Structural Biochemistry. Part XII.[1] Synthesis of Tobacco Mosaic Virus Protein Unit 150—158

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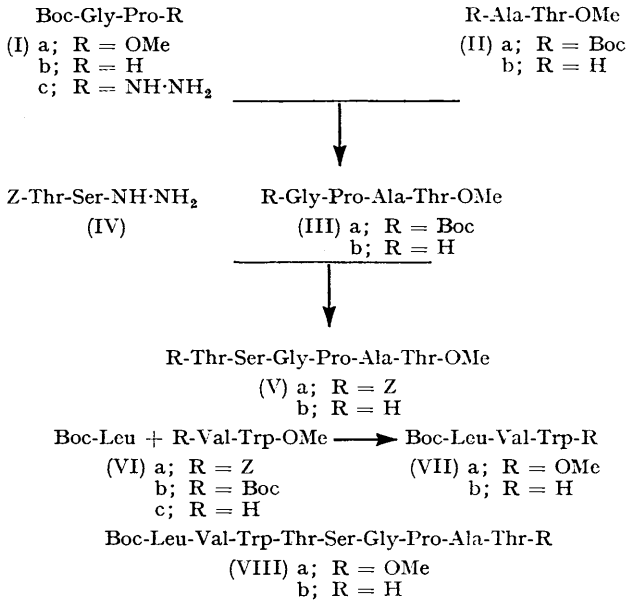
The *C*-terminal nonapeptide sequence of tobacco mosaic virus protein was synthesized by a fragment-condensation approach. Important steps in the synthesis involved coupling of N-benzyloxycarbonyl-L-threonyl-L-serine azide with glycyl-L-prolyl-L-alanyl-L-threonine methyl ester and condensing the mixed carbonic anhydride derived from *N*-t-butoxycarbonyl-L-leucyl-L-valyl-L-tryptophan and isobutyl chloroformate with the hexapeptide (Vb) to provide the nonapeptide *N*-t-butoxycarbonyl-L-leucyl-L-valyl-L-tryptophyl-L-threonyl-L-seryl-glycyl-L-prolyl-L-alanyl-L-threonine (VIIIb).

We have previously indicated our interest in synthesizing the complete TMV protein,[1] and now describe an approach to the Boc-protected *C*-terminal nonapeptide sequence corresponding to TMV protein 150—158.

After preparation of Boc-Gly-Pro, we evaluated methods for making Boc-Ala-Thr-OMe, employing 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (EDCI)[2] hydrochloride, mixed carbonic anhydride (MCA) with isobutyl chloroformate and *N*-methylmorpholine,[3] and azide (A) methods. The yields of the dipeptide (IIa) were respectively 79, 82, and 66%. Although m.p.s of protected dipeptide (IIa) samples prepared by each of the three methods varied, the optical rotation and t.l.c. data were in good agreement. The t-butoxycarbonyl group was easily removed with trifluoroacetic acid.

The protected tetrapeptide (IIIa) was obtained by two methods (MCA and A) from the dipeptides (Ib) and (IIb) and purified by ion-exchange and silica gel column chromatography. Treatment with trifluoroacetic acid afforded the tetrapeptide methyl ester (IIIb) which was treated with the azide derived from Z-Thr-Ser hydrazide (IV)[4] to afford the hexapeptide (Va) in 55% yield.

The initial approach to the dipeptide methyl ester (VIc) was based on condensing Z-Val with Trp-OMe using EDCI. However, subjecting the product (VIa) to catalytic hydrogenolysis led to only 56% yield of the dipeptide (VIc), accompanied by the corresponding dioxopiperazine (26%). To avoid piperazine formation the dipeptide (VIc) was prepared by using Boc for protection and trifluoroacetic acid for deblocking. Next, Boc-Leu was condensed (EDCI) with the dipeptide methyl ester (VIc) to yield the tripeptide methyl ester (VIIa), which was saponified and coupled by the mixed carbonic anhydride method with the hexapeptide methyl ester (Vb). The resulting nonapeptide methyl ester (VIIIa) was purified by ion-exchange chromatography and recrystallization to yield the Boc-protected TMV protein nonapeptide 150—158 (VIIIb). Essentially complete hydrolysis of the methyl ester occurred during the isolation and purification sequence and the nonapeptide (VIIIb) was characterized as the crystalline



dicyclohexylammonium salt. A quantitative amino-acid analysis employing an accelerated,[5] high-sensitivity[6] modification of the method of Spackman *et al.*[7] demonstrated the presence of threonine, serine, proline, glycine, alanine, valine, leucine, and tryptophan in the molar proportions 2·1, 1·0, 1·1, 1·0, 1·1, 0·8, 0·9, and 0·4, respectively. Homogeneity of the deblocked nonapeptide was established by an enzymatic hydrolysis with Aminopeptidase M. Analysis of the hydrolysate by t.l.c. indicated the absence of any unhydrolysed nonapeptide as well as the absence of any peptide fragments resulting from D-amino-acids. The homogeneity of the

[1] Part XI, G. R. Pettit and W. R. Jones, *J. Org. Chem.*, 1971, **36**, 870.

[2] Abbreviations are those recommended by the I.U.P.A.C. Commission on Nomenclature (*Biochemistry*, 1972, **11**, 1726); see also G. R. Pettit, 'Synthetic Peptides,' Van Nostrand-Reinhold, New York, vol. 1, 1970, vol. 2, 1971.

[3] G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, 1966, **88**, 1338.

[4] G. R. Pettit and S. K. Gupta, *J. Chem. Soc.* (*C*), 1968, 1208.

[5] J. V. Benson, jun., and J. A. Patterson, *Analyt. Chem.*, 1965, **37**, 1108.

[6] R. W. Hubbard and D. M. Kremen, *Analyt. Biochem.*, 1965, **12**, 593.

[7] D. H. Spackman, W. H. Stein, and H. Moore, *Analyt. Chem.*, 1958, **30**, 1190.

nonapeptide salt was further confirmed by a 60-tube counter-current distribution. The distribution of the peptide (VIIIb) among tubes 37—53 corresponded to that expected for a pure substance. A thin-layer comparison of material from each tube further substantiated this observation.

EXPERIMENTAL

The L-amino-acids employed as starting materials were used as received from Koch–Light of Nutritional Biochemicals Corp. Aminopeptidase M was obtained as a lyophilized powder from Henley and Co., Inc. Solvent extracts containing completely protected peptides were washed successively with water, 1% citric acid, water, 1% sodium carbonate, and water. Partially protected peptides were washed with a suitable combination of the foregoing solutions. All such extracts were dried over magnesium sulphate and removal of solvent was performed under reduced pressure by rotary evaporation at 35—40°. Cooling refers to ice-bath temperatures. Both dimethylformamide and tetrahydrofuran were passed successively through columns of silica gel (Merck) and basic alumina before use in peptide bond-forming reactions. The t-butoxycarbonyl-protected amino-acids were prepared with the help of Dr. R. Quinn.

Thin-layer chromatograms were prepared on microscope slides using silica gel G (Merck) and developed with iodine or ninhydrin reagent.[8] Each analytical sample was colourless and displayed a single spot on t.l.c. The identical composition of two substances was established by t.l.c., mixed m.p., and i.r. spectral comparisons. M.p.s were recorded with a Kofler hot-stage apparatus. Optical rotations were measured by Dr. P. Demoen, Analytical Dept., Janssen Labs, Beerse, Belgium; elemental microanalyses were obtained from Alfred Bernhardt, 5251 Elbach uber Engelskirchen, West Germany. The amino-acid analysis was performed by Dr. J. R. Cronin, of our department, using a Beckman-Spinco 121 amino-acid analyser.

Boc-Amino-acids.—The t-butoxycarbonyl-protected amino-acids used were prepared by reaction between t-butyloxycarbonyl azide (Aldrich) and the amino-acid on a mole to mole basis instead of the usual excess of azide reagent. The following synthesis of Boc-Gly illustrates the general method.

Glycine (13·0 g) was dissolved in aqueous sodium hydroxide (7·7 g in 100 ml). Dioxan (100 ml) was added, followed by t-butoxycarbonyl azide (25 g), and the mixture was stirred at room temperature for 24 h. The resulting solution was diluted with ice–water (200 ml) and extracted with ether (3 × 50 ml). The aqueous phase was acidified with solid citric acid to pH 3·0, saturated with sodium chloride, and extracted with ethyl acetate. The extract was washed with saturated sodium chloride solution (3 × 25 ml), dried, and evaporated to give an oil (22·4 g, 74%) which crystallized upon cooling (m.p. 88—89°). Recrystallization from benzene gave Boc-Gly, m.p. 88·5—89° (lit.,[9] 87—88°).

In several cases, precipitation occurred during the reaction and further quantities of aqueous sodium hydroxide (7·7 g in 25 ml) together with an equal volume of dioxan were added in order to maintain solution. Thus, alanine (15·0 g) required 14 g of sodium hydroxide to give 22·0 g (70%) of Boc-Ala, m.p. 82—84°. One recrystallization from ether–petroleum gave cubic crystals, m.p. 83·5—84°, $[\alpha]_D$ −27° (*c* 2·26 in AcOH) {lit.,[9] m.p. 83—84°, $[\alpha]_D$ −22·4° (AcOH)} (Found: C, 50·95; H, 8·05; N, 7·65. Calc. for $C_8H_{15}NO_4$: C, 50·75; H, 8·0; N, 7·4%). Similarly, leucine (22·8 g) required 7·7 g of sodium hydroxide to give Boc-Leu (8·29 g, 21%), m.p. 63—65° (lit.,[9] 67—72°). Valine (10·2 g) required 7·0 g of sodium hydroxide to give Boc-Val (13·0 g, 55%) as an oil which displayed one spot on t.l.c. (5 : 1 : 4 butan-1-ol–acetic acid–water). The oil (2 g) in ether (25 ml) was treated with ethereal dicyclohexylamine (10 ml of 20%). Removal of solvent gave a quantitative yield of crystalline Boc-Val dicyclohexylammonium salt, m.p. 142—142·5°. Two recrystallizations from ether–petroleum yielded *needles*, m.p. 140·5—141°, $[\alpha]_D$ 0·0° (MeOH) (Found: C, 66·0; H, 10·6; N, 6·95. $C_{22}H_{42}N_2O_4$ requires C, 66·3; H, 10·6; N, 7·05%).

Boc-Gly-Pro-OMe (Ia).—To a cold (−5°) solution of Boc-Gly (17·5 g) and *N*-methylmorpholine (11·2 ml) in tetrahydrofuran (200 ml), isobutyl chloroformate (12·8 ml) was added. The mixture was stirred for 15 min and then a cold solution of methyl prolinate hydrochloride (16·5 g) in dimethylformamide (15 ml) and *N*-methylmorpholine (11·2 ml) was added during 10 min. After being stirred for 2 h at *ca.* 0° and then at room temperature for 4 h, the solution was concentrated to an oil which was dissolved in ethyl acetate. The solution was washed and concentrated to an oily residue which solidified as cubic crystals (23·5 g, 82%), m.p. 65·5—67°. Recrystallization from petroleum afforded *needles*, m.p. 66—67°, $[\alpha]_D$ −69° (*c* 1·16 in $CHCl_3$) (Found: C, 54·5; H, 7·75; N, 9·6. $C_{13}H_{22}N_2O_5$ requires C, 54·55; H, 7·75; N, 9·8%).

When the reaction was repeated with triethylamine in place of *N*-methylmorpholine, the yield and m.p. respectively were 73% and 56—60°. However, t.l.c. showed only one spot.

Boc-Gly-Pro (Ib).—A solution prepared from water (25 ml), sodium hydroxide (6·0 g), methanol (150 ml), and the methyl ester (Ia) (15·0 g) was kept at room temperature for 1·5 h. The methanol was removed and the residue diluted with water (50 ml) and extracted with ether (100 ml). The aqueous phase was acidified with solid citric acid to pH 2·0, saturated with sodium chloride, and extracted with ethyl acetate. The extract was washed and concentrated to a crystalline solid (12·5 g, 88%), m.p. 159—161°. Two recrystallizations from ethyl acetate gave *needles*, m.p. 161—163°, $[\alpha]_D$ −57° (*c* 2·17 in $CHCl_3$) (Found: C, 53·2; H, 7·45; N, 10·4. $C_{12}H_{20}N_2O_5$ requires C, 52·95; H, 7·4; N, 10·3%).

Boc-Gly-Pro Hydrazide (Ic).—A solution prepared from methanol (25 ml), 99% hydrazine hydrate (2 ml), and the methyl ester (Ia) (5·0 g) was kept at room temperature for 10 h. Solvent was removed and the residual oil solidified upon storage in a desiccator. Recrystallization from ethyl acetate–petroleum provided *needles* (4·9 g, 98%), m.p. 177·5—179°. Two recrystallizations from methyl acetate–petroleum yielded material m.p. 199·5—200°, $[\alpha]_D$ −74·2° (*c* 1·24 in N-HCl) (Found: C, 50·25; H, 7·9; N, 19·6. $C_{12}H_{22}N_4O_4$ requires C, 50·35; H, 7·75; N, 19·55%).

Boc-Ala Hydrazide.—To a solution of methyl alaninate hydrochloride (14·0 g) in pyridine (50 ml), *N*-methylmorpholine (11 ml) and t-butoxycarbonyl azide (15·0 g)

[8] E. Stähl, 'Thin Layer Chromatography,' Academic Press, New York, 1965, p. 496.

[9] G. W. Anderson and A. C. McGregor, *J. Amer. Chem. Soc.*, 1957, **79**, 6180, and E. Schnabel, *Annalen*, 1967, **702**, 188.

were added. Stirring was continued at room temperature for 24 h and the solvent was removed. A solution of the residual syrup in ethyl acetate was washed and evaporated. The resulting oil was dissolved in methanol (75 ml) and treated with 99% hydrazine hydrate (10 ml) during 24 h at room temperature. Solvent was removed and the resulting oil crystallized in a desiccator to yield the *hydrazide* (13·0 g, 64%), m.p. 111·5—113°. Two recrystallizations from ethyl acetate–petroleum gave needles, m.p. 112·5—113°, $[\alpha]_D$ −45° (*c* 1·02 in N-HCl) (Found: C, 47·4; H, 8·35; N, 20·65. $C_8H_{17}N_3O_3$ requires C, 47·3; H, 8·45; N, 20·65%).

Thr-OMe.—To a cold (−5°) solution prepared by adding (dropwise) thionyl chloride (26 ml) to methanol (100 ml) was added threonine (12·0 g). The mixture was stirred for an additional 1 h at −5° and at room temperature for 10 h. Solvent was removed and the residual oil dissolved in methylene chloride (15 ml) was treated with triethylamine (15 ml) with ice-cooling. The solution was filtered; evaporation gave an oil which crystallized on cooling to yield material (13·8 g), m.p. 52—53°. Recrystallization from ether provided needles (13·3 g, 99%), m.p. 64—65°. Two recrystallizations from ether led to a sample, m.p. 64·5—65°, $[\alpha]_D$ −14° (*c* 0·65 in MeOH). The methyl ester was also purified by sublimation at 25° (2 mmHg), which provided cubic crystals, m.p. 64—65° {lit.,[10] m.p. 63—65°, $[\alpha]_D$ −21° (*c* 0·999 in N-HCl)} (Found: C, 45·05; H, 8·3; N, 10·65. Calc. for $C_5H_{11}NO_3$: C, 45·1; H, 8·35; N, 10·5%).

At room temperature the methyl ester slowly deteriorated and turned yellow. However, at *ca.* 0° it could be stored without change for several weeks.

Boc-Ala-Thr-OMe (IIa).—*Method A*. To a solution of Boc-Ala (15·2 g) and Thr-OMe (11·2 g) in cold (ice-bath) methylene chloride (200 ml) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (16·0 g). After 1 h at ice-bath temperature, stirring was continued an additional 1 h at room temperature. The mixture was washed and concentrated to an oil which crystallized upon storage in a desiccator to yield a solid (19·0 g, 79%), m.p. 102—103°. *Needles*, m.p. 104·5—105°, were obtained by two recrystallizations from ethyl acetate–petroleum, $[\alpha]_D$ −15·3° (*c* 2·61 in $CHCl_3$) (Found: C, 51·4; H, 7·7; N, 9·1. $C_{13}H_{24}N_2O_6$ requires C, 51·3; H, 7·95; N, 9·2%).

Method B. To a solution of Boc-Ala hydrazide (4·1 g) in ethyl acetate (40 ml) containing N-hydrochloric acid was added sodium nitrite (1·7 g) in water (5·0 ml). Both solutions were maintained at −5°. The mixture was stirred for 15 min and extracted with ethyl acetate. The cold extract was washed, dried (K_2CO_3), and filtered, and a solution of methyl threonate (2·66 g) in ethyl acetate (10 ml) and triethylamine (2·28 ml) was added. After being stirred at *ca.* 0° for 48 h the solution was washed and concentrated to an oil which slowly crystallized to yield material (4·0 g, 66%), m.p. 113—115°. Two recrystallizations from ethyl acetate–petroleum raised the m.p. to 117·5—118°. The fine needles exhibited $[\alpha]_D$ −15° (*c* 2·69 in $CHCl_3$) and displayed one spot on t.l.c. (9 : 1 chloroform–methanol).

Method C. When the preparation of Boc-Ala-Thr-OMe was repeated by the mixed anhydride procedure already noted for preparation of Boc-Gly-Pro-OMe, using 18·9 g of Boc-Ala, some racemization was evident. The initial product (25 g, 82%; m.p. 122—124°) was fractionally recrystallized from ethyl acetate–petroleum to yield cubic crystals, m.p. 126·5—127·5°, and an amorphous solid, m.p. 122·5—123·5°, in *ca.* 2% yield assumed to be the racemate. Further recrystallization of the higher melting material from ethyl acetate–petroleum gave a sample, m.p. 126·5—127·5°, $[\alpha]_D$ −11° (*c* 4·97 in $CHCl_3$) (Found: C, 51·25; H, 8·1%).

Ala-Thr-OMe (IIb) *Trifluoroacetate*.—A solution prepared from trifluoroacetic acid (20 ml) and the methyl ester (IIa) (4·1 g) was left at room temperature for 1 h. Solvent was removed and the residue, upon refrigeration with ether solidified to provide 4·0 g (90%), m.p. 179—180·5°, of Ala-Thr-OMe,CF_3CO_2H. Recrystallization from methanol–ether gave *prisms*, m.p. 182—183°, $[\alpha]_D$ −7° (*c* 2·97 in MeOH) (Found: C, 37·7; H, 5·45; N, 8·85. $C_{10}H_{17}F_3N_2O_6$ requires C, 37·75; H, 5·4; N, 8·8%).

Boc-Gly-Pro-Ala-Thr-OMe (IIIa).—*Method A*. To a solution of the hydrazide (Ic) (4·3 g) in N-hydrochloric acid (30 ml) at −5° was added sodium nitrite (1·3 g) in water (5 ml) with stirring during 10 min. The azide separated as a semi-solid that was extracted with cold ethyl acetate. The extract was washed, filtered, and added during 5 min to a solution of methyl alanylthreonate trifluoroacetate (4·7 g) in dimethylformamide (10 ml)–triethylamine (3·6 ml), with stirring at ice-bath temperature. The mixture was stirred for 24 h at 4° then for 24 h at room temperature. After removing the solvent the residue was diluted with 2% ammonium hydroxide and extracted with butan-1-ol previously equilibrated with water. The extract was washed with 2% citric acid and water. Both washing solutions had previously been equilibrated with butan-1-ol. Evaporation of the butanol gave an oil (6·0 g, 89%). Upon storing for a few days the oil began to crystallize; trituration with petroleum completed solidification. The solid melted at 95—97° and appeared as one spot on t.l.c. (9 : 1 chloroform–methanol). A solution of the product in methanol was passed through a column of IR-45 (OH^-) resin and finally through a column of silica gel. Evaporation and recrystallization of the residue from ether provided a *powder*, m.p. 107—110° (sintering at 100°), $[\alpha]_D$ −92° (*c* 3·26 in MeOH), $[\alpha]_D$ −62° (*c* 3·37 in $Me_2N{\cdot}CHO$) (Found: C, 52·6; H, 7·6; N, 12·4; O, 27·75. $C_{20}H_{34}N_4O_8$ requires C, 52·4; H, 7·45; N, 12·2; O, 27·9%).

Method B. Application of the mixed carbonic anhydride procedure [Boc-Gly-Pro (6·8 g), tetrahydrofuran (90 ml), triethylamine (2·9 ml), and isobutyl chloroformate (3·2 ml)] essentially as for preparation of the dipeptide (Ia), and using the isolation procedure of Method A provided the tetrapeptide (IIIa) (10 g, 89%). The crude product displayed one spot on t.l.c.; recrystallization from ether yielded a powder, m.p. 108—110° (sintering at 100°), identical with material obtained by the azide technique.

Gly-Pro-Ala-Thr-OMe (IIIb) *Trifluoroacetate and Hydrochloride*.—An exothermic reaction ensued from mixing the tetrapeptide (IIIa) (1·6 g) with trifluoroacetic acid (5 ml). The resulting solution was kept for 2 h at room temperature and then extracted with ether. The solid which separated was collected and washed with ether to provide the hygroscopic *trifluoroacetate* (1·64 g, 100%), purified by precipitation from methanol with ether. T.l.c. (5 : 1 : 4 butan-1-ol–acetic acid–water) showed one spot (Found: C, 43·15; H, 5·75; N, 11·75. $C_{17}H_{27}F_3N_4O_8$ requires C, 43·2; H, 5·75; N, 11·8%).

A solution of the trifluoroacetate (2·0 g) in methanol (10 ml) was passed through a column of IRA-400 (Cl^-)

[10] T. Fujii and K. Okawa, *Bull. Chem. Soc. Japan*, 1966, **39**, 1598.

resin. Evaporation of the eluate and trituration of the residue with ether provided a solid (1·6 g, 100%). Three crystallizations from methanol–ether afforded the *tetrapeptide* (IIIb) *hydrochloride*, m.p. 229—231°, $[\alpha]_D$ −91° (*c* 5·24 in MeOH), $[\alpha]_D$ −61° (*c* 5·46 in $Me_2N{\cdot}CHO$), showing one spot on t.l.c. (5 : 1 : 4 butan-1-ol–acetic acid–water) (Found: C, 46·3; H, 6·95; O, 24·3. $C_{15}H_{27}ClN_4O_6$ requires C, 45·9; H, 6·9; O, 24·3%).

Z-Thr-Ser-Gly-Pro-Ala-Thr-OMe (Va).—Benzoyloxycarbonylthreonylseryl hydrazide [4] (0·53 g) was dissolved in N-hydrochloric acid (10 ml) and the following steps were performed at −5°. Sodium nitrite (0·135 g) in water (2 ml) was added (dropwise). The mixture was stirred for 10 min and sodium chloride (3 g) was added to facilitate separation of the azide. An ethyl acetate extract containing the azide was washed and dried, and to it was added the tetrapeptide methyl ester (IIIb) trifluoroacetate (0·60 g) in dimethylformamide (5 ml)–triethylamine (0·34 ml). The mixture was stirred for 24 h at 4° and 24 h at room temperature. The product was isolated with butan-1-ol as for the preparation of the tetrapeptide (IIIa); yield 0·55 g (55%), m.p. 202—204°. When the reaction was repeated on larger scales, yields were consistently 50—52%. Two recrystallizations from methanol–ether provided *crystals*, m.p. 203—204°, $[\alpha]_D$ −33° (*c* 0·7 in $Me_2N{\cdot}CHO$) (Found: C, 53·05; H, 6·7; N, 12·2; O, 27·95. $C_{30}H_{44}N_6O_{12}$ requires C, 52·95; H, 6·5; N, 12·35; O, 28·2%).

Boc-Val-Trp-OMe (VIb).—Condensation of Boc-Val (7·0 g) with methyl tryptophanate hydrochloride (8·0 g) [11] in methylene chloride (75 ml)–triethylamine (4·4 ml) by use of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (6·4 g) was performed as for the preparation of Boc-Ala-Thr-OMe. The crude product crystallized to yield material (9·0 g, 70%), m.p. 145—147°. T.l.c. (9 : 1 chloroform–methanol) revealed one spot. Two recrystallizations from ethyl acetate–petroleum gave cubic *crystals*, m.p. 166—167°, $[\alpha]_D$ +52° (*c* 3·50 in $CHCl_3$) (Found: C, 63·15; H, 7·35; N, 9·95. $C_{22}H_{31}N_3O_5$ requires C, 63·3; H, 7·5; N, 10·05%).

Val-Trp-OMe (VIc) *Hydrochloride*.—*Method A*. Hydrogen was passed through a methanol (100 ml)–acetic acid (10 ml) solution of Z-Val-Trp-OMe [4] (VIa) (5·0 g) containing suspended 10% palladium–charcoal (0·50 g). Debenzyloxycarbonylation was complete in 3 h. The solution was filtered and the solvent removed. The oily residue was extracted with ethyl acetate followed by water. The aqueous phase was basified with dilute ammonium hydroxide, saturated with sodium chloride, and extracted with ethyl acetate. The organic extract was washed and concentrated to *ca.* 10 ml. To the cool solution was added ethereal hydrogen chloride whereupon the hydrochloride separated. Reprecipitation from methanol–ether provided a *powder*, m.p. 136—139° (2·2 g, 56%), $[\alpha]_D$ −9° (*c* 1·73 in MeOH) (Found: C, 54·75, 54·85; H, 7·9, 7·8. $C_{17}H_{24}ClN_3O_3,H_2O$ requires C, 54·9; H, 7·05%).

The original ethyl acetate extract was washed and concentrated to give 3-(*indol*-3-*ylmethyl*)-6-*isopropylpiperazine*-2,5-*dione* (0·80 g, 26%), decomp. 280—300°. Two recrystallizations from methanol gave needles, m.p. 275—278° (decomp. 306—308°) (Found: C, 67·4; H, 6·55; N, 14·8. $C_{16}H_{18}N_3O_2$ requires C, 67·6; H, 6·35; N, 14·8%).

Method B. To trifluoroacetic acid (50 ml) at room temperature was added Boc-Val-Trp-OMe (8·0 g). After 2 h the excess of trifluoroacetic acid was removed and the residual oil upon trituration with ether provided a solid which was dissolved in methylene chloride (25 ml) and treated with cold ethereal hydrogen chloride. The hydrochloride which separated was collected and washed with ether; yield 6·5 g (95%). The hydrochloride displayed one spot on t.l.c. (methanol) and was identical with the substance prepared by Method A.

Boc-Leu-Val-Trp-OMe (VIIa).—By means of the 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (4·5 g) technique [see (IIa)] Val-Trp-OMe hydrochloride (8·0 g) was coupled with Boc-Leu hydrate (5·7 g) in methylene chloride (100 ml)–triethylamine (3·2 ml). The crude oily product crystallized upon cooling to yield the *tripeptide* (VIIa) (9·4 g, 78%), m.p. 125—130° (sintering at 120°). Two recrystallizations from methanol–water afforded a specimen, m.p. 131—133°, $[\alpha]_D$ +13° (*c* 2·90 in $CHCl_3$) (Found: C, 63·4; H, 8·05; N, 10·65; O, 18·2. $C_{28}H_{42}N_4O_6$ requires C, 63·35; H, 8·0; N, 10·55; O, 18·1%).

Boc-Leu-Val-Trp (VIIb).—Aqueous (12 ml) potassium hydroxide (2·5 g) was added to the tripeptide methyl ester (VIIa) (5·0 g) in methanol (60 ml). After 1·5 h at room temperature, the excess of methanol was removed and a solution of the residue in water (50 ml) was acidified to pH 2·0 with citric acid. The mixture was extracted with ethyl acetate and the extract washed and concentrated to an oil which solidified upon drying; yield 4·5 g (93%), m.p. 150—155°. T.l.c. (methanol) of the oily carboxylic acid showed only one spot but the oil resisted crystallization. To a solution of the oil in ether (25 ml) was added ethereal 10% dicyclohexylamine (10 ml). Removal of solvent and trituration of the residue with petroleum gave the solid *dicyclohexylammonium salt* (6·0 g), m.p. 170—174°. Recrystallization from ether–petroleum gave a powder, m.p. 171—173°. A sample of m.p. 170—172°, $[\alpha]_D$ −5° (*c* 3·77 in MeOH), was obtained by recrystallization from methylene chloride–pentane (Found: C, 66·75; H, 9·2; N, 9·85. $C_{39}H_{63}N_5O_6$ requires C, 67·1; H, 9·1; N, 10·05%).

Boc-Leu-Val-Trp-Thr-Ser-Gly-Pro-Ala-Thr (VIIIb) *Dicyclohexylammonium Salt*.—A solution of Z-Thr-Ser-Gly-Pro-Ala-Thr-OMe (1·0 g) in methanol (25 ml)–acetic acid (10 ml) was subjected to hydrogenolysis over 10% palladium–charcoal (0·20 g) during 2 h. More catalyst (0·20 g) was added and hydrogenation was continued for another hour when evolution of carbon dioxide had ceased. The solution was filtered and concentrated to an oil, which solidified upon trituration with ether. T.l.c. of the hexapeptide acetate (Vb) (0·70 g, 80%) (methanol) showed only one spot. A solution of the hexapeptide acetate in dimethylformamide (10 ml) containing triethylamine (0·13 ml) was added to the mixed carbonic anhydride prepared by stirring a mixture of Boc-Leu-Val-Trp dicyclohexylammonium salt (0·82 g) and isobutyl chloroformate (0·15 ml) in tetrahydrofuran (25 ml) at −5° during 15 min. The mixture was stirred for 2 h at ice-bath temperature followed by 6 h at room temperature. Before adding ether (100 ml) the solution was reduced to a small volume. The solid (VIIIa) which separated upon cooling was collected and passed in 9 : 1 methanol–water through a column of IRA-400 (OH^-) resin. Elution with the same solvent gave a powder (1·2 g), m.p. 208—211°. The same solvent was used for ion-exchange chromatography of the peptide on Amberlite CG-4b (OH^-). Elution with the same solvent, treatment of the product with ethereal dicyclohexylamine, and recrystallization of the salt from methanol–ether

[11] R. A. Boissonnas, S. Guttmann, R. L. Huguenin, P. A. Jaquenoud, and E. Sandrin, *Helv. Chim. Acta*, 1958, **41**, 1867.

provided crystals, m.p. 208—210°. An additional two recrystallizations from the same solvent gave a *sample* of m.p. 210—212°, $[\alpha]_D$ −62° (*c* 1·16 in MeOH), $[\alpha]_D$ −40° (*c* 1·33 in $Me_2N{\cdot}CHO$) (Found: C, 59·55, 59·0; H, 8·65, 8·65; N, 12·2. $C_{48}H_{74}N_{10}O_{15},C_{12}H_{23}N$ requires C, 59·45; H, 8·05; N, 12·7%).

The salt (52 mg) was subjected to a 60-tube countercurrent distribution in 5 : 5 : 8 : 2 chloroform–toluene–methanol–water.[12] Tubes 37—53 were found to contain the peptide, with *ca.* 30 mg in tubes 43—47. The distribution curve corresponded to that of a pure specimen, and this was supported by t.l.c. (4 : 1 : 1 butan-1-ol–acetic acid–water; iodine development) examination of tubes 37—53. In each case the same single spot of identical mobility was detected. A total of 46 mg was collected from tubes 37—53.

The salt (VIIIb) (5 mg) dissolved in 6N-hydrochloric acid (1 ml) was hydrolysed at 110° for 20 h in an evacuated, sealed Pyrex tube. The solution was lyophilized and the residue dissolved in citrate buffer (1 ml) (' sample diluter '). The solution (50 μl) was diluted to 10 ml with sample diluter and a 1 ml sample (0·5 ml per column) of the new solution was used in the amino-acid analysis. The analysis showed the presence of threonine, serine, proline, glycine, alanine, valine, leucine, and tryptophan only, in the molar proportions 2·1, 1·0, 1·1, 1·0, 1·1, 0·8, 0·9, and 0·4, respectively.

A solution of the nonopeptide (VIIIb) (1·58 mg) in trifluoroacetic acid (0·5 ml) was set aside for 2 h. The solvent was removed *in vacuo* and the residue was dissolved in methanol (*ca.* 2 ml); the solution was evaporated to dryness. A solution of the residue in 0·03M-phosphate buffer, pH 7·5 (1 ml) was treated with 5000 munits of Aminopeptidase M dissolved in the same buffer solution. After 42 h at room temperature, the solution was made acidic with acetic acid (0·5 ml) and lyophilized. The residue was dissolved in water (0·5 ml) and a portion of the resulting solution was spotted on a 20 × 20 cm silica gel G thin-layer plate (250 μm thick). Two-dimensional development with chloroform–methanol–17% aqueous ammonia (2 : 2 : 1 w/w) and phenol–water (3 : 1 w/w) gave spots with the following R_F values only (amino-acid, R_{F1}, R_{F2}): Ser, 0·63, 0·11; Gly, 0·64, 0·17; Thr, 0·70, 0·22; Ala, 0·68, 0·27; Pro, 0·72, 0·28; Val, 0·72, 0·38; Leu, 0·75, 0·46; Trp, 0·76, 0·54.

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[1/1363 *Received*, 3*rd August*, 1971]

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