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STUDIES ON POLYPEPTIDES

XXI. Synthesis of Four Protected Peptide Fragments corresponding to Parts of the N-terminal 1–17 Sequence of Rat Pancreatic Ribonuclease*

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Syntheses of derivatives of the peptide sequences required for the synthesis of the N-terminal 1–17 part of rat pancreatic ribonuclease are described. These protected peptides are:

$$\overset{OBu^tBu^t}{\underset{|\ \ |}{\ }}\qquad\qquad\qquad\qquad\overset{OBu^tBu^tBu^t\quad OBu^t}{\underset{|\ \ |\ \ |\quad\ \ |}{\ }}$$

Boc-Gly-Glu-Ser-Arg-OH (sequence 1–4); Nps-Glu-Ser-Ser-Ala-Asp-OH

$$\overset{Boc\quad Boc}{\underset{|\qquad |}{\ }}$$

(sequence 5–9); Nps-Lys-Phe-Lys-Arg-OH (sequence 10–13) and

$$\overset{OBu^t}{\underset{|}{\ }}$$

Nps-Gln-His-Met-Asp-OBu^t (sequence 14–17).

In a previous communication we reported on the capacity of the 5–17 and 1–17 peptide sequences of rat pancreatic ribonuclease to bind to bovine S-protein, and on the enzymatic activities of the resulting complexes (Voskuyl-Holtkamp *et al.*, 1976). These sequences have been synthesized via convergent coupling of the appropriate peptide fragments. This report describes the synthesis in solution of the fully protected peptide fragments Nps-Gln-His-Met-Asp-(OBu^t)-OBu^t (sequence 14–17), Nps-Lys (Boc)-Phe-Lys(Boc)-Arg-OH (sequence 10–13), Nps - Glu(OBu^t) - Ser(Bu^t) - Ser(Bu^t) - Ala - Asp (OBu^t)-OH (sequence 5–9) and Boc-Gly-Glu

* All amino acids, exept glycine, were of the L-configuration. The following abbreviations are used: RNase S, subtilisin modified bovine pancreatic ribonuclease; S-protein, protein component obtained from bovine RNase S; S-peptide, polypeptide obtained from RNase S. The abbreviations for the amino acids and protecting groups are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, *Biochemistry* **11**, (1972) 1726–1732.
DCCI, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; HONSu, N-hydroxysuccinimide; HOBt, 1-hydroxybenzotriazole; TEA, triethylamine; DCHA, dicyclohexylamine; NEtM, N-ethylmorpholine; DMF, dimethylformamide; EtOAc, ethylacetate; Et_2O, ether; i-Pr_2O, diisopropyl ether; Pe, petroleum ether (40–60°); THF, tetrahydrofuran; TFA, trifluoroacetic acid; Met(O), methionine sulphoxide; Tos.OH, p-toluenesulphonic acid monohydrate; BMV, 2-benzoyl-1-methylvinyl; Nps, ortho-nitrophenysulphenyl.

Gly - Glu - Ser - Arg - Glu - Ser - Ser - Ala - Asp - Lys - Phe - Lys - Arg - Gln - His - Met - Asp - Thr - Glu - Gly - Pro - Ser - Lys
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

FIGURE 1

Amino acid sequence of rat "S-peptide", the 1–23 N-terminal part of rat pancreatic ribonuclease.

(OBu^t)-Ser(Bu^t)-Arg-OH (sequence 1–4) used in the synthesis of the 5–17 and 1–17 sequences of rat RNase. (Rat "S-peptide" Fig. 1). Figs. 2–5 provide orientation regarding the synthetic routes for the fragments.

The presence of Met in rat "S-peptide" forced us to protect the various functional side chains with Boc-, -OBu^t and -Bu^t groups that permit the use of mild acidolytic procedures for their removal. The imidazole group of His is left unprotected while the guanidine function of Arg is blocked as its "Zwitterion".

All fragments were synthesized by stepwise elongation starting from the C-terminal amino acid using DCCI/HOBt (König & Geiger, 1970) or HONSu-esters (Wünsch *et al.*, 1971; Itoh, 1972) in the coupling step. For protection of the α-amino function use was made of the Z-, BMV- and Nps-groups because these permit selective deblocking of this group at each step, by hydrogenolysis, treatment with Tos.OH (Southard *et al.*, 1971*a*) and HCl in dry MeOH (Poduška, 1968) respectively, without damage to the Boc-, -OBu^t and -Bu^t protecting groups. Purification of the protected peptides was achieved by crystallization or column chromatography. Homogeneity was carefully checked by thin-layer chromatography in different solvent systems and in appropriate cases by electrophoresis at different pH's.

In addition the purity of the four fragments was confirmed after deblocking with TFA in the presence of anisole. The resulting free peptides were homogeneous, as checked by thin-layer and paper chromatography and electrophoresis at different pH's and had the correct amino acid composition.

The synthesis of sequence 14–17 (Fig. 2) was achieved by stepwise elongation starting from H-Asp(OBu^t)-OBu^t.HCl applying the DCCI/HOBt (König & Geiger, 1970) procedure. As the presence of Met precluded convenient protection of the α-amino groups as their Z-derivatives, use was made of the BMV- and Nps-groups. The intermediate BMV-derivatives were deblocked with Tos.OH (Southard *et al.*, 1971*a*). In our first synthesis the imidazole side chain of His was protected by the tosyl group according to Sakakibara *et al.* (1969) but partial detosylation in the following deblocking and condensing steps forced us to leave the imidazole function unprotected (Fujii & Sakakibara, 1974; Schwartz & Katsoyannis, 1973).

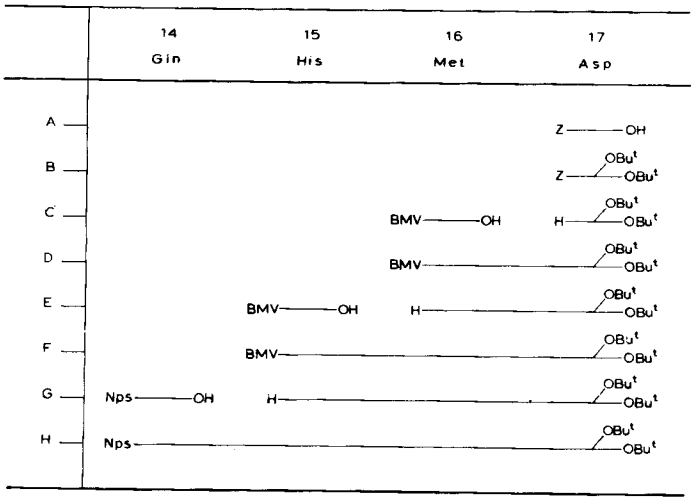

FIGURE 2

Outline of the synthesis of the protected peptide fragment 14–17.

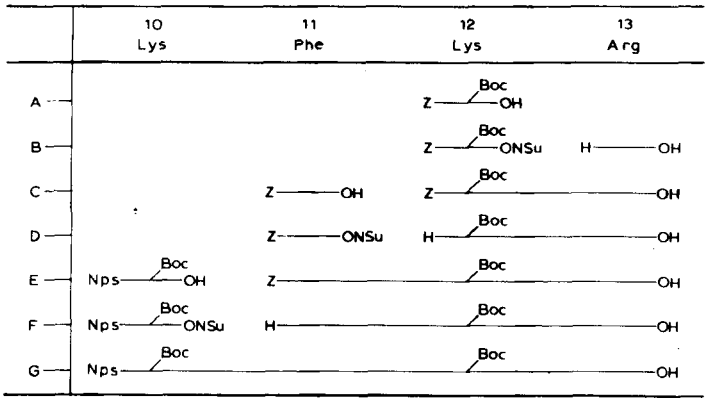

FIGURE 3
Outline of the synthesis of the protected peptide fragment 10–13.

Fig. 3 shows the synthesis of sequence 10–13 where the stepwise active ester method was used. Starting with free Arg we coupled successively with the HONSu-esters of Z-Lys(Boc)-OH, Z-Phe-OH and Nps-Lys(Boc)-OH in the presence of carbon dioxide and a tertiary base (Wünsch *et al.*, 1971; Itoh, 1972). The intermediate Z-derivatives were deblocked by catalytic hydrogenolysis.

The synthesis of sequence 5–9 presented no

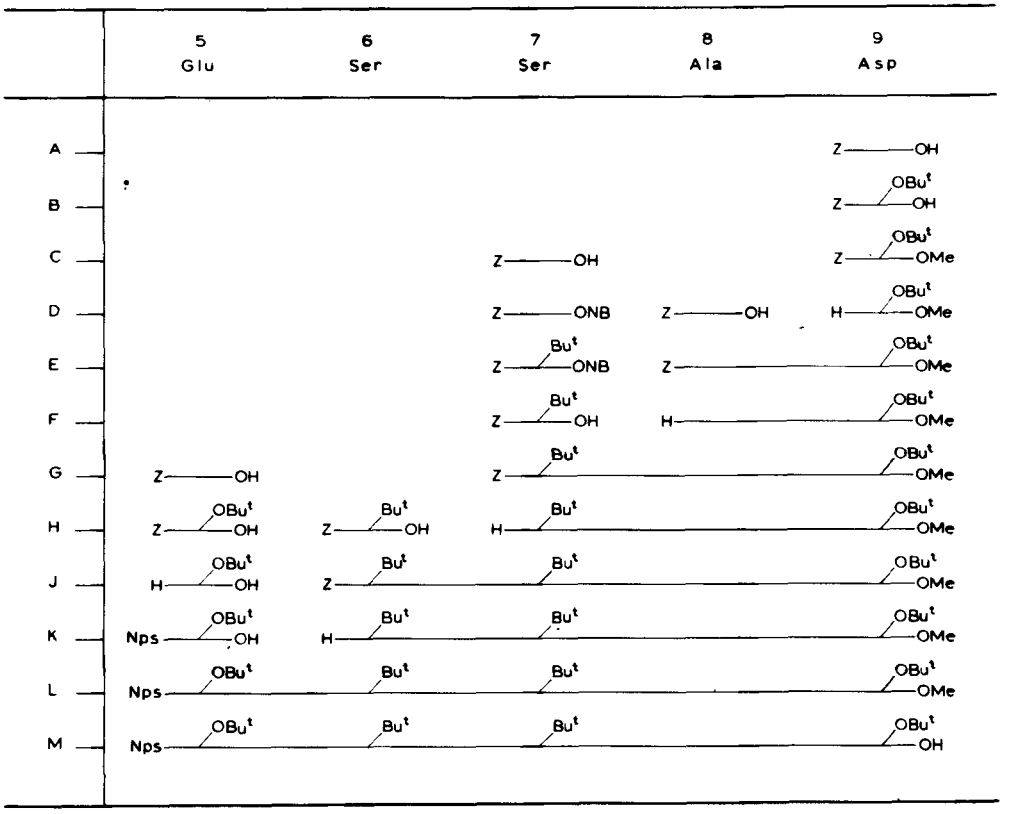

FIGURE 4
Outline of the synthesis of the protected peptide fragment 5–9.

special problems (Fig. 4). We chose DCCI/HOBt (König & Geiger, 1970) as condensing agent. The N^{α}-amino function was protected by the Z-group except for Glu where the Nps-group was used. The α-carboxyl group of Asp was converted into the methylester. The Z-groups were removed by catalytic hydrogenolysis and the C-terminal methylester was selectively saponified in the final step with the pH-stat at pH 11.4, analogous to the method of Wünsch *et al.* (1967).

The synthesis of sequence 1–4 was originally effected as for sequence 10–13 using HONSu-esters in the coupling step, but because of difficulties in the purification of the intermediates and end product, which lowered the yield considerably, a more efficient synthesis was devised (Fig. 5). The tetrapeptides prepared by these two routes were identical as judged by thin-layer chromatography, electrophoresis and optical rotation. H-Arg(NO_2)-OMe.HCl was stepwise condensed with BMV-Ser(Bu^t)-OH, BMV-Glu(OBu^t)-OH and Boc-Gly-OH applying the DCCI/HOBt (König & Geiger, 1970) procedure. The intermediate BMV-derivatives were deblocked with Tos.OH (Southard *et al.*, 1971*a*). Finally the fully protected tetrapeptide was hydrolysed under alkaline conditions and the nitro group was removed by catalytic hydrogenolysis. The subsequent paper will deal with the construction and purification of the 5–17 and 1–17 sequences of rat RNase.

EXPERIMENTAL PROCEDURES

Melting points were determined with the apparatus designed by Tottoli and are uncorrected. The optical rotations (room temperature) were measured photoelectrically with a Perkin-Elmer instrument model P-141.

Thin-layer chromatograms (t.l.c.) were run on silicagel GF-254 (Merck), paper chromatograms on Whatman No. 1 filter paper (descending technique) in the following systems: 1) $CHCl_3$-MeOH 9:1; 2) EtOH-benzene 3:7; 3) $CHCl_3$-MeOH-AcOH 85:10:5; 4) n-BuOH-AcOH-H_2O 4:1:1; 5) n-BuOH-AcOH:pyridine-H_2O 15:3:10:12; 6) sec.BuOH-3% NH_3 aq. 7:3; 7) $CHCl_3$-MeOH 8:2; (8) $CHCl_3$-MeOH 7:3;

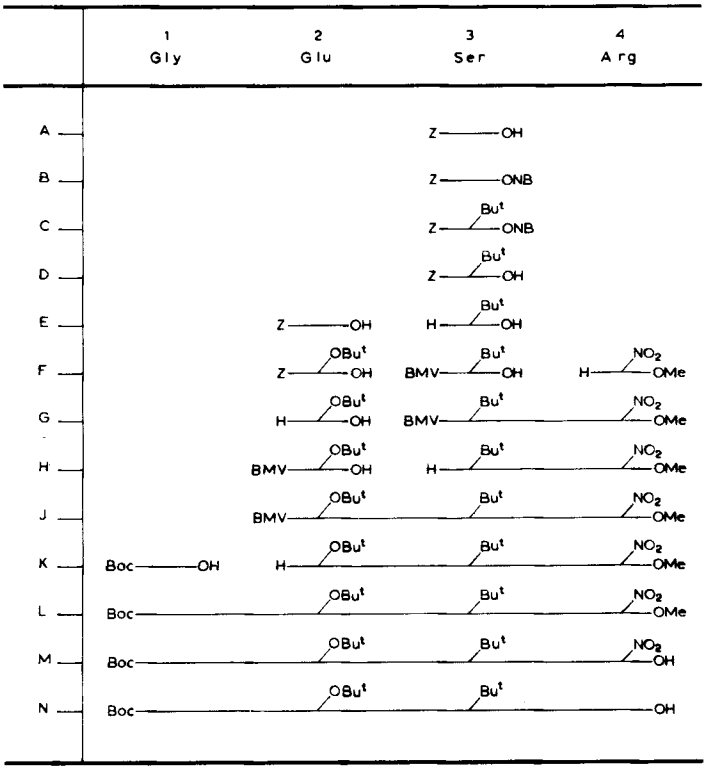

FIGURE 5
Outline of the synthesis of the protected peptide fragment 1–4.

9) $CHCl_3$-acetone 7:3; 10) $CHCl_3$-acetone 6:4.

Paper electrophoresis was carried out with a refrigerated Pherograph, type Mini 65 on MN paper No. 214 at 1500V and pH 1.7 (AcOH-formic acid-H_2O 150:50:800), pH.4.8 (AcOH-pyridine-H_2O 3:4.1:993) and pH 6.5 (AcOH-pyridine-H_2O 4:100:896). On chromatography and electrophoresis (50–150 μg amounts were spotted) the compounds were detected by u.v.-light (254 nm) and by spraying with the following reagents: ninhydrin (N), Reindel-Hoppe (Reindel & Hoppe, 1954) after chlorination (RH), Barton (Hais & Macek, 1963) for Met (B), Pauly (Stahl, 1967) for His (P) and phenantrene quinone (Yamada & Itano, 1966) for Arg (Guan).

Paper chromatographic mobilities are given relative to Tyr or His; electrophoretic mobilities relative to Phe (at pH 1.7), Lys (at pH 4.8) and His (at pH 6.5). Amino acid ratios are given in an acid hydrolysate, with Nle as an internal standard. All evaporations were performed *in vacuo* at 30–40° C. Hydrogenolyses were carried out with 0.5–1.0 g Pd 5% on C per 10 mmol of peptide derivative, in abs.EtOH at atmospheric pressure and room temperature unless otherwise stated. The completeness of the reductions was checked by thin-layer chromatography (u.v., N, RH). Unless otherwise stated, a 5% aqueous solution of potassium hydrogen sulfate was used (Spangenberg *et al.*, 1971) for acidification of DCHA salts of acid labile protected amino acids.

Synthesis of Nps-Gln-His-Met-Asp(OBu^t)-OBu^t (sequence 14–17) (Fig. 2)

Z-Asp-OH (A 17). Prepared from Asp on reaction with Z-chloride the pH being maintained at 9.8 by means of a pH-stat. The mixture was worked up as described (Bergman & Zervas, 1932). Yield 90%, m.p. 115–116°(dec), $(\alpha)_D$ + 8.5 (c 2, AcOH), homogeneous on t.l.c.-3 (u.v., N, RH). Lit. (Bergman & Zervas, 1932) m.p. 116° (dec), $(\alpha)_D$ + 9.6 (c 7.1, AcOH).

Z-Asp(OBu^t)-OBu^t (B 17). 13.4 g (50 mmol) of Z-Asp-OH were dissolved in 200 ml of dry CH_2Cl_2 containing 1.4 ml of conc. H_2SO_4 as a catalyst. At −20° 100 ml (1000 mmol) of isobutene were added and the solution was stirred in a stoppered flask at 5° for 4 days. After addition of 12 ml of TEA to the cooled solution the solvent was evaporated. The residue was dissolved in EtOAc and the organic layer was extracted with $KHSO_4/K_2SO_4$ (pH2) solution (Spangenberg *et al.*, 1971), M sodium bicarbonate solution and saturated sodium chloride solution. The oily product remaining after evaporation of the dried (Na_2SO_4) EtOAc layer was pure on t.l.c.-1, 3 (u.v., RH). Yield 12.9 g (68%).

H-Asp(OBu^t)-OBu^t (C 17). 12.9 g (34 mmol) of B17 were hydrogenated. The oily residue remaining after evaporation was pure on t.l.c.-1, 3 (N, RH). The product was dissolved in dry MeOH in the presence of 1 eq. of 0.03 N HCl, the solution was evaporated to a small volume and precipitation with Et_2O gave 6.9 g (72%) of crystalline C 17, m.p. 151°–152° (dec), $(\alpha)_D$ + 6.5 (c 1, MeOH) homogeneous on t.l.c.-1,3,4,5, 6 (N, RH) and on electrophoresis: $E^{1.7}_{Phe}$ 1,10, $E^{4.8}_{Lys}$ 0.79 (N, RH). Lit. (Taschner *et al.*, 1961) m.p. 152–154°.

BMV-Met-Asp(OBu^t)-OBu^t (D 16–17). To a stirred mixture of 11.6 g (24.5 mmol) of BMV-Met-OH.DCHA (Southard *et al.*, 1971*b*), 6.9 g (24.5 mmol) of C 17 and 3.8 g (28 mmol) of HOBt (König & Geiger, 1970) in 250 ml of CH_2Cl_2 cooled to 0°, 5.4 g (26 mmol) of DCCI were added. After 1 h at 0° and 18 h at room temperature the filtered solution was evaporated. The residue dissolved in EtOAc was extracted successively with H_2O, 2.2 M potassium bicarbonate solution and saturated sodium chloride solution. The dried (Na_2SO_4) solution was evaporated to dryness and yielded 12.4 g (97%) of the oily dipeptide derivative, pure on t.l.c.-1 (u.v., RH, B).

H-Met-Asp(OBu^t)-OBu^t.Tos.OH (E 16–17). 4.2 g (22 mmol) of Tos.OH were added to 12.4 g (23.8 mmol) of D 16–17 (Southard *et al.*, 1971*a*) in 750 ml of CH_2Cl_2 at 0°. The mixture was kept at room temperature overnight. The solvent was evaporated and the oily residue dissolved in water. The aqueous phase was extracted with two portions of Et_2O which were washed with two portions of water. The combined aqueous phases were lyophilised and yielded 11.0 g (85%) of E 16–17, m.p. ~ 35° (dec), homogeneous on t.l.c.-1 (N, RH, B) and

on electrophoresis: $E^{1.7}_{Phe}$ 0.87, $E^{4.8}_{Lys}$ 0.67 (N, RH, B).

BMV-His-OH.DCHA (E 15). Prepared using a slight modification of the procedure described by Southard *et al.* (1971*b*). 15.5 g (100 mmol) of His and 16.2 g (100 mmol) of benzoylacetone were refluxed in 100 ml of H_2O. After dropwise addition of 20 ml of DCHA (105 mmol) in 100 ml of EtOH the mixture was refluxed for 3 h before evaporation. EtOH was added to the residue, the solution was evaporated and the procedure repeated several times. Before final evaporation the EtOH insoluble, unreacted H-His-OH was filtered off (< 2%). The residue was dissolved in CH_2Cl_2 and the insoluble BMV-His-OH that is easily formed by decomposition of BMV-His-OH.DCHA was filtered off. The product, precipitated by the addition of Et_2O to the filtrate, was air-dried to prevent elimination of DCHA. Yield 82%, m.p. 162–164° (dec), $(\alpha)_D$ – 122° (c 0.5 DMF), pure on t.l.c.-6 (u.v., RH, P).

BMV-His-Met-Asp(OBu^t)-OBu^t (F 15–17). 5.3 g (11 mmol) of BMV-His-OH.DCHA were dissolved in EtOH and the insoluble BMV-His-OH (0.4 g) was filtered off. After evaporation of the filtrate 4.9 g (10.2 mmol) of BMV-His-OH.DCHA remained as an oil. This was coupled with 5.5 g (10 mmol) of E 16–17 in CH_2Cl_2 by the DCCI/HOBt (König & Geiger, 1970) procedure as described for D 16–17. The oily residue remaining after evaporation of the dried EtOAc was further purified by chromatography on silica (Merck, Kieselgel 60, 0.063–0.200 mm), using $CHCl_3$-MeOH 99:1–92:8 as eluent. Crystallization from CH_2Cl_2/i-Pr_2O gave 3.6 g (55%) of the trepeptide, m.p. 83°–84° (dec), $(\alpha)_D$ – 5.4 (c 1, MeOH), t.l.c.-1 (u.v., RH, B, P) one spot.

H-His-Met-Asp(OBu^t)-OBu^t.2Tos.OH (G 15–17). To a stirred solution of 3.9 g (6 mmol) of F 15–17 in 200 ml of CH_2Cl_2 2.3 g (12 mmol) of Tos.OH were added at 0° (Southard *et al.*, 1971*a*). After standing overnight at room temperature the solution was evaporated to dryness and the oily residue was triturated with i-Pr_2O several times. Precipitation from CH_2Cl_2 with i-Pr_2O yielded 4.5 g (87%) of G 15–17. Homogeneous on t.l.c.-4,5,6,8 (N, RH, B, P) and on electrophoresis: $E^{1.7}_{Phe}$ 1.44, $E^{4.8}_{Lys}$ 0.81 (N, RH, B, P).

Nps-Gln-OH.DCHA (G 14). Prepared from Nps-chloride and Gln at pH 8.5 in dioxan-H_2O 2:1 and isolated as its DCHA salt in accordance with the literature (Zervas *et al.*, 1963; Wünsch & Fontana, 1968). Yield 60%, m.p. 186–188° (dec), homogeneous on t.l.c.-3.6 (u.v., RH).

Nps-Gln-His-Met-Asp(OBu^t)-OBu^t (H 14–17). 2.5 g (5.3 mmol) of G 14 and 4.4 g (5.1 mmol) of G 15–17 in 50 ml of DMF were coupled after addition of 0.65 ml (5.1 mmol) of NEtM by the DCCI/HOBt method (König & Geiger, 1970) as above. After purification by short-column chromatography (Hunt & Rigby, 1967), $CHCl_3$-MeOH 8:2 serving as eluent, the tetrapeptide was crystallized from abs. EtOH, yield 45%, m.p. 178–183°, $(\alpha)_D$ –32.0 (c 1, DMF), homogeneous on t.l.c.-7 (u.v., RH, H, P).

10 mg of H 14–17 were completely deblocked by exposure to 1 ml of TFA containing anisole for 1 h. After evaporation the residue was suspended in dry toluene followed by re-evaporation. This procedure was repeated and the residue triturated with Et_2O. Filtration gave a white amorphous solid, the free tetrapeptide. T.l.c.-5; one major spot R_f 0.33 (N, RH, B, P) and a trace of the Met(0) derivative R_f 0.16 (N, RH, P). Paper chromatography 5: homogeneous R_f Tyr 0.48 (N, B, P). Electrophoresis: one major spot $E^{1.7}_{Phe}$ 1.41, $E^{4.8}_{Lys}$ 0.16, $E^{6.5}_{His}$ 0.00 (N, RH, B, P). In both cases traces of the sulphoxide as well as of the pyroglutamyl derivative could be spotted. Amino acid ratios in the free tetrapeptide: Gln 0.98; His 1.05; Met 0.96; Asp 1.00.

Synthesis of Nps-Lys(Boc)-Phe-Lys(Boc)-Arg-OH (Sequence 10–13) (Fig. 3)

Z-Lys(Boc)-Arg-OH (C 12–13). A stirred solution of 2.9 g (17 mmol) of Arg and 2.38 ml (17 mmol) of TEA in a mixture of H_2O (40 ml) and dioxan (40 ml) was adjusted to pH 8 by the addition of dry ice and 8.1 g (17 mmol)

of Z-Lys(Boc)-ONSu (Anderson *et al.*, 1964) in 40 ml of dioxan were added. After 2 h standing at room temperature the coupling reaction was complete (t.l.c.-3,4). The pH of the mixture was adjusted to 8.5 (TEA) and the dioxan was evaporated. The product was extracted with several portions of nOBuOH and the combined extracts were washed with H_2O. Evaporation of the butanol laver and crystallization of the residue from n-BuOH/Et_2O gave 7.5 g (85%) of the dipeptide derivative, m.p. 90°–93°, $(\alpha)_D$ –4.3 (c 1, MeOH), homogeneous on t.l.c.-3.4,6 (u.v., N, 0.10 (u.v., N, RH, Guan). After recrystallization from EtOH/H_2O a product was isolated that crystallized with 2 mol of water: m.p. 105–108° (dec), $(\alpha)_D$ –3.5 (c 1, MeOH).

H-Lys(Boc)-Arg-OH (D 12–13). 6.6 g (12.3 mmol) of C 12–13 were hydrogenated in aqueous EtOH (50%). The filtered solution was evaporated and the oily residue was dried by several azeotropic distillations with EtOH. Yield 100% of an oily product, homogeneous on t.l.c.-4,6 (N, RH) and on electrophoresis: $E^{1.7}_{Phe}$ 1.20, $E^{4.8}_{Lys}$ 0.66 (N, RH, Guan) except a minor spot (~ 1%) from H-Lys-(Boc)-OH (N, RH).

Z-Phe-Lys(Boc)-Arg-OH (E 11–13). 5.08 g (12.8 mmol) of Z-Phe-ONSu (Anderson *et al.*, 1964) and 6.6 g (12.3 mmol) of D 12–13 were coupled for 18 h as described for C 12–13. The pH of the mixture was adjusted to 8.5 (TEA), EtOH was added and the solution evaporated to dryness. The residue was washed with EtOAc and crystallized twice from EtOH/H_2O: yield 6.6 g (78%), m.p. 120–124° (dec), $(\alpha)_D$ –8.9 (c 1, MeOH), homogeneous on t.l.c.-3,4,6 (u.v., N, RH) except a minor spot (~ 1%) from Z-Phe-Lys(Boc)-OH (u.v., N, RH).

H-Phe-Lys(Boc)-Arg-OH (F 11–13). 4.1 g (6 mmol) of E 11–13 were hydrogenated as described for D 12–13. Yield 100% of an oily product, homogeneous on electrophoresis: $E^{1.7}_{Phe}$ 1.28, $E^{4.8}_{Lys}$ 0.46 (N, RH, Guan) except a minor spot (~ 1%) from H-Phe-Lys(Boc)-OH (N, RH).

Nps-Lys(Boc)-OH.DCHA (E 10). Prepared from H-Lys(Boc)-OH (Zervas & Hamalidis, 1965) on reaction with Nps-chloride in dioxan-H_2O 1:1, the pH being maintained at 8 by means of a pH-stat and isolated as its DCHA salt in accordance with the literature (Zervas *et al.*, 1963; Wünsch & Fontana, 1968; Zervas & Hamalidis, 1965). Yield 76%, m.p. 187–189° (dec), $(\alpha)_D$ –12.9 (c 0.5, EtOH), t.l.c.-3 (u.v., N, RH) one spot. A sample was recrystallized from DMF, m.p. 195–196° (dec). Lit. (Zervas & Hamalidis, 1965) m.p. 194–195°. Just before use the salt was converted into the free acid with 1 eq. of NH_2SO_4 in the usual manner. Yield 90% of an oily product, pure on t.l.c.-3 (u.v., N, RH).

Nps-Lys(Boc)-ONSu (F 10). To 2.9 g (7.2 mmol) of E 10 and 0.83 g (7.2 mmol) of HONSu (Anderson *et al.*, 1964) in 20 ml of stirred and cooled (–5°) dimethoxyethane, 1.48 g (7.2 mmol) of DCCI were added. After standing overnight at 0° the solution was filtered. Evaporation of the solvent yielded an oily product which was used as such in the next step. T.l.c.-1, 3; one major spot (u.v., N, RH) and one minor spot (< 5%) of the free acid (E 10) (u.v., N, RH).

Nps-Lys(Boc)-Phe-Lys(Boc)-Arg-OH (G 10–13). 3.3 g (6 mmol) of F 11–13 and F 10 (obtained from 7.2 mmol of E 10) were coupled for 48 h as described for E 11–13. After adjusting the pH to 8.5 (TEA) the reaction mixture was evaporated to dryness. The residue dissolved in 100 ml of EtOAc of 40° was washed three times with H_2O. The dried solution (Na_2SO_4) was evaporated and the product crystallized from acetone/Et_2O, to yield 3.4 g of the tetrapeptide (60%), m.p. 200°–201° (dec), $(\alpha)_D$ –4.6 (c 1, MeOH), homogeneous on t.l.c.-3.8 (u.v., N, RH) except a minor spot (~ 1%) from Nps-Lys(Boc)-Phe-Lys(Boc)-OH (u.v., N, RH).

10 mg of G 10–13 were deblocked with TFA in the presence of anisole as described for H 14–17 (Fig. 2). T.l.c.-5: R_f 0.33 (N, RH). Paper chromatography 5: R_f Tyr 0.43 (N, RH, Guan). Electrophoresis: $E^{1.7}_{Phe}$ 2.40, $E^{4.8}_{Lys}$ 1.06, $E^{6.5}_{His}$ 2.04 (N, RH, Guan). Electrophoresis showed a minor spot (~ 1%) from H-Lys-Phe-Lys-OH; $E^{1.7}_{Phe}$ 1.37, $E^{4.8}_{Lys}$ 0.43, $E^{6.5}_{His}$ 1.10 (N, RH). Amino acid ratios: Lys 2.00; Phe 1.06; Arg 0.94.

Synthesis of Nps-Glu(OBu^t)-Ser(Bu^t)-Ser(Bu^t)-Ala-Asp(OBu^t)-OH.DCHA (Sequence 5–9) (Fig. 4)

Z-Asp(OBu^t)-OMe (C 9). An ethereal solution of diazomethane (obtained from 5.15 g (50 mmol) of nitrosomethylurea in the usual way (Arndt, 1943) was added dropwise to a stirred, cooled (0°) solution of 3.3 g (10 mmol) of Z-Asp(OBu^t)-OH, prepared as described by Itoh (1969), until gas evolution ceased and the solution acquired a pale yellow colour. After decomposing the excess of diazomethane with a drop of glacial acetic acid the ethereal solution was washed with M sodium bicarbonate solution and H_2O. After drying (Na_2SO_4) and evaporating the solvent C 9 was obtained as an oil. Yield 3 g (90%), one single spot on t.l.c.-3 (u.v., RH).

H-Asp(OBu^t)-OMe.Tos.OH (D 9). 8.4 g (25 mmol) of C 9 were hydrogenated in aqueous MeOH (90%), the pH being maintained at 4.5 (autotitrator) by addition of a 0.5 M Tos.OH solution. The reduction was complete within 30 min (t.l.c.-1). The filtered solution was evaporated and the residue was dried by several azeotropic distillations with benzene. Trituration of the oily residue with Et_2O gave 9.5 g (75%) of D 9, pure on t.l.c.-1 (N, RH), m.p. ~ 100° (dec). A sample was recrystallized from MeOH/i-Pr_2O; m.p. 119–121° (dec) $(\alpha)_D$ + 11.0 (c 1, MeOH), homogeneous on t.l.c.-1 (N, RH) and on electrophoresis: $E^{1.7}_{Phe}$ 1.80, $E^{4.8}_{Lys}$ 0.86 (N, RH).

Z-Ala-Asp(OBu^t)-OMe (E 8–9). To a stirred mixture of 2.23 g (10 mmol) of Z-Ala-OH (Bergmann & Zervas, 1932) 3.75 g (10 mmol) of D 9, 1.26 ml (10 mmol) of NEtM and 1.5 g (11 mmol) of HOBt (König & Geiger, 1970) in 75 ml of THF cooled to 0°, 2.3 g (10.5 mmol) of DCCI in 25 ml of THF were added. After 1 h at 0° and 18 h at room temperature the filtered solution was evaporated to dryness. The oily residue was dissolved in EtOAc and extracted with $KHSO_4/K_2SO_4$ (pH2) solution (Spangenberg *et al.*, 1971), half saturated sodium chloride solution, M sodium bicarbonate solution and saturated sodium chloride solution. After drying (Na_2SO_4) and evaporating the solvent the residue was crystallized from EtOAc/Pe. Yield 3.7 g (90%), m.p. 72–74° (dec), $(\alpha)_D$ –16.4 (c 1, MeOH), t.l.c.-1,2,3 (u.v., RH) one spot. A sample was recrystallized from EtOAc/Pe: m.p. 74–76° (dec), $(\alpha)_D$ –16.4 (c 1, MeOH).

H-Ala-Asp(OBu^t)-OMe.HCl (F 8–9). 4.1 g (10 mmol) of E 8–9 were hydrogenated in the presence of 1 eq. of 0.03 N HCl in dry MeOH. The filtered solution was evaporated to yield an oil (100%), homogeneous on t.l.c.-1 (N, RH) and on electrophoresis: $E^{1.7}_{Phe}$ 1.05, $E^{4.8}_{Lys}$ 0.73 (N, RH).

Z-Ser(Bu^t)-Ala-Asp(OBu^t)-OMe (G 7–9). 2.95 g (10 mmol) of Z-Ser(tBu)-OH, prepared as described by Wünsch *et al.* (1964; 1966), and 3.1 g (10 mmol) of F 8–9 in 75 ml of THF were coupled after addition of 1.26 ml (10 mmol) of NEtM by the DCCI/HOBt method (König & Geiger, 1970) in the same way as E 8–9. Crystallization from i-Pr_2O/Pe gave 4.6 g (83%) of the tripeptide derivative, homogeneous on t.l.c.-1,3,9 (u.v., RH). A sample was recrystallized from i-Pr_2O: m.p. 88–90° (dec), $(\alpha)_D$ –15.8 (c 1, MeOH).

H-Ser(Bu^t)-Ala-Asp(OBu^t)-OMe (H 7–9). 5.52 g (10 mmol) of G 7–9 were hydrogenated. The filtered solution was evaporated to dryness and yielded 4.2 g (100%) of H 7–9 as an oil. Homogeneous on t.l.c.-1 (N, RH) and on electrophoresis: $E^{1.7}_{Phe}$ 0.85, $E^{4.8}_{Lys}$ 0.64 (N, RH).

Z-Ser(Bu^t)-Ser(Bu^t)-Ala-Asp(OBu^t)-OMe (J 6–9). 2.95 g (10 mmol) of Z-Ser(tBu)-OH, prepared as described by Wünsch *et al.* (1964; 1966) and 4.2 g (10 mmol) of H 7–9 in 100 ml of THF were coupled in the same way as E 8–9. Crystallization from acetone/H_2O gave 5.9 g (85%) of the tetrapeptide derivative, m.p. 126–128° (dec), $(\alpha)_D$ –8.8 (c 1, MeOH), homogeneous on t.l.c.-1,2,9 (u.v., RH). A sample was recrystallized from acetone: m.p. 145–146° (dec), $(\alpha)_D$ –8.8 (c 1, MeOH).

H-Ser(Bu^t)-Ser(Bu^t)-Ala-Asp(OBu^t)-OMe (K 6–9). The tetrapeptide J 6–9 was hydrogenated. Yield 100% of an oil, single spot on t.l.c.-1 (N, RH) and on electrophoresis: $E^{1.7}_{Phe}$ 0.69, $E^{4.8}_{Lys}$ 0.49 (N, RH).

H-Glu(OBu^t)-OH (J 5). 5.2 g (10 mmol) of Z-Glu(OBu^t)-OH, prepared from Z-Glu-OH (Bloemhoff & Kerling, 1975) as described by Itoh (1969), were hydrogenated in EtOH-H_2O 7:3 following the method of Zervas & Hamalidis (1965). Yield 100%, m.p. 182–183° (dec), $(\alpha)_D$ + 9.7 (c 1, H_2O), homogeneous on t.l.c.-3,6 (N, RH) and on electrophoresis: $E^{1.7}_{Phe}$ 0.94, $E^{4.8}_{Lys}$ 0.05 (N) Lit. (Zervas & Hamalidis, 1965) m.p. 182° $(\alpha)_D$ + 9.82 (c 2, H_2O).

Nps-Glu(OBu^t)-OH (K 5). Prepared from H-Glu (OBu^t)-OH on reaction with Nps-chloride in dioxan-H_2O 2:1, the pH being maintained at 8.5 by means of a pH-stat and isolated as its DCHA salt in accordance with the literature (Zervas *et al.*, 1963; Wünsch & Fontana, 1968; Zervas & Hamalidis, 1965). Yield 70%, t.l.c.-3, 6 (u.v., RH) one spot. A sample was recrystallized from EtOH: m.p. 172°–173° (dec) $(\alpha)_D$ –22.6 (c 1, MeOH) Lit. (Zervas & Hamalidis, 1965) m.p. 179–180° (dec), $(\alpha)_D$ –25.0 (c 4, MeOH). Just before use the salt was converted into the free acid with 1 eq. of N H_2SO_4. Yield 95% of an oily product, pure on t.l.c.-3 (u.v., RH).

Nps - Glu(OBu^t) - Ser(Bu^t) - Ser(Bu^t) - Ala - Asp (OBu^t)-OMe (L 5–9). 2 g (5.7 mmol) of K 5 and 3.2 g (5.7 mmol) of K 6–9 were coupled by the DCCI/HOBt method (König & Geiger, 1970). After 1 h at 0° and 18 h at room temperature the DCU was filtered off and the filtrate evaporated to dryness. Crystallization of the oily residue from EtOAc gave 3.0 g (55%) of the pentapeptide derivative m.p. 185–190° (dec), $(\alpha)_D$ –21.3 (c 0.25, MeOH), homogeneous on t.l.c.-3.9 (u.v., RH). A sample was recrystallized from acetone: m.p. 199–200° (dec), $(\alpha)_D$ –23.5 (c 0.25, MeOH).

Nps - Glu(OBu^t) - Ser(Bu^t) - Ser(Bu^t) - Ala - Asp (OBu^t)-OH.DCHA (M 5–9). To a solution of 2.4 g (2.7 mmol) of the foregoing methylester in 33 ml of dioxan-MeOH-H_2O (5:5:1) N-sodiumhydroxide was added, the pH being maintained at 11.4 (autotitrator). The saponification was completed within 2 h (t.l.c.-1). The reaction mixture was adjusted to pH 8.5 with $KHSO_4/K_2SO_4$ (pH2) solution (Spangenberg, 1971), n-BuOH added and the resulting clear solution was evaporated to dryness.

The residue was suspended in EtOAc-H_2O and after cooling (0°) acidified to pH3 with 0.1 N H_2SO_4. The EtOAc layer was separated and washed with half saturated sodium chloride solution. After drying (Na_2SO_4) 0.53 ml (2.7 mmol) of DCHA was added. The precipitated DCHA salt crystallized from EtOAc. Yield 2.4 g (84%), m.p. ~ 205° (dec), $(\alpha)_D$ –9.3 (c 1, MeOH), homogeneous on t.l.c.-1.3 (u.v., RH).

10 mg of M 5–9 were deblocked with TFA in the presence of anisole as described for H 14–17 (Fig. 2). T.l.c.-4 R_f 0.10, t.l.c.-5 R_f 0.16 (N, RH). Paper chromatography 5: R_f Tyr 0.35 (N, RH). Electrophoresis: $E^{1.7}_{Phe}$ 0.73, $E^{4.8}_{Lys}$ –0.57, (N, RH). Amino acid ratios: Glu 0.99; Ser 1.72; Ala 1.03; Asp 0.98.

Synthesis of Boc-Gly-Glu(OBu^t)-Ser(Bu^t)-Arg-OH (Sequence 1–4) (Fig. 5)

BMV-Ser(Bu^t)-Arg(NO$_2$)-OMe (G 3–4). 6.3 g (13 mmol) of BMV-Ser(tBu)-OH.DCHA, prepared from H-Ser(Bu^t)-OH (Wünsch & Jentsch, 1964) as described by Southard *et al.* (1971*b*), and 3.5 g (13 mmol) of H-Arg(NO$_2$)-OMe.HCl (Hofmann *et al.*, 1956) were coupled in THF/DMF 2:1 by the DCCI/HOBt method (König & Geiger, 1970) as described for BMV-Met-Asp (OBu^t)-OBu^t (D 16–17 (Fig. 2)). After purification by short-column chromatography (Hunt & Rigby, 1967) ($CHCl_3$-acetone 7:3 serving as eluent) the dipeptide was crystallized from CH_2Cl_2/i-Pr_2O. Yield 4.1 g (60%), m.p. ~ 80° $(\alpha)_D$ + 151.0 (c 0.25, MeOH), homogeneous on t.l.c.-1,2,9 (u.v., N, RH).

H-Ser(Bu^t)-Arg(NO$_2$)-OMe.Tos.OH (H 3–4). 3.83 g (7.37 mmol) of G 3–4 were deblocked with 1.4 g (7.37 mmol) of Tos.OH (Southard *et al.*, 1971*a*) as described for G 15–17 (Fig. 2). Precipitation from CH_2Cl_2 with i-Pr_2O yielded 4.1 g (100%) of H 3–4, homogeneous on t.l.c.-1 (u.v., N, RH) and on electrophoresis: $E^{1.7}_{Phe}$ 1.00, $E^{4.8}_{Lys}$ 0.64 (u.v., N, RH).

BMV-Glu(OBu^t)-OH.DCHA (H 2). Prepared from H-Glu(OBu^t)-OH (J 5 (Fig. 4)) according to the method of Southard *et al.* (1971*b*). The product was crystallized from CH_2Cl_2/Pe.

Yield 89% of a white crystalline product, m.p. 139–140° (dec), $(\alpha)_D$ + 20.4 (c 1, MeOH), t.l.c.-6 (u.v., N, RH) one spot.

BMV-Glu(OBu^t)-Ser(Bu^t)-Arg(NO$_2$)-OMe (J 2–4). 4.0 g (7.5 mmol) of H 2 and 4.1 g (7.37 mmol) of H 3–4 were coupled in THF as described for G 3–4. After purification by short-column chromatography (Hunt & Rigby, 1967) ($CHCl_3$-acetone 6:4 serving as eluent) the tripeptide derivative was crystallized from CH_2Cl_2/i-Pr_2O, yield 3.6 g (70%), m.p. ~ 90° (dec), $(\alpha)_D$ + 68.3 (c 1, MeOH), homogeneous on t.l.c.-1,2,9 (u.v., N, RH).

H - Glu(OBu^t) - Ser(Bu^t) - Arg(NO$_2$) - OMe. Tos.OH (K 2–4). 3.28 g (4.65 mmol) of the foregoing tripeptide were deblocked with Tos. OH as described for H 3–4. Crystallization from CH_2Cl_2/i-Pr_2O gave 3.5 g (100%) of K 2–4, homogeneous on t.l.c.-1,4,5,6 (u.v., N, RH) and on electrophoresis: $E^{1.7}_{Phe}$ 0.75, $E^{4.8}_{Lys}$ 0.44 (u.v., N, RH).

Boc - Gly - Glu(OBu^t) - Ser(Bu^t) - Arg(NO$_2$) - OMe (L 1–4). 0.88 g (5 mmol) of Boc-Gly-OH (Schnabel, 1967) and 3.5 g (4.65 mmol) of K 2–4 were coupled after addition of 0.59 ml (4.65 mmol) of NEtM by the DDCI/HOBt method (König & Geiger, 1970) as described in the preceding experiments. Crystallization from EtOAc gave 2.2 g (66%) of the tetrapeptide derivative homogeneous on t.l.c.-1, 2,3 (u.v., N, RH) except a minor spot from DCU. A sample was recrystallized from EtOAc: m.p. ~ 90° (dec), $(\alpha)_D$ –15.3 (c 1, MeOH).

Boc - Gly - Glu(OBu^t) - Ser(Bu^t) - Arg(NO$_2$) - OH (M 1–4). 1.87 g (2.6 mmol) of the preceding methylester was treated with 2.6 ml N-aq. sodium hydroxide in 23 ml of MeOH for 3 h (using thymolphtaleine as an indicator (Wünsch & Jentsch, 1964)). The pH of the mixture was adjusted to 8 with $KHSO_4/K_2SO_4$ (pH2) solution (Spangenberg *et al.*, 1971), the MeOH was evaporated off and the remaining solution was extracted with EtOAc. The aqueous layer was acidified to pH 3 with $KHSO_4/K_2SO_4$ (pH2) solution and extracted with EtOAc. The combined extracts were washed several times with water, dried and evaporated. Crystallization from EtOAc/i-Pr_2O gave the acid M 1–4. Yield 1.2 g (66%), m.p. ~ 90° (dec), $(\alpha)_D$ –8.76 (c 1, MeOH), t.l.c.-3 (u.v., N, RH) one spot.

Boc - Gly - Glu(OBu^t) - Ser(Bu^t) - Arg - OH (N 1–4). 0.85 g (1.2 mmol) of M 1–4 were hydrogenated in 25 ml of 70% acetic acid for 18 h. The catalyst was filtered off and after adding H_2O to the filtrate, the solution was lyophilised. Yield 0.64 g (79%), m.p. 130–131° (dec), $(\alpha)_D$ –9.1 (c 1, MeOH), homogeneous on t.l.c.-4, 5,6 (u.v., N, RH)

10 mg of N 1–4 were deblocked with TFA in the presence of anisole as described for H 14–17 (Fig. 2). Paper chromatography 5:R_f His 0.65 (N, Guan, RH). Electrophoresis: $E^{1.7}_{Phe}$ 1.50, $E^{4.8}_{Lys}$ 0.18, $E^{6.5}_{His}$ 0.54. Amino acid ratios: Gly: 1.01; Glu 1.01; Ser 0.88; Arg 0.98.

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