CHROM. 10,754

PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THYROTROPIN-RELEASING HORMONE ANALOGUES

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(Received November 14th, 1977)

SUMMARY

A high-performance liquid chromatographic system for the preparative separation of peptide mixtures using strongly acidic ion-exchange resins and volatile elution media is described. About 5 % of the effluent is subjected to continuous basic partial hydrolysis in order to increase the sensitivity and then detected with ninhydrin. Several examples have demonstrated that this method is suitable for the isolation of peptides with high purity from multi-component mixtures.

INTRODUCTION

Pyroglutamic acid (Pyr) peptides have received increasing attention recently because of their natural occurrence as biologically active compounds. Eledoisin (Pyr–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met–NH$_2$)[1], physalaemin (Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH$_2$)[2], thyrotropin-releasing hormone (Pyr–His–Pro–NH$_2$)[3] and follicle-stimulating hormone-releasing hormone (Pyr–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH$_2$)[4,5] are some examples of naturally occurring pyroglutamyl peptides.

Several syntheses of the tripeptide hormone Pyr–His–Pro–NH$_2$ (TRH) have been reported using glutamyl (Glu) or glutaminyl (Gln) peptides as intermediates. The first synthesis of TRH was achieved by the treatment of Glu(OCH$_3$)–His–Pro–OCH$_3$ with ammonia[3]. In the TRH syntheses of Rivaille and Milhaud[6], Folkers *et al.*[7] and Inouye *et al.*[8], Glu–His–Pro–NH$_2$ was treated with acid to give the desired tripeptide TRH. Several TRH syntheses were based on the discovery of König and Geiger[9] that N-4,4′-dimethoxybenzhydryl(Mbh)-L-glutamine derivatives form pyroglutamic acid in boiling trifluoroacetic acid. For example, Z–Gln(Mbh)–His–Pro–NH$_2$ (Z=benzyloxycarbonyl) can be converted into TRH in good yields in boiling trifluoroacetic acid[9].

In the natural occurring pyroglutamyl peptides, the Pyr residue is always attached to the N-terminal side of the chain. For investigations of the structure–activity relationship, Pyr^2–TRH or Pyr^3–TRH analogues are of general interest[10,11]. Therefore several tripeptides with Glu, Gln and isoglutaminyl (Isogln) residues at the C-terminal end or at position 2 were synthesized and treated with trifluoroacetic acid

(TFA) or hydrogen fluoride in order to achieve the formation of the pyroglutamic acid residue. In most instances, however, the crude products could not be purified by conventional methods used in peptide chemistry such as recrystallization, ion-exchange chromatography on CM-cellulose or partition chromatography on Sephadex. To solve this problem, we have developed a preparative high-performance liquid chromatographic separation system.

EXPERIMENTAL

Reagents for peptide synthesis

For the synthesis of the peptides, all solvents were purified according to ref. 12. Dimethylformamide (DMF) and tetrahydrofuran (THF) were products of Merck (Darmstadt, G.F.R.). All amino acids except pyroglutamic acid, which was a product of Aldrich-Europe (Beerse, Belgium), were purchased from Merck. For the syntheses of amino acid derivatives and peptides, the following reagents were used: dicyclohexylamine (Merck), dicyclohexylcarbodiimide (DCC, Merck), 2,4,5-trichlorophenol (Merck), isobutyl chloroformate (Merck), 1-hydroxybenzotriazole (Aldrich-Europe) and N-hydroxysuccinimide (Fluka, Buchs, Switzerland). *tert.*-Butyloxycarbonyl(Boc) azide and benzyloxycarbonyl chloride were synthesized according to refs. 13 and 14. For thin-layer chromatography, Kieselgel 60 F_{254} plates (Merck) and the solvent systems *n*-butanol–acetic acid–water (3:1:1) and chloroform–methanol–benzene–water (60:40:40:5) were used.

Syntheses of the peptide materials

TRH (Pyr–His–Pro–NH$_2$). TRH was synthesized as described in ref. 15.

D-*His^2–TRH (L-Pyr–D-His–L-Pro–NH$_2$).* The synthesis of D-*His^2*–TRH was carried out according to ref. 16.

Phe^2–TRH (Pyr–Phe–Pro–NH$_2$). The synthetic tripeptide[11] had the following characteristics: m.p., 182–185°; $[\alpha]_D^{23}$, −43.06° (c = 1.46/MeOH).

Reaction products after treatment of Boc–Phe–Glu(OBzl)–Pro–NH$_2$ with trifluoroacetic acid and hydrogen fluoride[11]. The starting material Boc–Phe–Glu(OBzl)–Pro–NH$_2$ had the following characteristics: m.p., 57–61°; $[\alpha]_D^{23}$, −15.3° (c = 1.04/DMF). Elemental analysis: calculated, C 64.12, H 6.94, N 9.65%; found, C 63.00, H 7.12, N 8.66%.

A 400-mg amount of Boc–Phe–Glu(OBzl)–Pro–NH$_2$ was dissolved in 45 ml of TFA, the mixture was refluxed for 2.5 h, evaporated to dryness and the residue subjected to chromatography. In a second study, 580 mg of Boc–Phe–Glu(OBzl)–Pro–NH$_2$ were dissolved in 45 ml of liquid hydrogen fluoride and the mixture was stirred for 2 h at room temperature. After evaporation, the product was dried at 10^{-2} torr over solid potassium hydroxide and separated by chromatography. To obtain the reference peptide H–Phe–Glu(OH)–Pro–NH$_2$, Boc–Phe–Glu(OBzl)–Pro–NH$_2$ was treated with TFA at room temperature for 90 min. After evaporation to dryness, the resulting product, H–Phe–Glu(OBzl)–Pro–NH$_2$, was saponified with a 2 M excess of potassium hydroxide for 90 min. The subsequent neutralization was effected with 1 N hydrochloric acid. After evaporation to dryness, the product was separated by chromatography.

Reaction products after treatment of Boc–Pro–Phe–Isogln(OBzl) with tri-fluoroacetic acid[11]. The starting material Boc–Pro–Phe–Isogln(OBzl) had the following characteristics: m.p., 158–162°; $[\alpha]_D^{23}$, −57.17° (c = 1.2/DMF). Elemental analysis: calculated, C 64.12, H 6.94, N 9.65%; found, C 63.27, H 7.12, N 9.90%.

A 250-mg amount of Boc–Pro–Phe–Isogln(OBzl) was refluxed in 35 ml of TFA for 2.5 h. After evaporation to dryness, the product was subjected to chromatographic separation.

Mass spectrometry

All mass spectra were obtained with a Varian-MAT F11 instrument operating at 70 eV.

Materials for chromatographic separation

Pyridine was distilled over solid potassium hydroxide and then at least three times over ninhydrin in order to remove all impurities that react with the reagent. For the separations, the cation-exchange resin DC-1A (Durrum, Palo Alto, Calif., U.S.A.), which is an 8% crosslinked polystyrene of particle size 18 $\pm$ 3 μm, was used. Pyridine–acetic acid buffers were prepared from pyridine and deionized, doubly distilled water. The pH was adjusted with distilled acetic acid. For partial hydrolysis after chromatographic separation, 5 N sodium hydroxide solution was used. The ninhydrin solution was prepared from a mixture of the reagent prepared by Spackman et al.[17] and distilled acetic acid (1:1). The peptide mixture (up to 250 mg) was dissolved in 2–5 ml of the starting buffer.

Amino acid analysis

The fractions from the fraction collector were evaporated to dryness, hydrolysed with 6 N hydrochloric acid at 110° for 12 h and used for amino acid analysis.

RESULTS AND DISCUSSION

Fig. 1 shows a schematic diagram of the preparative peptide analyser, developed in collaboration with Biotronik, Frankfurt, G.F.R.

A Milton Roy Dosapro micro-pump, which is connected with the buffer reservoirs, magnetic valves and an air-bubble trap, pumps the pyridine–acetic acid buffers (1.5 ml/min) via a 2 $\times$ 3-way valve on a Biotronik glass-jacketed high-performance glass column (550 $\times$ 9 mm) filled with DC-1A cation-exchange resin. To the effluent from the column, distilled water is added (1:2.5) in order to reduce the loss of separated peptides. From this diluted effluent a small amount (0.2 ml/min) is used for detection. After dilution with 5 N sodium hydroxide solution (1:2), partial hydrolysis in a PTFE reaction coil (20 m $\times$ 0.7 mm) at 100° is carried out and the mixture is subjected to reaction with ninhydrin reagent. The colour is finally developed in a second PTFE reaction coil (30 m $\times$ 0.7 mm) at 100° and detected and recorded with a Biotronik photometer at 570 nm. About 95% of the effluent is brought to a fraction collector.

Figs. 2 and 3 show chromatograms of synthetic TRH and TRH derivatives with an N-terminal pyroglutamic acid residue. The synthetic steps used for the samples subjected to chromatographic separation are also given in Figs. 2 and 3. The

chromatograms demonstrate the high purity of TRH and the derivatives and thus the suitability of the strategy chosen for their synthesis. The chromatogram of D-His²-TRH shows clearly that the compound is contaminated with about 8% of the natural hormone (L-Pyr-L-His-L-Pro-NH₂).
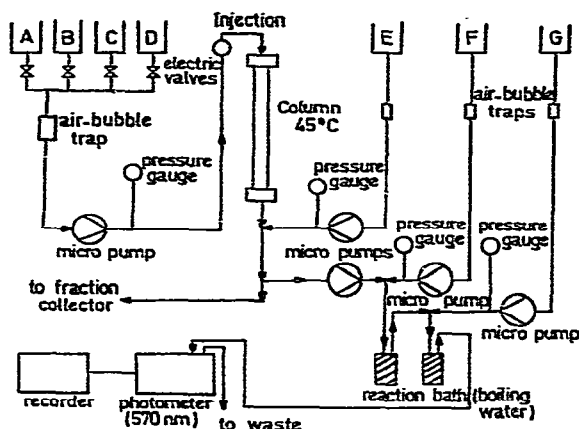

Fig. 1. Schematic diagram of the peptide analyser. A, B, D, D = Buffer reservoirs; E = water reservoir; C = 5 N NaOH reservoir; D = ninhydrin reagent–acetic acid (1:1) reservoir.
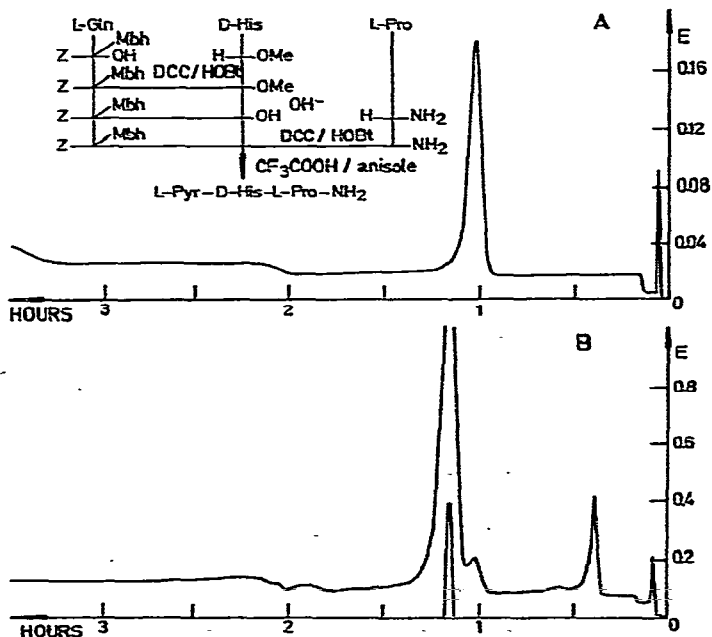

Fig. 2. Preparative chromatograms of TRH and D-His²–TRH. Column: 550 × 9 mm, DC-1A resin (18 ± 3 μm). Elution buffers: A, 0.1 M pyridine acetate, pH 3.2 (15 min); B, 0.2 M pyridine acetate, pH 3.5 (45 min); C, 0.3 M pyridine acetate, pH 4.0 (60 min); D, 0.6 M pyridine acetate, pH 4.2 (60 min); E, 1.0 M pyridine acetate, pH 4.6 (80 min). Flow-rate, 1.5 ml/min; back-pressure, 70–90 bar; temperature, 43°; detection with ninhydrin (570 nm) after partial hydrolysis (5 N NaOH). Samples: A, 8.7 mg; B, 250 mg. Sensitivity: A, 0.2 a.u.; B, 1.0 a.u.
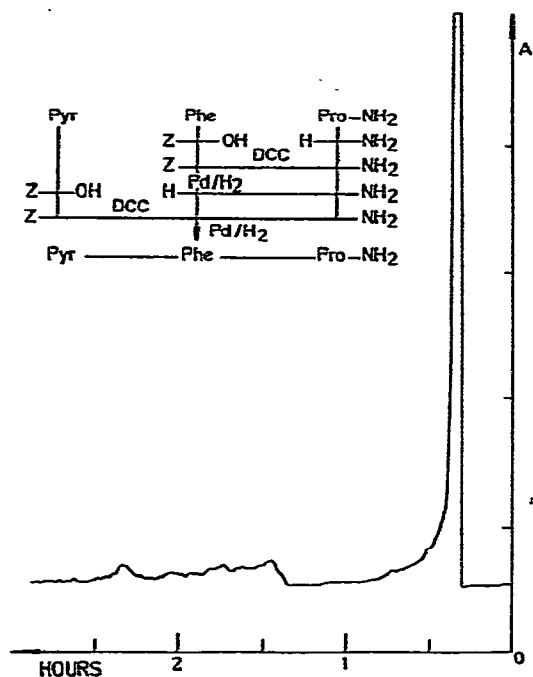
Fig. 3. Chromatogram of synthetic Phe^2–TRH. Column: 550 × 9 mm, DC-1A resin. Pyridine–acetic acid buffers: A, 0.2 M pyridine, pH 3.4 (60 min); B, 0.5 M pyridine, pH 4.25 (120 min); C, 1.0 M pyridine, pH 5.0 (120 min); D, 4.0 M pyridine, pH 5.6 (180 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 = 4.4% loss of sample for detection; recorder range, 0–0.5 A; amount injected, 150 mg.

According to several workers[10], D-His^2–TRH should have considerable biological activity. However, we were able to demonstrate that the chromatographically pure D-His^2–TRH is completely inactive. Hence this example clearly shows the suitability of our chromatographic system for separating peptide diastereomers.

Contrary to the synthesis of Pyr^1-tripeptides, that of Pyr^2-tripeptides seems to be much more difficult, as is demonstrated in Figs. 4–6.

Figs. 4 and 5 demonstrate the wide variety of compounds that are formed when Glu^2-tripeptides react with boiling TFA (Fig. 4) or hydrogen fluoride (Fig. 5). Therefore, it is not surprising that the reaction of TFA or hydrogen fluoride with Boc–Phe–Glu(OBzl)–Pro–NH$_2$ yields reaction products that could not be purified by crystallization[11]. Under similar conditions, Glu^1-tripeptides form Pyr^1-tripeptides in large amounts. The formation of the pyroglutamic acid ring seems to be sterically hindered in Glu^2-tripeptides and fission and rearrangement products seem to be formed instead (Fig. 7).

According to amino acid analysis, many peaks correspond to fractions with the expected amino acid content Phe–Glu–Pro = 1:1:1 (Figs. 4 and 5, solid line). However, most of the fractions contain peptides with protected amino functions and therefore give rise to no peaks in the chromatogram performed without partial hydrolysis (Fig. 4).
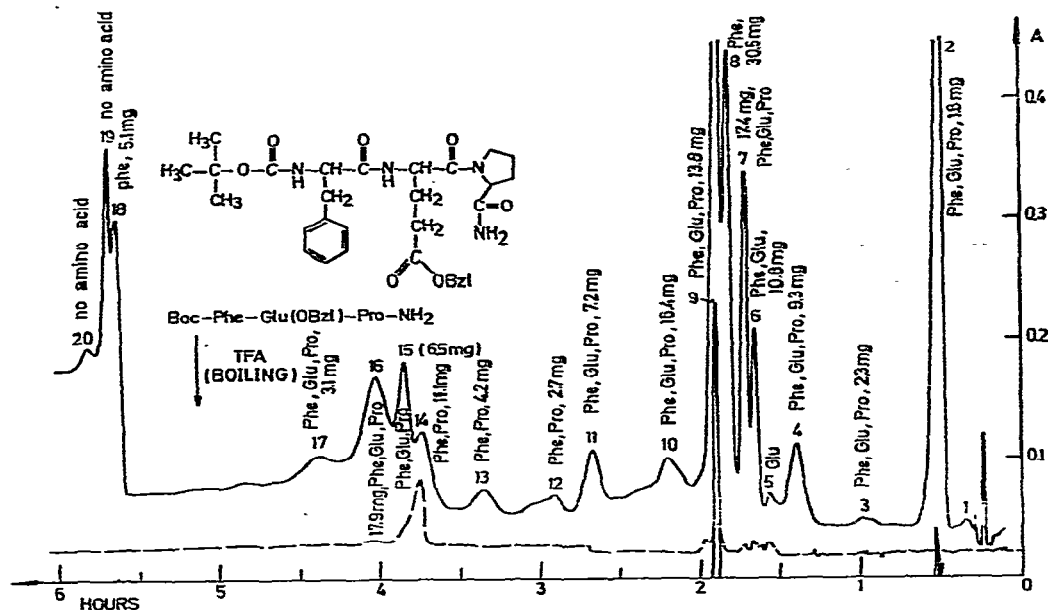
Fig. 4. Chromatogram of the reaction mixture of Boc–Phe–Glu(OBzl)–Pro–NH₂ after treatment with boiling TFA (2.5 h). Column: 550 × 9 mm, DC-1A resin. Pyridine–acetic acid buffers: A, 0.2 M pyridine, pH 3.4 (60 min); B, 0.5 M pyridine, pH 4.25 (120 min); C, 1.0 M pyridine, pH 5.0 (120 min); D, 4.0 M pyridine, pH 5.6 (180 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 = 4.4% loss of sample for detection; recorder range, 0–0.5 A. Solid line: amount injected, 166 mg; separated material collected, 155 mg; detection after partial hydrolysis. Broken line: amount injected, 109 mg; detection without partial hydrolysis.
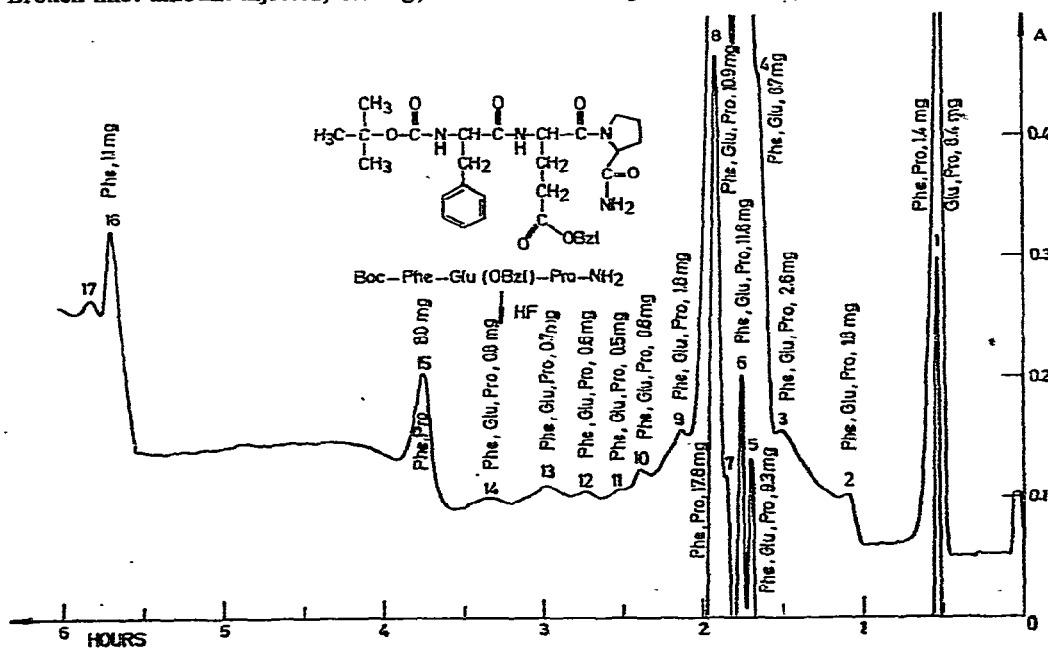

Fig. 5. Chromatogram of the reaction mixture of Boc–Phe–Glu(OBzl)–Pro–NH₂ after treatment with hydrogen fluoride. Column: 550 × 9 mm, DC-1A resin. Pyridine–acetic acid buffers: A, 0.2 M pyridine, pH 3.4 (60 min); B, 0.5 M pyridine, pH 4.25 (120 min); C, 1.0 M pyridine, pH 5.0 (120 min); D, 4.0 M pyridine, pH 5.6 (180 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 = 4.4% loss of sample for detection; recorder range, 0–0.5 A; amount injected, 139.5 mg; separated material collected, 86.1 mg.
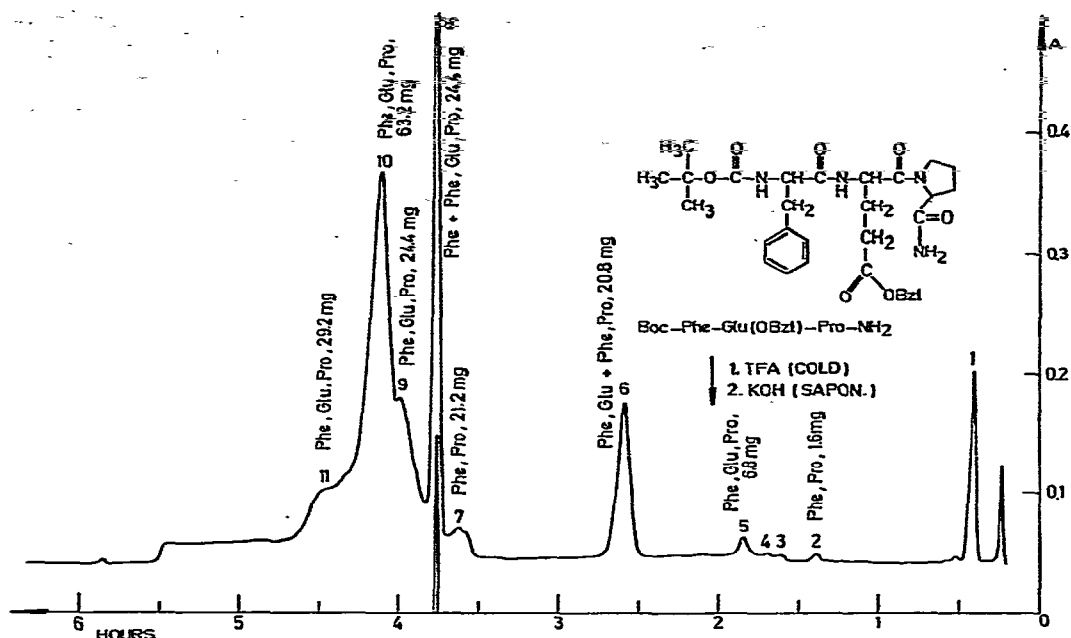
Fig. 6. Chromatogram of the reaction mixture of Boc–Phe–Glu(OBzl)–Pro–NH₂ after treatment (1) with TFA in the cold and (2) with 1 N KOH solution. Column: 550 × 9 mm, DC-1A resin. Pyridine–acetic acid buffers: A, 0.2 M pyridine, pH 3.4 (60 min); B, 0.5 M pyridine, pH 4.25 (120 min); C, 1.0 M pyridine, pH 5.0 (120 min); D, 4.0 M pyridine, pH 5.6 (180 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 = 4.4% loss of sample for detection; recorder range, 0–0.5 A; amount injected, 207 mg; separated material collected, 192 mg.
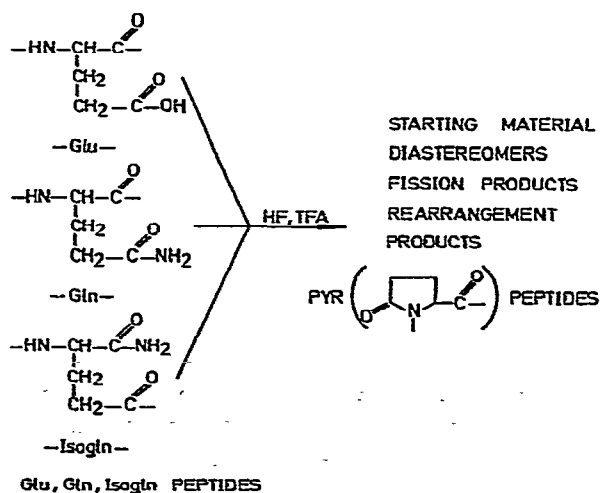

Fig. 7. Possible reaction products of Glu, Gln and Isogln peptides.

Common reactions in peptide chemistry are cleavage of the *tert.*-butyloxy-carbonyl group by dichloromethane–TFA or saponification of esters with $1\,N$ potassium hydroxide solution. Boc–Phe–Glu(OBzl)–Pro–NH$_2$ was subjected to both reactions and the resulting product separated by ion-exchange chromatography (Fig. 6). Although the chromatogram in Fig. 6 shows a much smaller number of peaks than those in Figs. 4 and 5, it demonstrates clearly that commonly used peptide reagents may also lead to cleavage of the peptide chain.

The products of several fractions corresponding to peaks in the chromatograms in Figs. 4 and 5 were investigated by mass spectrometry. These studies proved unequivocally that peak 7 in Fig. 4 is caused by the pyroglutamylpeptide Phe–Pyr–Pro–NH$_2$ (Fig. 8).
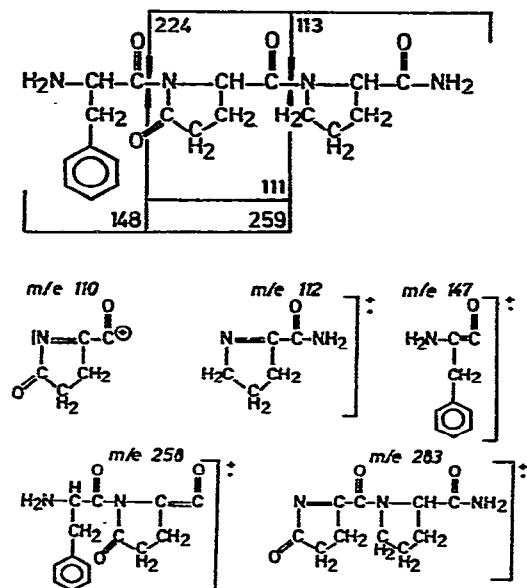

Fig. 8. Principal ions formed upon electron impact of the peptide fraction corresponding to peak 7 in Fig. 4.

The mass spectrum of the peptide fraction corresponding to peak 7 in Fig. 4 shows ions at m/e 110, 111, 112, 113, 147, 148, 224, 258, 259 and 283. The peaks at m/e 111, 224 and 259 are characteristic of Pyr, Pyr–Pro–NH$_2$ and Phe–Pyr moieties.

These investigations clearly demonstrate that Glu^2-tripeptides form partly Pyr^2-tripeptides in boiling TFA, which can be separated by the chromatographic technique described here.

Contrary to glutamyl^2-tripeptides, C-terminal glutamyl^3-tripeptides form more readily the pyroglutamyl residue in boiling TFA, as is demonstrated in Fig. 9.

In boiling TFA, Boc–Pro–Phe–Isogln(OBzl) forms a product that shows one main peak (peak 6) in the chromatogram in Fig. 9. Mass spectrometric studies led to the unequivocal conclusion that this peak is caused by the pyroglutamyl peptide Pro–Phe–Pyr–NH$_2$ (see Fig. 10, the principal ions upon electron impact of the peptide fraction corresponding to peak 6 in Fig. 9).
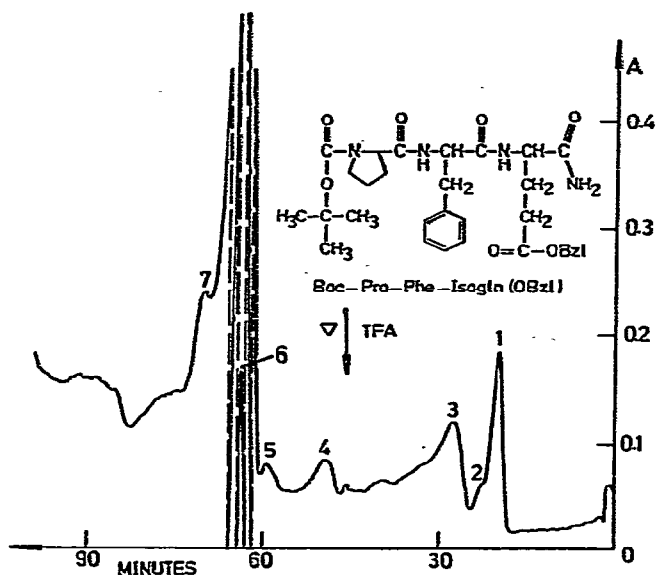
Fig. 9. Chromatogram of the reaction mixture of Boc–Pro–Phe–Isogln(OBzl) after treatment with boiling TFA (3 h). Column: 550 × 9 mm, DC-1A resin. Pyridine acetic acid buffers: A, 0.2 M pyridine, pH 3.2 (30 min); B, 0.5 M pyridine, pH 4.6 (30 min); C, 1.0 M pyridine, pH 5.9 (60 min); D, 4.0 M pyridine, pH 5.2 (60 min). Flow-rate, 1.5 ml/min; temperature, 45°; pressure, 80–95 bar; ratio of effluent splitting, 1:22 = 4.4% loss of sample for detection; recorder range, 0–0.5 A; amount injected, 200 mg.
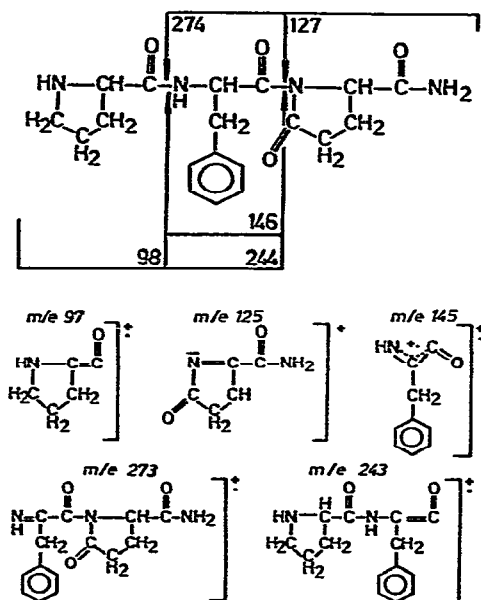

Fig. 10. Principal ions formed upon electron impact of the peptide fraction corresponding to peak 6 in Fig. 9.

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