[GLY^2]LRF AND DES-HIS^2-LRF. THE SYNTHESIS, PURIFICATION, AND CHARACTERIZATION OF TWO LRF ANALOGUES ANTAGONISTIC TO LRF.

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SUMMARY: Two peptide analogues of LRF (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), [Gly^2]LRF and des-His^2-LRF were synthesized by solid phase techniques on a benzhydrylamine resin. The peptides, purified by ion-exchange chromatography (CMC-32) and by partition chromatography, were characterized by amino acid analysis, u. v. analysis, tlc in seven systems, and optical rotation. As agonists [Gly^2]LRF has low specific biological activity and des-His^2-LRF has no detectable activity at the same doses. Both peptides inhibit LRF-induced release of LH *in vitro*.

The primary structure of porcine (1, 2) and ovine (3, 4) hypothalamic luteinizing hormone releasing factor (LRF) has been shown to be pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I). We published the solid phase synthesis (on a benzhydrylamine resin) and purification of LRF and established that the purified LRF obtained has the biological activity of natural LRF and is active in rats at nanogram levels (5). Several publications have now appeared describing synthesis of this decapeptide by exclusively solid phase techniques (6, 7, 8), partial solid phase methods (8, 9), and by fragment condensation (10).

In order to evaluate the relative importance of the functional groups in LRF, we have synthesized its glycine substituted analogues and a series of shorter analogues. One of these analogues, [Gly^2]LRF (II) which has a low specific biological activity, was discovered to exhibit antagonism towards LRF in its release of LH *in vitro* in dispersed rat pituitary cells (11). This observation led us to investigate other LRF analogues substituted in the 2-position. One of these analogues, des-His^2-LRF (III), has

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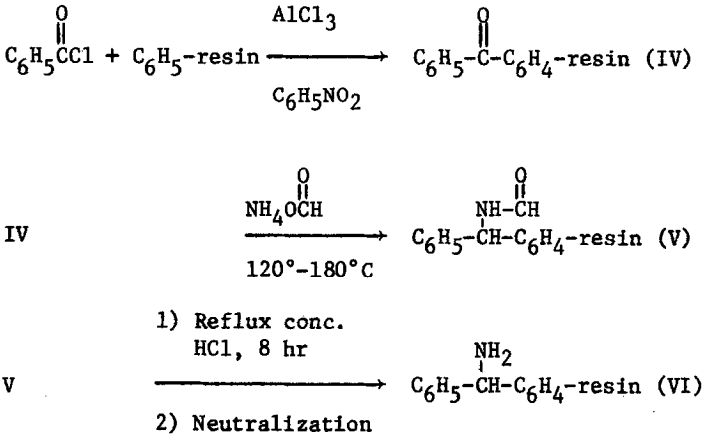
no statistically significant biological activity as an agonist at the doses tested, and also is an antagonist of LRF (11).

The syntheses of [Gly^2]LRF and des-His^2-LRF were carried out on a benzhydrylamine resin (12) prepared from BIO-RAD SX-1 resin beads by the following sequence:



The C-terminal residue was coupled to VI via dicyclohexylcarbodiimide (DCC) by standard solid phase techniques as were succeeding residues (13). The L-amino acids were coupled as the N^{α}-t-butoxycarbonyl(Boc) protected derivatives. Side chain protecting groups were necessary for Arg(N^{G}-nitro), Tyr(OBenzyl) and Ser(OBenzyl). ⌐Glu was coupled as carbobenzoxy-⌐Glu. These amino acid derivatives, purchased from either Bachem or Fox Chemical Company, were evaluated for purity by thin-layer chromatography, melting point, mass spectral analysis and nmr analysis, and further purified when necessary.

Deprotection of the N^{α}-Boc group was achieved with TFA(47.5):CH_2Cl_2 (47.5):1,2-dithioethane(5.0) followed by neutralization with 12.5% triethylamine in DMF. The resulting protected [Gly^2]LRF decapeptide-resin (prepared by M. M.) was hydrolyzed for 2 hours at 130° in concentrated HCl(1.0)-propionic acid(1.0) (14). The hydrolysate was evaporated and subjected to amino acid analysis on a Beckman model 119 amino acid analyzer. The result of this analysis is: Trp(-), NH_3(3.9), Arg(.9),

Ser(.8), Glu(.8), Pro(1.0), Gly(3.9), Leu(1.0), Tyr(1.1).

The protected decapeptide-resin (0.5 g) was treated with 0.5 ml anisole and ≈ 5 ml anhydrous HF for 1 hr at 25°C. After evaporation of the HF in vacuo at 25°C, the deprotected cleaved peptide was dissolved in glacial acetic acid and separated from the resin by filtration. Evaporation of the filtrate by rotary evaporation (35°C) left an oily residue which was washed several times with benzene, dried, taken up in 2 ml 0.2N HOAc; a small amount of insoluble material was removed with a 25 mμ millipore filter. Evaporation of the solvent gave 182 mg of an oily residue.

After this material was dissolved in ≈ 10 ml water, the pH was adjusted to 4.5 with ammonia and the solution was applied to a cation exchange column (Whatman CMC-32) of 34 ml bed volume (1.8 cm dia. X 13.5 cm long), equilibrated to pH 4.5. The peptide was eluted with a 0-0.4M, pH 7.2, linear aqueous ammonium acetate gradient (reservoir and mixing vessels 300 ml each). Analysis of the effluent from the column by measurement of u.v. absorbance at 280 nm revealed one major peptide zone eluting at 0.12-0.15M buffer concentration. The fractions in this zone were combined and lyophilized to yield 60 mg of white powder, which, after hydrolysis (6N HCl containing .5% thioglycolic acid, 20 hr, 110°C, sealed at 10 μ Hg pressure), gave the following amino acid analysis: Trp(.6), NH_3(1.5), Arg(1.1), Ser(.9), Glu(.8), Pro(1.0), Gly(2.8), Leu(1.0), Tyr(.9), 60% peptide by weight.

This material was applied to a BAW(n-Butanol (4.0):Acetic acid (1.0): H_2O (5.0)) partition column prepared on a bed of Sephadex G-25 fine (.9 cm dia. X 190 cm long) and eluted with upper phase (15). The fractions were analyzed in the same way as above with the product (II) eluting in the region 77-92 ml. The combined fractions in this zone were collected, and then lyophilized to yield 16.1 mg of II with the amino acid analysis shown: Trp(.7), NH_3(1.1), Arg(1.0), Ser(.9), Glu(.9), Pro(.9), Gly(3.0), Leu(1.0), Tyr(1.0), 96% peptide mono-acetate by weight.

Des-His^2-LRF was synthesized and purified (by J. R.) in essentially the same way as [Gly^2]LRF except that a step-gradient was used at the CMC-32 stage. A CMC-32 column of 44 ml bed volume (2.5 cm dia. X 9 cm long), equilibrated as above, was prepared. The product was applied to the column, washed with 50 ml water, and then with .075M ammonium acetate, with the product eluting between 180-240 ml of the ammonium acetate buffer. The correct product fractions were located by spot test (Pauly), spectrophotometric analysis, and nmr analysis of the fractions. The desired peptide fractions were combined, lyophilized, and applied to a BAW partition column (1.6 cm dia. X 95 cm long) and eluted with upper phase. Analysis of the fractions revealed one major peptide zone eluting at 125 ml-150 ml. Removal of the solvent by rotary evaporation, and subsequent lyophilization yielded 95 mg of III (from 1.1 g of peptide resin), a white powder having

Table I

tlc System*	R_f Value		
	des-His^2-LRF	[Gly^2]LRF	LRF†
I	.41-.44	.37-.41	.39-.43
II	.82-.87	.86-.93	.77-.83
III	.54-.60	.48-.54	.50-.56
IV	.74-.80	.70-.74	.64-.69
V	.76-.80	.70-.81	.78-.82
VI	.43-.46	.39-.45	.28-.36
VII	.53-.59	.53-.60	.49-.56

*I, n-BuOH(1), i-PrOH(1), 1N NH_3(2.5), EtOAc(1). II, i-PrOH(2), 1N AcOH(1). III, 0.1% AcOH(11), n-BuOH(5), Pyr(3). IV, EtOAc(5), Pyr(5), AcOH(1), H_2O(3). V, i-PrOH(2), 1N NH_3(1). VI, n-BuOH(4), AcOH(1), H_2O(5). VII, i-AmylOH(7), Pyr(7), H_2O(6).

Peptide solutions were spotted (20 µg) on Eastman #6061 Silica Gel Plates, developed at 25°C (minimum 15 cm run) in sandwich tanks, and were examined under ultraviolet light, and then exposed successively to iodine vapor, ninhydrin and Pauly reagents (no ninhydrin positive material was observed).

†LRF prepared as in reference 5.

the amino acid content: Trp(.8), NH_3(1.1), Arg(.9), Ser(.9), Glu(1.0), Pro(1.0), Gly(2.0), Leu(1.0), Tyr(1.0), 93% peptide mono-acetate by weight.

Characterization. II and III were submitted to tlc analysis on seven systems (Table I), and were homogeneous (one spot, fluorescent under u.v. light, I_2 and Pauly positive, and ninhydrin negative) on each system. Optical rotations were measured on a Perkin-Elmer model 141 polarimeter: II, $\alpha]_D^{25} = -45°$ (c = 0.703, 1% HOAc); III, $\alpha]_D^{25} = -47°$ (c = 0.951, 1% HOAc). Ultraviolet spectra were obtained with a Beckman Acta III recording spectrophotometer in 0.1N NaOH. Broad maxima are observed for both compounds at 244 nm and 280 nm, with a shoulder at 288 nm. Absorption coefficients at these wavelengths and at 294 nm are shown in Table II.

Table II

Compound*	E_{244}	E_{280}	E_{288}	E_{294}
[Gly^2]LRF	12200	5740	5610	4100
des-His^2-LRF	10465	5310	5177	3706

*u.v. absorbances measured in 0.1N NaOH.

Discussion. [Gly^2]LRF was bioassayed in vitro and was found to have a low level of activity compared to the parent peptide, LRF. Des-His^2-LRF has no statistically significant biological activity in the same system at the doses tested. These results, together with the bioassay for antagonism towards LRF are described in a separate publication (11).

Many reasons can be postulated to explain the low specific activity of [Gly^2]LRF, the lack of activity of des-His^2-LRF, and the ability of either to negate the action of LRF. All or part of these effects could be due to conformational requirements involving the histidine in the receptor-LRF complex. At the same time, histidine may be required as a nucleophilic or electrophilic catalyst (in a rate process involving release of LH by the receptor-peptide complex). Also, the elimination of histidine may reduce

or eliminate the effect of a thermodynamic parameter (e.g. bonded or non-bonded electrostatic interactions due to the acid-base or aromatic character of the imidazole function in histidine). Any one, or all of these factors might exert their effects by altering binding in the pituitary cell, effectiveness at the active site, or access to the active site (transport phenomena).

[Gly^2]LRF and des-His^2-LRF are the first examples of synthetic peptide analogues exhibiting antagonism toward LRF; knowledge of the importance of the His^2-position in the action of LRF provides a basis for the preparation of other LRF analogues.

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