# APPLICABILITY OF GLUTAMINYL-tRNA CYCLOTRANSFERASE IN THE PEPTIDE SYNTHESIS*

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Peptides with amino-terminal glutamine and their derivatives on treatment with crude papain preparation, containing glutaminyl-tRNA cyclotransferase (E.C. 2.3.2.5.), are converted into the corresponding compounds with pyroglutamic adid. This enzymic cyclization does not take place with glutamic acid or its γ-derivatives. The reaction has been optimized for the preparation of pyroglutamic acid phenylhydrazide. Glutaminyl-leucine phenylhydrazide and glutaminyl--histidyl-proline amide reacted to give pyroglutamyl-leucine phenylhydrazide and TRH, respectively. Treatment of crude papain with iodoacetamide completely inhibited its activity without affecting the glutaminyl-cyclotransferase activity.

Amino-terminal pyroglutamic acid represents a frequent structural element of biologically active peptides (eledoisin, physalemin, thyreotropin-releasing hormone, bombesin, litorin, gastrin, neurotensin, caerulein *etc.*; refs[1,2]). Chemical syntheses of these peptides utilize either amino-protected pyroglutamic acid** or amino-terminal glutamine peptides which are converted into the end-products by heating in water, in some cases in the presence of organic acids. Both these procedures may lead to formation of side-products whose removal is difficult.

In 1964, glutamine cyclotransferase was isolated from dried papaya latex by chromatography on CM-Sephadex[5-7]. Principal physico-chemical activities of this enzyme have been described and its activity tested on glutamine and some of its derivatives and peptides. The enzymic reaction was studied quantitatively by colorimetric determination of the liberated ammonia. The conversion of glutaminyl-tRNA into pyroglutamyl-tRNA was also described[8].

This communication concerns the possible use of crude papain which contains this enzyme (glutamine cyclotransferase; recommended name: glutaminyl-tRNA cyclotransferase; systematic name: L-glutaminyl-tRNA γ-glutamyltransferase (cycli-

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** Nomenclature and symbols of the amino acids and peptides obey the published recommendations[3,4]; Glp denotes a pyroglutamic acid residue. All the chiral amino acids belong to the L-series.

zing[9]; E.C. 2.3.2.5.) for the preparation of peptides with amino-terminal pyroglutamic acid starting from glutamine precursors. The cyclization reaction was studied with glutamine phenylhydrazide as a model compound. Using the HPLC technique, we followed the effect of pH, organic solvents and the amount of the crude enzyme on time course of the reaction. The dependence on pH, given in Table I, shows that the reaction is fastest in neutral or weakly basic media. Since the cyclization proceeds also to some extent spontaneously, the yields of the formed pyroglutamic acid phenylhydrazide in Table I are given both for the total (enzymically catalyzed) reaction and for the reaction proceeding without the enzyme (blank). The effect of various organic solvents is seen from Table II. Whereas dimethylformamide, and particularly dimethyl sulfoxide, reduce the yield, other studied solvents (acetonitrile, 2-propanol, methanol and dioxane) have no significant effect on the reaction course. The reaction rate increases with increasing amounts of the enzyme (Table III).

With other compounds, the cyclization reaction was performed at pH 7 in the absence of organic solvents at concentration 0·1 mol $l^{-1}$ of the starting compound and 2 mg of papain in 1 ml of the reaction mixture. The unprotected α-carboxyl

TABLE I

Effect of pH on cyclization of glutamine phenylhydrazide catalyzed with crude papain

| pH | Yield[a] Glp-$N_2H_2$—$C_6H_5$, % | | | |
|---|---|---|---|---|
| | 10 min | 1 h | 2 h | 4 h |
| 4·5 | 10·4 | 23·0 | 33·0 | 45·1 |
| | (4·1) | (9·7) | (9·2) | (14·5) |
| 5·0 | 16·9 | 38·4 | 54·2 | 70·6 |
| | (6·7) | (7·1) | (9·7) | (15·5) |
| 6·0 | 17·1 | 53·5 | 72·8 | 88·8 |
| | (5·1) | (5·5) | (6·2) | (7·5) |
| 7·0 | 26·8 | 91·5 | 97·1 | 97·8 |
| | (4·1) | (6·6) | (7·5) | (10·7) |
| 8·0 | 21·4 | 88·2 | 98·2 | 98·9 |
| | (4·6) | (5·6) | (7·6) | (8·6) |
| 9·0 | 35·9 | 78·6 | 93·7 | 97·6 |
| | (5·8) | (8·0) | (10·8) | (14·4) |

[a] Numbers in parentheses denote the blank (in the absence of the enzyme). The starting compound contained 3·5% of the cyclic derivative. The values were obtained by HPLC. Reaction conditions: 1 mg of crude papain in 0·1 mol $l^{-1}$ solution of the starting compound (0·5 ml).

of glutamine slows down the cyclization. Dipeptides and tripeptides with amino-terminal glutamine afford very good yields of cyclization products (Table IV). Also in these cases some (less than 10%) spotaneous cyclization occurs. It seems that cyclizations, catalyzed with glutamine cyclotransferase, require the presence of an amide group at the γ-carboxyl (see also ref.[7]). The only exception, glutamic acid α-phenylhydrazide γ-methylamide, afforded only very low yield (5 – 10% after 24 h) of pyroglutamic acid phenylhydrazide. α-Phenylhydrazides of glutamic acid, its γ-anilide or γ-methyl ester did not react at all (in the last-mentioned case corrected for the spontaneous cyclization of the γ-methyl ester).

On the preparative scale, the catalysis with crude papain has been utilized for the preparation of pyroglutamic acid phenylhydrazide, pyroglutamyl-leucine phenylhydrazide and pyroglutamyl-histidyl-proline amide (TRH).

We observed that treatment of crude papain with iodoacetamide completely inhibited the cleavage of $N^{\alpha}$-benzoylarginine ethyl ester. However, the thus-inactivated crude papain, under identical conditions, was fully active in the cyclization of glutamine phenylhydrazide.

TABLE II

Effect of organic solvents on cyclization of glutamine phenylhydrazide catalyzed with crude papain

| Solvent | % v/v | Yield[a] Glp-$N_2H_2$—$C_6H_5$, % | | | |
|---|---|---|---|---|---|
| | | 10 min | 1 h | 2 h | 4 h |
| None | — | 16·6 | 62·0 | 92·8 | 97·8 |
| Acetonitrile | 20 | 16·9 | 65·3 | 96·6 | 97·8 |
| | 40 | 13·3 | 61·3 | 90·3 | 97·5 |
| Dimethylformamide | 20 | 11·5 | 38·0 | 62·5 | 87·8 |
| | 40 | 7·6 | 17·4 | 28·1 | 45·9 |
| Dimethyl sulfoxide | 20 | 8·5 | 19·6 | 31·7 | 53·0 |
| | 40 | 6·1 | 8·2 | 9·8 | 13·2 |
| 2-Propanol | 20 | 17·1 | 66·4 | 95·1 | 98·2 |
| | 40 | 18·1 | 74·8 | 96·4 | 97·5 |
| Methanol | 20 | 16·8 | 66·4 | 96·1 | 98·3 |
| | 40 | 18·8 | 73·1 | 98·5 | 98·8 |
| Dioxane | 20 | 18·1 | 67·6 | 95·6 | 97·9 |
| | 40 | 17·9 | 69·7 | 95·5 | 98·1 |

[a] Reactions performed at pH 6·5, other conditions same as described in Table I.

## EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. Reaction mixtures were evaporated on a rotatory evaporator at bath temperature 30°C. Thin-layer chromatography (TLC) was performed on silica gel plates (Silufol, Kavalier) in the following systems: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1), 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S2), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4). Electrophoresis was carried out in a moist chamber on a Whatman 3MM

TABLE III

Effect of enzyme amount and substrate concentration on cyclization of glutamine phenylhydrazide, catalyzed with crude papain

| Concentration | | Yield[a] Glp-$N_2H_2$—$C_6H_5$, % | | | |
|---|---|---|---|---|---|
| Enzyme mg/0·5 ml | Substrate mol $l^{-1}$ | 10 min | 1 h | 2 h | 4 h |
| 0·1 | 0·1 | 0·5 | 7·9 | 17·7 | 31·6 |
| 0·2 | 0·1 | 2·6 | 12·5 | 24·6 | 45·5 |
| 0·5 | 0·1 | 5·3 | 31·8 | 57·4 | 82·4 |
| 1·0 | 0·1 | 22·7 | 84·8 | 89·6 | 87·1 |
| 2·0 | 0·1 | 20·8 | 87·3 | 89·8 | 88·1 |
| 1·0 | 0·2 | 6·7 | 34·9 | 65·1 | 83·3 |

[a] Differences between the yields in the presence and in the absence of the enzyme; reaction carried out at pH 7 under conditions specified in Table I.

TABLE IV

Cyclization of glutamine and its peptides, catalyzed with crude papain

| Product[a] | Yield ,% | | | | |
|---|---|---|---|---|---|
| | 10 min | 1 h | 2 h | 4 h | 24 h |
| Glp | 11·3 | 18·3 | 26·9 | 59·7 | 92·2[b] |
| Glp-Gly | 32·5 | 97·4 | — | — | 72·5[c] |
| Glp-Gly-Gly | — | 99·4 | — | — | 96·0[c] |
| Glp-Leu-$N_2H_2$.$C_6H_5$ | 12·7 | 71·5 | 95·0[d] | 95·5 | — |
| Glp-His-Pro-$NH_2$ | 10·1 | 90·8 | 96·6 | — | 52·7[c] |

[a] No enzymic cleavage of peptide bonds was observed in any reaction; [b] the reaction proceeds also spontaneously (10·6% after 4 h, 45·8% after 24 h); with other peptides the yields of the spontaneous cyclization were substantially lower; [c] reaction time 30 min; [d] after 2 h some of the product precipitated.

paper at 20 V/cm for 1 h in 1 mol $l^{-1}$ acetic acid (pH 2·4) or a pyridine–acetate buffer (pH 5·7). Compounds were detected with ninhydrin or by the chlorination method. Samples for amino acid analysis were hydrolyzed in 6 mol $l^{-1}$ HCl at 105°C for 20 h; the analyses were performed on an automatic analyzer AAA 339 (Mikrotechna). Optical rotations were measured on a Perkin–Elmer 161 MCA polarimeter. High performance liquid chromatography (HPLC) was carried out on a Spectra Physics SP 8700 instrument equipped with SP 8400 UV-detector and SP 4100 integrator. Column chromatography was performed on columns of Separon SIX C-18 (Laboratorní přístroje, Prague). Analyses were done on a 15 × 0·4 cm column (flow rate 42 ml/h), preparative experiments on 25 × 0·8 cm and 25 × 1·27 cm columns (120 ml/h and 240 ml/h, respectively). The compounds were detected at 224 nm and eluted with a mixture of methanol and water containing 0·05% of trifluoroacetic acid (the percentage of methanol is given in parentheses for each $k'$ value). The trifluoroacetates used in the analytical studies were prepared from the corresponding tert-butyloxycarbonyl derivatives and trifluoroacetic acid. Their homogeneity was followed by paper electrophoresis and HPLC. The employed crude papain was an Enzymase (Belgium) product.

Tert-butyloxycarbonylglutamine Phenylhydrazide

A solution of tert-butyloxycarbonylglutamine (2·46 g) in a 0·2 mol $l^{-1}$ acetate buffer, pH 4·8 (50 ml) was mixed with phenylhydrazine (2 ml), ethylenediaminetetraacetic acid (15 mg) and cysteine hydrochloride (50 mg) and the solution was adjusted to pH 4·8 with acetic acid. Papain (210 mg) was added and the mixture was incubated at 38°C for 24 h. The precipitate was filtered, washed successively with a hydrogen sulfate buffer (pH 2), water, sodium hydrogen carbonate solution and water, and dried. Crystallization from ethyl acetate–light petroleum afforded 1·8 g (53·5%) of the product, m.p. 162°C; $[\alpha]_D$ −26·2° (*c* 0·3, methanol); $k' = 3{\cdot}96$ (50% methanol); $R_F$ 0·68 (S1), 0·57 (S2), 0·66 (S3), 0·69 (S4). For $C_{16}H_{24}N_4O_4$ (336·4) calculated: 57·13% C, 7·19% H, 16·65% N; found: 57·13% C, 7·22% H, 16·71% N.

Tert-butyloxycarbonylglutamic Acid α-Phenylhydrazide

Phenylhydrazine (2 ml), followed by ethylenediaminetetraacetic acid (15 mg) and cysteine hydrochloride (50 mg), was added to a solution of tert-butyloxycarbonylglutamic acid (2·47 g) in a 0·2 mol $l^{-1}$ acetate buffer, pH 4·8, (50 ml). The mixture was adjusted to pH 4·8 with acetic acid and papain (210 mg) was added. After incubation at 38°C for 24 h and acidification with a hydrogen sulfate buffer, pH 2, the product was taken up in ethyl acetate, then in 0·5 mol $l^{-1}$ sodium hydrogen carbonate and, after acidification with cold 1 mol $l^{-1}$ HCl, again in ethyl acetate. The ethyl acetate solution was washed with water, dried, evaporated and crystallized from ethyl acetate–light petroleum to give 2·73 g (81%) of the product, m.p. 127°C. $R_F$ 0·80 (S1), 0·17 (S2), 0·78 (S3), 0·66 (S4), $k' = 4{\cdot}44$ (50% methanol). An analytical sample was crystallized from ethyl acetate–light petroleum; m.p. 128°C; $[\alpha]_D$ −27·8° (*c* 0·3, methanol). For $C_{16}H_{23}N_3O_5$ (337·4) calculated: 56·95% C, 6·87% H, 12·45% N; found: 56·94% C, 6·76% H, 12·46% N.

Tert-butyloxycarbonylglutamic Acid α-Phenylhydrazide γ-Methyl Ester

Tert-butyloxycarbonylglutamic acid α-phenylhydrazide (0·5 g) was esterified with diazomethane. Crystallization from ethyl acetate–light petroleum gave 440 mg (84·5%) of the title product, m.p. 83°C, $R_F$ 0·78 (S1), 0·75 (S2), 0·75 (S3), 0·77 (S4); $k' = 7{\cdot}61$ (50% methanol). An analytical sample was crystallized from ethyl acetate–light petroleum, m.p. 85°C; $[\alpha]_D$ −25·5° (*c* 0·3, methanol). For $C_{17}H_{25}N_3O_5$ (351·4) calculated: 58·10%C, 7·17% H, 11·96% N; found: 57·64% C, 7·13% H, 12·01% N.

Tert-butyloxycarbonylglutamic Acid α-Phenylhydrazide γ-N-Methylamide

Methylamine hydrochloride (136 mg) and 1-hydroxybenzotriazole (271 mg) were added to a solution of tert-butyloxycarbonylglutamic acid α-phenylhydrazide (675 mg) in dimethylformamide (5 ml). The mixture was cooled to −15°C, made alkaline with N-ethylpiperidine, mixed with dicyclohexylcarbodiimide (413 mg) and stirred at −10°C for 1 h and at room temperature overnight. After evaporation of dimethylformamide, the residue was dissolved in ethyl acetate and the separated dicyclohexylurea filtered. The filtrate was washed successively with hydrogen sulfate buffer (pH 2), water, 0·5 mol $l^{-1}$ sodium hydrogen carbonate and water, dried and taken down. The residue was crystallized from ethyl acetate–light petroleum; yield 270 mg (38·5%) of the product, m.p. 171–172°C. The melting point did not change on further crystallization. $R_F$ 0·67 (S1), 0·59 (S2), 0·65 (S3), 0·70 (S4); $k' = 4·47$ (50% methanol); $[\alpha]_D$ −23·7° (*c* 0·3, methanol). For $C_{17}H_{26}N_4O_4$ (350·4) calculated: 58·27% C, 7·48% H, 15·99% N; found: 58·33% C, 7·58% H, 15·67% N.

Tert-butyloxycarbonylglutamic Acid α-Phenylhydrazide γ-Anilide

Aniline (90 μl) and 1-hydroxybenzotriazole (136 mg) were added to a solution of tert-butyloxycarbonylglutamic acid α-phenylhydrazide (338 mg) in dimethylformamide (2·5 ml) and the mixture was cooled to −15°C. After addition of N-ethylpiperidine and dicyclohexylcarbodiimide (210 mg), the mixture was worked up as described in the preceding experiment. Crystallization from ethyl acetate–light petroleum gave 180 mg (43·5%) of the product, m.p. 75–80°C which narrowed to 78–80°C on recrystallization from the same solvent mixture. $R_F$ 0·69 (S1), 0·59 (S2), 0·70 (S3), 0·74 (S4); $k' = 4·98$ (50% methanol); $[\alpha]_D$ −69·4° (*c* 0·3, methanol). For $C_{22}H_{28}N_4O_4 \cdot H_2O$ (430·5) calculated: 61·37% C, 7·02% H, 13·02% N; found: 61·38% C, 7·06% H, 12·51% N.

Tert-butyloxycarbonylglutaminyl-leucine Phenylhydrazide

Ethylenediaminetetraacetic acid (1·5 mg), followed by cysteine hydrochloride (5 mg), was added to a solution of tert-butyloxycarbonylglutamine (124 mg) and leucine phenylhydrazide trifluoroacetate (168 mg) in a 0·2 mol $l^{-1}$ acetate buffer, pH 4·8 (5 ml). The solution was adjusted to pH 4·8 with 4 mol $l^{-1}$ NaOH. After addition of papain (10·5 mg), the mixture was incubated at 37°C for 24 h. The precipitate was filtered and washed in succession with 20% citric acid, water, 0·5 mol . $l^{-1}$ sodium hydrogen carbonate, water and ether, affording 143 mg (63·5%) of the product, m.p. 196°C which did not rise upon crystallization from methanol–ether; $[\alpha]_D$ −47·7° (*c* 0·3, methanol). Amino acid analysis: Glu 1·01, Leu 1·00. $k' = 4·08$ (60% methanol); $R_F$ 0·74 (S1), 0·68 (S2), 0·75 (S3), 0·76 (S4). For $C_{22}H_{35}N_5O_5$ (449·6) calculated: 58·77% C, 7·85% H, 15·58% N; found: 58·90% C, 7·73% H, 15·27% N.

Cyclization of Glutamine Phenylhydrazide by Action of Papain under Various Reaction Conditions

The reaction was studied on an analytical scale by HPLC (25% methanol). For Gln-$N_2H_2$—$C_6H_5$ $k'$ 1·25, for Glp-$N_2H_2$—$C_6H_5$ $k' = 2·42$. The samples were taken after 10, 60, 120 and 240 min. For studies of the pH-dependence the following buffers were used: 0·2 mol $l^{-1}$ acetate buffer (for pH 4·5 and 5·0), 0·2 mol $l^{-1}$ Tris-maleate buffer (pH 6·0, 7·0, 8·0) and 0·2 mol $l^{-1}$ carbonate-bicarbonate buffer (pH 9·0).

A solution of glutamine phenylhydrazide trifluoroacetate (35·5 mg) in a given buffer (1 ml) was adjusted to the required pH. To half of this solution was added papain (1 mg), the second half was used without the enzyme as a blank. The incubations were carried out at 37°C. For the

study with organic solvents we used 0·1 mol$^{-1}$ NaOH as an aqueous medium to which the given organic solvent (100 or 200 µl) was added. The reactions were carried out at pH 6·5 in a total volume of 0·5 ml, the concentration of all the components being as given above. Simultaneously, the reaction was run in the absence of solvent. The effect of the enzyme–substrate ratio was studied in a Tris-maleate buffer, pH 7·0.

Cyclization of Glutamine and Its Peptides on Analytical Scale

The starting compound (0·1 mmol) was dissolved in 0·05 or 0·1 mol l$^{-1}$ NaOH (1 ml) and the solution was adjusted to pH 7·0—7·2. Papain (1 mg) was added to one half of this solution whereas the second half was used as a blank and the mixture was incubated at 37°C for the time specified in Table IV. The reactions were followed by HPLC (0—40% of methanol in the mobile phase). For Gln $k' = 0·33$ (0% methanol), Glp $k' = 2·66$ (0% methanol), Gln-Gly $k' = 0·71$ (0% methanol), Glp-Gly $k' = 2·50$ (0% methanol), Gln-Gly-Gly $k' = 0·92$ (0% methanol), Glp-Gly-Gly $k' = 2·55$ (0% methanol), Gln-Leu-$N_2H_2$—$C_6H_5$ $k' = 4·41$ (40% methanol), Glp-Leu-$N_2H_2$—$C_6H_5$ $k' = 5·86$ (40% methanol), Gln-His-Pro-$NH_2$ $k' = 1·03$ (10% methanol), Glp-His-Pro-$NH_2$ $k' = 2·36$ (10% methanol).

Cyclization of Glutamic Acid Derivatives

Papain (1 mg) was added to one half of a solution of the starting compound (0·1 mmol) in 0·05 or 0·1 mol l$^{-1}$ NaOH (1 ml), the other half being used as a blank. Incubation at 37°C for 4—24 h. Glutamic acid α-phenylhydrazide trifluoroacetate ($k' = 1·89$; 25% methanol) was tested at pH 4·5 and 6·8, glutamic acid α-phenylhydrazide γ-methyl ester trifluoroacetate ($k' = 4·88$; 25% methanol) at pH 4·5, 6·8 and 8·8, glutamic acid α-phenylhydrazide γ-anilide trifluoroacetate ($k' = 1·06$; 30% methanol) at pH 6·8. Glutamic acid α-phenylhydrazide γ-methylamide trifluoroacetate ($k' = 1·73$; 30% methanol) was cyclized at pH 6·9 for 24 h; in this last case, pyroglutamic acid phenylhydrazide was detected also by TLC.

Experiments with Iodoacetamide-Inhibited Crude Papain

Crude papain (2 mg) was dissolved in 10 mmol l$^{-1}$ aqueous iodoacetamide (100 µl) and the solution was set aside for 15 min at room temperature. *A*) The solution (50 µl) was added to 0·1 mmol l$^{-1}$ glutamine phenylhydrazide trifluoroacetate in 0·2 mol l$^{-1}$ Tris-maleate buffer, pH 7·0 (0·5 ml). The mixture was incubated for 2 h at 37°C. According to HPLC, the reaction proceeded as in the case of uninhibited papain. *B*) The papain solution (50 µl) prepared as described above was added to a solution of $N^{\alpha}$-benzoylarginine ethyl ester hydrochloride (0·1 mol l$^{-1}$) in the same buffer (0·5 ml) as described under *A*). The mixture was incubated for 1 h at 37°C. As found by HPLC, the substrate was not cleaved (for Bz-Arg-OEt $k' = 1·98$ (50% methanol), for Bz-Arg-OH $k' = 0·78$ (50% methanol)). The same experiment was performed with uninhibited papain. Samples, taken after 5 min and 75 min showed 50% and 90% cleavage, respectively.

Pyroglutamic Acid Phenylhydrazide

The tert-butyloxycarbonyl group was removed from the protected glutamine derivative with trifluoroacetic acid. Papain (20 mg) was added to a solution of glutamine phenylhydrazide trifluoroacetate (351 mg) in 0·1 mol l$^{-1}$ NaOH (10 ml) which had been adjusted to pH 7·5. After incubation at 37°C for 2 h, the mixture was filtered through a column of Dowex 50 (10 ml) and the eluate was freeze-dried. The residue was triturated with ether, affording 185 mg (84·4%) of the product, m.p. 184°C. $R_F$ 0·51 (S1), 0·34 (S2), 0·51 (S3), 0·63 (S4). $k' = 2·42$ (25% methanol).

A sample was crystallized from methanol–ether; m.p. 190–191°C; $[\alpha]_D$ −12·1° (*c* 0·3, 1M-HCl). For $C_{11}H_{13}N_3O_2.0{\cdot}5\ H_2O$ (228·2) calculated: 57·89% C, 6·18% H, 18·41% N; found: 58·07% C, 6·05% H, 18·34% N.

Pyroglutamyl-leucine Phenylhydrazide

The tert-butyloxycarbonyl group was removed from tert-butyloxycarbonylglutaminyl-leucine phenylhydrazide by treatment with trifluoroacetic acid. The obtained peptide trifluoroacetate (80 mg) was dissolved in 0·1 mol $l^{-1}$ NaOH (1·7 ml) and papain (3·5 mg) was added at pH 7·5. After incubation at 37°C for 2 h, the mixture was filtered through a column of Dowex 50 (2 ml), the eluate was freeze-dried and the residue triturated with ether to give 50 mg (87·4%) of the product, m.p. 182–184°C. $R_F$ 0·64 (S1), 0·59 (S2), 0·66 (S3), 0·70 (S4). $k' = 5{\cdot}86$ (40% methanol). A sample was crystallized from methanol–ether; m.p. 186–187°C; $[\alpha]_D$ −39·3° (*c* 0·3, methanol). Amino acid analysis: Glu 1·00, Leu 0·96. For $C_{17}H_{24}N_4O_3.H_2O$ (350·4) calculated: 58·27% C, 7·48% H, 15·99% N; found: 58·11% C, 7·01% H, 16·01% N.

Tert-butyloxycarbonylglutaminyl-histidyl-proline Amide

A solution of histidyl-proline amide hydrobromide (333 mg) in dimethylformamide (4 ml) was cooled to 0°C and made alkaline with N-ethylpiperidine. Tert-butyloxycarbonylglutamine *p*-nitrophenyl ester (368 mg) was added and the mixture was stirred at 0°C for 2 h and at room temperature for 20 h. After evaporation of dimethylformamide, the residue was triturated with light petroleum and ethyl acetate to give 610 mg of product which was further purified by HPLC. The pertinent fractions were freeze-dried and the product was triturated with ether. Yield 186 mg (39%) of the title compound, m.p. 127–129°C; $k' = 5{\cdot}04$ (25% methanol); $R_F$ 0·14 (S1), 0·23 (S2), 0·10 (S3), 0·51 (S4). Amino acid analysis: His 1·00, Glu 1·01, Pro 0·90. $[\alpha]_D$ −40° (*c* 0·25; methanol). For $C_{21}H_{33}N_7O_6.CF_3COOH.2\ H_2O$ (629·6) calculated: 43·87% C, 6·08% H, 15·57% N; found: 43·76% C, 5·78% H, 15·70% N.

Pyroglutamyl-histidyl-proline Amide Trifluoroacetate

Tert-butyloxycarbonylglutaminyl-histidyl-proline amide (126 mg) was dissolved in trifluoroacetic acid (1 ml). After standing at room temperature for 40 min, the solution was coevaporated with toluene. Trituration of the residue with ether afforded 132 mg of the product; $E_{2.4}^{Gly}$ 1·14, $E_{5.7}^{His}$ 1·19.

A solution of this compound (100 mg) in 0·1 mol $l^{-1}$ NaOH (2 ml) was incubated with papain (4 mg) at 37°C for 2 h (pH 6·9) and worked up using either of the following procedures. *A*) A part (1 ml) of the mixture was purified by HPLC (5% of methanol in the mobile phase). The product-containing fraction was freeze-dried and triturated with ether, affording 11 mg (30%) of the compound; $R_F$ 0·05 (S1), 0·14 (S3), 0·03 (S3), 0·27 (S4). $k' = 2{\cdot}36$ (10% methanol); $[\alpha]_D$ −48·1° (*c* 0·4; water). Amino acid analysis: Glu 1·07, Pro 1·00, His 0·96. The product was chromatographically identical with the sample prepared according to ref.[10]. *B*) A part (1 ml) of the mixture was filtered through a column of Dowex 50 (0·5 ml). The column was washed with water, the eluate was freeze-dried and the residue was triturated with ether to give 20 mg (54·5%) of the product, identical with the compound obtained by procedure *A*).

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REFERENCES

1. Schröder E., Lübke K.: *The Peptides*, Vol. II. Academic Press, New York 1966.
2. Jakubke H.-D., Jeschkeit H.: *Aminosäuren — Peptide — Proteine*. Akademie-Verlag, Berlin, 1982
3. *Biochemical Nomenclature and Related Documents*. International Union of Biochemistry, London 1978.
4. *Nomenclature and Symbolism for Amino Acids and Peptides*. Recommendation 1983. Eur. J. Biochem. *138*, 9 (1984).
5. Messer M.: Nature (London) *197*, 1299 (1963).
6. Messer M., Ottesen M.: Biochim. Biophys. Acta *92*, 409 (1964).
7. Messer M., Ottesen M.: C. R. Trav. Lab. Carlsberg *35*, 1 (1965).
8. Bernfield M. R., Nestor L.: Biochem. Biophys. Res. Commun. *33*, 843 (1968).
9. *Enzyme Nomenclature* 1978. Academic Press, New York 1979.
10. Kasafírek E., Semonský M., Felt V., Krejčí I.: This Journal *42*, 1903 (1977).

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