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Synthesis of the Thyrotropin-Releasing Hormone†

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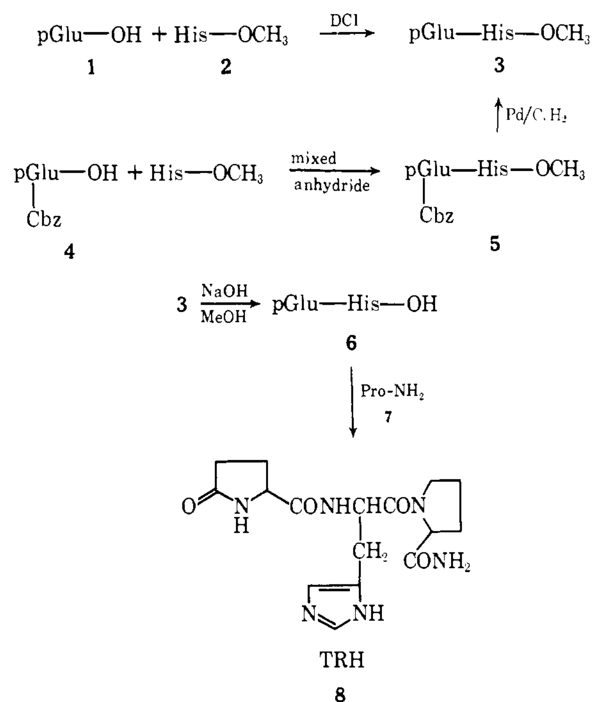
Two syntheses of the thyrotropin-releasing hormone (TRH), pGlu-His-Pro-NH_2, were an integral phase of the structural elucidation of this hormone. The first synthesis consisted in the conversion of Glu-His-Pro to TRH by reaction with HCl in MeOH and then NH_3. The second synthesis was based on combining pyroglutamic acid and histidine without the advantage of any N-protecting group followed by reaction of the dipeptide with Pro-NH_2 to give TRH. This second synthesis was subsequently modified by using *N*-carbobenzoxypyroglutamic acid in the form of a mixed anhydride to result in an improved total yield of TRH.

The porcine hypothalamic releasing hormone was isolated by Schally, *et al.*,[1] from a total of about 265,000 fragments. The several fractions of the hormone from the isolation which amounted to about 5–7 mg presumably ranged in purity from about 30 to 50%. Some techniques of classical structural elucidation were impossible for use on porcine TRH and other procedures were severely restricted because of the paucity of hormone, the impurities, and the cost and time involved. Nevertheless, useful spectral data on TRH were obtained; particularly, the probable relevance of 3 amino acids, which were in the sequence of Glu-His-Pro, to the structure of the hormone had been proposed by Schally, *et al.*[2] However, the peptide concept of TRH, as based on studies on the ovine hormone by Guillemin, *et al.*,[3] had been doubted in early efforts to gain clues on the chemical nature of the hormone. Also, the behavior of the porcine hormone in gel filtration led to consideration of a molecular weight which would be compatible with a yield of about 30% of the 3 amino acids, Glu, His, and Pro. On this basis, up to 70% of the structure of the hormone remained unaccounted and this concept was reasonable if the isolated porcine hormone were 90% or higher in purity and had the appropriate molecular weight. Preference was given to the interpretation that the porcine hormone was closer to about 25–50% purity rather than 90% or so on the basis of a report by Guillemin, *et al.*,[4] that the ovine TRH yielded 80–85% of the 3 amino acids in comparison with the 30% yield of these amino acids from the porcine TRH. Direct comparison of the yields could be based on the probability that the porcine and ovine hormones would be proven to be identical.

The discovery[5] that synthetic pGlu-His-Pro-NH_2 (8) from the methylation and ammonolysis of Glu-His-Pro exhibited the hormonal activities of the porcine TRH in the nanogram dosage range unexpectedly reoriented conceptional and experimental efforts on the structural elucidation of the porcine TRH. Since only a few milligrams of the free tripeptide, Glu-His-Pro, was available at the critical time of discovery of the hormonal activity of pGlu-His-Pro-NH_2, a second synthesis of pGlu-His-Pro-NH_2 was urgently needed. The second synthesis was promptly achieved by using the 3 amino acids without any protecting group for any N atom. It was expedient to treat directly the readily available pyroglutamic acid (**1**) with histidine Me ester (**2**) to give the dipeptide pGlu-His-OCH_3 (**3**). Conversion of the Me ester to the free acid, pGlu-His-OH (**6**), and coupling with prolinamide (**7**) gave pGlu-His-Pro-NH_2 (**8**). For the objective of the communication,[6] the nmr spectral data of the pGlu-His-Pro-NH_2 (**8**) from this second synthesis was reported. The details of our second synthesis of pGlu-His-Pro-NH_2 (**8**) are described herein. The synthetic pGlu-His-Pro-NH_2 (**8**) from both of the syntheses possessed identical R_f values and these R_f values in turn were identical with those of the porcine TRH in 17 diversified systems as described.[6]



The initial procedure of the second synthesis which involved the direct reaction between L-pyroglutamic acid (**1**) and L-histidine Me ester (**2**) gave pyroglutamylhistidine Me ester (**3**), in a yield of approximately 55%, and employed the *N,N'*-dicyclohexylcarbodiimide (DCI) method.[7]

† Hypothalamic Hormones. 15.

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This second synthesis was subsequently modified. *N*-Carbobenzoxypyroglutamic acid[8] (**4**), in the form of a mixed anhydride[9], was allowed to react with L-histidine Me ester (**2**) to give *N*-carbobenzoxypyroglutamylhistidine Me ester (**5**) which was then subjected to hydrogenolysis with 5% Pd/C for removal of the Cbz. Pyroglutamylhistidine Me ester (**3**) was obtained in an overall yield of 74%. The melting points and specific rotations of ester **3**, which were obtained by both routes, were identical. The structural assignment for pyroglutamylhistidine Me ester (**3**) was supported by a correct mass for the molecular ion and by the nmr spectrum which showed the presence of the imidazole nucleus and the Me group of the ester.

The pyroglutamylhistidine Me ester (**3**) was saponified by NaOH in MeOH and the resulting pyroglutamylhistidine (**6**) was converted into the corresponding dicyclohexylamine salt. This salt was coupled with prolinamide (**7**)·HCl,[10] using the DCI procedure[7] to afford pyroglutamylhistidylprolinamide (**8**), or TRH in a yield of about 60%.

The pGlu-His-Pro-NH_2 (**8**) which was prepared by this second synthesis showed the hormonal activities of natural TRH and the synthetic product of the first synthesis.[6,11]

Additional details on the relationship of our second synthesis of TRH to the first synthesis and to the structural studies are described in a companion paper by Enzmann, *et al.*;[11] in this paper, there are citations to other investigators who have synthesized TRH which are not repeated here.

Experimental Section

Melting points were obtained with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Microanalyses were performed by the Galbraith Laboratories, Inc., Knoxville, Tenn., and by the Mikroanalytisches Laboratorium, Bonn, West Germany, on samples which 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 (silica gel G), $R_f{}^1$ and $R_f{}^2$ values refer to the system of *n*-BuOH-glacial AcOH-EtOAc-H_2O (1:1:1:1) and $CHCl_3$-MeOH-30% NH_4OH (60:45:20), resp. The location of the peptide spot was revealed by the ninhydrin reagent, the Pauly reagent, or the Cl method.[12] The nmr spectra were obtained at 60 Hz on a Varian Associates spectrometer and HA-100 nmr spectrometer with Me_4Si as internal reference, the chemical shifts are expressed in τ values. The mass spectrum was taken on a Bell and Howell 21-491 mass spectrometer. The specific rotations were obtained with a Perkin-Elmer Model 141 digital readout polarimeter using a microcell.

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Pyroglutamylhistidine Me Ester (3). Coupling by a Carbodiimide.—L-Histidine Me ester (**2**)·2HCl (1.2 g) and L-pyroglutamic acid (0.64 g) in dry MeCN (20 ml) were treated with Et_3N (1.4 ml) and DCI (1.24 g) in dry MeCN (3 ml) at 0°. After being stirred at room temp for 24 hr, the reaction mixt was filtered. The white ppt, which was collected, was dissolved in MeOH and the dicyclohexylurea was removed by filtration. The mother liquor was concd and pyroglutamylhistidine Me ester (**3**) was pptd by addition of Et_2O. This ppt was removed by filtration and recrystd twice from MeOH to afford the pure **3** (0.77 g, yield 55%), mp 210-212 dec.

Coupling by the Mixed Anhydride.—To a soln of *N*-carbobenzoxypyroglutamic acid[8] (1.98 g) in dry THF (20 ml), which was magnetically stirred at 0°, was successively added Et_3N (1.2 ml), ethyl chloroformate (0.8 ml), and after 1 hr, L-histidine Me ester (**2**)·2HCl (1.61 g) and Et_3N (2.4 ml) in $CHCl_3$ (10 ml). After the mixt was stirred at room temp during 24 hr, the solvent was removed *in vacuo* and the reaction mixt was added to H_2O (50 ml). After being extd with $CHCl_3$ (2 × 50 ml), the ext was dried ($MgSO_4$), filtered, and evapd to give an oily dipeptide (3.10 g). A portion (85 mg) of this material was purified by prep silica gel plates using $CHCl_3$-MeOH (9:1, v/v) to afford the pure *N*-carbobenzoxypyroglutamylhistidine Me ester (**5**) (65 mg): $R_f{}^1$ 0.75, single Pauly and Cl positive spots; τ_{CDCl_3} 2.70 ppm, s, 5 H, arom protons, 4.80 ppm, s, 2 H, benzylic CH_2 protons, and 6.38 ppm, s, 3 H, Me protons.

The crude *N*-carbobenzoxypyroglutamylhistidine Me ester (**5**) (2.6 g), in abs MeOH (100 ml) containing 5% Pd/C as catalyst (500 mg), was hydrogenated during 24 hr at room temp and at 1 atm pressure. The reaction mixt was filtered and was evapd *in vacuo* to give the dipeptide ester (1.3 g, yield 74%), mp 205-207° dec. This material was recrystd from MeOH to afford the pure **3**: mp 210-212° dec; $[\alpha]^{22}D$ −1.5° (*c* 1.27, MeOH); $R_f{}^1$ 0.36 and $R_f{}^2$ 0.79, single Pauly and Cl positive spots; *m/e* 280; τ_{MeOH-d_4} 2.41 ppm, s, 1 H, 3.12 ppm, s, 1 H, imidazole protons, and 6.30 ppm, s, 3 H, Me protons. *Anal.* ($C_{12}H_{16}N_4O_4$) C, H, N.

Pyroglutamylhistidine (6) Dicyclohexylamine Salt.—Compd **3** (380 mg), was dissolved in abs MeOH (50 ml) contg NaOH (300 mg). After the soln was stirred at room temp during 1 hr, the reaction mixt was neutralized to pH 7.5 with dil HCl, and then evapd to dryness *in vacuo*. The solid residue was thoroughly suspended in abs MeOH (10 ml) and was filtered through a cotton plug. The filtrate was treated with dicyclohexylamine (300 mg) and **6** was collected as a white ppt after addn of Et_2O; wt 310 mg; $R_f{}^1$ 0.24 (free acid) and $R_f{}^2$ 0.47 (free acid), single Pauly and Cl pos spots. This dipeptide salt was not further characterized, but coupled directly with prolinamide·HCl.

Pyroglutamylhistidylprolinamide (8) (Thyrotropin-Releasing Hormone).—A soln of pyroglutamylhistidine (**6**) dicyclohexylamine salt (244 mg) and prolinamide (**7**)·HCl (82 mg),[10] in DMF (10 ml), was treated with DCI (150 mg). After being stirred at room temp during 48 hr, the reaction mixt was filtered and the filtrate was evapd *in vacuo* to dryness. The residue was purified by chromatography on silica gel with development by $CHCl_3$-MeOH (8:2, v/v) to afford the pure pyroglutamylhistidylprolinamide (**8**): 116 mg; yield 59%; $[\alpha]^{22}D$ −42.4° (*c* 1.00, MeOH); $R_f{}^1$ 0.23 and $R_f{}^2$ 0.64, single Pauly and Cl pos spots.

The HCl salt of **8** was prepd in the usual manner. It was recrystd from MeOH-Et_2O, mp 180° (soften), 200° (darkens), 250° dec. *Anal.* ($C_{16}H_{22}N_6O_4$·HCl) C, H, Cl; N, calcd 21.07; found 20.41.

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