Insulin Peptides. Part XXIII.[1] The Synthesis of a Hexadecapeptide Derivative Related to the B Chain of Human Insulin

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A protected hexadecapeptide with the amino-acid sequence found at the *C*-terminal region of the human insulin B chain has been prepared by condensation of *N*-terminal hexapeptide and *C*-terminal decapeptide fragments, which were prepared stepwise. All the protecting groups employed are labile to hydrogen fluoride.

WE have reported previously [1,2] the synthesis of the B chains of human and sheep insulin and their isolation in the *S*-sulphonated form. In these syntheses the final steps consisted of the construction of the protected triacontapeptide containing the amino-acid sequence of the B chain, the removal of the protecting groups with sodium in liquid ammonia, and the oxidative sulphitolysis of the reduced product. The yield of the desired material however was low, since a number of by-products were formed and their removal required application of rigorous purification procedures. We felt that some of these by-products were probably formed as a result of the sodium in liquid ammonia deblocking step; we therefore investigated alternative routes for B chain synthesis which would not require the use of sodium in liquid ammonia. An alternative route involved the use of protecting groups that could be removed by liquid hydrogen fluoride, a reagent originally used successfully by Sakakibara [3] for deprotection during peptide synthesis.

The present report describes the synthesis of a hexadecapeptide derivative (XVI) containing the amino-acid sequence found at the *C*-terminal region of the B chain of human insulin (sequence B^{15}—B^{30}) and with its secondary functions protected with groups removable by liquid hydrogen fluoride. This peptide derivative is the key intermediate for a novel synthesis of the human B chain, described in the following paper,[4] and for the synthesis of analogues of that chain.

For the construction of the hexadecapeptide derivative (XVI) the *C*-terminal partially protected decapeptide (IXa) (sequence B^{21}—B^{30}) and the adjacent partially protected hexapeptide (XV) (sequence B^{15}—B^{20}) were prepared and then condensed. Details are given in the Scheme.

The *C*-terminal decapeptide derivative (IX) was synthesized by the stepwise approach. The t-butoxycarbonyl group was used exclusively for the protection of the α-amino-function, whereas the benzyloxycarbonyl group was employed for the protection of the ε-amino-function of lysine. The guanidino-group of arginine and the hydroxy- and carboxy-functions were protected by use of the nitro- and the benzyl group, respectively. Activation of the acylamino-acid used at each synthetic step was accomplished by conversion into the corresponding *p*-nitrophenyl esters (in one instance the *N*-succinimidyl ester). The t-butoxycarbonyl-L-threonine and the N^{α}-t-butoxycarbonyl-N^{ω}-nitro-L-arginine, however, were activated by the dicyclohexylcarbodiimide–*N*-hydroxysuccinimide method of Weygand.[5] In the latter case, only 0·5 equiv. of *N*-hydroxysuccinimide was used and the products were obtained in good yields.

Removal of the t-butoxycarbonyl group at each synthetic step was carried out by exposure to 98% formic acid.[6] Deblocking by trifluoroacetic acid was avoided since in preliminary experiments we have observed that the ε-benzyloxycarbonyl group of lysine is not stable to treatment with trifluoroacetic acid. Similar experiences have been reported.[7] Interaction in the presence of triethylamine of formic acid salts of the deblocked peptide intermediates with acylamino-acid activated esters led however occasionally to ninhydrin-negative products; amino-acid analysis after acidic hydrolysis indicated that these products did not contain the acylamino-acid used for chain elongation. It is possible that formylation of the peptide derivative occurred through an activated formic acid intermediate. We have overcome this complication by first isolating the free base of the peptide intermediate and then allowing it to react with the activated acylamino-acid.

In the construction of the decapeptide (IX), the tri-, tetra-, and penta-peptide intermediates were obtained as oils and no attempt was made to convert them into crystalline derivatives for further characterization. All the other peptide intermediates and the final product were solids, and were characterized by elemental analysis and t.l.c.

The stepwise method, with *p*-nitrophenyl or *N*-hydroxysuccinimidyl esters used for peptide bond formation, was also employed for the construction of the *N*-terminal hexapeptide fragment (XV). The t-butoxycarbonyl group was employed for the protection of the α-amino-function and the diphenylmethyl group for blocking the thiol function.[8] Removal of the α-amino-group protector was carried out by treatment with trifluoroacetic acid (in one instance with formic acid).

[1] Part XXII, P. G. Katsoyannis, J. Ginos, C. Zalut, M. Tilak, S. Johnson, and A. Trakatellis, *J. Amer. Chem. Soc.*, 1971, **93**, 5877.

[2] (*a*) P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *J. Amer. Chem. Soc.*, 1964, **86**, 930; (*b*) P. G. Katsoyannis, A. Tometsko, J. Ginos, and M. Tilak, *ibid.*, 1966, **88**, 164; (*c*) P. G. Katsoyannis, C. Zalut, A. Tometsko, M. Tilak, S. Johnson, and A. Trakatellis, *ibid.*, 1971, **93**, 5871.

[3] S. Sakakibara, in 'Chemistry and Biochemistry of Amino Acids, Peptides and Proteins,' ed. B. Weinstein, Marcel Dekker, New York, 1971, p. 51.

[4] G. P. Schwartz and P. G. Katsoyannis, following paper.

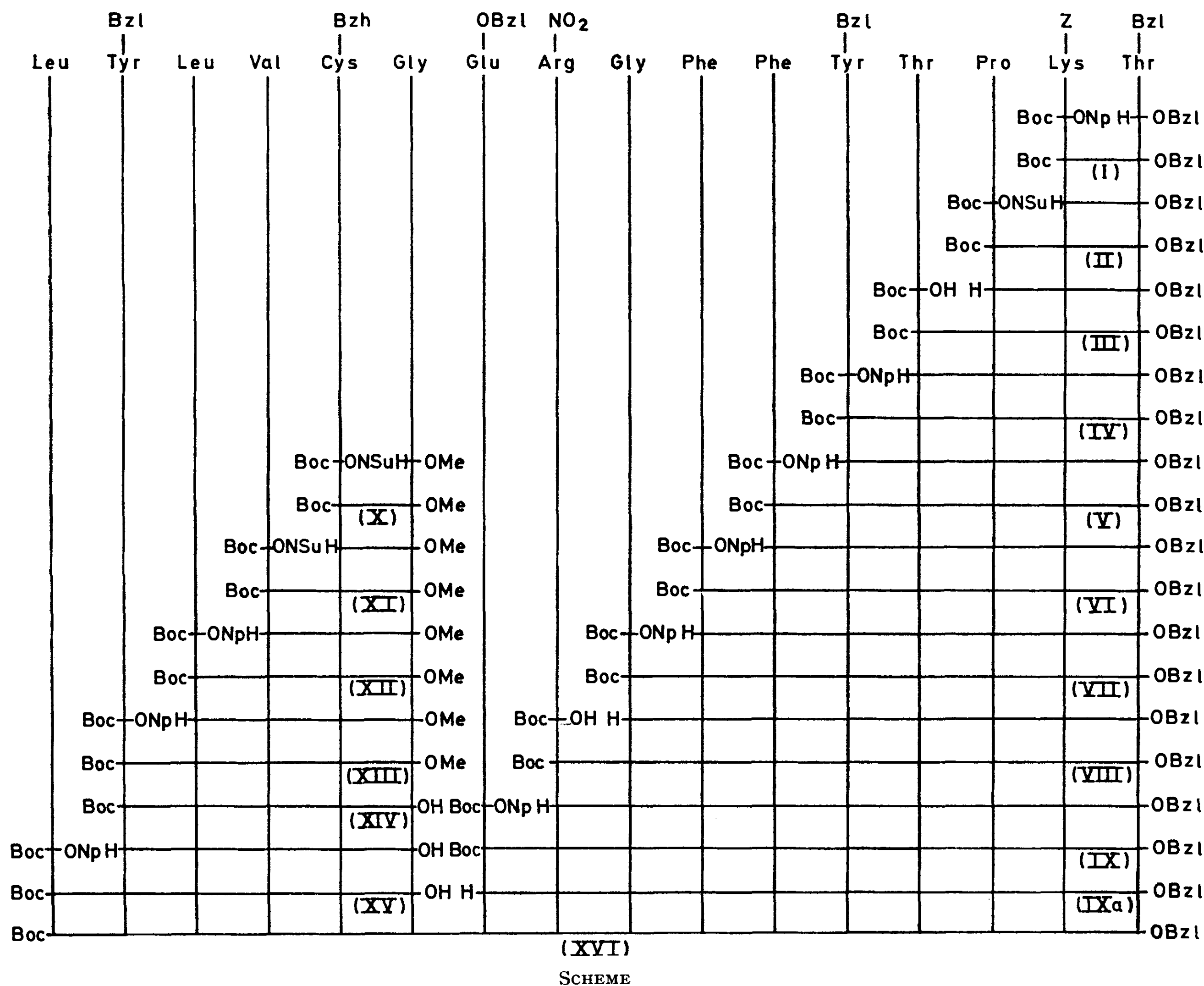
[5] F. Weygand, D. Hoffman, and E. Wünsch, *Z. Naturforsch.*, 1966, **21b**, 426.

[6] B. Halpern and D. E. Nitecki, *Tetrahedron Letters*, 1967, 3031.

[7] A. Yarn and S. F. Schlossmen, *Biochemistry*, 1968, **7**, 2673.

[8] I. Photaki, J. Taylor-Papadimitriou, C. Sakarellos, P. Mazarakis, and L. Zervas, *J. Chem. Soc.* (*C*), 1970, 2683.

For the synthesis of the hexadecapeptide derivative (XVI), the *N*-terminal hexapeptide fragment (XV) was condensed with the *C*-terminal decapeptide fragment (IXa), which was obtained by deblocking of the fully protected derivative (IX) with formic acid. The condensation was effected *via* the hydroxybenzotriazole–dicyclohexylcarbodi-imide method of Konig and Geiger.[9] Specifically, the hydroxybenzotriazole ester of the hexapeptide fragment (XV) was formed *in situ* and then treated with the amino-component (IXa). Complete and rapid coupling was achieved by employing an excess of the active ester at sufficiently high concentration, such that its level did not fall below 0·05M at completion.



SCHEME

EXPERIMENTAL

T.l.c. was used to confirm the purity of all intermediates. Thus, the protected peptides were deblocked by exposure to trifluoroacetic acid or formic acid and the products were chromatographed on 6060 silica gel (Eastman Chromatogram Sheet, Eastman Kodak Co., Rochester, New York). The following solvent systems were used: A, butan-1-ol–acetic acid–water (4 : 1 : 1); B, butan-1-ol–acetic acid–water–pyridine (60 : 6 : 24 : 20). Compounds were located with ninhydrin reagent. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. Amino-acid analyses of acid hydrolysates were carried out according to the method of Spackman *et al.*,[10] with a Beckman–Spinco amino-acid analyser (model 120C) equipped with a digital readout system (model CRS 12 AB, Infotronics Corp., Houston, Texas). The reported values for serine, tyrosine, and half-cystine are not corrected. All operations said to be carried out in the cold were performed at 4 °C unless otherwise stated. In all synthetic steps, coupling of the fragments was followed by detection of the amino-component present with ninhydrin; completion of the reaction was indicated by a negative ninhydrin test. The t-butoxycarbonylamino-acids used were obtained from the Cyclo Chemical Corporation, Los Angeles, California. The following abbreviations are used: Z, benzyloxycarbonyl;

[9] W. Konig and R. Geiger, *Chem. Ber.*, 1970, **103**, 788.
[10] D. H. Spackman, W. H. Stein, and S. Moore, *Analyt. Chem.*, 1958, **30**, 1190.

Bzl, benzyl; Me, methyl; Np, *p*-nitrophenyl; NSu, *N*-succinimidyl; Boc, t-butoxycarbonyl; DMF, dimethylformamide; Me_2SO, dimethyl sulphoxide; Bzh, diphenylmethyl.

N^{α}-*t-Butoxycarbonyl*-N^{ε}-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (I).—*O*-Benzyl-L-threonine hemioxalate [11] (25·6 g) was partitioned between ethyl acetate and M-potassium carbonate. The organic layer was washed with water, dried ($MgSO_4$), and concentrated to dryness *in vacuo*. The residue was dissolved in DMF (120 ml) and N^{α}-t-butoxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester [12] (33 g) was added. After stirring for 24 h, the mixture was poured into ethyl acetate (500 ml) and water (100 ml). The organic layer was separated, washed (successively with N-NH_4OH, 0·2N-HCl, and water), dried ($MgSO_4$), and concentrated to a small volume *in vacuo*. Upon addition of petroleum, the product crystallized. Recrystallization from ethyl acetate–petroleum gave the *product* (36·5 g, 83%), m.p. 97—98°; $[\alpha]_D^{26}$ −9·1° (*c* 1·0 in DMF); homogeneous in solvent A after formic acid deblocking (Found: C, 67·4; H, 7·05; N, 6·4. $C_{37}H_{47}N_3O_8$ requires C, 67·2; H, 7·15; N, 6·4%).

N-*t-Butoxycarbonyl*-L-*prolyl*-N^{ε}-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (II).—A solution of (I) (35 g) in 98% formic acid (200 ml) was stored at room temperature for 3 h and then the solvent was removed *in vacuo*. The oily residue was dissolved in a mixture of cold ethyl acetate (1 l) and cold water (500 ml) and 2N-ammonia was added until the pH of the aqueous layer was 9·0. The organic layer was separated, washed with water until neutral, dried ($MgSO_4$), and concentrated to dryness under reduced pressure. The remaining oil was dissolved in DMF (150 ml) and *N*-t-butoxycarbonyl-L-proline *N*-hydroxysuccinimide ester [13] (16·7 g) was added. After 2 days, the mixture was poured into ethyl acetate (1 l) and washed (N-NH_4OH, 0·2N-HCl, and water). The organic layer was dried ($MgSO_4$) and concentrated to dryness to give an oil (34·3 g, 99%), homogeneous on t.l.c. in solvent A, after formic acid deblocking.

N-*t-Butoxycarbonyl*-L-*threonyl*-L-*prolyl*-N^{ε}-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (III).—The oily compound (II) (35 g) was treated with 98% formic acid (200 ml) for 3 h. The formic acid was removed *in vacuo* and the remaining oil was dissolved in ethyl acetate and treated with aqueous ammonia as described previously. The resulting peptide bearing a free amino-group was dissolved in acetonitrile (300 ml) and *N*-t-butoxycarbonyl-L-threonine (11·6 g) was added. To this solution, cooled to 4°, *N*-hydroxysuccinimide (3 g) and dicyclohexylcarbodi-imide (11 g) were added. After 2 days at room temperature, the precipitated dicyclohexylurea was filtered off and the filtrate concentrated to dryness. A solution of the residue in ethyl acetate was washed (N-NH_4OH, 0·2N-HCl, and water), dried ($MgSO_4$), and concentrated to dryness to give an oil (40 g, 98%). After formic acid deblocking, the peptide was homogeneous on t.l.c. in solvent A. Amino-acid analysis after acid hydrolysis: $Lys_{1·0}Thr_{1·9}Pro_{1·0}$.

N-*t-Butoxycarbonyl*-O-*benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N^{ε}-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (IV).—The tetrapeptide ester (III) (40 g) was treated with 98% formic acid (200 ml) and the deblocked product was worked up as described previously. The resulting peptide derivative bearing a free amino-group was dissolved in DMF (130 ml) and t-butoxycarbonyl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester [14] (24 g) was added. After stirring for 2 days at room temperature, the mixture was poured into ethyl acetate and the resulting solution was washed (N-NH_4OH, 0·2N-HCl, and water), dried ($MgSO_4$), and concentrated under reduced pressure to give an oil (45 g, 90%). After deblocking with formic acid, the material gave a single spot on t.l.c. in solvent A.

N-*t-Butoxycarbonyl*-L-*phenylalanyl*-O-*benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N^{ε}-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (V).—The pentapeptide ester (IV) (45 g) was deblocked in 98% formic acid (200 ml) and the free base was isolated as described previously. This product (44 g) was dissolved in DMF (100 ml) and *N*-t-butoxycarbonyl-L-phenylalanine *p*-nitrophenyl [15] ester (17 g) was added. After 24 h at room temperature, the mixture was diluted with ethyl acetate and the solution was washed (N-NH_4OH, 0·2N-HCl, and water), dried ($MgSO_4$), and concentrated *in vacuo*. The oily residue solidified after trituration with cold petroleum and crystallized on reprecipitation from 95% ethanol; yield 38 g (85%), m.p. 161—162°; $[\alpha]_D^{26}$ −32·4° (*c* 1·0 in DMF); homogeneous in solvent A after formic acid deblocking (Found: C, 67·1; H, 6·65; N, 7·8. $C_{71}H_{85}N_7O_{14}$ requires C, 67·6; H, 6·8; N, 7·8%).

N-*t-Butoxycarbonyl*-L-*phenylalanyl*-L-*phenylalanyl*-O-*benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N^{ε}-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (VI).—The hexapeptide derivative (V) (27 g) was treated with 98% formic acid (200 ml) for 3 h. The solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate (1 l) and the solution was washed with aqueous ammonia as described previously. During the washing of the organic layer with water, the free base of the hexapeptide began to precipitate. This material was collected by filtration and the ethyl acetate filtrate was dried and concentrated to a small volume. Addition of petroleum caused precipitation of more of the free base. The two fractions were combined and dried (P_2O_5) *in vacuo*. To a solution of this product (24 g) in DMF (100 ml) *N*-t-butoxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (7·5 g) was added. After 24 h the mixture was poured into cold water (2 l) saturated with sodium chloride. The precipitated product was collected and washed (0·5N-NH_4OH, 2% acetic acid, and water). The wet solid was suspended in acetone (400 ml) and ether (400 ml) was added. After cooling for several hours, the precipitated *product* was collected by filtration and on trituration with hot 95% ethanol crystallized; yield 24 g (80%); m.p. 207—208°; $[\alpha]_D^{26}$ −28·9° (*c* 1·0 in DMF). After formic acid deblocking the peptide gave a single spot on t.l.c. in solvent A (Found: C, 68·1; H, 6·65; N, 8·0. $C_{80}H_{94}N_8O_{15}$ requires C, 68·25; H, 6·7; N, 7·95%).

N-*t-Butoxycarbonylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-O-*benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N^{ε}-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (VII).—The heptapeptide (VI) (24 g) was deblocked on exposure to 98% formic acid (200 ml) for 3 h. The solvent was removed

[11] T. Mizoguchi, G. Levin, D. W. Woolley, and J. M. Stewart, *J. Org. Chem.*, 1968, **33**, 903.
[12] K. Suzuki, *Chem. and Pharm. Bull.* (*Japan*), 1966, **14**, 909.
[13] G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, 1964, **86**, 1839.
[14] H. Zahn, W. Danho, and B. Gutte, *Z. Naturforsch.*, 1966, **21*b***, 763.
[15] E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, 1963, **46**, 1637.

under reduced pressure and the residue was solidified upon trituration with ether. A solution of this solid in DMF (70 ml) was mixed with N-ammonia (30 ml) and immediately poured into cold water (1 l) saturated with sodium chloride. The pH of the mixture was adjusted to 9·5 with N-NH_4OH and the precipitated free base of the hexapeptide derivative was collected, washed with water until neutral, and dried (P_2O_5) *in vacuo*. To a solution of this material in DMF (125 ml), t-butoxycarbonylglycine *p*-nitrophenyl ester [15] (4 g) was added. After 24 h the mixture was poured into a cold saturated solution of sodium chloride containing N-ammonia (20 ml). The precipitated product was isolated, washed with water and ether, and crystallized on trituration with acetone. Recrystallization from 95% ethanol gave the *product* (21 g, 84%), m.p. 198—200°; $[\alpha]_D^{26}$ −31·8° (*c* 1 in DMF). After deblocking with formic acid, the peptide was homogeneous on t.l.c. in solvent A (Found: C, 67·5; H, 6·7; N, 8·6. $C_{82}H_{97}N_9O_{16}$ requires C, 67·2; H, 6·7; N, 8·6%).

N$^{\alpha}$-*t-Butoxycarbonyl*-N$^{\omega}$-*nitro*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-O-*benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N$^{\varepsilon}$-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (VIII).—The fully protected octapeptide (VII) (19 g) was deblocked by 98% formic acid in the usual way. The solvent was removed and the residue solidified on trituration with ether. This solid was dissolved in cold DMF (100 ml) and the solution was diluted with N-ammonia (20 ml) and immediately poured into cold water (1 l) saturated with sodium chloride. The pH of the mixture was adjusted to 9·5 (N-NH_4OH) and the precipitated product was filtered off, washed with cold water until neutral, and dried (P_2O_5). To a cold solution of this product (18·4 g) in DMF (100 ml), N^{α}-t-butoxycarbonyl-N^{ω}-nitroarginine (4·3 g) was added, followed by *N*-hydroxysuccinimide (1·6 g) and dicyclohexylcarbodi-imide (2·8 g). After 24 h at room temperature, the precipitated dicyclohexylurea was filtered off and the filtrate poured into cold water saturated with sodium chloride. The precipitated product was collected by filtration and washed (N-NH_4OH, 2% acetic acid, and water). The wet *solid* was reprecipitated from propan-2-ol and subsequently from 95% ethanol; yield 16 g (75%); m.p. 189—191°; $[\alpha]_D^{26}$ −31·2° (*c* 1·0 in DMF); on t.l.c. it was homogeneous in solvent A after formic acid deblocking (Found: C, 63·2; H, 6·35; N, 11·6. $C_{88}H_{108}N_{14}O_{19}$ requires C, 63·4; H, 6·55; N, 11·8%).

N-*t-Butoxycarbonyl*-γ-*benzyl*-L-*glutamyl*-N$^{\omega}$-*nitro*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-O-*benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N$^{\varepsilon}$-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (IX).—The nonapeptide (VIII) (16 g) was deblocked on exposure to 98% formic acid (200 ml) and the free base was isolated as described in the synthesis of (VIII). To a solution of the free base of the nonapeptide (14·8 g) in DMF (100 ml), *N*-t-butoxycarbonyl-γ-benzyl-L-glutamic *p*-nitrophenyl ester [15] (5·5 g) was added. After 24 h the mixture was poured into cold water (1 l) saturated with sodium chloride. The *precipitate* was collected by filtration, washed (N-NH_4OH, water, and ether), reprecipitated from acetone–ether (100 : 600), and purified further by trituration with hot 95% ethanol and acetone; yield 15·5 g (86%), m.p. 184—186°; $[\alpha]_D^{26}$ −29·1° (*c* 1·0 in DMF), and was homogeneous on t.l.c. in solvents A and B after formic acid deblocking (Found: C, 63·5; H, 6·6; N, 10·9. $C_{100}H_{121}N_{15}O_{22}$ requires C, 63·7; H, 6·45; N, 11·1%). Amino-acid analysis after acid hydrolysis gave the following composition expressed in molar ratios: $Lys_{1·2}Arg_{1·0}Thr_{2·0}Glu_{1·0}Pro_{1·0}Gly_{1·0}Phe_{2·0}Tyr_{0·7}$.

N-*t-Butoxycarbonyl*-S-*diphenylmethyl*-L-*cysteinylglycine Methyl Ester* (X).—*N*-t-Butoxycarbonyl-*S*-diphenylmethyl-L-cysteine *N*-hydroxysuccinimide ester [16] (34 g) was added to a cold solution of glycine methyl ester hydrochloride (12 g) in DMF (100 ml) containing triethylamine (14 ml). The mixture was stirred overnight at room temperature and then poured into ethyl acetate (300 ml). The organic phase was washed (M-$NaHCO_3$, 0·2N-HCl, and water), dried ($MgSO_4$), and concentrated to a small volume. Addition of petroleum caused crystallization of the *product*; yield 26·6 g (87%), m.p. 120—121°; $[\alpha]_D^{26}$ −24·6° (*c* 1·0 in DMF); homogeneous on t.l.c. in solvent A after formic acid deblocking (Found: C, 62·9; H, 6·8; N, 6·2. $C_{24}H_{30}N_2O_5S$ requires C, 62·9; H, 6·6; N, 6·1%).

N-*t-Butoxycarbonyl*-L-*valyl*-S-*diphenylmethyl*-L-*cysteinylglycine Methyl Ester* (XI).—The dipeptide (X) (22 g) was dissolved in 98% formic acid (200 ml). After 3 h the solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate and treated with aqueous ammonia as described previously. The resulting free base of the dipeptide was dissolved in DMF (100 ml) and *N*-t-butoxycarbonyl-L-valine *N*-hydroxysuccinimide ester [13] (16 g) was added. After 48 h the mixture was poured into cold water (1 l) containing N-ammonia (20 ml). The *precipitate* was collected by filtration, washed (N-NH_4OH, water, 2% acetic acid, and water), and crystallized from 95% ethanol; yield 23 g (83%); m.p. 159—161°; $[\alpha]_D^{26}$ −33·6° (*c* 1·0 in DMF); homogeneous in solvent A after trifluoroacetic acid deblocking (Found: C, 62·7; H, 7·05; N, 7·7. $C_{29}H_{39}N_3O_6S$ requires C, 62·5; H, 7·05; N, 7·5%).

N-*t-Butoxycarbonyl*-L-*leucyl*-L-*valyl*-S-*diphenylmethyl*-L-*cysteinylglycine Methyl Ester* (XII).—A solution of the tripeptide (XI) (23 g) in trifluoroacetic acid (25 ml) was stored at room temperature for 1 h and then poured into cold ether (1 l). The precipitate was collected by filtration, washed with ether, and dried. To a solution of this product in cold DMF (100 ml) containing triethylamine (5·6 ml), *N*-t-butoxycarbonyl-L-leucine *p*-nitrophenyl ester [17] (14 g) was added. After 48 h the mixture was poured into cold water (800 ml) containing N-ammonia (20 ml). The *precipitate* was collected, washed (N-NH_4OH, water, and ether), and reprecipitated from a solution in acetone (200 ml) and ether (700 ml) by addition of petroleum (80 ml); yield 19 g (73%), m.p. 181—182°; $[\alpha]_D^{26}$ −31·0° (*c* 1·0 in DMF); homogeneous in solvent A after trifluoroacetic acid deblocking (Found: C, 62·6; H, 7·55; N, 9·1. $C_{35}H_{50}N_4O_7S$ requires C, 62·6; H, 7·5; N, 8·4%).

N-*t-Butoxycarbonyl*-O-*benzyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*diphenylmethyl*-L-*cysteinylglycine Methyl Ester* (XIII).—A solution of the tetrapeptide (XII) (21·3 g) in trifluoroacetic acid (25 ml) was stored for 1 h at room temperature and then poured into cold ether (800 ml). The precipitated trifluoroacetate was collected by filtration, washed with ether, and dried. To a solution of this material in DMF (100 ml), *N*-t-butoxycarbonyl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester [14] (15·7 g) and triethylamine (4·2 ml) were added. After 48 h the mixture was poured into cold water (1 l) saturated with sodium chloride. The *precipitate* was collected by filtration, washed (N-NH_4OH, water, 2%

[16] R. G. Hiskey, L. N. Beacham, and V. G. Matl, *J. Org. Chem.*, 1972, **37**, 2472.

[17] K. Vogler, R. O. Studer, P. Lanz, W. Lergier, and W. Bohni, *Helv. Chim. Acta*, 1965, **48**, 1161.

acetic acid, and water), reprecipitated from 95% ethanol, and then triturated with hot acetone; yield 23 g (78%); m.p. 223—225°; $[\alpha]_D^{26}$ −21·1° (*c* 1·0 in DMF); homogeneous in solvent A after trifluoroacetic acid deblocking (Found: C, 66·1; H, 7·05; N, 7·6. $C_{51}H_{65}N_5O_9S$ requires C, 66·3; H, 7·1; N, 7·6%).

N-*t-Butoxycarbonyl*-O-*benzyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*diphenylmethyl*-L-*cysteinylglycine* (XIV).—To a suspension of the protected pentapeptide ester (XIII) (3 g) in a mixture of acetone (75 ml) and 95% ethanol (25 ml), N-sodium hydroxide (4 ml) was added during 1 h. The mixture was stirred for an additional 20 min, cooled to 0°, diluted with N-hydrochloric acid (4 ml), and poured into cold water (1 l) saturated with sodium chloride. The precipitated partially protected *pentapeptide* was isolated, washed with water, and dried. On reprecipitation from ethyl acetate–ether, 2·5 g (85%) of product was obtained; m.p. >250° (decomp.); $[\alpha]_D^{26}$ −23·2° (*c* 1·0 in DMF). After deblocking with trifluoroacetic acid, the peptide showed a single spot on t.l.c. in solvent A (Found: C, 65·5; H, 6·95; N, 7·5. $C_{50}H_{63}N_5O_9S$ requires C, 66·0; H, 6·95; N, 7·7%).

N-*t-Butoxycarbonyl*-L-*leucyl*-O-*benzyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*diphenylmethyl*-L-*cysteinylglycine* (XV).—A solution of the partially protected pentapeptide (XIV) (2·5 g) in trifluoroacetic acid (15 ml) was stored at room temperature for 30 min and then poured into cold ether (400 ml). The trifluoroacetate salt of the peptide was collected by filtration, washed with ether, and dried. To a solution of this product in DMF (30 ml), *N*-t-butoxycarbonyl-L-leucine *p*-nitrophenyl ester (0·8 g) and triethylamine (0·56 ml) were added, followed by hexamethylphosphoramide (15 ml). After 48 h the mixture was diluted with ether (600 ml) and petroleum (600 ml) and cooled overnight. The precipitated hexapeptide derivative was collected by filtration, reprecipitated twice from acetone–ether, and triturated with warm propan-2-ol to give the *product* (2·4 g, 85%); m.p. >260°; $[\alpha]_D^{26}$ −32·6° (*c* 1·0 in DMF). After deblocking with trifluoroacetic acid, the peptide was homogeneous on t.l.c. in solvents A and B (Found: C, 65·0; H, 7·45; N, 8·4. $C_{56}H_{74}N_6O_{10}S$ requires C, 65·7; H, 7·3; N, 8·2%). The following amino-acid ratios were found after acidic hydrolysis: $Leu_{2\cdot0}Val_{1\cdot1}Gly_{1\cdot0}Cys_{0\cdot7}Tyr_{0\cdot6}$.

N-*t-Butoxycarbonyl*-L-*leucyl*-O-*benzyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*diphenylmethyl*-L-*cysteinylglycyl*-γ-*benzyl*-L-*glutamyl*-N^{ω}-*nitro*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-O-*benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N^{ϵ}-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (XVI).—A solution of the protected decapeptide (IX) (6·0 g) in 98% formic acid (150 ml) was stored at room temperature for 3 h. The solvent was then removed under reduced pressure and the residue solidified on trituration with ether. A solution of this product in DMF (60 ml) was cooled, diluted with N-ammonia (15 ml), and immediately poured into cold water (700 ml) saturated with sodium chloride. The pH of the mixture was adjusted to 9·5 (N-NH_4OH) and the precipitated decapeptide ester bearing a free α-amino-group was isolated by filtration, washed with cold water, and dried. This solid was then added to a solution in DMF of the hexapeptide derivative (XV), which was activated as follows: to a cold solution of the partially protected hexapeptide (XV) (5·8 g) in DMF (35 ml) 1-hydroxybenzotriazole (1 g) was added, followed by dicyclohexylcarbodi-imide (1·2 g). After 2 h, to this solution was added the free base of the decapeptide ester prepared as just described. The mixture was stirred for 24 h and then poured into a saturated solution of sodium chloride (500 ml) containing N-ammonia (20 ml). The precipitated protected *hexadecapeptide* (XVI) was isolated, washed with water and ether, and reprecipitated from DMF–propan-2-ol and from DMF–water; yield 7·1 g (80%); m.p. >260°; $[\alpha]_D^{26}$ −18·6° (*c* 1·0 in Me_2SO) (Found: C, 63·7; H, 6·65; N, 10·3; O, 18·1. $C_{151}H_{185}N_{21}O_{29}S,3H_2O$ requires C, 63·8; H, 6·8; N, 10·3; O, 18·0%). The following amino-acid ratios were found after acidic hydrolysis: $Lys_{1\cdot1}Arg_{0\cdot8}Thr_{2\cdot0}Glu_{1\cdot0}Pro_{1\cdot0}Gly_{2\cdot1}Val_{1\cdot0}Leu_{2\cdot0}Phe_{1\cdot9}Cys_{0\cdot5}Tyr_{0\cdot7}$.

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