# USE OF THE AMMONIUM SALT OF PYROGLUTAMIC ACID FOR THE SYNTHESIS OF TRF

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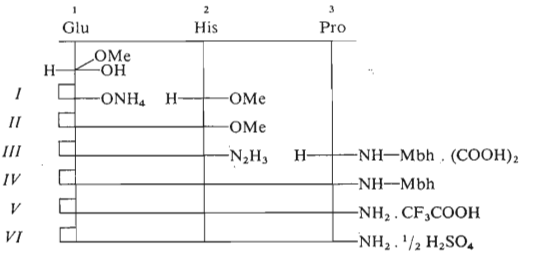
During the synthesis of TRF (thyroliberin) the starting compound used was the ammonium salt of pyroglutamic acid obtained by cyclization of the γ-ester of glutamic acid in ammoniacal ethanol. The amide pyroglutamyl-histidyl-proline (TRF) was isolated as a somewhat hygroscopic sulfate.

Pyroglutamic acid, 5-pyrrolidone-2-carboxylic acid, is the N-terminal component of the releasing factors of the thyrotropic hormone (TRF) and of gonadotropic hormones (LRF/FRF). The synthesis of TRF by fragment condensation in solution is based on the application of free[1-7] or N-protected pyroglutamic acid[8,9] or on the synthesis of glutamine peptides and their subsequent cyclization to the pyroglutamic moiety[10,11].

During the direct synthesis of N-unprotected peptides of pyroglutamic acid one must isolate free pyroglutamic acid[12-14]. In view of the difficulties connected with its high water solubility, we decided to use its ammonium salt for the synthesis of N-pyroglutamate peptides (*I*),* the salt being readily prepared in a pure state from the γ-ester of glutamic acid[18]. Cyclization is done in ammonia solution of a lower alcohol (ethanol, 2-propanol, and the like) from which the ammonium salt *I* crystallizes directly without by products. Methanol is not suitable for the cyclization since *I* is readily soluble in it. By conversion of *I* on a Zerolit 225 ($H^+$ form) resin one can obtain free pyroglutamic acid. The ammonium salt *I* was used for the synthesis of TRF, *i.e.* the pyroglutamyl-histidyl-proline amide. The synthetic procedure is shown in Scheme 1. The ammonium salt *I*, subjected to the N,N'-dicyclohexylcarbodiimide method, reacts with the histidine methyl ester dihydrochloride and one equivalent of N-ethylpiperidine in dimethylformamide to the dipeptide *II* which was isolated as a hydrochloride-monohydrate. The dipeptide ester *II* was obtained from pyroglutamic acid under similar conditions[2]. Hydrazinolysis of *II* with 80% hydrazine hydrate yielded the corresponding hydrazide[1] *III*. To protect

* Symbols and names of compounds follow the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature[15-17]. All amino acids are of the L-configuration. □Glu residue of pyroglutamic acid, Mbh 4,4'-dimethoxybenzhydryl group.

the amide group of benzyloxycarbonylproline we used 4,4'-dimethoxybenzhydrol[11]; by hydrogenolysis we isolated the free amide as an oxalate[8]. In a 10% excess, hydrazide *III* reacts in the azide method[19] with proline 4,4'-dimethoxybenzhydrylamide to the protected tripeptide *IV*. In the isolation we made use of its low solubility in water in the region of pH 9·0–9·2 to separate by-products. Free tripeptide was obtained by boiling with trifluoroacetic acid and the trifluoroacetate *V* obtained was converted to the acetate, sulfate or to the base.




SCHEME 1

The biological activity of TRF was determined radioimmunologically on cattle, sheep, monkeys and rats and it corresponded to data in the literature[20].

## EXPERIMENTAL

The melting points were determined in Kofler's block and are not corrected. Samples for analysis were dried at 0·5 Torr over $P_2O_5$ at 105°C. Optical rotation was measured in a photoelectric Perkin-Elmer polarimeter in methanol (*c* 0·2). Chromatography was done on a thin layer of silica gel (Kieselgel G., Merck) in butanol–acetic acid–water (4 : 1 : 1) ($S_1$) and in butanol–acetic acid–pyridine–water (15 : 3 : 10 : 6) ($S_2$). Electrophoresis was done on Whatman No 3 MM at a potential gradient of 23 V/cm for 50 min, or in a thin layer of silica gel (Kieselgel G. Merck) at a potential gradient of 40 V/cm, for 8 min in pyridine – acetic acid (pH 5·7). Detection was done with ninhydrin, Pauly's reagent, and chlorine and tolidine. Solutions were evaporated on a rotatory evaporator at reduced pressure.

### Ammonium Salt of Pyroglutamic Acid (*I*)

Suspension of the γ-methyl ester of glutamic acid (16·1 g; 100 mmol) in 2-propanol (200 ml) and ammonia (40 g) was stirred for 3 h at room temperature. The original suspension clears after some 20 min and *I* precipitates after 2 h. After 1 h the salt was filtered and dried *in vacuo* over $P_2O_5$. A total of 11·2 g (77%) product melting at 125–132°C was obtained. Sample for analysis was crystallized from methanol and ether; m.p. 138–140°C. For $C_5H_{10}N_2O_2$ (146·2) calculated:

41·09% C, 6·90% H, 19·17% N; found: 41·20% C, 6·89% H, 18·82% N. $[\alpha]_D^{20}$ −9·9°. $R_F$: 0·47 ($S_1$), 0·41 ($S_2$).

Methyl Ester of Pyroglutamyl-histidine (*II*)

Suspension of *I* (14·6 g; 100 mmol) in dimethylformamide (300 ml) and of histidine methyl ester dihydrochloride (24·2 g; 100 mmol) together with 14 ml N-ethylpiperidine was shaken for 30 min at room temperature, N,N'-Dicyclohexylcarbodiimide (22 g) was added to the suspension at 0°C. After 12 h of standing at 0°C, N,N'-dicyclohexylurea was filtered, the filtrate was evaporated and the residue was crystallized from 2-propanol (100 ml) and dichloromethane (500 ml). A total of 18·3 g (55%) product melting at 148—152°C was obtained. Sample for analysis was crystallized from dimethylformamide and dichloromethane, m.p. 184—186°C (it softens at 123°C). For $C_{12}H_{16}N_4O_4.HCl.H_2O$ (334·8) calculated: 43·06% C, 5·72% H, 16·74% N; found: 43·16% C, 5·32% H, 16·89% N. $[\alpha]_D^{20}$ −24·3° (*c* 1). $R_F$: 0·23 ($S_1$), 0·52 ($S_2$). Calculated: 10·89% HCl; found: 10·59% HCl (argentometrically), *i.e.* content per dry weight was 99·05%. Determination of the base by titration with perchloric acid showed 98·58%, *i.e.* content per dry weight of 100·3%. Determination of water (K. Fischer) showed 7·06% (calculated 5·38%). Ref.[1] reports for the m.p. of □Glu-His-OMe 199—201°C, $[\alpha]_D^{25}$ −4·3° (*c* 1, methanol), ref.[3] gives a m.p. of 210—212°C (decomposition), ref.[7] gives a m.p. of □Glu-His-OMe. 1/2 $H_2O$ as 208—209°C, $[\alpha]_D^{22}$ −42° (*c* 1·1, methanol).

Hydrazide of Pyroglutamyl-histidine (*III*)

A solution of *II* (20·1 g; 60 mmol) in methanol (150 ml) and 80% hydrazine hydrate (21 ml) was left to stand for 12 h at room temperature. The precipitated product was filtered, washed with methanol (m.p. 213—216°C) and crystallized from methanol (200 ml) and water (40 ml). The yield was 11·2 g (66%), m.p. 238—240°C. Sample for analysis was crystallized similarly; m.p. 243—245°C. For $C_{11}H_{16}N_6O_3$ (280·2) calculated: 47·14% C, 5·75% H, 29·98% N; found: 46·81% C, 5·65% H, 29·97% N.

4,4'-Dimethoxybenzhydrylamide of Pyroglutamyl-histidyl-proline (*IV*)

A solution of *III* (6·16 g; 22 mmol) in dimethylformamide (100 ml) and concentrated HCl (8 ml) was combined at −20°C with a solution of sodium nitrite (1·625 g) in water (6·5 ml). After 10 min the solution was neutralized with N-ethylpiperidine (about 12·5 ml) to pH 6·9 and combined with a solution of proline 4,4'-dimethoxybenzhydrylamide oxalate (8·6 g; 20 mmol; m.p. 219 to 221°C, ref.[8] reporting a m.p. of 201—202°C) in dimethylformamide (100 ml) neutralized with N-ethylpiperidine (6·2 ml). After 12 h of standing at 0°C, the solution was evaporated, the residue was dissolved in water (200 ml) and the pH adjusted with dilute NaOH to 9·0. The separated emulsion was left to stand for 12 h at 0°C. The aqueous phase was decanted, the residue was triturated with water and finally, by sequential dissolving and evaporating in ethanol, and in ethanol and benzene, an amorphous product was obtained; 8·6 g (73%), giving a single spot positive for Pauly's reagent; $R_F$ 0·37 in $S_1$, 0·68 in $S_2$.

Pyroglutamyl-histidyl-proline Amide Trifluoroacetate (*V*)

A solution of *IV* (9·2 g; 15·6 mmol) in trifluoroacetic acid (40 ml) and anisol (4 ml) was boiled for 10 min and precipitated with ether to a sediment which was washed with tetrahydrofuran to yield 6·1 g (81%) product. A sample for analysis was crystallized from methanol and ether,

m.p. 145—150°C. For $C_{16}H_{22}N_6O_4.CF_3COOH$ (476·4) calculated: 45·38% C, 4·87% H, 17·64% N; found: 45·82% C, 5·41% H, 16·77% N. $R_F$: 0·13 ($S_1$), 0·43 ($S_2$). Amino acid analysis[21] (after hydrolysis with 6M-HCl at 110°C, 20 h): His 0·98, Glu 1·00, Pro 1·00.

Pyroglutamyl-histidyl-proline Amide Sulfate (*VI*)

A solution of *V* (715 mg; 1·5 mmol) in water (10 ml) was passed through a column of Zerolit FF ($SO_4^-$ cycle) (1·5 cm × 25 cm). The eluate (200 ml) was partly evaporated and freeze-dried. The residue was dissolved in methanol (35 ml) and poured under stirring into 100 ml ether. The precipitate was filtered, washed with ether and dried over $P_2O_5$. A total of 570 mg (80%) product was obtained. A sample for analysis was precipitated in a similar way, m.p. 197—199°C. For $C_{16}H_{22}N_6O_4.1/2\,H_2SO_4.H_2O$ (429·5) calculated: 44·75% C, 5·87% H, 19·57% N, 3·73% S; found: 44·64% C, 5·53% H, 19·19% N, 4·17% S. $[\alpha]_D^{20}$ −42·5°. The acetate and the base hydrate were obtained in a similar way.

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