In most instances, TRH stimulated only release of TSH. There were some notable exceptions in which the serum levels of 1 or 2 of the other hormones measured rose coincidently with the TSH rise. The latter changes were neither related to the dose of TRH nor the magnitude of the TSH response. Further studies will be necessary to elucidate the possible importance of the secretion of more than one pituitary hormone in response to TRH.

As has been found in animal studies,[5] whether or not T_3 inhibits the response to TRH will depend probably on its dose and time of administration as well as the dose of TRH. It will be of interest to determine what factor(s) may alter the sensitivity of this inhibitory effect of T_3.

TRH is indeed active when given orally to man[5,21] as was also found in mice.[5] The high sustained rise in the TSH level stimulated by oral administration of TRH indicates that this may be the method of choice in producing a prolonged effect. The relative absence in changes of the serum levels of the other hormones again shows the specificity of the TRH effect.

Acknowledgment.—Authors are very grateful for RIA preparations supplied by National Pharmacological Association and National Institutes of Health endocrine study section and the expert help of Louyse Lee and G. A. Reynolds. Supported by U. S. Public Health Service Grant AM-06164-08 and AM08943.

(21) J. M. Hershman and J. A. Pittman, Jr., *J. Clin. Endocrinol.*, **31**, 457 (1970).

Syntheses of Pyroglutamylhistidylprolinamide and Unusual Mass Fragmentation†

JAW-KANG CHANG, HANS SIEVERTSSON, CONNY BOGENTOFT, BRUCE CURRIE, KARL FOLKERS,*

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

AND G. DOYLE DAVES, JR.

Oregon Graduate Center, Portland, Oregon 97225

Received December 29, 1970

The thyrotropin-releasing hormone, which is pGlu-His-Pro-NH_2, has been synthesized by a new procedure. This synthesis has also served particularly as a general procedure for the synthesis of many analogs of TRH to study structure-activity relationships. TRH was also synthesized by a solid phase method which couples a dipeptide, pyroglutamylhistidine, to a proline-resin. Low- and high-resolution mass spectra show that the fragmentation pattern of TRH is more specific to the histidine characteristics of this tripeptide than it is to the behavior of peptides in general.

Two syntheses of the porcine thyrotropin-releasing hormone (TRH), which is pGlu-His-Pro-NH_2 (**1**), and citations to pertinent publications of other investigators on the synthesis of TRH are detailed in companion papers.[1,2] These several syntheses of pGlu-His-Pro-NH_2 were a part of, or stemmed from, the structural elucidation of both porcine and ovine TRH. The TRH of all mammalian species[3] which have been reported upon to date has the same structure (**1**).

We have now synthesized TRH by a new procedure, which has been effective not only in making available larger amounts of the pure hormone, but the key steps have very effectively served for synthesis of many structural analogs of TRH.[4] This synthesis (of general utility for TRH and related tripeptides) has the advantage of providing two intermediates, **5** and **6**, which are generally crystalline and provide intermediate purifications. These advantages became increasingly apparent as many of the analogs were being synthesized.[4] These peptides were desired to elucidate the structure–activity relationships of a tripeptide which shows such astounding hormonal activities at nanogram and picogram levels.

By this procedure, *N-tert*-butyloxycarbonyl-N^{im}-benzylhistidine (**2**)[5] was coupled with prolinamide (**3**)[6] by using *N,N'*-dicyclohexylcarbodiimide (DCI) to obtain in 92% yield the protected dipeptide, *N-tert*-butyloxycarbonyl-N^{im}-benzylhistidylprolinamide (**4**). Treatment of this dipeptide with glacial AcOH saturated with HBr to remove the *N-tert*-butyloxycarbonyl group gave a 72% yield of the crystalline N^{im}-benzylhistidylprolinamide·2HBr (**5**). This dihydrobromide was readily recrystallized for purification.

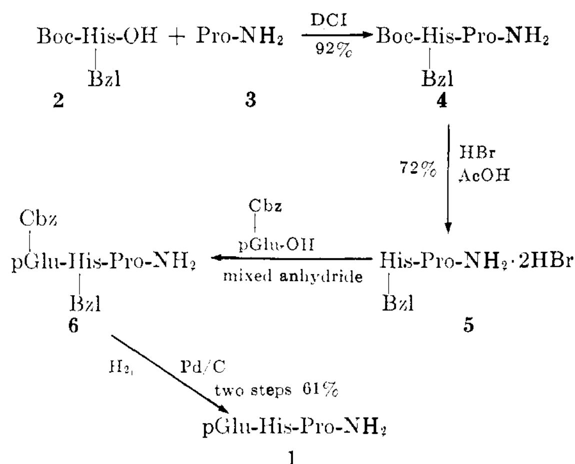
After drying the dipeptide·2HBr (**5**) *in vacuo* for 24 hr, it was coupled with *N*-carbobenzoxypyroglutamic acid[7] by the mixed anhydride method.[8] The resulting protected tripeptide, *N*-carbobenzoxypyroglutamyl-N^{im}-benzylhistidylprolinamide (**6**) was subjected to catalytic hydrogenolysis to remove the benzyl and carbobenzoxy groups to afford the pyroglutamylhistidylprolinamide (TRH) (**1**) in a yield of 61% over the last 2 steps. The chromatographic behavior, the spectroscopic properties, and the hormonal activities of TRH from this synthesis were identical with those corresponding characteristics of pGlu-His-Pro-NH_2 which have been described in the two companion papers.[1,2]

† Hypothalamic Hormones. 16.

(1) F. Enzmann, J. Bøler, K. Folkers, C. Y. Bowers, and A. V. Schally, *J. Med. Chem.*, **14**, 469 (1971).
(2) J. Bøler, J.-K. Chang, F. Enzmann, and K. Folkers, *ibid.*, **14**, 475 (1971).
(3) C. Y. Bowers, A. V. Schally, A. Weil, G. A. Reynolds, and K. Folkers, *Curr. Top. Thyroid Res., Proc. Int. Thyroid Conf. 6th*, **1970** (1970).
(4) Unpublished data.
(5) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).
(6) R. W. Chambers and F. H. Carpenter, *J. Amer. Chem. Soc.*, **77**, 1522 (1955).
(7) H. Gibian and E. Klieger, *Justus Liebigs Ann. Chem.*, **640**, 145 (1961).
(8) K. Hofmann, W. Haas, M. J. Smithers, R. D. Wells, Y. Wolmann, N. Yanaihara, and G. Zanetti, *J. Amer. Chem. Soc.*, **87**, 620 (1965).

The thyrotropin-releasing hormone was also synthesized by the solid phase method of Merrifield.[9] In this synthesis, we coupled a dipeptide to an amino acid resin instead of coupling the amino acids one by one in the usual manner. Thus, the dipeptide, pyroglutamylhistidine, was separately prepared[10] and coupled to a proline-resin.[11] The cleavage of the tripeptide was accomplished by ammonolysis[11] at room temp.

The pGlu-His-Pro-NH_2 (**1**) which was obtained by this procedure was found to have the same tlc mobilities in 4 different systems which had previously been recorded for samples of this tripeptide synthesized by the previous procedures.[1,2]



Unusual Fragmentation in Mass Spectrometry.—The behavior of pGlu-His-Pro-NH_2 upon electron impact is that of a small peptide and such low molecular weight peptides have not been thoroughly investigated in mass spectrometry. Many peptides upon electron impact cleave primarily at the peptide-NHCO bond,[12,13] which makes an amino acid sequence analysis possible. TRH is not primarily characterized by this behavior. The dominant feature of the spectrum consists of fragments derived from the histidine moiety of the molecule. Consequently, the mass spectral data on TRH have interest for peptides in general, and specific interest for the new field of hypothalamic hormones.

The lower limit of the size of a sample of TRH has generally been 10 μg of synthetic TRH. Spectra with a molecular ion could be readily reproduced, but it was necessary to have the temp of the probe in the range of 210–220°, even though this range caused some thermal degradation. The diketopiperazine ion, *m/e* 234 of His-Pro is evidence of the thermal reactions at such temperatures.

The principle mass spectral peaks of low-resolution spectra of synthetic pGlu-His-Pro-NH_2 are listed in Table I.

The ion *m/e* 70, which is the base peak, corresponds to structure **7**, which is derived from the proline moiety. Prominent fission occurred as indicated in **8**, which gives rise to the ions at *m/e* 84, *m/e* 278, *m/e* 221, and *m/e* 249.

(9) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963).

(10) J.-K. Chang, H. Sievertsson, B. L. Currie, K. Folkers, and C. Y. Bowers, *J. Med. Chem.*, **14**, 484 (1971).

(11) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman and Company, San Francisco, Calif., 1969.

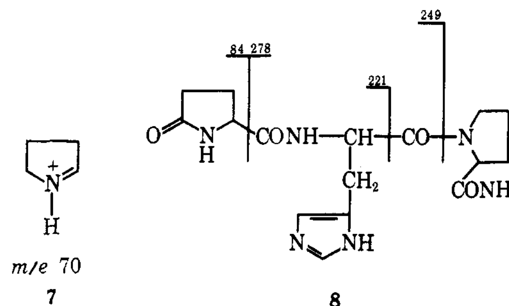
(12) M. M. Semyakin, *Pure Appl. Chem.*, **17**, 313 (1968).

(13) J. H. Jones, *Quart. Rev., Chem. Soc.*, **22**, 302 (1968).

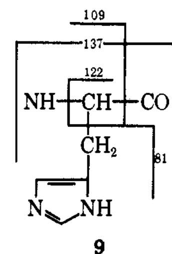
Table I

Principal Mass Spectral Peaks of pGlu-His-Pro-NH_2

m/e	Relative intensity	*m/e*	Relative intensity	*m/e*	Relative intensity
362	0.2	221	3.8	109	10.9
278	1.4	154	15.7	94	10.0
249	2.9	153	8.6	84	61.0
248	2.4	137	10.0	82	71.5
235	5.7	136	7.2	81	32.0
234	7.6	122	8.6	70	100.0
		110	11.4		



The charge appears to be preferentially localized on the imidazole ring as judged from the series of abundant peaks which correspond to ions formed by cleavage at different sites in the histidine moiety, according to *m/e* 81, *m/e* 82, *m/e* 94, *m/e* 109, *m/e* 122, *m/e* 136, and *m/e* 137. These ions are depicted in **9**. This pattern of peaks is also found in the fragmentation of several related tripeptides having the pGlu-His moiety.[14]



High-resolution spectra were also recorded and all the expected peaks, including the parent ion at *m/e* 362, were observed. The accurate masses of some of the important ions are listed in Table II.

Table II

High-Resolution Mass Spectra of pGlu-His-Pro-NH_2

m/e found	*m/e* calcd	Formula
362.170	362.168	$C_{16}H_{22}N_6O_4$
278.1253	278.1230	$C_{12}H_{16}N_5O_3$
234.1117	234.1222	$C_{11}N_{14}N_4O_2$
154.0742	154.0736	$C_7H_{10}N_2O_2$
153.0664	153.0670	$C_7H_9N_2O_2$
84.0449	84.0449	C_4H_6NO

Experimental Section

Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Microanalyses were performed by the Mikro analytisches Laboratorium, Bonn, West Germany, and all samples for microanalyses were dried at 60° for 24 hr at 0.5 mm. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values. On tlc with silica gel G, R_f^1, R_f^2, R_f^3, and R_f^4 values refer to the following

(14) Unpublished data.

systems: (1) n-BuOH-AcOH-EtOAc-H_2O (1:1:1:1); (2) $CHCl_3$-MeOH-30% NH_4OH (60:45:20); (3) EtOH-H_2O (7:3); (4) n-PrOH-30% NH_4OH (7:3). The location of the peptide spots was revealed either by the ninhydrin reagent, the Pauly reagent, or the Cl method.[15] The nmr spectra were measured at 60 Hz on a Varian Associates A-60 spectrometer (Me_4Si) and the chemical shifts are expressed in τ values. The optical rotations were obtained with a Perkin-Elmer Model 141 digital readout polarimeter using a microcell. The low-resolution mass spectra were obtained from a CEC 21-491 using a direct probe heated to 210-220°. The ionization energy was maintained at 70 eV and the temp of the ion source was kept at 260°. The high resolution spectra for all peaks except m/e 362 were recorded on photo plates using a CEC 21-110 mass spectrometer. Peak m/e 362 was measured by peak matching.

***N*-tert-Butyloxycarbonyl-N^{im}-benzylhistidylprolinamide (4).**—N-*tert*-Butyloxycarbonyl-N^{im}-benzylhistidine[5] (7.25 g) and prolinamide[6] (2.4 g), in dry MeCN (200 ml), were treated with DCI (5 g) in dry MeCN (20 ml) at 0°. After being stirred at room temp during 12 hr, the reaction mixt was filtered and the filtrate was evapd *in vacuo* to dryness. The residue was dissolved in $CHCl_3$ (250 ml) which was washed well with H_2O (2 × 50 ml). The org layer was dried ($MgSO_4$), filtered, and finally evapd to give the crude oily product (10.3 g). This was purified by column chromatography on silica gel with elution by $CHCl_3$-MeOH (9:1 v/v) to afford pure **4** (8.5 g yield 92%): $[\alpha]^{22}D$ −18.7° (c 1.10 $CHCl_3$); $R_f{}^1$ 0.64; single Cl-tolidine-positive spot. The nmr data on **4** were satisfactory.

N^{im}-Benzylhistidylprolinamide·2HBr (5).—To **4** (1 g) was added a satd soln of dry HBr in glacial HOAc (10 ml). After being stirred during 20 min at room temp the reaction mixt was treated with anhyd Et_2O (50 ml). The solvent was decanted and the ppt was recrystd twice from MeOH-Et_2O to afford pure **5** (0.82 g yield 72%): mp 196-198° dec; $[\alpha]^{22}D$ −26.8° (c 1.00, MeOH), $R_f{}^1$ 0.30 (free base) and $R_f{}^2$ 0.85 (free base); single ninhydrin- and Cl-tolidine-positive spot. *Anal.* ($C_{18}H_{23}N_5O_2$·2HBr) C, H, N, Br.

A portion of this salt (0.27 g) was dissolved in $CHCl_3$ (10 ml) and NH_3 was bubbled through the soln at 0°, during 15 min. The NH_4Br salt was filtered off and the filtrate was concd *in vacuo* to give the free base (0.15 g): τ_{CDCl_3} 2.70 ppm, s, 5 H, arom protons and 4.95 ppm, s, 2 H, benzylic CH_2 protons.

***N*-Carbobenzoxypyroglutamyl-N^{im}-benzylhistidylprolinamide (6).**—To N-carbobenzoxypyroglutamic acid[7] (1.1 g), in dry THF (30 ml) magnetically stirred at 0° were successively added Et_3N (0.7 ml), ethyl chloroformate (0.5 ml), and after 30 min N^{im}-benzylhistidylprolinamide·2HBr (**5**) (1.9 g) and Et_3N (1 ml) in dry THF (10 ml). After being stirred at room temp during 20 hr, the solvent was evapd *in vacuo* to dryness. The residue was triturated with H_2O (50 ml) and the ppt was collected (2.8 g), dried, and recrystd twice from $CHCl_3$-EtOAc-Et_2O to afford pure **6** (2 g, yield 90%): mp 149-152°; $[\alpha]^{22}D$ −25.3° (c 0.87, $CHCl_3$); $R_f{}^1$ 0.58; single Cl-tolidine-positive spot. *Anal.* ($C_{31}H_{34}N_6O_6$) H, N; C, calcd, 63.47, found, 62.86.

Pyroglutamylhistidylprolinamide (1).—Compd **6** (2 g) in abs MeOH (200 ml) was hydrogenated at room temp and 1 atm pressure in the presence of 5% Pd/C (5 g). After 24 hr, the reaction mixt was filtered and evapd to the tripeptide 1 (950 mg). This was purified by column chromatography on silica gel with elution by $CHCl_3$-MeOH (7:3, v/v) to afford pure 1 (840 mg, yield 68%): M^+ calcd, 362.1680; found, 362.1700; single Cl-tolidine-, and Pauly-positive spot. $[\alpha]^{22}D$ and chromatographic mobilities ($R_f{}^1$, $R_f{}^2$, $R_f{}^3$, and $R_f{}^4$) were completely identical with those of the compd derived from the previous series.[1,2]

Pyroglutamylhistidylprolinamide (TRH) by Solid Phase Method.—The Boc-proline resin (chloromethylated polystyrene 200-400 mesh capacity, 0.94 mequiv of Cl/g) was prepd in the usual manner[11] and the proline was detd to be 0.35 mmole/g of resin. In the deprotection and coupling steps the following scheme was used: (1) washing the proline-resin (0.30 g, equiv to 0.10 mmole of proline) with dioxane (3 × 10 ml); cleavage of the Boc-group with 4 N HCl in dioxane (15 ml) for 30 min; (3) washing with dioxane (3 × 10 ml); (4) washing with CH_2Cl_2 (3 × 10 ml); (5) neutralization of the hydrochloride with Et_3N (2.5 ml) in CH_2Cl_2 (10 ml) for 15 min; (6) washing with DMF (3 × 10 ml); (7) addition of pGlu-His[10] (1 mmole) in DMF (5 ml) and mixing for 10 min; (8) addition of DCI (1 mmole) in DMF (1 ml), followed by a reaction period of 4 hr; (9) washing with DMF (3 × 10 ml); (10) washing with EtOH (3 × 10 ml); each washing period was for 5 min. The wt of the resin after drying over KOH pellets *in vacuo* for 24 hr was 0.31 g.

The pGlu-His-Pro resin was then suspended in anhyd MeOH, satd with NH_3 at 0°, and stirred in a tightly stoppered flask for 48 hr. After filtration and removal of the solvent, a white ppt was left (20 mg), which in tlc showed 3 products. One major and one minor product was Pauly pos and the third product was identified as prolinamide.

Purification of the reaction mixt as previously described for 1 gave pyroglutamylhistidylprolinamide (1): 7.5 mg; 21% yield. It was identified by having the same tlc mobilities as authentic pGlu-His-Pro-NH_2 in 4 different solvent systems ($R_f{}^1$, $R_f{}^2$, $R_f{}^3$, and $R_f{}^4$). The product showed one spot by the Pauly and the Cl-tolidine reagents.

Acknowledgment.—Gratitude is expressed to Dr. David Isaksson of the Aktiebolaget/Kabi, Stockholm, Sweden, for his interest and support of a part of this research, and to The Robert A. Welch Foundation for additional support.

(15) E. Stahl, "Dünnschicht-Chromatographie," Springer-Verlag, Berlin, West Germany, 1962.