Synthesis of Peptides Analogous to the *N*-Terminal Eicosapeptide Sequence of Ribonuclease A. Part III.[1] A New Synthesis of the *C*-Terminal Octapeptide 13—20

By **Fernando Marchiori, Raniero Rocchi, Luigi Moroder, Giorgio Vidali,** and **Ernesto Scoffone,** Istituto di Chimica Organica dell'Università, Sezione VIII del Centro Nazionale di Chimica delle Macromolecole del C.N.R., Padova, Italy

Synthesis is described of the octapeptide corresponding to the 13—20 sequence of the bovine pancreatic ribonuclease A. The pentapeptidebenzyloxycarbonyl-L-seryl-L-threonyl-L-seryl-L-alanyl-L-alanine t-butyl ester was built up by coupling, by the azide procedure, benzyloxycarbonyl-L-seryl-L-threonine hydrazide with L-seryl-L-alanyl-L-alanine t-butyl ester.

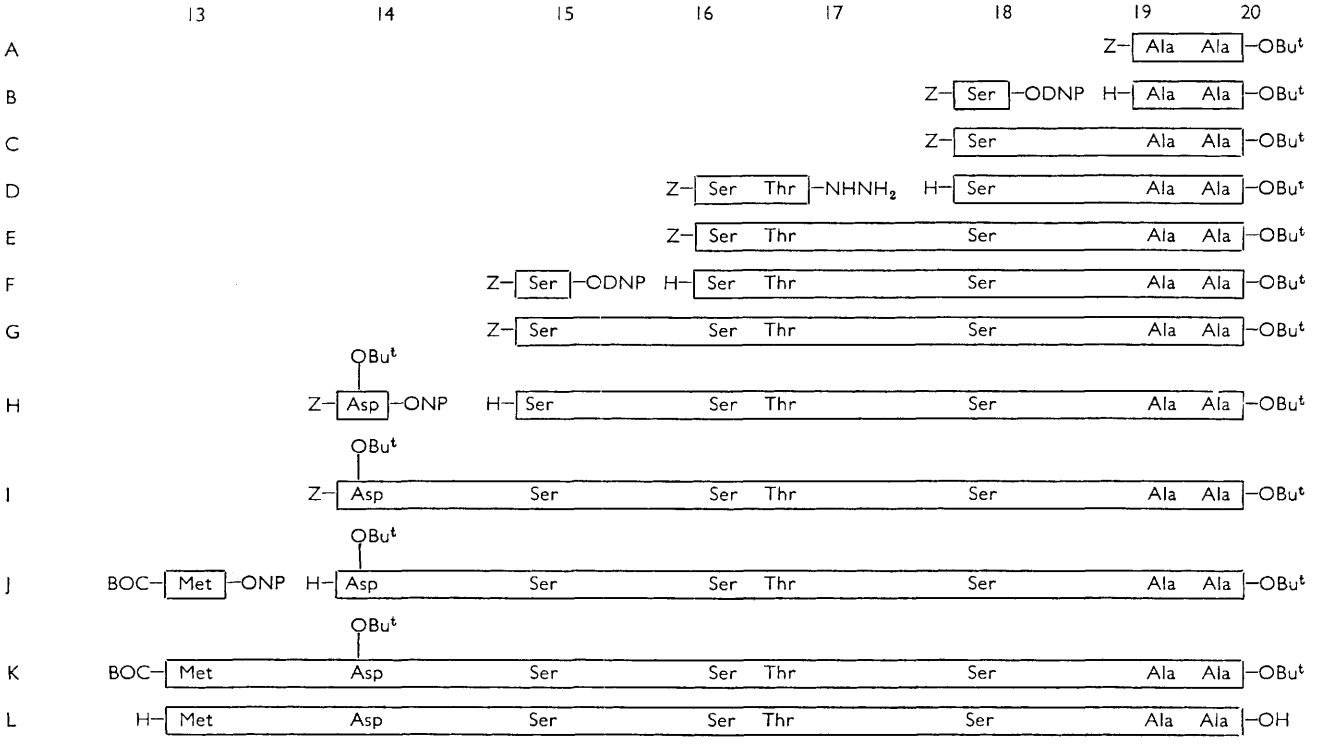
The subsequent elongation to the octapeptide was carried out stepwise by the *p*-nitrophenyl ester method. Exposure of the protected peptide to trifluoroacetic acid yielded L-methionyl-L-aspartyl-L-seryl-L-seryl-L-threonyl-L-seryl-L-alanyl-L-alanine.

We have described [2,3] the synthesis of the protected octapeptide corresponding to the *C*-terminal sequence of the S-peptide, *i.e.*, the eicosopeptide obtained from the subtilisin-modified bovine pancreatic ribonuclease A.

The synthetic route is shown in the Scheme.* For the synthesis of benzyloxycarbonyl-L-seryl-L-alanyl-L-alanine t-butyl ester (C, 18—20) the benzyloxycarbonyl-L-serine 2,4-dinitrophenyl ester [2] was coupled with L-alanyl-L-alanine t-butyl ester (B, 19—20), obtained by hydrogenolysis of benzyloxycarbonyl-L-alanyl-L-alanine t-butyl ester [4] (A, 19—20).

Scheme

Synthesis of the 13—20 sequence



Hofmann *et al.*[4] reported the preparation of the *C*-terminal nonapeptide of the S-peptide; they used the t-butyl ester as *C*-protecting group both for the α- and ω-carboxyl groups. We now report [5] new synthesis of the 13—20 sequence.

* The peptides and the peptide derivatives mentioned are of L-configuration. For a simpler description the customary L-designation for individual amino-acid residues is omitted. Z, Benzyloxycarbonyl; BOC, t-butyloxycarbonyl; OBu^t, t-butyl ester; ONP, *p*-nitrophenyl ester; ODNP, 2,4-dinitrophenyl ester.

[1] Parts I and II, preceding Papers.

[2] F. Marchiori, R. Rocchi, and E. Scoffone, *Gazzetta*, 1963, **93**, 834.

[3] E. Scoffone, F. Marchiori, A. M. Tamburro, and R. Rocchi, *Gazzetta*, 1964, **94**, 695.

[4] K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *J. Amer. Chem. Soc.*, 1965, **87**, 631.

[5] For a preliminary communication see E. Scoffone, F. Marchiori, R. Rocchi, G. Vidali, A. M. Tamburro, A. Scatturin, and A. Marzotto, *Tetrahedron Letters*, 1966, **9**, 943.

The protected tripeptide ester (C, 18—20) was hydrogenated and condensed by the azide procedure with benzyloxycarbonyl-L-seryl-L-threonine hydrazide [2] (D, 16—17) to give benzyloxycarbonyl-L-seryl-L-threonyl-L-seryl-L-alanyl-L-alanine t-butyl ester (E, 16—20).

The elongation to octapeptide (K, 13—20) was carried out by the stepwise procedure by the *p*-nitrophenyl ester method (2,4-dinitrophenyl ester for the serine residue).

The protected pentapeptide ester (E, 16—20) was hydrogenated and condensed with the activated ester of benzyloxycarbonyl-L-serine [2] to give benzyloxycarbonyl-L-seryl-L-seryl-L-threonyl-L-seryl-L-alanyl-L-alanine t-butyl ester [4] (G, 15—20) which, after hydrogenolysis, was coupled with α-*p*-nitrophenyl-β-t-butylbenzyloxycarbonyl-L-aspartic acid.

The benzyloxycarbonyl group was removed from the resulting benzyloxycarbonyl-β-t-butyl-L-aspartyl-L-seryl-L-seryl-L-threonyl-L-seryl-L-alanyl-L-alanine t-butyl ester [4] (I, 14—20) by hydrogenolysis and the ensuing partially protected eptapeptide (J, 14—20) was then allowed to react with t-butoxycarbonyl-L-methionine *p*-nitrophenyl ester [3,4] to give t-butoxycarbonyl-L-methionyl-β-t-butyl-L-aspartyl-L-seryl-L-seryl-L-threonyl-L-seryl-L-alanyl-L-alanine t-butyl ester (K, 13—20).

Treatment with trifluoroacetic acid transformed the protected octapeptide (K, 13—20) into L, 13—20.

EXPERIMENTAL

The melting points were determined by Tottoli's capillary apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. Acid hydrolyses were carried out in 6N-hydrochloric acid for 22 hr. at 110°.

The amino-acid composition of the acid hydrolysate was determined by Spackman, Moore, and Stein's method.[6]

Ascending thin layer chromatography was performed on silica-gel (Merck) with the following solvent systems: R_{f1}, n-butanol–glacial acetic acid–water, 4 : 1 : 1; R_{f2}, ethyl acetate–pyridine–glacial acetic acid–water, 60 : 20 : 6 : 14; R_{f3}, chloroform–benzene–glacial acetic acid, 95 : 1 : 4.

The chlorine test was carried out as in the literature.[7] Leucine-aminopeptidase (LAP) digest was prepared as described by Hill *et al.*[8] Unless stated otherwise, solvents were evaporated at a bath temperature of 40—50° in a rotatory evaporator.

Benzyloxycarbonyl-L-alanyl-L-alanine t-Butyl Ester (A, 19—20) [Z–Ala–Ala–OBu^t].—5·81 g. (40 mmoles) of H–Ala–OBu^t [4] were dissolved in pyridine (75 ml.) and 6·5 ml. of triethylamine and 13·43 g. (39 mmoles) of Z–Ala–ONP [9] were added. The mixture was kept at room temperature overnight, the solvent was evaporated under reduced pressure and the residue, taken up in chloroform, was thoroughly washed with 1N-sodium hydroxide, 5% citric acid, and water, dried (Na_2SO_4), and evaporated to dryness.

Recrystallisation twice from ether–light petroleum gave the crystalline protected dipeptide ester (11·6 g.; 85%), m. p. 70—71°, $[\alpha]_D^{20}$ −46·8° ± 0·2° (*c* 1·0 in ethanol), $[\alpha]_D^{20}$ −54·1° ± 0·2° (*c* 2·0, in methanol), R_{f1} 0·8, R_{f3} 0·5, single ninhydrin-negative and chlorine-positive spot {lit.,[4] m. p. 69·71°, $[\alpha]_D^{20}$ −54·2° (*c* 2·0 in methanol)}.

Benzyloxycarbonyl-L-seryl-L-alanyl-L-alanine t-Butyl Ester (C, 18—20) (Z–Ser–Ala–Ala–OBu^t).—8·5 g. (24·2 mmoles) of the above protected dipeptide (A, 19—20) were hydrogenated for 2 hr. over 10% palladised charcoal in ethanol (150 ml.) containing a few drops of glacial acetic acid. The catalyst was filtered off and the filtrate evaporated to dryness. The residue (R_{f1} 0·52, single ninhydrin-positive spot) was dissolved in chloroform (150 ml.) containing 3·5 ml. of triethylamine and 9·80 g. (24 mmoles) of Z–Ser–ODNP [2] were added. After one night at room temperature the solution was thoroughly washed with 1N-sodium hydroxide, 5% citric acid, and water, dried (Na_2SO_4), and evaporated to dryness. Crystallisation from ethanol–light petroleum gave 7·8 g. (74%) of the crystalline, protected *tripeptide ester* (C, 18—20), m. p. 166—167°, $[\alpha]_D^{20}$ −44·5° ± 0·2° (*c* 1·0 in ethanol), R_{f1} 0·85, R_{f2} 0·95, single ninhydrin-negative and chlorine-positive spot (Found: C, 58·0; H, 7·2; N, 9·6. $C_{21}H_{31}N_3O_7$ requires C, 57·7; H, 7·1; N, 9·6%).

Benzyloxycarbonyl-L-seryl-L-threonyl-L-seryl-L-alanyl-L-alanine t-Butyl Ester (E, 16—20) (Z–Ser–Thr–Ser–Ala–Ala–OBu^t).—*Procedure* (*a*). Sodium nitrite (0·5 g.) in water (3 ml.) was added to a cooled (−20°) solution of 1·76 g. (5 mmoles) of Z–Ser–Thr–$NHNH_2$ [2] (D, 16—17) in a mixture of 2N-hydrochloric acid (20 ml.) and glacial acetic acid (20 ml.).

After 15 min., ice-cold water (20 ml.) was added and the mixture was extracted with one 80 ml. and two 25 ml. portions of ethyl acetate, cooled to −10°. The combined organic layers were washed with saturated potassium carbonate solution and dried (Na_2SO_4). At the same time 2·19 g. (5 mmoles) of Z–Ser–Ala–Ala–OBu^t (C, 18—20) were hydrogenated for 2 hr. over 10% palladised charcoal in ethanol (45 ml.) containing a few drops of glacial acetic acid. The catalyst was filtered off and the filtrate evaporated to dryness. The residue (R_{f1} 0·5, single ninhydrin- and chlorine-positive spot), dissolved in dimethylformamide (35 ml.), was added to the ethyl acetate solution of the protected dipeptide azide. The mixture, after evaporation at 0° to remove most of the ethyl acetate, was kept at 5° for 4 days, then the solvent was removed under reduced pressure. The *residue* was dissolved in methanol containing a little dimethylformamide and crystallisation occurred when ether was added; the product (2·07 g.; 66%) had m. p. 205—206°, $[\alpha]_D^{24}$ −3·9° ± 0·2° (*c* 1·0 in dimethylformamide) R_{f1} 0·7, single ninhydrin-negative and chlorine-positive spot (Found: C, 53·0; H, 7·0; N, 11·2. $C_{28}H_{43}N_5O_{11}$ requires C, 53·8; H, 6·9; N, 11·2%).

Procedure (*b*).[10] t-Butyl nitrite (0·83 ml.) was added to a stirred solution, cooled to −20°, of 2·4 g. (6·8 mmole) of Z–Ser–Thr–$NHNN_2$ [2] (D, 16—17) in dimethylformamide (13 ml.) containing 4·5 ml. of 5N-hydrochloric acid in ether. The solution was stirred at −20° for 10 min., 80 ml. of ethyl acetate at −10° were added, and the mixture was washed with ice-cold sodium hydrogen carbonate and saturated sodium chloride and dried (Na_2SO_4). The solution of the protected dipeptide azide was added to a solution of

[6] D. H. Spackman, S. Moore, and W. H. Stein, *Analyt. Chem.*, 1958, **30**, 1190.
[7] H. N. Rydon and P. Smith, *Nature*, 1952, **196**, 922.
[8] R. L. Hill, D. H. Spackman, D. M. Brown, and E. L. Smith, *Biochem. Preparations*, 1958, **6**, 35.
[9] F. Marchiori, R. Rocchi, and E. Scoffone, *Ricerca Sci.*, 1962, **32** (II-A), 647.
[10] J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, 1961, **26**, 2333.

H–Ser–Ala–Ala–OBu^t (D, 18—20) obtained by hydrogenolysis of 3 g. (6·8 mmoles) of Z–Ser–Ala–Ala–OBu^t (C, 18—20) as described in (*a*). The combined solutions, allowed to react as previously, gave 2·8 g. (61·4%) of the same product as obtained by the standard azide procedure.

*Benzyloxycarbonyl-*L*-seryl-*L*-seryl-*L*-threonyl-*L*-seryl-*L*-alanyl-*L*-alanine t-Butyl Ester* (G, 15—20) (Z–Ser–Ser–Thr–Ser–Ala–Ala–OBu^t).—1·78 g. (2·85 mmoles) of the above protected pentapeptide ester (E, 16—20) were hydrogenated for 2 hr. over 10% palladised charcoal in dimethylformamide containing a few drops of glacial acetic acid. The catalyst was filtered off and the filtrate evaporated to dryness. The residue (R_{f1} 0·65, single ninhydrin- and chlorine-positive spot) was dissolved in dimethylformamide (60 ml.) and 1·216 g. (3·0 mmole) of Z–Ser–ODNP [2] and 0·5 ml. of triethylamine were added. The solution was kept at room temperature overnight, then the solvent was removed under reduced pressure, the residue was dissolved in methanol, and crystallisation occurred when ether was added. Recrystallisation from methanol–dimethylformamide (1 : 1 v/v)–ether gave 1·03 g. (51%) of the protected hexapeptide ester (G, 15—20), m. p. 235—236° (decomp.), $[\alpha]_D^{20}$ −3·5° ± 0·2° (*c* 1·0 in dimethylformamide), R_{f1} 0·75, single ninhydrin-negative and chlorine-positive spot (Found: C, 51·6; H, 6·9; N, 11·6. Calc. for $C_{31}H_{48}N_6O_{13}$: C, 52·2; H, 6·8; N, 11·8%) {lit.,[4] m. p. 236—238° (decomp.), $[\alpha]_D^{29}$ −3·4° (*c* 0·52 in dimethylformamide)}.

*Benzyloxycarbonyl,*β*,t-butyl,*L*-aspartyl-*L*-seryl-*L*-seryl-*L*-threonyl-*L*-seryl-*L*-alanyl-*L*-alanine, t-Butyl Ester* (I, 14—20) (Z–Asp(OBu^t)–Ser–Ser–Thr–Ser–Ala–Ala–OBu^t).—2·53 g. (3·54 mmoles) of Z–Ser–Ser–Thr–Ser–Ala–Ala–OBu^t (G, 15—20) were hydrogenated for 2 hr. over 10% palladised charcoal in methanol–dimethylformamide (1 : 1 v/v) containing a few drops of glacial acetic acid. The catalyst was filtered off and the filtrate evaporated to dryness. The residue (R_{f1} 0·45, single ninhydrin- and chlorine-positive spot) was dissolved in pyridine (150 ml.), and 1·76 g. (39·5 mmoles) of Z–Asp(OBu^t)–ONP [11,12] and 0·56 ml. of triethylamine were added. The solution was kept at room temperature for 24 hr. and precipitation occurred when ether was added. The precipitate was dissolved in 125 ml. of dimethylformamide–water (4 : 1 v/v), Dowex 50 × 12 (25 ml., settled in water), in the hydrogen form, was added and the mixture was stirred for 30 min. at room temperature. The resin was filtered off, the filtrate evaporated to dryness, and the residue crystallised from pyridine–light petroleum; the product (2·0 g.; 64%), m. p. 213°, $[\alpha]_D^{20}$ −15·1° ± 0·2° (*c* 1·0 in dimethylformamide), R_{f1} 0·7, R_{f2} 0·85, single ninhydrin-negative and chlorine-positive spot (Found: C, 52·5; H, 7·0; N, 11·1. Calc. for $C_{39}H_{61}N_7O_{16}$: C, 53·0; H, 7·0; N, 11·1%) {lit.,[4] m. p. 230—232°; $[\alpha]_D^{26}$ −4·3° (*c* 1·0 in dimethylformamide)}.

*t-Butoxycarbonyl-*L*-methionyl-*β*-t-butyl-*L*-aspartyl-*L*-seryl-*L*-seryl-*L*-threonyl-*L*-seryl-*L*-alanyl-*L*-alanine t-Butyl Ester* (K, 13—20) [BOC–Met–Asp(OBu^t)–Ser–Ser–Thr–Ser–Ala–Ala–OBu^t].—2·48 g. (2·8 mmoles) of Z–Asp(OBu^t)–Ser–Ser–Thr–Ser–Ala–Ala–OBu^t (I, 14—20) were hydrogenated for 3 hr. over 10% palladised charcoal in dimethylformamide–methanol (1 : 1 v/v) containing a few drops of glacial acetic acid. The catalyst was filtered off and the solvent evaporated under reduced pressure. The residue (R_{f1} 0·6, single ninhydrin- and chlorine-positive spot) was dissolved in pyridine (100 ml.) and 1·11 g. (3 mmoles) of BOC–Met–ONP,[3,4] and 0·4 ml. of triethylamine were added.

The solution was kept at room temperature for 36 hr., then ether was added, and the precipitate was collected and dried (2·31 g.).

The crude product was dissolved in 100 ml. of dimethylformamide–water (4 : 1 v/v), Dowex 50 × 12 (25 ml. settled in water) in the hydrogen form was added and the mixture stirred for 30 min. at room temperature. The resin was filtered off, the filtrate evaporated to dryness, and the *residue* crystallised from dimethylformamide–ether; the product (2·12 g.; 77%) had m. p. 212—213°, $[\alpha]_D^{20}$ −11·80° ± 0·2° (*c* 1·1 in dimethylformamide), R_{f1} 0·75, R_{f2} 0·83, single ninhydrin-negative and chlorine-positive spot, amino-acid ratios in acid hydrolysate $met_{0.90}asp_{1.03}ser_{2.90}thr_{0.95}ala_{2.10}$ (Found: C, 49·8; H, 7·5; N, 11·2; S, 3·3. $C_{41}H_{72}N_8O_{17}S$ requires C, 50·2; H, 7·4; N, 11·4; S, 3·3%).

*L-Methionyl-*L*-aspartyl-*L*-seryl-*L*-seryl-*L*-threonyl-*L*-seryl-*L*-alanyl-*L*-alanine Monoacetate Trihydrate* (H–Met–Asp–Ser–Ser–Thr–Ser–Ala–Ala–OH, $CH_3{\cdot}CO_2H,3H_2O$).—0·2 g. (0·2 mmole) of the above protected octapeptide ester (K, 13—20) were dissolved in 1 ml. of anhydrous trifluoroacetic acid and the solution was kept at room temperature for 90 min. whereupon the solvent was removed under reduced pressure.

The residue was taken up in ethanol and evaporated to dryness (3 times) and dissolved in water (3 ml.). Amberlite IR-4B (OH) (1 ml. settled in water) was added to the solution and the mixture stirred for 30 min. at room temperature. The resin was filtered off and the filtrate, evaporated under reduced pressure to 1·5 ml., was added, with stirring, to 20 ml. of acetone.

The precipitate was collected, washed with ether, and dried (P_2O_5,NaOH) (yield 0·092 g.; 58·6%). A sample of this crude material (77 mg.) was purified by passage through an Amberlite CG 50 column (1·8 × 90 cm., 200 mesh) with 0·2M-sodium phosphate (pH 6·5) as eluent.

Fractions (2 ml.) were collected at a flow rate of approximately 2 ml./min. and the desired peptide was located by the colorimetric test for methionine.[13]

These eluates were pooled, the solvent was evaporated under reduced pressure to a small volume, and the resulting solution was added to a Sephadex G-25 column (1·8 × 140 cm.) and eluted with 5% acetic acid. The desired and desalted peptide was located [13] in the eluate and these fractions were pooled and lyophilised from small volumes of water; the product (44 mg.; 50%) had $[\alpha]_D^{20}$ −65·8° ± 2° (*c* 0·208 in water), R_{f1} 0·2, single ninhydrin- and chlorine-positive spot, single component on paper electrophoresis at pH 1·9, 6·5, and 8·0 (Found: C, 40·0; H, 6·5; N, 12·9; S, 3.7. $C_{30}H_{58}N_8O_{20}S$ requires C,40·8; H, 6·6; N, 12·7; S, 3·6%). Amino-acid ratios in acid hydrolysate: $met_{0.90}asp_{1.05}ser_{2.95}thr_{0.95}ala_{2.00}$; amino-acid ratios in LAP digest: $met_{0.9}asp_{0.98}ser_{3.05}thr_{1.00}ala_{2.10}$.

We thank Dr. E. Celon for microanalyses.

[6/573 *Received, April 12th,* 1966]

[11] E. Wunsch and A. Zwick, *Physiol. Chem.*, 1963, **333**, 108.
[12] L. Bernardi, G. Bosisio, O. Goffredo, and R. De Castiglione, *Experientia*, 1964, **20**, 490.
[13] T. E. McCarthy and M. X. Sullivan, *J. Biol. Chem.*, 1941, **141**, 871.