ANALOGS OF LUTEINIZING HORMONE RELEASING FACTOR (LRF) SYNTHESIS AND BIOLOGICAL ACTIVITY OF [(N^{α}-Me)Leu^7]LRF and [D-Ala^6,(N^{α}-Me)Leu^7]LRF

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SUMMARY: [(N^{α}-Me)Leu^7]LRF, (pGlu-His-Trp-Ser-Tyr-Gly-(N^{α}-Me)Leu-Arg-Pro-Gly-NH_2), and [D-Ala^6,(N^{α}-Me)Leu^7]LRF, (pGlu-His-Trp-Ser-Tyr-D-Ala-(N^{α}-Me)Leu-Arg-Pro-Gly-NH_2), were synthesized by solid-phase methodology. The peptides were assayed by the in vitro system against LRF and found to have ca. 102% and 560% the potency of LRF respectively. The biological results are interpreted in terms of the conformational aspects of LRF.

We recently reported the synthesis of [D-Ala^6]LRF (Monahan et al.,1973), a highly potent analog of luteinizing hormone releasing factor, LRF (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). Subsequent to our publication other laboratories have confirmed our observation that substitution of a D-amino acid in the sixth position of LRF greatly enhanced its biological potency (Coy et al., 1974; Vilchez-Martinez et al., 1974; Fujino et al., 1974a; Fujino et al., 1974b). In our paper (Monahan et al., 1973) we had attributed the increased activity of [D-Ala^6]LRF to a restriction in the conformational degrees of freedom. More specifically, a β-II bend involving the sequence -Ser-Tyr-D-Ala-Leu- with a hydrogen bond between the carbonyl oxygen of serine and the α-amino hydrogen of leucine was suggested as the mechanism for stabilizing the molecule in a preferred conformation. It was then speculated that the pituitary cell receptors probably preferred LRF in a specific conformational state incorporating the β-II type bend.

We have undertaken the testing of this hypothesis by synthesizing [(N^{α}-Me)Leu^7]LRF and [D-Ala^6,(N^{α}-Me)Leu^7]LRF and determining their biological activities. Replacement of Leucine by N^{α}-methyl-leucine at the seventh position of LRF would prevent the formation of the proposed hydrogen bond (Monahan et al., 1973) and thus disrupt the stabilized β-II type bend.

MATERIALS AND METHODS

Synthesis and characterization. The two peptides were synthesized by solid-phase methodology (Rivier et al., 1973) on a benzhydrylamine resin (6g,

0.052 mmole glycine per gram resin). Both L and D amino acid derivatives used in the synthesis were purchased from Bachem Inc. The t-BOC-(N^{α}-Me)-L-Leu was prepared from t-BOC-L-Leu with MeI and NaH according to the method of Benoiton and Coggins (1972). Starting with 25 g of t-BOC-L-Leu, 18.8g (76% yield) of crystalline (from benzene) t-BOC-(N^{α}-Me)-L-Leu was obtained, m.p. 57-58°, $[\alpha]_D^{23}$ -25.3° in DMF (c = 0.010 g/ml). Proton magnetic resonance spectrometry showed the expected N-methyl singlet at 2.76 ppm and no N-H proton. Anal. Calculated mass for $C_{12}H_{23}NO_4$: 245. Found (M^+): 245. The methylated compound contained less than 1% D-isomer as determined by the method of Coggins and Benoiton (1970).

The assembled peptide was cleaved from the resin with HF containing 1.5 ml anisole per gram resin. After washing the cleavage product with Et_2O, the free peptide was extracted with 1N HOAc. The residue obtained from lyophilization of the acetic acid extract was purified in the following manner: First it was passed through an ion-exchange column (2.1 X 18 cm) of Whatman microgranular CM-32 carboxymethylcellulose which had been equilibrated with 0.01M NH_4OAc buffered at pH 7. A concentration gradient was used to elute the product by introducing 0.1M NH_4OAc (pH 7) into a 500 ml mixing flask containing 400 ml of the 0.01 M NH_4OAc (pH 7). The peptides in the effluent fractions were evaluated by tlc on Eastman No. 6060 silica gel sheets in a solvent system consisting of the upper phase of 1-BuOH:HOAc:H_2O (4:1:5) and visualized by Pauly reagent. The fractions containing the major compound were pooled and lyophilized twice. Partition chromatography on a (3 X 100 cm) G-25F Sephadex column was further used to purify the product in a solvent system of 2-BuOH:0.1N HOAc (1:1). The fractions were evaluated by tlc using the upper phase of the 2-BuOH:0.1N HOAc (1:1) system.

[(N^{α}-Me)Leu^7]LRF was obtained in 35% yield (142 mg, based on the coupling yield of the first amino acid) $[\alpha]_D^{23}$ -60.3° in 1% HOAc (c = 0.010 g/ml). It appeared homogeneous in six tlc systems (see Table 1). Amino acid analysis (Rivier et al., 1973) gave: Glu 1.00, His 1.01, Trp 0.95, Ser 0.80, Tyr 1.00, Gly 2.01, Arg 0.91, Pro 0.91, NH_3 1.09. The sequence of the peptide was confirmed by mass spectrometry (Ling et al., 1973) utilizing CD_3I instead of CH_3I for the permethylation.

[$D\text{-}Ala^6$,(N^{α}-Me)Leu^7]LRF after purification gave 193 mg (47% yield), $[\alpha]_D^{23}$ -55.9° in 1% HOAc (c = 0.010 g/ml). On tlc it appeared homogeneous in six solvent systems (see Table 1). Amino acid analysis gave: Glu 1.00, His 1.07, Trp 0.92, Ser 0.82, Tyr 1.01, Ala 0.99, Arg 0.92, Pro 0.96, Gly 1.02, NH_3 1.15. Its sequence was also confirmed by mass spectrometry (Ling et al., (1973).

TABLE I. Rf Values of LRF Analogs*

Analog	Solvent System**					
	(I)	(II)	(III)	(IV)	(V)	(VI)
[(N^{α}-Me)Leu^{7}]LRF	0.55	0.13	0.38	0.33	0.28	0.63
[D-Ala^{6},(N^{α}-Me)Leu^{7}] LRF	0.60	0.18	0.39	0.39	0.32	0.70

* Rf values were determined on Eastman No. 6060 silica gel sheets. The spots were detected by UV light and Pauly reagent.

** Solvent Systems: (I) 1-BuOH:pyridine:0.1% HOAc (5:3:11); (II) EtOAc:pyridine:HOAc:H_2O (10:5:1:6); (III) 1-BuOH:2-PrOH:1N NH_4OH: EtOAc (1:1:2.5:1); (IV) 1-BuOH:HOAc:H_2O (4:1:5); (V) 2-BuOH:0.1N HOAc (1:1); (VI) 1-BuOH:EtOAc:HOAc:H_2O (1:1:1:1). For the two phases solvent systems, only the upper phase was used.

TABLE II. EFFECT of LRF or LRF ANALOGS on SECRETION of LH BY PRIMARY CULTURES OF RAT PITUITARY CELLS.

	ADDITIONS	ng LH SECRETED per DISH-HOUR	± SEM*	P**
	SALINE	267	± 45	
Exp.A	0.3 nM LRF	908	± 35	<.01
	1.0 nM LRF	1294	± 59	<.01
	3.0 nM LRF	1614	± 80	<.01
	0.3 nM [(N^{α}-Me)Leu^{7}]LRF	806	± 66	<.01
	1.0 nM [(N^{α}-Me)Leu^{7}]LRF	1167	± 117	<.01
	3.0 nM [(N^{α}-Me)Leu^{7}]LRF	1596	± 99	<.01
	SALINE	150	± 13	
Exp.B	0.3 nM LRF	198	± 11	>.05
	1.0 nM LRF	336	± 24	<.01
	3.0 nM LRF	663	± 28	<.01
	10.0 nM LRF	795	± 31	<.01
	0.03 nM [D-Ala^{6},(N^{α}-Me)Leu^{7}]LRF	167	± 27	>.05
	0.1 nM [D-Ala^{6},(N^{α}-Me)Leu^{7}LRF	314	± 19	<.01
	0.3 nM [D-Ala^{6},(N^{α}-Me)Leu^{7}LRF	510	± 31	<.01
	1.0 nM [D-Ala^{6},(N^{α}-Me)Leu^{7}LRF	710	± 15	<.01

* SEM: Standard error of the mean.
** P: Probability of statistical difference from control (saline treatment).

Biological testing. The biological assay (Vale et al., 1972; Vale and Grant, 1974) is based on the ability of LRF and its analogs to stimulate the secretion of radioimmunoassayable LH by primary cultures of enzymatically dissociated rat anterior pituitary cells. This test is highly quantitative

as well as specific and has been used in the routine testing of over 100 LRF analogs (Vale and Rivier, 1974). In each assay, the response to three or more concentrations of an LRF analog is compared to the response of three or more concentrations of LRF. Relative potencies are calculated using the program HUBA (Harvard University Biological Assay).

A typical assay result is shown in Table II. In experiment A the dose response of [(N^{α}-Me)Leu^7] is compared with LRF while in experiment B [D-Ala^6, (N^{α}-Me)Leu^7]LRF is compared with LRF. Several independent assays were performed on each analog. The average relative potencies of the two analogs are presented in Table III.

BIOLOGICAL RESULTS AND DISCUSSION

The analog [(N^{α}-Me)Leu^7]LRF exhibited an average potency of 102% relative to that of LRF while [D-Ala^6,(N^{α}-Me)Leu^7]LRF had an average of 560% of LRF's potency. Highest concentrations of LRF and of the two analogs resulted in the same LH secretion rate indicating that both analogs possess full intrinsic activity (Vale and Rivier, 1974) and the dose response function of each analog is parallel to that of LRF suggesting that the differences in biological activities are related to differences in affinity for the LRF receptor. The biological activity of these two peptides cannot be explained by traces of LRF and [D-Ala^6]LRF present in these two samples respectively since, based on the amino acid analysis, each analog contained less than 1 nmole leucine per 100 nmole peptide.

One effect of substituting (N^{α}-Me)Leu for Leu in these two LRF analogs is to eliminate the possibility of the hydrogen bond between the N-H of Leu^7 and the C=O of Ser^4 in LRF. In the absence of this hydrogen bond, the stabilized β-II type bend as described by Venkatachalam (1968) is unlikely to exist in LRF. Yet the analog [(N^{α}-Me)Leu^7]LRF possesses identical potency as LRF which would suggest that a β-II type bend in LRF involving the sequence -Ser-Tyr-Gly-Leu- is not required for full biological activity.

TABLE III. RELATIVE POTENCIES OF LRF ANALOGS

ANALOG	N*	AVERAGE RELATIVE POTENCY (LRF = 100%)	±SEM**
[(N^{α}-Me)Leu^7]LRF	4	102% ± 17	
[D-Ala^6,(N^{α}-Me)Leu^7]LRF	5	560% ± 110	

* N: Number of independent bioassays contributing to average potencies.
** SEM: Standard error of the mean.

The potency of [D-Ala^6,(N$^\alpha$-Me)Leu^7]LRF (560%) is in the same range as that observed for [D-Ala^6]LRF (450%, Monahan et al., 1973), using the same assay method. These results suggest that the enhanced potency of [D-Ala^6]LRF, while probably related to the influence of a D amino acid at the sixth position on the conformation of the molecule, is not dependent upon the formation of the stabilized β-II type bend incorporating the Ser^4....Leu^7 (C=O...H-N) hydrogen bond. However, the possibility remains that conformers of both [D-Ala^6]LRF and [D-Ala^6,(N$^\alpha$-Me)Leu^7]LRF exist with folds (Grant and Vale, 1972) similar to the β-II type bend but without intramolecular stabilization by a hydrogen bond involving the N$^\alpha$-hydrogen of leucine in the seventh position.

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