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Synthesis and Biological Properties of the 2-L-β-(Pyrazolyl-1)alanine Analogs of Luteinizing Hormone-Releasing Hormone and Thyrotropin-Releasing Hormone

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The luteinizing hormone-releasing hormone (LH-RH) analog, <Glu-Pyr(1)Ala-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, and the thyrotropin-releasing hormone (TRH) analog, <Glu-Pyr(1)Ala-Pro-NH_2, were synthesized by azide couplings of the dipeptide hydrazide, <Glu-Pyr(1)Ala-$NHNH_2$, to the C-terminal octapeptide of LH-RH and to proline amide, respectively. In an ovariectomized, steroid-blocked rat assay, the LH-RH analog was found to have only 1% of the LH-releasing activity of the natural hormone. The TRH analog was 1.5 times more effective than TRH itself in releasing TSH in vivo from the anterior pituitary of mice. This peptide is one of two synthetic peptides so far discovered which are more potent than TRH.

β-(Pyrazolyl-1)alanine (A) and β-(pyrazolyl-3)alanine (B) are amino acids possessing a pyrazole ring system which is isomeric with, but in both cases considerably less basic[1] than, the imidazole nucleus of histidine (C). The β-(pyrazolyl-3)alanine analogs of a number of biologically active peptides,[2] including LH-RH[2] and TRH,[3,4] have been synthesized in order to investigate the importance of the histidine residues in maintaining activity. β-(Pyrazolyl-1)alanine has only been incorporated in the place of histidine in a RNase S-peptide analog[1] which was unable to produce an enzymatically active complex with the S-protein. We considered it worthwhile, therefore, to continue our studies on the function of histidine in LH-RH by preparing and assaying [L-β-(pyrazolyl-1)alanine^2]-LH-RH and, at the same time, synthesizing [L-β-(pyrazolyl-1)alanine^2]-TRH which, it appeared, could be made via a common starting material (see Figure 1).

Synthesis. Racemic β-(pyrazolyl-1)alanine was synthesized by the method of Reimlinger and Oth[5] and resolved into its L isomer by the method described by Sugimoto et al.[6] The L-amino acid methyl ester[1] was then used to prepare the dipeptide hydrazide, <Glu-Pyr(1)Ala-$NHNH_2$ (IV), which was coupled by the azide method to proline amide and to the C-terminal octapeptide (I)[2,7] of LH-RH to produce the tripeptide V and decapeptide VI, respectively.

The LH-RH analog was purified by partition chromatography on Sephadex G-25, followed by ion-exchange chromatography on CM-cellulose, and the TRH analog by chromatography on silica gel and on CM-cellulose.

Biological Results and Discussion. [Pyr(1)Ala^2]-LH-RH was assayed (Table I) for LH-releasing properties at doses of 50 and 250 ng in ovariectomized rats pretreated with estrogen and progesterone, followed by radioimmunoassay[8] for serum LH. LH levels were compared with those obtained in control animals given saline and 1- and 5-ng amounts of LH-RH. The LH-RH analog was found to have only 1% of the activity of LH-RH itself, which is considerably lower than the 19% activity found previously[2] for the closely related [Pyr(3)Ala^2]-LH-RH. The pyrazole groups of Pyr(1)Ala and Pyr(3)Ala and the imidazole ring of histidine have pK values[1] of 2.2, 2.5, and 6.0, respectively. Thus, reduction in the basicity of the side chain of the position two amino acid of the LH-RH decapeptide appears to lead to a concomitant lowering of gonadotropin-releasing activity. The side group of the Pyr(1)Ala-peptide is substituted via the pyrazole NH moiety and it is possible that its 1% of LH-RH activity is derived only from the aromatic character of the pyrazole ring, since [Phe^2]-LH-RH[9] has virtually identical activity in this same assay system.

The TRH activity of [Pyr(1)Ala^2]-TRH was determined by the procedure of Redding and Schally,[10] based on its ability to release TSH from the pituitary gland of mice pretreated with ^{125}I. The elevation of endogenous TSH increases the rate of release of labeled thyroid hormone from the thyroid gland, measured by the increase in blood radioactivity over that of control groups, and compared (Table II) to the response elicited by TRH. [Pyr(1)Ala^2]-TRH was about 50% more active than TRH and thus about 30 times more active than [Pyr(3)Ala^2]-TRH[3,4] which has only 5% of the activity of the parent hormone. Only one other analog, [(N^{im}-Me-His)^2]-TRH,[11] has been found to be more potent than TRH. This peptide was about eight times more active than TRH and, like the Pyr(1)Ala peptide, contained a substitution on one of the nitrogen atoms of the aromatic ring system. In the same report,[11] it was demonstrated that a roughly linear relationship exists between decreasing basicity of the imidazole group in a varying environment in a number of analogs and increasing biological activity. This appears to be the reverse of the situation existing with LH-RH peptides and does not explain fully the surprisingly low activity of the less basic [Pyr(3)Ala^2]-TRH.

Experimental Section

Asymmetric amino acids were of the L configuration. Melting points were uncorrected. Microanalyses were carried out by PCR, Inc., Gainesville, Fla. Amino acid analyses were performed on a Beckman Model 119 amino acid analyzer equipped with a System AA computing integrator using the single-column method. Peptides were hydrolyzed (110°, 18 hr) in vacuo in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole.[12] Pyr(1)Ala was eluted in the same position as serine and, under the normal hydrolysis conditions using either methanesulfonic acid or 6 M HCl, was approximately 30% destroyed. The Pyr(1)Ala-LH-RH and TRH peptides were, therefore, hydrolyzed for 18, 36, and 72 hr and nanomoles of Pyr(1)Ala were calculated by extrapolation to zero time. R_f^1, R_f^2, R_f^3, R_f^4, R_f^5, and R_f^6 values refer to TLC on silica gel using the solvent systems $CHCl_3$–MeOH (25:1), 1-BuOH-

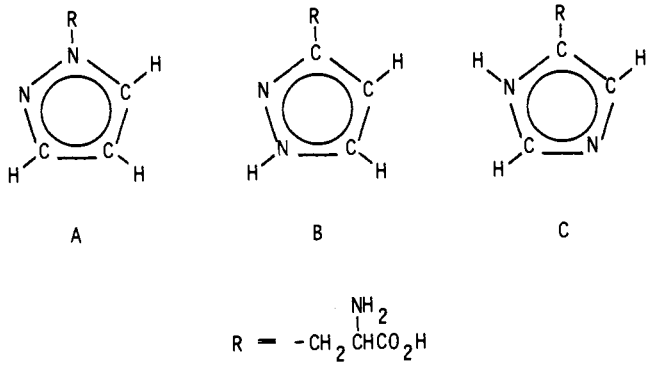

Figure 1. Structures of β-(pyrazolyl-1)alanine (A), β-(pyrazolyl-3)alanine (B), and histidine (C).

$AcOH-H_2O$ (4:1:5), 1-BuOH–AcOH–H_2O–EtOAc (1:1:1:1), 2-PrOH–1 *M* AcOH (2:1), EtOAc–pyridine–AcOH–H_2O (5:5:1:3), and 1-BuOH–AcOH–H_2O (4:1:1), respectively.

H-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I). The octapeptide was prepared by a solid-phase method which we have described previously.[2]

Carbobenzoxy-<Glu-Pyr(1)Ala-OMe (II). To a suspension of Pyr(1)Ala-OMe·2HCl[1] (1.1 g, 5.0 mmol) in $CHCl_3$ (20 ml) was added $CHCl_3$ (3 ml) saturated with NH_3 at 0°. The mixture was stirred for 30 min at 0°, NH_4Cl filtered off, and the solution evaporated in vacuo. The residue was dissolved in THF (20 ml) and Cbz-<Glu (2.3 g, 5.0 mmol) and DCC (1.3 g, 6.5 mmol) were added. The mixture was stirred at room temperature for 18 hr, the precipitated urea removed by filtration, and the solution evaporated in vacuo. The residue was dissolved in $CHCl_3$, washed with 5% $NaHCO_3$ and water, and dried over $MgSO_4$. After removal of the solvent, the residue was crystallized from EtOAc–hexane to give pure peptide II: 1.80 g; yield 86%; mp 145–146°; $[\alpha]^{23}D$ −29° (*c* 2.1, MeOH); R_f[1] 0.6. Anal. ($C_{20}H_{22}O_6N_4$) C, H, N.

<Glu-Pyr(1)Ala-OMe (III). A mixture of the Cbz dipeptide II (0.83 g, 2.0 mmol) in methanol (50 ml) containing 10% Pd on charcoal (300 mg) was hydrogenated (18 hr). After filtration and removal of the solvent, the residue was crystallized from EtOAc–hexane to give pure peptide III: 0.48 g; yield 79%; mp 141–142°; $[\alpha]^{23}D$ −24° (*c* 1.26, MeOH); R_f[1] 0.3. Anal. ($C_{12}H_{16}O_4N_4 \cdot 0.25CH_3CO_2C_2H_5$) C, H, N.

<Glu-Pyr(1)Ala-$NHNH_2$ (IV). A solution of III (0.28 g, 1.0 mmol) and hydrazine hydrate (0.1 ml) in MeOH (5 ml) was stirred at room temperature for 24 hr. The crystalline material which precipitated was filtered and washed with ether to give the hydrazide IV: 0.22 g; yield 89%; mp 237–242° dec; $[\alpha]^{23}D$ −29° (*c* 1.1, H_2O); R_f[2] 0.70. Anal. ($C_{11}H_{10}O_3H_6$) C, H, N.

<Glu-Pyr(1)Ala-Pro-NH_2 (V). Sodium nitrite (35 mg) in water (400 μl) was added slowly to a solution of IV (125 mg, 0.5 mmol) in 6 *M* HCl (350 μl) cooled to −15°. THF (5 ml), DMF (1 ml), $NaHCO_3$ (200 mg), and $MgSO_4$ (1.0 g) were then added at −20°. The organic phase was concentrated in vacuo at −10° and proline amide (57 mg, 0.5 mmol) in DMF (1 ml) was added at 0° and the mixture stirred for 24 hr. Solvents were removed in vacuo and the residue was fractionated on a column (1.5 × 50 cm) of silica gel in 10% $CHCl_3$–MeOH. The tripeptide V was eluted with 20% $CHCl_3$–MeOH but contained a small amount of proline amide and was repurified on a column (1.5 × 90 cm) of CM-cellulose by elution with an exponential gradient of ammonium acetate buffer (0.002 *M*, pH 4.6, to 0.1 *M*, pH 7.0) using buffer volumes and conditions described previously.[2] [Pyr(1)Ala²]-TRH (V) was eluted between 150 and 200 ml and, after lyophilization to constant weight, 100 mg of powder was obtained: $[\alpha]^{24}D$ −45° (*c* 0.63, 0.1 *M* AcOH); R_f[6] 0.45. Amino acid analysis gave Glu, 1.00; Pyr(1)Ala, 1.03; Pro, 1.00; NH_3, 1.04.

<Glu-Pyr(1)Ala-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (VI). <Glu-Pyr(1)Ala-$NHNH_2$ (IV, 20 mg, 0.08 mmol) was converted to the azide under the conditions described in the synthesis of V. Reaction with the octapeptide I (35 mg) in DMF (1 ml) containing triethylamine (6 μl) at 0° (24 hr), followed by removal of the solvents in vacuo, gave a residue which was subjected to partition chromatography on a column (1.5 × 95 cm) of Sephadex G-25 (fine) previously equilibrated with the lower phase, followed by the upper phase of the 1-BuOH–AcOH–H_2O (4:1:5) system. Elution with the upper phase displaced a major peak after 100–130 ml. These fractions were evaporated to dryness in vacuo and the residual oil was applied to a column (1.4 × 90 cm) of CM-cellulose, which was eluted with the gradient system described above. [Pyr(1)Ala²]-LH-RH emerged in fractions corresponding to 660–670 ml and after lyophilization to constant weight gave the homogeneous decapeptide VI: 36 mg; $[\alpha]^{25}D$ −22° (*c* 0.7, 0.1 *M* AcOH); R_f[2] 0.27, R_f[3] 0.56, R_f[4] 0.62, R_f[5] 0.68. Amino acid analysis gave Glu, 0.97; Pyr(1)Ala + Ser, 1.85; Trp, 0.98; Tyr, 1.00; Gly, 1.98; Leu, 1.00; Arg, 1.00; Pro, 0.99; NH_3, 1.28.

Table I. LH-Releasing Activity of [Pyr(1)Ala²]-LH-RH Compared with LH-RH in Ovariectomized, Steroid-Blocked Rats

Sample	Dose, ng/rat[a]	Mean plasma LH, ng/ml ± SE	Potency (%) with 95% limits
Saline		7.0 ± 1.9	
LH-RH[b]	1	11.1 ± 1.0	
	5	31.1 ± 4.5	
[Pyr(1)Ala²]-LH-RH	50	12.4 ± 1.1	1 (0.4–2.0)
	250	18.7 ± 2.0	

[a] Four per group. [b] Natural LH-RH (AVS 77-33, no. 215-269).

Table II. TRH Activity of [Pyr(1)Ala²]-TRH Compared with TRH in Mice

Sample	Dose, ng/mouse[a]	Δ, cpm (^{125}I)	Potency (%) with 95% limits
TRH	4	181 ± 22	
	16	1070 ± 78	
[Pyr(1)Ala²]-TRH	4	208 ± 50	153 (94–281)
	16	1802 ± 402	

[a] Five per group.

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