# SYNTHESIS OF $^{14}$C-LABELLED THYROTROPIN RELEASING HORMONE [PYROGLUTAMYLHISTIDYL-$^{14}$C(U)-PROLINEAMIDE]•

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SUMMARY

*A simplified synthesis of pyroglutamylhistidylprolineamide (TRH) was developed for the purpose of making labelled TRH. Pentachlorophenyl pyroglutamate was reacted with histidine to make pyroglutamylhistidine, and the dipeptide was coupled* in situ *with prolineamide by means of dicyclohexylcarbodiimide to yield the desired hormone. Both the dipeptide and the hormone were purified by chromatography on silica gel. This method of synthesis was conveniently adapted to synthesize TRH-$^{14}$C starting with histidine-$^{14}$C(U). Purification of labelled TRH was accomplished by preparative TLC.*

Since the elucidation of structure of the ovine[1] and porcine[2] thyrotropin releasing hormone (TRH) as the tripeptide pGlu-His-Pro-$NH_2$**, there has been great interest in the availability of an isotopically labelled

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• All aminoacid residues are of the L configuration.

** The following abbreviations have been used: pGlu = pyroglutamyl residue, PCPE = pentachlorophenyl ester, DCU = dicyclohexylurea, DCC = dicyclohexylcarbodiimide.

preparation of this hormone for metabolic and other biological determinations. As the level of activity of TRH is detectable in the picrogram level in vitro and at the nanogram level in vivo[3], it appeared desirable to synthesize the hormone with a very high level of radioactivity to facilitate detection in metabolic studies.

Uniformly labelled histidine-$^{14}C$ (labelled in all six carbon atoms) was selected to label TRH, inasmuch as the hormone would probably be degraded by a chemical transformation or a degradation from the N- or C-terminus, and in all cases, histidine would be expected to appear as part of the main degradation product. No isotopic dilution with cold histidine was planned in order to keep the specific activity as high as possible.

The synthesis of TRH previously accomplished[4] involved several steps which would make manipulations of radioactively labelled intermediates laborious and subject to large losses on the small scale required. Therefore a new synthetic sequence was devised which would allow a minimum of manipulation of intermediates.

For the synthesis pGlu-PCPE was coupled in DMF with histidine in the presence of NaOH which led to the dipeptide pGlu-His. The dipeptide acid was identical to a sample prepared by hydrolysis of pGlu-His-OMe. However, pGlu-His was usually not isolated from the reaction mixture but was coupled in situ with Pro-$NH_2$.HCl in the presence of N-hydroxysuccinimide (to inhibit the possible epimerization of the histidyl residue during carboxyl group activation[5]) and DCC[6] (100% excess) as the coupling reagent. The reaction mixture was usually allowed to stand for 16 hr and then subjected to the isolation procedure to yield TRH. When 3 mmoles of histidine was converted to TRH by this method, purification by column chromatography with silica gel as previously described[4] gave a 38-40% yield of the hormone. The synthetic TRH had nmr spectrum, chromatographic (TLC) and electrophoretic mobilities identical to those found for an authentic sample of TRH, and bioassay with rat pitui-

taries in vitro[7] revealed full biological activity*.

Thus, this method yields material of the same quality as the stepwise method of synthesis[4], and it has the added convenience of rendering unnecessary the isolation of intermediates, and thus results in shorter purification procedures.

For the synthesis of labelled TRH, 1.22 mg, 2.0 mCi, of histidine-$^{14}C(U)$ with specific activity of 255 mCi/mmole was converted to TRH by the sequence of steps described above for the "cold" TRH. For purification, preparative TLC was employed. The product obtained had the same chromatographic mobility as TRH by TLC, and scanning of the TLC plate with a Radiochromatogram Scanner revealed a single symmetric peak. A bioassay with rat pituitaries in vitro[7] showed this material to possess biological activity equivalent to that of cold TRH*. In vivo assays of $^{14}C$-labelled TRH showed that it retained essentially its full biological activity when compared to the natural porcine TRH[8]. Scintillation counting on an aliquot of the tagged hormone showed recovery of 0.741 mCi, from the initial 2 mCi, of the starting histidine-$^{14}C(U)$ (37% yield of TRH-$^{14}C$).

The high resolution mass spectrum of labelled TRH showed molecular ions at 374.1884, 372.1853 and 362.1698 (physical scale) which are consistent with TRH containing $^{14}C$-histidine containing 6, 5 or 0 atoms of $^{14}C$.

The fragmentation patterns were consistent with the structure and showed a pronounced isotopic effect which will be further described at a latter date.

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## EXPERIMENTAL SECTION *

*Pyroglutamylhistidine methyl ester.* A solution of His-OMe.2HCl (7.26 g, 30 mmoles) in $H_2O$-MeOH was treated with Rexyn 201 ($OH^-$), and the suspension was filtered. The filtrate was evaporated to a residual oil which was dissolved in DMF (30 ml), and the solution was treated with pGlu-PCPE (11.325 g, 30 mmoles). After 16 hr the clear solution which had formed was evaporated to an oil which was taken up in a small volume of MeOH, and $Et_2O$ was added to it. The crystals formed were collected and washed with $Et_2O$; yield 4.2 g, mp 198-201°. This material was recrystallized from MeOH yielding 2.3 g, mp 211-213°, $[\alpha]_D^{25°}$ -53.5° (c 1, AcOH). The nmr spectrum ($C_5D_5N$) was consistent with the structure.

Anal. ($C_{12}H_{16}N_4O_4$) Calcd: C, 51.42; H, 5.75; N, 19.99 Found: C, 51.52; H, 5.91; N, 20.00

*Pyroglutamylhistidine.* - Method A. To a solution of pGlu-PCPE (11.325 g, 30 mmoles) in DMF (75 ml) was added a solution of histidine (4.65 g) in 2 N NaOH (15 ml). After 1 hr 1N HCl (30 ml) was added, and the clear solution was evaporated to a gummy residue. For further purification, a solution of the crude material in the least amount of $H_2O$-MeOH was applied to a column con-

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* A Thomas-Hoover apparatus was used for melting point determinations in capillary tubes. Where analyses are indicated only by the symbols of the elements, analytical results were obtained for the elements within ± 0.4% of the theoretical value. For electrophoresis Brinkmann precoated thin-layer cellulose sheets were employed with pyridine-AcOH buffer, pH 6.3 (25-45 min). The nmr spectra were obtained at 100 MHz using a Varian Associates HA-100 spectrometer. Mass spectra were recorded in an AEI MS-902 mass spectrometer.

taining silica gel (80 g) previously packed in 5% MeOH-$CHCl_3$. Elution was performed with MeOH-$CHCl_3$ combinations, and detection of the product in the eluent was accomplished by TLC (silica gel G) of all fractions with MeOH-$CHCl_3$ (2:1) containing 1% AcOH as the developing solvent and $Cl_2$-tolidine as the color spray. Faster moving by-products such as DCC, DCU and pentachlorophenol could be eluted with combinations of 5-35% MeOH-$CHCl_3$. Elution with MeOH-$CHCl_3$ (2:1), then MeOH, and finally 90% MeOH yielded fractions containing the dipeptide. The combined fractions were evaporated to a foam which was dissolved in MeOH (40 ml). The amorphous solid which formed overnight was collected, washed with MeOH and air dried; yield 4.84 g, mp 209-210°. Crystallization from the least amount of $H_2O$ yielded 3.8 g, mp 164-167°, $[\alpha]_D^{25°}$ + 10.38° (c 2, AcOH). The nmr spectrum ($D_2O$) was consistent with the structure. Anal. ($C_{11}H_{14}N_4O_4 \cdot H_2O$). Calcd: C, 46.47; H, 5.67; N, 19.71 Found: C, 46.54; H, 5.80; N, 19.75

Method B. To 2N NaOH (1 ml) was added pGlu-His-OMe (561 mg, 2 mmoles) while stirring. After a few min a clear solution resulted. After 1 hr almost all of the ester was hydrolyzed as ascertained by TLC (silica gel G) with MeOH-$CHCl_3$ (2:1) containing 1% AcOH as the developing solvent, and using $Cl_2$-tolidine as the color spray. The solution was neutralized with 1N HCl (2 ml) and evaporated to dryness. The residue obtained was digested with hot DMF, the suspension was filtered to remove solids, and the filtrate was evaporated to a gummy residue. A MeOH solution deposited an amorphous solid, 510 mg, mp 160-170°. Crystallization from $H_2O$ yielded 379 mg of pGlu-His, mp 169-171°, $[\alpha]_D^{25°}$ + 2.79° (c 1, $H_2O$), $[\alpha]_D^{25°}$ + 10.4° (c 1, AcOH).

Method C. Refluxing a 0.1M solution of pGlu-His-OMe in $H_2O$ for 7hr led to yields and quality of pGlu-His similar to those obtained by method B.

*Pyroglutamylhistidylprolineamide (TRH)*- Method A. To a solution of pGlu-PCPE (1.134 g, 3 mmoles) in DMF (7.5 ml) was added a solution of histidine (465 mg) in 2N NaOH (1.5 ml). After 1.5 hr prolineamide.HCl (498 mg), N-

hydroxysuccinimide (381 mg) and after an additional 10 min DCC (1.238 g, 6 mmole) were added. The resulting suspension was allowed to stand for 16 hr at $0^{\circ}$. The reaction mixture was filtered and the collected solid was washed with DMF.

The combined filtrate was evaporated to an oil which was dissolved in the least amount of MeOH-$H_2O$, and this solution was applied to a column containing silica gel (30 g) previously packed in 5% MeOH-$CHCl_3$. The effluent from the column was monitored by TLC (silica gel G) with MeOH-$CHCl_3$ (2:1) developing solvent and $Cl_2$-tolidine color spray. Components with Rf higher than TRH were removed with 15% MeOH-$CHCl_3$, and TRH was eluted with MeOH-$CHCl_3$ (1:2). Fractions of TRH which were contaminated with prolineamide were purified by repeating the chromatography, eluting the TRH directly with MeOH after application of the sample. The fractions containing TRH (Rf identical to an authentic sample) were combined and evaporated to a foam. A solution of this residue in MeOH was treated with Rexyn 201 ($OH^-$), the suspension was filtered, and the filtrate was evaporated _in vacuo_ to a foam. An aqueous solution of the foamy residue was lyophilized. The fluffy powder thus obtained weighed 415 mg (38%), and in another run 430 mg (40%); $[\alpha]_D^{25^{\circ}}$ -64.8° (_c_ 1.25, $H_2O$). The nmr spectrum ($C_5D_5N$) was identical to that of another synthetic sample obtained previously by a different route[4], which had $[\alpha]_D^{25^{\circ}}$ -65.6° (_c_ 1, $H_2O$). The electrophoretic mobilities of both samples were also identical. The bioassay of the synthetic material was performed with rat pituitaries _in vitro_[7]. The activity of this preparation was identical to TRH reference standards.

Method B. Crystallized pGlu-His could be converted to TRH under equivalent conditions of method A. The yields of product were at least as good or better (40-45%) and the final TRH was of comparable quality.

*Pyroglutamylhistidyl-$^{14}$C(U)-prolineamide.*- A solution of histidine-$^{14}$C(U)* (1.22 mg, 2 mCi) in 4.5 µl of 2 N NaOH and 4 µl of $H_2O$ was treated with 40 µl of a solution made by dissolving pGlu-PCPE (150 mg) in DMF and diluting to 2 ml. After 2 hr Pro-$NH_2$.HCl (1.6 mg) and N-hydroxysuccinimide (1.4 mg) were added. After 10 min DCC (4 mg) was added, and the reaction mixture was agitated gently overnight at $0^o$. To the reaction mixture was added 0.8 ml of MeOH-$CHCl_3$ (1:2), and the resulting solution was applied to several silica gel G plates with additional MeOH-$CHCl_3$ for washings. One of the plates had only a small aliquot of the reaction mixture and a TRH standard, and it was used to ascertain the position of the labelled TRH. The plates were developed with MeOH-$CHCl_3$ (2:1). The plate with TRH standard was treated with $Cl_2$ and then sprayed with tolidine, by the usual method. This plate was now scanned with a Radiochromatogram Scanner which revealed that there was a major radioactive component with Rf identical to that of TRH and a minor radioactive component appearing where pGlu-His usually shows in this TLC solvent system. The other plates more heavily loaded with labelled TRH were scanned manually with the aid of a thin window Geiger-Mueller Survey Meter with the detection tube covered by aluminum foil with a narrow slit for better resolution. The readings obtained in the recording scale in cpm were conveniently plotted against length of the plate and the exact position of labelled TRH was thus conveniently detected. Elution of the appropriate portion of the plate was performed in a chromatographic column with MeOH-$CHCl_3$ (1:2). The eluent was evaporated to dryness, and the residue was dissolved in 20 ml

* Uniformly labelled histidine-$^{14}$C (with $^{14}$C possible in all C atoms) 1.22 mg, 2 mCi, was purchased from New England Nuclear, Boston, Mass. Scanning of TLC plates with radioactive materials was performed with a Vanguard glass Scanner 885 with range of up to 100,000 cpm and with a portable Geiger-Mueller Survey Meter with a 3 mg/cm$^2$ thin window.

of 3% EtOH in $H_2O$. Aliquots (10 $\mu$l) of the 20 ml aqueous solution of the TRH-$^{14}C$ were assayed for $^{14}C$-activity by the liquid scintillation counting technique, using a Packard Instrument Company series 2000 counter and external standards to correct for quenching errors. The theoretical specific activity of the TRH-$^{14}C$ was 255 mCi/mmole. The total activity of the TRH-$^{14}C$ was found to be 740.5 $\mu$Ci of $^{14}C$, representing 1.05 mg of the tripeptide. The labelled TRH had identical Rf to reference TRH on TLC (silica gel G), with MeOH-$CHCl_3$ (2:1) and $Cl_2$-tolidine spray. A chromatogram scan of this TLC plate with a Vanguard glass plate scanner showed one symmetrical peak reading 75,000 counts at the maximum.

The high resolution mass spectrum of TRH-$^{14}C$ showed molecular ions with m/e 374.188$\underline{4}$, 372.185$\underline{3}$, and 362.169$\underline{8}$ (Physical scale), and fragmentation patterns consistent with the structure.

The bioassay of TRH-$^{14}C$ *in vitro* showed this preparation to have the same level of biological activity as that of TRH standard[7]. The bioassay *in vivo* also showed full activity for this preparation[8].

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