(6) C. Caradonna and M. L. Stein, *Farmaco, Ed. Sci.*, **15**, 647 (1960); *Chem. Abstr.*, **58**, 2441c (1963).
(7) W. G. Thurman, G. Bloedow, C. D. Howe, W. C. Levin, P. Davis, M. Lane, M. P. Sullivan, and K. M. Griffith, *Cancer Chemother. Rep.*, **29**, 103 (1963); I. H. Krakoff, M. L. Murphy, and H. Savel, *Proc. Am. Assoc. Cancer Res.*, **4**, 35 (1963).
(8) (a) U.S. Patent 3 254 984 (June 7, 1966); *Chem. Abstr.*, **65**, 5398b (1966); (b) U.S. Patent 3 166 587 (Jan 19, 1965); *Chem. Abstr.*, **62**, 7649d (1966).
(9) K. Kobashi, J. Hase, and K. Uehara, *Biochim. Biophys. Acta*, **65**, 380 (1962).
(10) K. Tonita, N. Mitsuo, and N. Nakamura (Sankyo Co., Ltd.), Japanese Patent 6 816 136 (Cl. 16 E 342) (July 8, 1968); *Chem. Abstr.*, **70**, P57817w (1969).
(11) A. R. Katritzky and S. Oksne, *Proc. Chem. Soc., London*, 387 (1961).
(12) (a) P. Bravo, G. Gaudians, A. Ricca, and A. Quilico, *Gazz. Chim. Ital.*, **91**, 47 (1961); (b) A. R. Gagneux and F. Hafliger (Geigy Chemical Corp.), U.S. Patent 3 397 209 (C1206-307) (August 13, 1966); *Chem. Abstr.*, **60**, P10669w (1968).
(13) *Bielstein*, 2nd ed, **2**, 640 (1942).
(14) A. J. Boulton, A. R. Katritzky, A. M. Hamid, and S. Oksne, *Tetrahedron*, **20**, 2935 (1964).
(15) E. H. Huntress, T. E. Lesslie, and J. Bornstein, "Organic Syntheses", Collect. Vol. IV, Wiley, New York, N.Y., 1963, p 329.
(16) *Bielstein*, **2**, 744 (1920).
(17) J. E. Baldwin, *J. Chem. Soc., Chem. Commun.*, 734, 736, 738 (1976).
(18) We are indebted to Dr. Glen R. Gale, Veterans Administration Hospital, and Department of Pharmacology, Medical College of South Carolina, Charleston, S.C., for the bacterial testing.

Inhibition of the Activity of the Luteinizing Hormone-Releasing Hormone (LH-RH) by Analogues with Variations at Positions 2, 3, and 6 and the Carboxyl Terminus[1]

John Humphries, Yieh-Ping Wan, Karl Folkers,*

Institute for Biomedical Research, The University of Texas at Austin, Austin, Texas 78712

and Cyril Y. Bowers

Tulane University School of Medicine, New Orleans, Louisiana 70112. *Received December 28, 1976*

In the isolated rat pituitary assay, [Thr^2,Leu^3]-LH-RH, [Leu^2,Ala^3,D-Ala^6]-LH-RH, and des-Gly^{10}-[Abu^2,Ala^3,D-Ala^6]-LH-RH ethylamide inhibited the LH release due to 0.3 ng/mL of added LH-RH at a 10 μg/mL dosage. Under these same assay conditions, des-Gly^{10}-[Ile^2,Ala^3,D-Ala^6]-LH-RH ethylamide was about one-tenth as active, and no inhibition was observed by [Leu^2,Ser^3]-LH-RH or [Leu^2,Asn^3]-LH-RH at a 100 μg/mL dosage. The corresponding results from FSH inhibition assays, in vitro, are also reported.

An inhibitory LH-RH sequence formed by dual structural variation at the biologically important 2 and 3 positions[2a] appears to be one of considerable promise for analogue design in the search for more potent and effective LH-RH inhibitors and antiovulation agents. The first lead to inhibitors based on this sequence was [Leu^2,Leu^3]-LH-RH. Humphries et al.[2b] reported that this analogue inhibited the release of LH and FSH, in vitro, as induced by 0.3 ng/mL of LH-RH from isolated rat pituitaries, at a dosage of 100 μg/mL. No agonist activity was observed up to a 100 μg/mL dosage with this analogue. Vale et al.[3] had earlier reported that des-His^2-LH-RH showed weak inhibition with a monolayer culture in vitro technique.

Later studies by Wan et al.[4] showed that [Leu^2,Leu^3,-D-Ala^6]-LH-RH and [Val^2,Leu^3,D-Ala^6]-LH-RH inhibited, in vitro, at a dosage of 10 μg/mL and had a ratio of inhibitor–LH-RH of 30 000:1.

Bowers et al.[5] found that [chlorambucil^1,Leu^2,Leu^3,D-Ala^6]-LH-RH irreversibly inhibited the action of LH-RH, in vitro, on isolated pituitaries.

We now report six new LH-RH analogues, having sequences related to the dually substituted [Leu^2,Leu^3]-LH-RH.[2b] Some of these new analogues contain a D-Ala residue in the 6 position[6] and a C-terminal Pro^9-NHEt unit[7] in an attempt to increase potency or duration of inhibition.

After this work was completed, Rees et al.[8] reported that [D-Phe^2]-LH-RH inhibited, in vitro, in a monolayer assay system at a higher potency than des-His^2-LH-RH.

Experimental Section

Amino acid derivatives were purchased from Beckman Instruments, Palo Alto, Calif., or from Bachem Inc., Marina del Rey, Calif. Amino acid analyses, on samples hydrolyzed in 6 N HCl at 110 °C for 18 h, were performed with a Beckman Model 119 amino acid analyzer equipped with an Infotronics Model CRS-210 automatic digital integrator. Optical rotations were measured on a Perkin-Elmer 141 digital read-out polarimeter.

During column chromatography, the peptide peaks were located at 254 nm using an ISCO UV monitor. The product of the major peak was examined by TLC and fraction cuts were made, prior to lyophilization, for purity at the expense of product yield. Product yields, based on starting amino acid-resin, therefore, will tend to be low.

Homogeneity of the peptides was demonstrated on silica gel plates, using baths from the following TLC systems: R_f^1 EtOAc–H_2O–AcOH–1-BuOH (1:1:1:1); R_f^2 EtOH–H_2O (7:3); R_f^3 $CHCl_3$–MeOH–concentrated NH_4OH (60:45:20); R_f^4 EtOAc–pyridine–AcOH–H_2O (5:5:1:3); R_f^5 1-BuOH–pyridine–AcOH–H_2O (30:20:6:24); R_f^6 2-propanol–1 N AcOH (2:1). The solvent fronts were allowed to travel for 10–15 cm and spots were negative to ninhydrin and positive to chlorine–*o*-tolidine reagent and to iodine. The peptides were homogeneous on thin-layer electrophoresis on silica gel plates at 500 V with the systems pyridine–AcOH–H_2O (30:1:270) (pH 6.5) and 1 M AcOH (pH 2.8).

Coupling Program. Reactions were performed with a Beckman Model 990 peptide synthesizer. The swollen resin, on to which was covalently attached the C-terminal amino acid as its N^{α}-Boc derivative, was washed three times with CH_2Cl_2. After a 2-min prewash with 30% trifluoroacetic acid (TFA) in CH_2Cl_2 (v/v), the N^{α}-protecting group was removed by stirring the resin with 30% TFA in CH_2Cl_2 (v/v) for 30 min. The amino acid-resin-trifluoroacetate salt remaining was given six washes (2 min each) with CH_2Cl_2 and two prewashes (2 min each) with 10% NEt_3 (redistilled from NaOH pellets) in CH_2Cl_2 (v/v). The resin salt was neutralized by stirring the resin with 10% NEt_3 in CH_2Cl_2 (v/v) for 10 min. The resulting amino acid-resin was washed five times (2 min each) with CH_2Cl_2. A solution of the next amino acid derivative to be incorporated, as its N^{α}-Boc derivative and

Table I. In Vitro Agonist and Antagonist Activity in the Analogues

Peptide analogue	Doses[a] Peptide, μg/mL of medium	Doses[a] LH-RH, ng/mL of medium	LH Δ, ng/mL of medium	LH SEM	LH p value	FSH Δ, ng/mL of medium	FSH SEM	FSH p value
[Thr²,Leu³]-LH-RH		0.3	408	±59		4848	±1124	
	10	0.3	180	±70	~0.02	3080	±551	ns
	100	0.3	180	±37	<0.01	3232	±340	ns
	100		14	±18	ns	699	±562	ns
			5	±21		220	±103	
[Leu²,Asn³]-LH-RH		0.3	257	±56		3107	±390	
	100	0.3	185	±13	ns	2298	±396	ns
	100		18	±4	<0.01	−1271	±101	0.02
			−42	±14		−704	±189	
[Leu²,Ser³]-LH-RH		0.3	106	±12		8559	±444	
	10	0.3	88	±38	ns	6545	±1137	ns
	100	0.3	90	±19	ns			
	100		−7	±2	ns	−367	±199	ns
			−8	±4		−168	±263	
Des-Gly¹⁰-[Abu²,-Ala³,D-Ala⁶]-LH-RH ethylamide		0.3	282	±89		5929	±605	
	1	0.3	157	±22	ns	2611	±317	<0.001
	10	0.3	56	±31	<0.05	2966	±708	<0.01
	100	0.3	62	±16	<0.05	1775	±514	<0.001
	100		−3	±7	ns	184	±206	~0.001
			18	±5		1389	±357	
Des-Gly¹⁰-[Ile²,-Ala³,D-Ala⁶]-LH-RH ethylamide		0.3	293	±68		6371	±636	
	100	0.3	49	±11	<0.01	1909	±344	<0.001
	100		42	±8	ns	1017	±153	<0.01
			15	±20		252	±162	
[Leu²,Ala³,D-Ala⁶]-LH-RH		0.3	332	±76		9002	±1023	
	1	0.3	230	±28	ns	7019	±473	ns
	10	0.3	149	±32	0.05	8153	±1175	ns
	100	0.3	115	±19	0.02	5852	±628	0.02
	100		13	±7	ns	−351	±143	ns
			12	±11		−543	±63	

[a] Intermediate dose levels have been omitted for brevity.

with any reactive side-chain functionality blocked with an HF-labile protecting group, was added to the resin in about a threefold excess in either CH_2Cl_2 or $HCONMe_2$ solution. The mixture was stirred for 10 min and then a solution of dicyclohexylcarbodiimide (DCC, threefold excess) in CH_2Cl_2 (10% solution) was added and coupling was allowed to proceed to completion when the ninhydrin color test[9] was negative. The Boc-peptide-resin was washed twice with CH_2Cl_2, four times with CH_2Cl_2–EtOH (10:1), and four times with CH_2Cl_2 (2 min each wash).

It was very seldom that the ninhydrin color test remained positive during the coupling step. If so, a double coupling program was performed, which involved repeating the above coupling program but omitting all of the operations up to the NEt_3 prewash step.

[Thr²,Leu³]-LH-RH. Benzhydrylamine (BHA) resin hydrochloride (Beckman Instruments, Inc., of 0.5 mequiv of amine/g) was neutralized with NEt_3 in CH_2Cl_2 (25%, v/v), was washed with CH_2Cl_2, and then was acylated with a 2.5-fold excess of Boc-Gly and DCC in CH_2Cl_2 to give Boc-Gly-BHA-resin (11 g, 0.4 mM/g of Gly).

Coupling cycles were performed with Boc-Pro, Boc-Arg(Tos), Boc-Leu, and Boc-Gly. The resulting Boc-Gly-Leu-Arg(Tos)-Pro-Gly-BHA resin amounted to 13.6 g.

A portion of the pentapeptide resin (2 g) was coupled successively with Boc-Tyr(*o*-Br-Z), Boc-Ser(Bzl), Boc-Leu, Boc-Thr(Bzl), and <Glu-OH to give the protected decapeptide resin.

Cleavage of the peptide from the resin and simultaneous deblocking of side-chain protecting groups occurred when the decapeptide resin (ca. 2.6 g) was stirred with anhydrous (CoF_3) liquid HF, containing 10–20% anisole,[10] for 1 h at 0 °C. The reaction mixture was evaporated to dryness and washed with Et_2O and with EtOAc. The [Thr²,Leu³]-LH-RH was extracted from the mixture of peptide and resin by 10% AcOH. The combined acetic acid extracts were washed with EtOAc and Et_2O, and the peptide was obtained as a white, fluffy solid after lyophilization.

Ion-exchange chromatography on Bio-Gel CM-2 (25 × 1.5 cm) with NH_4OAc buffers (1 mM, pH 4.5, to 100 mM) followed by chromatography on Sephadex LH-20 (90 × 1.5 cm) with 1-BuOH–H_2O (6:100) gave 300 mg (41%) of [Thr²,Leu³]-LH-RH: $[\alpha]^{24}_D$ −48.93° (*c* 9.38, MeOH). Amino acid analysis: Glu 1.01, Thr 0.87, Leu 2 × 1.09, Ser 0.86, Tyr 0.99, Gly 2 × 1.05, Arg 0.97, Pro 1.02. TLC data: R_f^1 0.42, R_f^2 0.37, R_f^4 0.77, R_f^5 0.60, R_f^6 0.32.

[Leu²,Ser³]-LH-RH. A portion of (2 g) of the pentapeptide resin synthesized above was coupled with Boc-Tyr(*o*-Br-Z), Boc-Ser(Bzl), Boc-Ser(Bzl), Boc-Leu, and <Glu-OH. The resulting decapeptide resin was treated with anhydrous HF and purification, as for the Thr analogue, gave 209.6 mg (29%) of [Leu²,Ser³]-LH-RH: $[\alpha]^{24}_D$ −42.86° (*c* 11.245, MeOH). Amino acid analysis: Glu 1.5, Leu 2 × 1.09, Ser 2 × 0.97, Tyr 0.97, Gly 2 × 1.02, Arg 0.99, Pro 0.80. TLC data: R_f^1 0.40, R_f^2 0.35, R_f^4 0.77, R_f^5 0.60, R_f^6 0.30.

[Leu²,Asn³]-LH-RH. A portion (2 g) of the pentapeptide resin described above was elongated with Boc-Tyr(*o*-Br-Z) and Boc-Ser(Bzl). The Asn residue was next incorporated by the active ester method using Boc-Asn-ONP. The active ester coupling program was the same as the above-described DCC coupling program, except that the DCC addition step was omitted. Further coupling steps with Boc-Leu and <Glu-OH gave the decapeptide resin.

Treatment with anhydrous HF and purification similar to the Thr analogue gave [Leu²,Asn³]-LH-RH (250 mg, 34%): $[\alpha]^{24}_D$ −43.56° (*c* 9.32, MeOH). Amino acid analysis: Glu 0.99, Leu 2 × 1.10, Asp 0.86, Ser 0.8, Tyr 0.98, Gly 2 × 1.06, Arg 1.00, Pro 1.05. TLC data: R_f^1 0.60, R_f^3 0.72, R_f^5 0.65.

[Leu²,Ala³,D-Ala⁶]-LH-RH. Boc-Gly-BHA-resin (1.85 g, 0.49 mM/g of Gly) was coupled with Boc-Pro, Boc-Arg(Tos), Boc-Leu, Boc-D-Ala, Boc-Tyr(2,4-Cl_2-Bzl), Boc-Ser(Bzl), and Boc-Ala. The resulting octapeptide-resin weighed 2.58 g and a portion (1.29 g) was further extended with Boc-Leu and Z-<Glu-OH to yield 1.3 g of final peptide resin. Treatment with HF and purification by partition chromatography over Sephadex G-25 (100 × 1.5 cm) with the system 1-BuOH–AcOH–H_2O (4:1:5) gave 192 mg of product which was further purified over Sephadex LH-20 and gave 102 mg (20%) of [Leu²,Ala³,D-Ala⁶]-LH-RH: $[\alpha]^{24}_D$ −48.64° (*c* 9.867, MeOH).

Amino acid analysis: Glu 1.00, Leu 2 × 1.07, Ala 2 × 1.07, Ser 0.89, Tyr 0.91, Arg 0.94, Pro 1.01, Gly 0.97. TLC data: R_f^1 0.65, R_f^2 0.69, R_f^3 0.79, R_f^4 0.88, R_f^6 0.72.

Des-Gly^{10}-[Abu^2,Ala^3,D-Ala^6]-LH-RH Ethylamide. The chloromethylated resin (Sigma Chemical Co.) was esterified with Boc-Pro[11] to give Boc-Pro-resin ester (0.5 mM/g of Pro). The Boc-Pro resin (10.2 g) was coupled with Boc-Arg(Tos), Boc-Leu, Boc-D-Ala, Boc-Tyr(*o*-Br-Z), and Boc-Ser(Bzl) and gave the hexapeptide resin ester (12 g). A portion of this resin (2 g) was coupled with Boc-Ala, Boc-Abu, and <Glu-OH. The resulting nonapeptide resin ester was stirred with anhydrous ethylamine for 6 h at 0 °C to form the nonapeptide ethylamide. Excess ethylamine was evaporated and the product was separated into MeOH. Concentration, under reduced pressure, of the MeOH extract, followed by precipitation of the protected peptide ethylamide with EtOAc, gave 620 mg of solid. Deprotection with HF and purification over columns of Sephadex LH-20 and CM-52 (30 × 1.5 cm) gave 179.3 mg (20%) of the product: $[\alpha]^{24}_D$ −59.64° (*c* 9.373, MeOH). Amino acid analysis: Glu 1.01, Ala 2 × 1.15, Ser 0.91, Tyr 0.87, Leu 0.90, Arg 1.00, Pro 1.01. TLC data: R_f^1 0.65, R_f^2 0.65, R_f^3 0.78, R_f^4 0.88, R_f^5 0.82, R_f^6 0.69.

Des-Gly^{10}-[Ile^2,Ala,D-Ala^6]-LH-RH Ethylamide. A portion (2 g) of the hexapeptide resin used for making the Abu analogue was coupled with Boc-Ala, Boc-Ile, and <Glu-OH. The peptide ethylamide (550 mg) was obtained with anhydrous ethylamine. Treatment with HF, and chromatography as for the Abu analogue, gave 50 mg (5.5%) of product: $[\alpha]^{24}_D$ −52.73° (*c* 9.255, MeOH). Amino acid analysis: Glu 0.98, Ile 0.83, Ala 2 × 1.13, Ser 0.88, Tyr 0.97, Leu 0.99, Arg 1.06, Pro 1.03. TLC data: R_f^1 0.71, R_f^2 0.71, R_f^3 0.83, R_f^4 0.87, R_f^5 0.87, R_f^6 0.79.

Biological Assays. All hormonal activities were obtained from in vitro studies using pituitaries of 20-day-old female Sprague–Dawley rats (Charles River Laboratory). To determine the LH and FSH agonist and antagonist activities two pituitaries were incubated at 37 °C in 1 mL of lactated Ringer's solution (Travenol Laboratories) in 10-mL Teflon beakers in a Dubnoff shaker. Pituitaries were incubated for a total of 6 h. Medium was removed each hour for RIA[12] for LH and FSH and fresh medium was added. After two preincubation periods (P_1, P_2), the LH-RH analogue was added to the incubation medium (I_3, I_4, I_5, I_6). LH-RH was added at I_5 and I_6. When both peptides were added together the LH-RH analogue was always added to the incubation medium 5 min before LH-RH. The values for these assays were calculated in terms of nanograms of the following standards: LH-LER-1240-2 (0.60 NIH-LH-SI unit/mg) and FSH (2.1 × NIH-FSH-SI units/mg).

Results and Discussion

The in vitro agonist and inhibitor data for these analogues are given in Table I. Under our assay conditions, none of these analogues have shown any significant agonist activity.

[Thr^3,Leu^3]-LH-RH inhibited at dosages of 10 and 100 μg/mL the LH response due to 0.3 ng/mL of LH-RH. The analogues, [Leu^2,Ser^3]-LH-RH and [Leu^2,Asn^3]-LH-RH, which have sequences related to [Leu^2,Leu^3]-LH-RH, with variations at position 3, resulted in no inhibition up to 100 μg/mL. We had earlier observed a reduction in inhibitory potency of about tenfold when Leu in position 3 of [Leu^2,Leu^3,D-Ala^6]-LH-RH was replaced by a Val residue.[4]

[Leu^2,Ala^3,D-Ala^6]-LH-RH and des-Gly^{10}-[Abu^2,Ala^3,-D-Ala^6]-LH-RH ethylamide inhibited at a dosage of 10 μg/mL, whereas des-Gly^{10}-[Ile^2,Ala^3,D-Ala^6]-LH-RH ethylamide was only about one-tenth as active as an inhibitor.

Acknowledgment. Appreciation is expressed to Dr. Marvin Karten and for the support of Contract NIH NICHD 72-2713 of the National Institutes of Health, for Public Health Service Research Grant No. CA-14200-02 from the National Cancer Institute, and for grants from the Rockefeller Foundation and the Robert A. Welch Foundation. The RIA reagents for FSH were distributed by NIAMD, NIH. We are grateful to Dr. G. Niswender, Dr. L. E. Reichert, and Dr. Albert Parlow for their RIA preparations and procedures.

References and Notes

(1) Peptide Hormones. 95.
(2) (a) J. Humphries, Y. P. Wan, G. Fisher, K. Folkers, and C. Y. Bowers, *Biochem. Biophys. Res. Commun.*, **57**, 675 (1974); (b) J. Humphries, G. Fisher, Y. P. Wan, K. Folkers, and C. Y. Bowers, *J. Med. Chem.*, **17**, 569 (1974).
(3) W. Vale, G. Grant, J. Rivier, M. Monahan, M. Amoss, R. Blackwell, R. Burgus, and R. Guillemin, *Science*, **176**, 933 (1972).
(4) Y. P. Wan, J. Humphries, G. Fisher, K. Folkers, and C. Y. Bowers, *J. Med. Chem.*, **19**, 199 (1976).
(5) C. Y. Bowers, Y. P. Wan, J. Humphries, and K. Folkers, *Biochem. Biophys. Res. Commun.*, **61**, 698 (1974).
(6) M. W. Monahan, M. S. Amoss, H. A. Anderson, and W. Vale, *Biochemistry*, **12**, 4216 (1973).
(7) M. Fujino, S. Kobayashi, M. Obayashi, S. Shinagawa, T. Fukuda, C. Kitada, R. Nakayama, J. Yamazaki, W. F. White, and R. H. Rippel, *Biochem. Biophys. Res. Commun.*, **49**, 863 (1972).
(8) R. W. A. Rees, T. J. Foell, S. Y. Chai, and N. Grant, *J. Med. Chem.*, **17**, 1016 (1974).
(9) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. T. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(10) L. Lenard and A. B. Robinson, *J. Am. Chem. Soc.*, **89**, 3045 (1967).
(11) J. M. Stewart and J. D. Young, "Solide Phase Peptide Synthesis", W. H. Freeman, San Francisco, Calif., 1969.
(12) G. D. Niswender, A. R. Midgeley, Jr., S. E. Monroe, and L. E. Reichert, *Proc. Soc. Exp. Biol. Med.*, **128**, 807 (1968).