).
8. Sakakibara, S., et al., Proc. of 9th symposium of peptide chemistry in Japan, p. 90 (1971).
9. Yanaihara, N. et al., Proc. of 9th symposium of peptide chemistry in Japan, p. 96 (1971).
10. Flouret, G., Arnold, W., Cole, W., Morgan, R., White, W., Hedlund, M., and Rippel, R., J. Med. Chem., in press.
11. Fujino, M., Shinagawa, S., Nishimura, O. and Fukuda, T., Chem. Pharm. Bull. (Tokyo), 20, 1017 (1972).
12. Fujino, M. and Shinagawa, S., Chem. Pharm. Bull. (Tokyo), 20, 1021 (1972).
13. Fujino, M., Fukuda, T., Kobayashi, S. and Obayashi, M., Chem. Pharm. Bull. (Tokyo), submitted for publication.
14. Fujino, M., Kobayashi, S., Fukuda, T., Obayashi, M. and Shinagawa, S., in preparation.
15. Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M. and Sugihara, H., Bull. Chem. Soc. Japan, 40, 2164 (1967).
16. Obayashi, M., Fukuda, T., Kobayashi, S., Kitada, C. and Fujino, M., in preparation.
17. Kobayashi, S., Shinagawa, S., Fukuda, T., Obayashi, M. and Fujino, M., in preparation.
18. Fujino, M., Kobayashi, S., Shinagawa, S., Fukuda, T. and Obayashi, M., in preparation.
19. Yamazaki, I. and Nakayama, R., submitted for publication.
20. Steelman, S. L. and Pohley, F. M., Endocrinology, 53, 604 (1953).
21. Parlow, A. F., in "Human Pituitary Gonadotropins" p. 300, A. Albert ed., Charles C. Thomas, Springfield, Ill. (1961).

22. Niswender, G. D., Midgley, A. R., Jr., Monroe, S. E. and Reichert, L. E., Proc. Soc. Exp. Biol. Med., 128, 807 (1968).
23. Baba, Y., Arimura, A. and Schally, A. V., J. Biol. Chem., 246, 7581 (1971).
24. Amoss, M., Burgus, R., Blackwell, R., Vale, W., Fellows, R. and Guillemin, R., Biochem. Biophys. Res. Commun., 44, 205 (1971).
25. Schally, A. V., Arimura, A., Bara, Y., Nair, R. M. G., Matsuo, H., Redding, T. W. and Debeljik, L., Biochem. Biophys. Res. Commun., 43 393 (1971).
26. Monahan, M. W., Rivier, J., Vale, W., Guillemin, R. and Burgus, R., Biochem. Biophys. Res. Commun., 47, 551 (1972).
27. Chang, J. K., Williams, R. H., Humphries, A. J., Johansson, N., Folkers, K., and Bowers, C. Y., Biochem. Biophys. Res. Commun., 47, 727 (1972).