Polypeptides. XLVII. Effect of the Pyrazole–Imidazole Replacement on the Biological Activity of Thyrotropin-Releasing Hormone[1,2]

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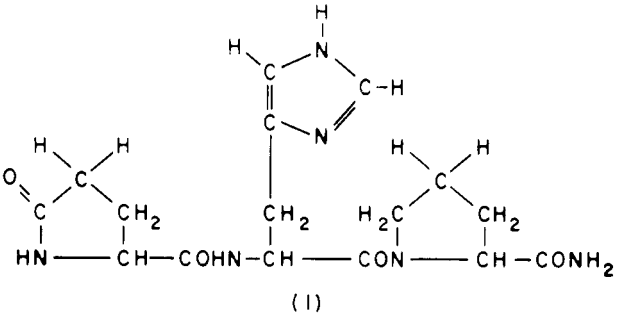
L-Pyroglutamyl-L-β-(pyrazolyl-3)-alanyl-L-prolinamide, an analog of thyrotropin-releasing hormone (TRH) in which the imidazole residue is replaced by L-β-(pyrazolyl-3)-alanine, has been synthesized and evaluated biologically. The analog exhibited the characteristic biological responses of natural or synthetic TRH; its activity was approximately 5% that of the parent hormone. These results suggest that the acid–base properties of the imidazole ring are not essential for thyrotropin-releasing activity.

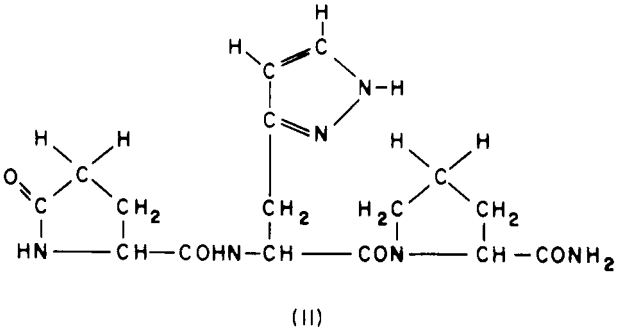
In a previous communication[3] it was suggested that the replacement of histidine by L-β-(pyrazolyl-3)-alanine [Pyr(3)Ala] may provide valuable information regarding the importance for function of the characteristic imidazole pK of histidine in biologically active peptides containing this residue. β-(Pyrazolyl-3)-alanine is isosteric with histidine but the acid–base behavior of the pyrazole ring differs markedly from that of imidazole. The validity of this concept was verified experimentally by the observation that β-(pyrazolyl-3)-alanine[12] S-peptide$_{1-14}$ was a powerful competitive inhibitor of RNase-S.[4,5] The analog combines with S-protein as firmly as does S-peptide but the pyrazole ring fails to function catalytically because of its inability to accept or donate protons under the conditions of assay.

In order to gain information regarding the functional importance of histidine for hormone activity the Pyr(3)Ala analogs of Val^5-angiotensin II[6–8] and of Gln^5-β-corticotropin$_{1-20}$amide[9,10] were prepared and tested for biological activity. Since both analogs possessed significant levels of biological activity it was concluded that the acid-base characteristics of the imidazole ring cannot be essential for the function of these hormones.

The recent disclosure of the covalent structure of a thyrotropin-releasing hormone TRH (TRF) (I)[11–14] provided another opportunity to test the importance of histidine dissociation for hormonal activity by the pyrazole–imidazole substitution approach. This communication describes a synthesis of L-pyroglutamyl-L-β-(pyrazolyl-3)-alanyl-L-prolinamide (II) [Pyr(3)Ala TRH] and relates observations pertaining to certain biological properties of this molecule.



(I)



(II)

Preparative Aspects.—The synthetic route to the pyrazole analog of TRH takes into account the well-known tendency of dipeptides containing proline to undergo cyclization with formation of diketopiperazines.[15] N^{α}-Benzyloxycarbonyl-L-β-(pyrazolyl-3)-alanine[16] was treated with *tert*-butoxycarbonylhydrazine in the presence of DCI to give an amorphous *tert*-butoxycarbonylhydrazide which was subjected to hydrogenolysis over Pd in MeOH. The ensuing crystalline L-β-(pyrazolyl-3)-alanine *tert*-butoxycarbonylhydrazide was then acylated with 2,4,5-trichloro-

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phenyl *N*-benzyloxycarbonyl-L-pyroglutamate[17] to give *N*-benzyloxycarbonyl-L-pyroglutamyl-L-β-(pyrazolyl-3)-alanine *tert*-butoxycarbonylhydrazide. The *tert*-butoxycarbonylhydrazide was converted into the hydrazide TFA salt by treatment with 90% TFA and the latter *via* the azide was coupled with L-prolinamide.[18] The reaction product was distributed between *n*-BuOH and 2% AcOH and crude *N*-benzyloxycarbonyl-L-pyroglutamyl-L-β-(pyrazolyl-3)-alanyl-L-prolinamide was isolated from the *n*-BuOH phases. This material was subjected to hydrogenolysis over Pd in MeOH–AcOH to give crude II which was purified on a Sephadex G 50 partition column with *n*-BuOH–AcOH–H_2O (4:1:5) lower phase as the stationary and the upper phase as the moving component. The process of separation was monitored by tlc using the Cl test for identification of products. Lyophilization from H_2O afforded the monhydrate of II in the form of a highly water-soluble, colorless, amorphous powder.

Experimental Section

A. General Procedures.—Melting points are uncorrected. Rotations were determined with a Zeiss precision polarimeter. Measurements were carried out with a Hg lamp at 546 and 578 mμ and extrapolated to the 589-mμ Na line. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.; the O value was actually determined and not computed by difference. The amino acid composition of acid hydrolysates was determined with a Beckmann-Spinco Model 120 amino acid analyzer.[19] Acid hydrolyses were performed in constant boiling HCl at 110° for 24 hr in evacuated tubes; values are not corrected for amino acid destruction. See Hofmann, *et al.*,[20] for designation of solvent systems for tlc.

B. Preparation of Peptides. L-β-(Pyrazolyl-3)-alanine *tert*-Butoxycarbonylhydrazide.—DCI (2.06 g) was added to an ice-cold soln of N^{α}-benzyloxycarbonyl-L-β-(pyrazolyl-3)-alanine[16] (2.89 g) and *tert*-butoxycarbonylhydrazine (1.32 g) in EtOAc (90 ml) and DMF (30 ml). The suspension was stirred for 5 days at room temperature, the DCU was removed by filtration, and the filtrate was evapd. The residue was dissolved in EtOAc and the soln was washed with ice-cold 1 *N* citric acid, satd aq $NaHCO_3$, and H_2O, and dried. Evaporation of the solvent gave an oily residue which was hydrogenated for 12 hr over Pd in 60 ml of MeOH. The filtrate from the catalyst was evaporated to give a syrup which soon deposited rosettes of prisms; these were washed with MeOH–Et_2O (1:10) and recrystd from the same solvent: yield 1.47 g (55%); mp 156–157°; $[\alpha]^{27}D$ +6.4° (*c* 1.15, MeOH). *Anal.* ($C_{11}H_{19}N_5O_3$).

***N*-Benzyloxycarbonyl-L-pyroglutamyl-L-β-(pyrazolyl-3)-alanine *tert*-Butoxycarbonylhydrazide.**—TEA (0.278 ml) was added to an ice-cold soln (20 ml) containing 2,4,5-trichlorophenyl *N*-benzyloxycarbonyl-L-pyroglutamate[17] (886 mg) and L-β-(pyrazolyl-3)-alanine *tert*-butoxycarbonylhydrazide (538 mg). The mixture was stirred at 0° for 30 min and at room temp for 20 hr. The solvent was then removed, the residue was dissolved in EtOAc, and the soln was washed in the usual manner. Evaporation afforded an oil which was triturated with petroleum ether. The ensuing precipitate was collected and purified by 2 pptn from a small vol of EtOH with Et_2O: yield 618 mg (60%); sinters at 120°; mp 140–142° dec; $[\alpha]^{27}D$ −49.3° (*c* 1.09, MeOH); R_f^{VI} 0.6; R_f^{VII} 0.8, single Cl and hydrazide-positive ninhydrin negative spot; amino acid ratios in acid hydrolysate, $Glu_{1.1}$Pyr(3)$Ala_{0.9}$; recovery 95%. *Anal.* ($C_{24}H_{30}N_6O_7$).

L-Pyroglutamyl-L-β-(pyrazolyl-3)-alanyl-L-prolinamide (II).—*N*-Benzyloxycarbonyl-L-pyroglutamyl-L-β-(pyrazolyl-3)-alanine *tert*-butoxycarbonylhydrazide (320 mg) was converted into the free hydrazide trifluoroacetate salt by treatment with 90% TFA in the usual manner; yield 310 mg. This hydrazide salt was dissolved in DMF (3 ml), and 6.91 *N* HCl in dioxane (0.42 ml) followed by *t*-BuONO (0.074 ml) was added with stirring at −25 to −30°. After 15 min at this temperature the hydrazide test was negative. The soln was cooled at −60° and TEA (0.488 ml) was added followed by a soln containing prolinamide·HCl[18] (88 mg), TEA (0.081 ml) in H_2O (0.2 ml), and DMF (2 ml). This mixture was stirred at 5° for 20 hr then the solvent was evapd. This material was distributed between *n*-BuOH and 2% AcOH, the *n*-BuOH phases were evaporated and the residue was triturated with Et_2O. The ensuing solid (308 mg) was hydrogenated over Pd in MeOH (3 ml) and 20% AcOH (1.5 ml) and the product was lyophilized from H_2O; yield 190 mg. This product (100 mg) was purified on a Sephadex G 50 partition column (0.5 × 140 cm) with *n*-BuOH–AcOH–H_2O (4:1:5) lower phase as the stationary and the upper phase as the mobile component. Fractions (3 ml each) were collected at a flow rate of 3 ml/hr and the progress of the separation was monitored by tlc using the Cl test as the indicator. Fractions containing the desired compd (R_f^I 0.4) were pooled, the solvent evapd, and the residue lyophilized from H_2O: colorless, fluffy, electrostatic, highly water-soluble powder; yield 80 mg; $[\alpha]^{22}D$ −62.3° (*c* 1.41, H_2O) R_f^{VI} 0.4; R_f^{VII} 0.5; amino acid ratios in acid hydrolysate, $Glu_{1.0}Pyr(3)Ala_{1.0}Pro_{0.9}$; recovery 99.4%. *Anal.* ($C_{16}H_{22}N_6O_4 \cdot H_2O$) calcd, C, 50.5; H, 6.4; N, 22.1; O, 21.03. Found, C, 51.0; H, 6.2; N, 21.8; O, 21.13.

Biological Results and Discussion

Linear and parallel dose–response curves (Table I) were obtained with 20, 60, and 90 mμg of Pyr(3)Ala TRH and 1,3, and 9 mμg of synthetic TRH in triiodothyronine T_3–TRH assay mice. Larger doses of the analog only slightly increase the response. In this assay, the ^{125}I blood levels are measured before and 2 hr after iv injection of TRH or the analog. The results, recorded as blood ^{125}I Δcpm, represent the difference in these values and are proportional to the amount of TSH released from the pituitary.[21]

Table I
Dose–Responses to TRH and Pyr(3)Ala TRH in T_3–TRH Assay Mice[a]

TRH, mμg/mouse				Pyr(3)Ala TRH, mμg/mouse						
Saline	1	3	9	10	20	60	180	540	1620	10,000
^{125}I, Δcpm				^{125}I, Δcpm						
118	591	2319	3961	125	403	2133	4274	4497	5956	5890

[a] Each result is mean obtained from 5 mice.

When the T_3–TRH assay mice were pretreated with 0.2 μg of T_3 2 hr before the injection of Pyr(3)Ala TRH or TRH, the responses were inhibited (Table II). Thus, as has been shown for TRH, the magnitude of the response of the Pyr(3)Ala TRH depends on the amount of T_3 present. Studies on the interaction of T_3 and TRH in regulation of TSH secretion have been described.[22,23]

Table II
Inhibition of TRH and Pyr(3)Ala TRH Responses by Triiodothyronine in T_3–TRH Assay Mice

	^{125}I Δcpm	^{125}I Δcpm[a]	*p* value
TRH, 9 mμg	4100	20	0.001
Pyr(3)Ala TRH, 60 mμg	2670	−100	0.001

[a] T_3 (0.2 μg) was administered sc 2 hr before iv TRH or analog; each result is mean obtained from 5 mice.

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To demonstrate that the site of action of Pyr(3)Ala TRH is the anterior pituitary gland the *in vitro* method was used.[24] In this assay corresponding halves of rat anterior pituitary glands are incubated in two beakers (control and experimental) containing Krebs–Ringer bicarbonate solution. The test substance is added to the experimental beaker and after a 1-hr incubation period the amount of TSH in the medium of the control and experimental beakers is measured in T_3–TSH assay mice. The results are recorded as the change in the blood level of ^{125}I. The observation (Table III), that more TSH is present in the experimental (E) than in the control (C) medium shows that Pyr(3)Ala TRH, like TRH, stimulates release of TSH into the medium.

TABLE III
COMPARISON OF TRH AND PYR(3)ALA TRH ACTIVITY IN THE *in Vitro* RAT ANTERIOR PITUITARY INCUBATION ASSAY[a]

	C ^{125}I Δcpm	E ^{125}I Δcpm	*p* value
TRH, 9 mμg	327	1506	0.001
Pyr(3)Ala TRH, 180 mμg	203	3887	0.001

[a] Values recorded represent amount of TSH present in medium without (C) and with added hormones (E). TSH measured in T_3–TSH assay mice; each result is the mean obtained from 5 mice.

Incubation of the analog in human serum for 30 min at 37° destroyed biological activity (Table IV). For comparison the analog was added to the serum and assayed without incubation. Parallel experiments were performed using synthetic TRH.

TABLE IV
INACTIVATION OF TRH AND PYR(3)ALA TRH BY INCUBATION IN HUMAN SERUM[a]

	C ^{125}I Δcpm	E ^{125}I Δcpm	*p* value
TRH, 9 mμg	3740	592	0.001
Pyr(3)Ala TRH, 180 mμg	3480	−53	0.001

[a] Each 0.2 ml of human serum obtained from a normal adult male contained TRH or analog as indicated. Serum without (C) or with (E) incubation (30 min at 37°) was assayed in T_3–TRH assay mice; each result is mean obtained from 5 mice.

The ability of Pyr(3)Ala TRH to inhibit the response of TRH in T_3–TRH assay mice was also investigated. Since previous studies indicated that the onset of the TRH response, TRH disappearance rate from plasma, and TRH inactivation by serum were rapid, the *in vivo* inhibitory effect of the analog was assessed by administering it at the same time or shortly before the TRH injection. When mice received subactive doses (10–15 mμg) of the analog in amounts 3–5 times the administered dose of TRH (3 mμg), there was no inhibiton of the TRH response (Table V, expt 1 and 2). Inhibitory studies were also performed (expt 3) using active doses of Pyr(3)Ala TRH. When TRH and Pyr(3)Ala TRH were both given to the same mouse, their effects were additive.

TABLE V
INHIBITION OF THE TRH RESPONSE IN T_3–TRH MICE BY PYR(3)ALA TRH[a]

Min −5	Min −2	Min 0	Expt 1 ^{125}I Δcpm	*p* value S *vs.* P	Expt 2 ^{125}I Δcpm	*p* value S *vs.* P	Expt 3 ^{125}I Δcpm	*p* value S *vs.* P
S		TRH	4015		1221			
P		TRH	3438	ns	1191	ns		
	S	TRH	2571		1000		2425	
	P	TRH	3048	ns	1230	ns	3637	0.01
		S + TRH			1643		1900	
		P + TRH			1334	ns	4100	0.001
		S	368		37		−184	

[a] Albumin (1%)–0.9% NaCl soln (S) or Pyr(3)Ala TRH (P) was injected iv before (at times recorded) or with synthetic TRH. All mice given TRH received 3 mμg while 10 and 15 mμg of analog were administered in expt 1 and 2, respectively. Mice in expt 3 received 3 mμg of TRH and 10 μg of Pyr(3)Ala TRH according to the above design. The difference in the magnitude of the TRH response is due to variations in the sensitivity of the mice; each result is mean obtained from 5 mice.

From the results presented in this communication it can be concluded that under the conditions of assay Pyr(3)Ala TRH stimulates the release of TSH from the pituitary of the mouse and that it possesses approximately 5% the biological activity of natural or synthetic TRH. The analog fails to compete with TRH and when it is administered in conjunction with the natural hormone exhibits an additive effect. Just as in the case of the natural hormone, the analog is inactivated when incubated with human serum and its effect is inhibited by T_3. Thus its spectrum of activities as far as it has been investigated parallels that of TRH, albeit at a lower level.

These experimental results eliminate the characteristic acid–base behavior of the imidazole ring of histidine as an essential feature for TSH release. The situation is similar to that which has been observed with Val^5-angiotensin II and Gln^5-β-corticotropin$_{1-20}$amide where the replacement of the histidine residue by Pyr(3)Ala resulted in analogs which retained biological activity but at a lower level.

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