Insulin Peptides. XVIII. The Synthesis of a Partially Protected Heneicosapeptide Containing the C-Terminal Sequence of the B Chain of Insulin[1,2]

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Abstract: The synthesis of a partially protected heneicosapeptide derivative with the amino acid sequence found at the carboxyl terminus of the B chain of insulin from various species is described. This synthesis was accomplished by two routes: (a) the coupling of the C-terminal heptadecapeptide fragment with the N-terminal tetrapeptide fragment and (b) more efficiently by the coupling of the C-terminal hexadecapeptide fragment with the N-terminal pentapeptide fragment.

The amino acid sequence of the carboxyl terminus of the B chain of bovine insulin (positions 10–30) as determined by Sanger and coworkers is histidylleucylvalylglutamylalanylleucyltyrosylleucylvalylcysteinylglycylglutamylarginylglycylphenylalanylphenylalanyltyrosylthreonylprolyllysylalanine.[3] The same amino acid sequence represents the carboxyl terminal portion of the B chain of sheep and porcine insulins.[4] In a previous communication, we have reported the synthesis of a heptadecapeptide derivative corresponding to the C-terminal seqence of the B chain of insulin (positions 14–30).[5] The present study relates synthetic routes to the partially protected heneicosapeptide N^{α}-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine (XIV) and its partially deblocked derivative XV. This heneicosapeptide fragment contains the C-terminal sequence of the B chain of insulin and served as the key intermediate in the synthesis of that chain.[2,6] In the early synthetic studies the heneicosapeptide derivative was prepared by coupling the C-terminal heptadecapeptide fragment with the N-terminal tetrapeptide fragment.[2] In subsequent studies, however, it was found that a more efficient method to prepare the desired heneicosapeptide derivative is the condensation of the C-terminal hexadecapeptide fragment with the N-terminal pentapeptide fragment. This latter route was also used successfully for the synthesis of the respective heneicosapeptide fragment of the B chain of human insulin.[7,8]

For the synthesis of the intermediate peptide derivatives the "fragment condensation" approach was employed.[9] Peptide subunits were prepared stepwise and then condensed to form larger peptide sequences. The benzyloxycarbonyl group was used[10] exclusively for the protection of the α-amino function and the *p*-nitrophenyl ester method[11,12] was employed to bring about peptide bond formation. Activation of the carboxyl group for fragment condensation was carried out by the azide method and in two instances by the carbonyldiimidazole[13] and the *p*-nitrophenyl ester methods.

The overall scheme which was employed for the synthesis of the partially protected heneicosapeptide XV by both routes is summarized in Chart I.

Hydrogenolysis of N^{α}-benzyloxycarbonyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine methyl ester[14] and coupling of the ensuing product with *N*-benzyloxycarbonyl-γ-*tert*-butyl-L-glutamic acid *p*-nitrophenyl ester[15] afforded the protected decapeptide I. The synthesis and the proof of stereochemical homogeneity of the same decapeptide derivative but with a benzyl ester group replacing the *tert*-butyl ester in the glutamic acid residue was discussed in a previous communication.[14] Decarbobenzoxylation of I by catalytic hydrogenation and coupling of the resulting product Ia with *N*-benzyloxycarbonyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycine[5] (II), using the *N,N'*-carbonyldiimidazole method, yielded the protected heptadecapeptide III in 54% yield. The crystalline N^{α}-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-L-glutamic acid α-ethyl-γ-*tert*-butyl ester (IV) was prepared by the *N,N'*-dicyclohexylcarbodiimide coupling of N^{α}-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucine[16] with the product obtained by catalytic hydrogenation of *N*-benzyloxycarbonyl-L-valyl-L-glutamic acid α-ethyl-γ-*tert*-butyl ester.[17] On exposure to

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(2) A preliminary report of part of the results described in this paper has appeared: P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *J. Amer. Chem. Soc.*, **86**, 930 (1964).

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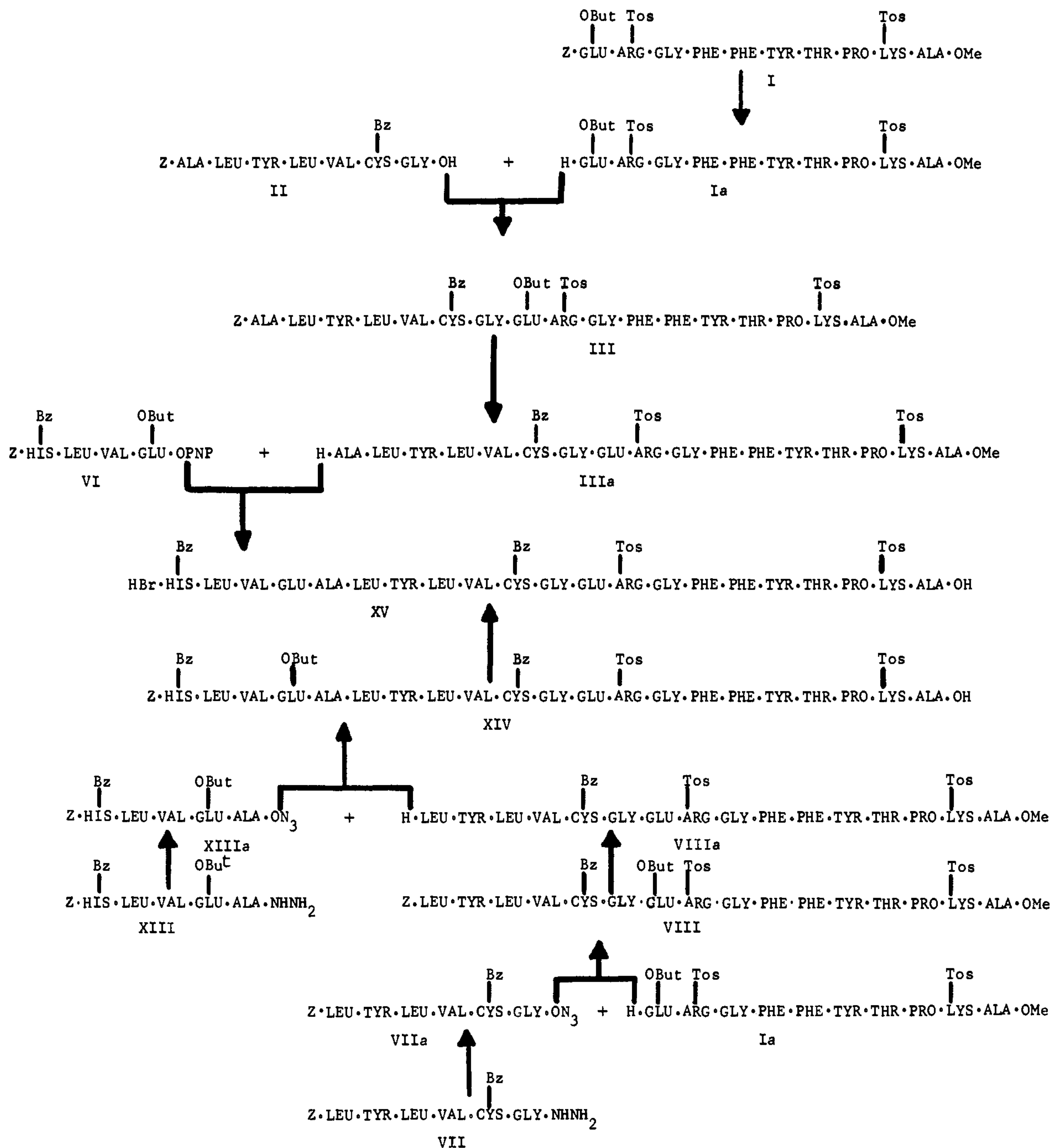
Chart I



NaOH the α-ethyl ester group was removed and the resulting partially protected tetrapeptide V was converted to the corresponding p-nitrophenyl ester VI. Interaction of the latter compound with the product IIIa obtained by HBr in acetic acid treatment of the heptadecapeptide fragment III yielded the protected heneicosapeptide derivative; this in turn, on exposure to NaOH and then to HBr in trifluoroacetic acid, afforded the desired partially protected heneicosapeptide XV as a white hygroscopic powder. Amino acid analysis after acid hydrolysis of XV gave a composition expressed in molar ratios in very good agreement with the values expected by theory. Attempts to obtain paper chromatographic data were unsuccessful since compound XV was practically insoluble in all the usual paper chromatographic systems employed in these studies. Similarly, the heneicosapeptide derivative XV was insoluble in the leucine aminopeptidase incubating medium[18] and hence no proof of its stereochemical homogeneity could be obtained. The last coupling step, however, in the synthesis of XV, namely the condensation of the tetrapeptide p-nitrophenyl ester VI with the partially protected heptadecapeptide derivative IIIa, is a point where racemization could have occurred. Consequently, we decided to prepare the heneicosapeptide derivative XV by a different route which involves synthetic steps that are known to proceed without any detectable racemization.

N-Benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinylglycine hydrazide (VII), which was prepared from the corresponding hexapeptide ester[5] on exposure to hydrazine, was converted to the solid azide VIIa. Interaction of the latter product with the decapeptide derivative Ia afforded the C-

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terminal protected hexadecapeptide VIII in 74% yield. N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanine methyl ester (XII) was prepared by the azide coupling of N^{α}-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucine with the product obtained by hydrogenolysis of *N*-benzyloxycarbonyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanine methyl ester (X). On exposure to hydrazine the protected pentapeptide ester XII is converted to the corresponding hydrazide XIII and this, in turn, in the usual way, is transformed to the solid azide XIIIa. Upon treatment with HBr in trifluoroacetic acid the protected hexadecapeptide VIII is converted to the partially protected derivative VIIIa. Interaction of VIIIa with the pentapeptide azide XIIIa yields the protected heneicosapeptide, which in turn, on exposure to NaOH, affords the partially protected derivative XIV in analytically pure form. Based on the amount of the protected hexadecapeptide VIII used, the overyield in the synthesis of the heneicosapeptide derivative XIV is 87% of theory. On exposure to HBr in trifluoroacetic acid, the protected heneicosapeptide XIV is converted to the derivative XV bearing a free amino group. Since all the coupling steps involving peptide fragments in this new approach were carried out by azide method, which experience has shown to proceed without any detectable racemization, it is assumed that the optical purity of the constituent amino acids has been preserved during the synthesis of the heneicosapeptide fragment. A more direct proof regarding the stereochemical homogeneity of this fragment, however, is provided by the fact that the B chain synthesized using the heneicosapeptide as the key intermediate was completely digested by aminopeptidase M;[6] furthermore, the insulin formed by combination of the synthetic B chain with natural or synthetic A chain was identical with the natural hormone.[19]

Experimental Section

Melting points for all compounds were taken in capillary tubes and are not corrected.

For paper chromatography the protected peptides were deblocked on exposure to 2 *N* HBr in acetic acid. The resulting hydrobromides were chromatographed on Whatman No. 1 filter paper. R_f^1 values refer to the Partridge system;[20] R_f^2 values refer to the system[21] 1-butanol–pyridine–acetic acid–water, 30:20:6:24 and are expressed as a multiple of the distance traveled by a histidine marker. The amino acid analyses of acid hydrolysates were carried out by the method of Moore, Spackman, and Stein[22] with a Beckman-Spinco amino acid analyzer, Model 120B. Acid hydrolysis was performed in constant-boiling HCl under nitrogen at 108° for 24 hr. To calculate the molar ratios given in this and the following articles, the average micromoles of glutamic acid, glycine, and leucine found were assumed to be equal to the theoretical number of residues in accordance with the known number of each of these residues in the compounds to be analyzed. Optical rotations were taken with a Rudolf precision polarimeter, Model 80, except if otherwise indicated. Enzymatic analysis by leucine aminopeptidase (LAP) was performed according to the procedure of Hill and Smith[18] using a crystalline enzyme (Worthington).

For taking infrared spectra, 2 mg of sample was thoroughly mixed with 250 mg of KBr and converted to a pellet under 18,000 lb of total load pressure with a Carver laboratory press, Model B. The infrared spectrum was taken with a Perkin-Elmer Infrared, Model 337. All operations were performed at 25°.

The following abbreviations are used: Z, benzyloxycarbonyl; Bz, benzyl; But, *tert*-butyl; Tos, *p*-toluenesulfonyl (tosyl); N_3, azide; PNP, *p*-nitrophenyl; Me, methyl; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HMPA, hexamethylphosphoramide.

N-Benzyloxycarbonyl-γ-*tert*-butyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine Methyl Ester (I). A solution of N^{α}-benzyloxycarbonyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine methyl ester (6.6 g) in methanol (200 ml) containing 1 *N* HCl (4.5 ml) was hydrogenated for 5 hr over 10% palladium/charcoal catalyst (3 g). The catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo*. The solid residue was dried by the addition of methanol followed by evaporation. To a solution of this solid in DMF (50 ml), triethylamine (0.75 ml) was added, followed by *N*-benzyloxycarbonyl-γ-*tert*-butyl-L-glutamic acid *p*-nitrophenyl ester (2.75 g). After 24 hr the reaction mixture was poured into ice-cold 0.5 *N* NH_4OH (300 ml). The precipitated product was isolated by filtration, washed successively with 1 *N* NH_4OH, water, 1% acetic acid, and water, and dried. On reprecipitation from methanol, 5.2 g (70%) of product was obtained: mp 206–209°; $[\alpha]^{27}D$ −25° (*c* 1, DMF).

Anal. Calcd for $C_{85}H_{110}N_{14}O_{21}S_2$: C, 59.1; H, 6.42; N, 11.4. Found: C, 59.5; H, 6.17; N, 11.1.

After treatment with HBr in acetic acid: R_f^1 0.90, R_f^2 4.43 × His, single ninhydrin-positive spot.

N-Benzyloxycarbonyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-γ-*tert*-butyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine Methyl Ester (III). A suspension of finely powdered I (1.5 g) in methanol (200 ml) containing acetic acid (0.5 ml) was hydrogenated for 6 hr over 10% palladium/charcoal catalyst (1.5 g). The catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo*. The residue was dried by the addition of methanol followed by evaporation. This product was subsequently used for condensation with the partially protected heptapeptide *N*-benzyloxycarbonyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycine (II). To a cooled (0°) solution of II (0.85 g) in DMF (20 ml) was added *N,N'*-carbonyldiimidazole (0.16 g). The reaction mixture was stirred at 0° for 2.5 hr and then diluted with a solution of the deblocked decapeptide ester Ia in DMF prepared as follows: the acetic acid salt of the decapeptide ester which was prepared as described above was dissolved in DMF (20 ml) containing triethylamine (0.14 ml) and then added to the activated carboxyl component prepared as described previously. After 24-hr stirring at room temperature the reaction mixture was diluted with DMF (20 ml) and poured into a mixture consisting of saturated $KHCO_3$ (20 ml), water (280 ml), and methanol (60 ml). The precipitated product was isolated by centrifugation and washed twice with a mixture of dimethylformamide–methanol–water (1:1:7) and finally with methanol: 1.17 g (54%); mp 256–259°. A sample for analysis was reprecipitated from dimethylformamide–water: melting point unchanged; $[\alpha]^{27}D$ −33.6° (*c* 1, DMF).

Anal. Calcd for $C_{126}H_{169}N_{21}O_{29}S_3H_2O$: C, 59.2; H, 6.74; N, 11.5; O, 18.8. Found: C, 58.7; H, 6.90; N, 11.5; O, 19.2.

For paper chromatography a sample was exposed to 2 *N* HBr in acetic acid: R_f^1 0.93, R_f^2 6.58 × His, single ninhydrin-positive spot. Amino acid analysis of an acid hydrolysate showed the expected composition expressed in molar ratios: $Lys_{1.1}Arg_{1.0}$-*S*-benzylcysteine$_{0.9}$$Thr_{0.9}Glu_{1.0}Gly_{2.1}Ala_{1.9}Val_{1.1}Leu_{2.2}Pro_{1.0}Tyr_{2.0}$-$Phe_{1.9}$.

N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid α-Ethyl-γ-*tert*-butyl Ester (IV). *N*-Benzyloxycarbonyl-L-valyl-L-glutamic acid α-ethyl-γ-*tert*-butyl ester (4.65 g) was dissolved in ethanol (80 ml) and hydrogenated for 2 hr over 10% palladium/charcoal catalyst (1 g). The catalyst was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. To a solution of the residue in tetrahydrofuran (60 ml), cooled to 0°, was added N^{α}-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucine (4.9 g) and *N,N'*-dicyclohexylcarbodiimide[23] (2.5 g). After 24 hr at 0° the precipitated *N,N'*-dicyclohexylurea was filtered off and the filtrate was evaporated to dryness *in vacuo*. Trituration of the

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(21) S. G. Waley and G. Watson, *ibid.*, **55**, 328 (1953).
(22) S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

(23) J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, **77**, 1067 (1955).

residue with ethanol resulted in the crystallization of the product which subsequently was recrystallized from 95% ethanol: 6 g (73%); mp 168–172°; $[\alpha]^{28}D$ −20.3° (*c* 1.26, DMF); $R_f{}^1$ 0.91 (chlorine test[24]); after HBr in acetic acid, $R_f{}^1$ 0.73, single ninhydrin-positive spot.

Anal. Calcd for $C_{43}H_{60}N_6O_9H_2O$: C, 62.8; H, 7.59; N, 10.2; O, 19.4. Found: C, 62.8; H, 7.42; N, 10.5; O, 19.0.

N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamic Acid (V). To a solution of IV (4.1 g) in dioxane (25 ml) was added, with stirring, 1 *N* NaOH (6 ml). The reaction mixture was allowed to stand at room temperature for 2.5 hr, cooled to 0°, and diluted with water (150 ml) and acetic acid (9 ml). The precipitated product was isolated by filtration, washed with water, and dried. This solid was dissolved in absolute ethanol (50 ml) and the undissolved material was removed by filtration. Upon addition of water (50 ml) to the filtrate, the product was precipitated: 2.7 g (71%); mp 178–182°; $[\alpha]^{29}D$ −16.5° (*c* 0.77, DMF); after HBr in acetic acid treatment, $R_f{}^1$ 0.67, single ninhydrin-positive spot.

Anal. Calcd for $C_{41}H_{56}N_6O_9$: C, 63.4; H, 7.27; N, 10.8. Found: C, 63.5; H, 7.34; N, 10.9.

N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-L-glutamic Acid γ-*tert*-Butyl-α-*p*-nitrophenyl Ester (VI). To a suspension of V (1.25 g) in acetonitrile (60 ml) cooled to 0° was added *p*-nitrophenol (0.3 g) and *N,N'*-dicyclohexylcarbodiimide (0.53 g). The reaction mixture was stirred at 2° for 24 hr and the precipitated *N,N'*-dicyclohexylurea was filtered off and washed with acetonitrile (15 ml). The combined filtrates were concentrated to dryness *in vacuo* and the solid obtained was washed with absolute ether (50 ml). This solid was dissolved in warm ethyl acetate (15 ml) and the solution was cooled to 15°. Removal by filtration of an additional amount of *N,N'*-dicyclohexylurea which was precipitated and mixing of the filtrate with petroleum ether (60 ml) resulted in the precipitation of the product. Upon reprecipitation from ethyl acetate–petroleum ether (1:3) 0.6 g of the protected tetrapeptide *p*-nitrophenyl ester was obtained: mp 113–116°; $[\alpha]^{27}D$ −14.5° (*c* 1, DMF).

Anal. Calcd for $C_{47}H_{62}N_7O_{11}$: C, 62.7; H, 6.90; N, 10.7. Found: C, 62.2; H, 6.43; N, 10.5.

***N*-Benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycine Hydrazide (VII).** To a solution of *N*-benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycine ethyl ester (40.8 g) in DMF (180 ml), hydrazine hydrate (27 ml) was added. After 4 days at room temperature, the reaction mixture was poured into cold water (1.5 l.) and the precipitated product was isolated by filtration, washed thoroughly with water, and dried. Upon trituration with hot methanol, 39 g (95%) of product was obtained; mp 285° dec. A sample for analysis was reprecipitated from dimethylformamide–water: melting point unchanged; $[\alpha]^{28}D$ −43° (*c* 1, DMF).

Anal. Calcd for $C_{46}H_{64}N_8O_9S$: C, 61.0; H, 7.13; N, 12.4. Found: C, 61.1; H, 7.31; N, 12.3.

Amino acid analysis after acid hydrolysis: $Gly_{1.0}Val_{1.0}Leu_{2.0}$-$Tyr_{0.8}$*S*-benzylcysteine$_{0.9}$.

***N*-Benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-γ-*tert*-butyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine Methyl Ester (VIII).** A solution of I (6.3 g) in a mixture of methanol (350 ml) and DMF (70 ml) containing acetic acid (1.6 ml) was hydrogenated for 4 hr over 10% palladium/charcoal catalyst (4 g). The catalyst was filtered off and washed thoroughly with methanol (200 ml) and DMF (50 ml). The combined filtrates were concentrated to a small volume *in vacuo* and then mixed with ether. The precipitated product was isolated by filtration, washed with ether, and dried. This product was dissolved in DMF (110 ml) containing triethylamine (0.30 ml), cooled to 0°, and allowed to react with *N*-benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycine azide prepared as follows. The hexapeptide hydrazide VII (3.5 g) was dissolved in DMF (60 ml) and 2 *N* HCl (9.1 ml) was added. After cooling this solution to −15° (Dry Ice–acetone), $NaNO_2$ (266 mg) dissolved in cold water (0.5 ml) was added. The reaction mixture was stirred at −15° for 6 min and then poured into a cold mixture consisting of a saturated solution of NaCl (200 ml) and sodium bicarbonate (20 ml). The precipitated hexapeptide azide was isolated by filtration, washed with water (0°), and dried for 2 hr over P_2O_5 at 0° *in vacuo.* The infrared spectrum of the dry product exhibited the characteristic azide band at 4.75 μ without any trade of the isocyanate band.[25] This azide was then added to the solution of the decarbobenzoxylated decapeptide prepared as described previously along with DMF (25 ml). The reaction mixture was stirred at 0° for 48 hr, diluted with DMF (25 ml), and then poured into cold water (600 ml) containing 1 *N* HCl (2 ml). The precipitated protected hexadecapeptide was isolated by filtration washed with water, triturated with hot methanol (250 ml in three portions), and reprecipitated from dimethylformamide–ether: 6.3 g (73%); mp 257–259°; $[\alpha]^{28}D$ −30° (*c* 1, DMF).[26]

Anal. Calcd for $C_{123}H_{164}N_{20}O_{28}S_3$: C, 59.9; H, 6.70; N, 11.4. Found: C, 59.4; H, 6.85; N, 11.5.

For paper chromatography a sample of the protected hexadecapeptide was deblocked on exposure to HBr in acetic acid; $R_f{}^1$ 0.92, $R_f{}^2$ 3.0 × His. Amino acid analysis of an acid hydrolysate showed the following composition in molar ratios: $Lys_{0.7}Arg_{0.9}$-$Thr_{0.9}Glu_{1.0}Pro_{1.0}Gly_{2.0}Ala_{0.9}Val_{1.0}Leu_{2.1}Tyr_{1.7}Phe_{1.9}$*S*-benzylcysteine$_{0.9}$.

***N*-Benzyloxycarbonyl-γ-*tert*-butyl-L-glutamyl-L-alanine Methyl Ester (IX).** To a solution of *N*-benzyloxycarbonyl-γ-*tert*-butyl-L-glutamic acid hydrazide[27] (35.1 g) in DMF (140 ml) cooled to −5°, 1 *N* HCl (80 ml) was added followed by $NaNO_2$ (7 g) in cold water (3 ml). After 5 min at −5° the solution was diluted with cold water (500 ml) and then extracted three times with cold ethyl acetate (total amount: 600 ml). The combined ethyl acetate extracts were washed successively with 1 *N* $NaHCO_3$ and water, dried with $MgSO_4$, and then added to a cold solution of L-alanine methyl ester hydrochloride (15 g) in methylene chloride (300 ml) containing triethylamine (15 ml). After 24 hr at 0° the reaction mixture was washed successively with cold 1 *N* acetic acid, water, 1 *N* $NaHCO_3$, and water, and dried over $MgSO_4$. Upon removal of the solvent *in vacuo* and reprecipitation of the residue from methanol–water the product was obtained in crystalline form: 38 g (90%); mp 102°; $[\alpha]^{28}D$ −15.5° (*c* 1, DMF)[26] (lit.[28] mp 102–104°; $[\alpha]^{20}D$ −13° (*c* 1, DMF)). After HBr in acetic acid cleavage $R_f{}^1$ 0.55, $R_f{}^2$ 2.64 × His, single ninhydrin-positive spot.

***N*-Benzyloxycarbonyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanine Methyl Ester (X).** This compound was prepared[2] from IX by the *p*-nitrophenyl ester method in essentially the same way described subsequently by Meienhofer.[28]

N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucine Hydrazide (XI). *N*-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucine benzyl ester[29] (11 g) was dissolved in methanol (60 ml) and treated with hydrazine hydrate (4.5 ml) at room temperature for 72 hr. Upon removal of the solvent *in vacuo* and reprecipitation of the residue from methanol–water, 9 g (94%) of product was obtained; mp 109°. A sample for analysis was crystallized from methanol–ether: mp 110–111°; $[\alpha]^{26}D$ −18.9° (*c* 1, acetic acid).[26]

Anal. Calcd for $C_{27}H_{34}N_6O_4$: C, 64.0; H, 6.69; N, 16.6. Found: C, 64.0; H, 7.00; N, 16.6.

N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanine Methyl Ester (XII). A solution of X (10.5 g) in methanol (250 ml) containing acetic acid (1.2 ml) was hydrogenated over 10% palladium/charcoal catalyst (2 g). After 1.5 hr the catalyst was filtered off, the filtrate was concentrated *in vacuo*, and the residue was dried by the addition of methanol followed by evaporation under reduced pressure. To a solution of the residue in methylene chloride (150 ml) containing triethylamine (2.8 ml) and cooled to 0° was added the azide of *N*-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucine prepared as follows: the dipeptide hydrazide XI (10.1 g) was dissolved in DMF (60 ml) and 2 *N* HCl (30 ml) was added. After cooling the solution to −10°, $NaNO_2$ (1.5 g) dissolved in water (2 ml) was added. The reaction mixture was stirred at −10° for 10 min and then poured into a precooled solution consisting of one-half saturated NaCl (300 ml) and saturated $NaHCO_3$ (30 ml). The precipitated dipeptide azide was extracted from the reaction mixture with cold ethyl acetate (500 ml). The ethyl acetate extract was washed with one-half saturated NaCl solution dried with $MgSO_4$ and subsequently added to the solution of the deblocked tripeptide ester prepared as described previously. After 48 hr at 0° the reaction mixture was concentrated to dryness

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in vacuo. The residue was dissolved in hot methanol (200 ml) and upon cooling of the solution the pentapeptide ester was precipitated: 13 g (75%); mp 176–178°. A sample for analysis was reprecipitated from methanol: mp 180–181°; $[\alpha]^{28}D$ −22° (*c* 1, DMF).[26]

Anal. Calcd for $C_{45}H_{63}N_7O_{10}$: C, 62.7; H, 7.36; N, 11.4. Found: C, 62.9; H, 7.64; N, 11.3.

For paper chromatography a sample was deblocked on exposure to HBr in acetic acid; R_f^1 0.84, R_f^2 3.5 × His; single ninhydrin-positive spot. Amino acid analysis of LAP digest of the deblocked material by the automatic analyzer gave the following composition expressed in molar ratios: $Glu_{1.0}Ala_{0.9}Val_{1.1}Leu_{1.1}$; *im*-benzylhistidine was not determined.

N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanine Hydrazide (XIII). To a solution of XII (30.2 g) in DMF (300 ml), hydrazine hydrate (15 ml) was added. After 72 hr the reaction mixture was cooled to 0° and the precipitated product was isolated by filtration and washed thoroughly with methanol: 27.2 g (90%); mp 243–244°; $[\alpha]^{28}D$ −17° (*c* 1, DMF).[26]

Anal. Calcd for $C_{44}H_{63}N_9O_9$: C, 61.4; H, 7.36; N, 14.6. Found: C, 61.2; H, 7.22; N, 14.5.

N^{α}-Benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine (XIV). A suspension of the hexadecapeptide derivative VIII (2 g) in acetic acid (18 ml) was treated with 4 *N* HBr in acetic acid (20 ml). After 1.5 hr the resulting solution was poured into ether (200 ml) and the precipitated peptide hydrobromide was isolated, washed with ether, and dried over KOH *in vacuo*. To a solution of this solid in DMF (75 ml), cooled to 0°, triethylamine (0.4 ml) was added followed by N^{α}-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanine azide prepared as follows: a suspension of the protected pentapeptide hydrazide XIII (2 g) in DMF (50 ml) was cooled to −15° and then diluted with 2 *N* HCl (6.5 ml). To the resulting solution was subsequently added $NaNO_2$ (190 mg) dissolved in cold water (1 ml). The reaction mixture was stirred at −15° for 5 min and then poured into a cold mixture consisting of one-half saturated aqueous solution of NaCl (200 ml) and a saturated solution of $NaHCO_3$ (10 ml). The precipitated pentapeptide azide was filtered off, washed with cold water, and dried for 2 hr at 0° over P_2O_5 *in vacuo*. The infrared spectrum of the dry material exhibited the characteristic azide band at 4.75 μ without any trace of the isocyanate band.[25] The dry azide was added to the solution of the amino component prepared as described previously. After 48 hr at 2° the reaction mixture was diluted with DMF (30 ml) and poured into methanol (700 ml) containing acetic acid (1 ml). The precipitated product was isolated by centrifugation, washed with methanol, and dried. A suspension of this material in a mixture of HMPA (55 ml) and DMF (35 ml) was stirred with occasional warming to 40° for 1 hr. To the resulting solution, cooled to 0°, was added over a period of 1 hr 1 *N* NaOH (5 ml). The reaction mixture was further stirred at 0° for 30 min and then diluted with cold water (500 ml) containing 1 *N* HCl (5.2 ml). The precipitated saponified heneicosapeptide was isolated by centrifugation, washed with water, and dried: 2.2 g (87%); mp 257–259°. A sample for analysis was reprecipitated from dimethyl sulfoxide–water: melting point unchanged; $[\alpha]^{27}D$ −25° (*c* 1, DMSO).[26]

Anal. Calcd for $C_{155}H_{209}N_{27}O_{35}S_3$: C, 59.9; H, 6.80; N, 12.2. Found: C, 59.7; H, 7.12; N, 12.2.

Amino acid analysis after acid hydrolysis showed the following composition expressed in molar ratios: $Lys_{1.1}Arg_{1.0}Thr_{1.0}Glu_{2.0}Pro_{1.1}Gly_{2.2}Ala_{1.9}Val_{1.7}Leu_{2.7}Tyr_{1.8}Phe_{2.2}$*S*-benzylcysteine$_{0.8}$ (im-benzylhistidine was not determined).

***im*-Benzyl-L-histidyl-L-leucyl-L-valyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine Hydrobromide (XV).** A. XIV (2.1 g) was dissolved in trifluoroacetic acid (20 ml) containing water (0.5 ml) and HBr was passed through the solution for 1 hr at 10°. Addition of ether to the reaction mixture caused the partially protected heneicosapeptide hydrobromide to precipitate. This material was isolated by filtration, washed with ether, and reprecipitated from dimethyl sulfoxide–ethyl acetate: 2.0 g (94%); mp 267–268°; $[\alpha]^{27}D$ −23° (*c* 0.9, DMF).[26] Amino acid analysis of the heneicosapeptide derivative after acid hydrolysis gave the following amino acid ratios in agreement with the theoretically expected values: $Lys_{0.9}Arg_{1.1}Thr_{1.0}Glu_{2.0}Pro_{0.9}Gly_{2.1}Ala_{1.8}Val_{1.8}Leu_{2.7}Tyr_{1.8}Phe_{2.1}$*S*-benzylcysteine$_{0.8}$. *im*-Benzylhistidine was not determined. In none of the usual chromatographic systems employed in these studies did this peptide move from the origin; hence no paper chromatographic criteria could be obtained.

B. Compound III (1.15 g) was suspended in acetic acid (7 ml) and treated with 4 *N* HBr in acetic acid (8 ml) for 1 hr. The resulting solution was poured into anhydrous ether (100 ml) and the precipitated product was isolated by filtration, washed with ether, and dried over KOH *in vacuo*. To a solution of this product in DMF (50 ml) containing triethylamine (0.15 ml) and cooled to 0° was added compound VI (1 g) along with DMF (10 ml). After 2 hr at 0° and 45 hr at room temperature the reaction mixture was poured into methanol (450 ml) containing acetic acid (0.4 ml) and the resulting mixture was stirred for 1.5 hr at 0°. The precipitated product was isolated by centrifugation and washed thoroughly with methanol. This material was suspended in HMPA (100 ml) and stirred until all the solid was dissolved (*ca.* 1 hr). To this solution, cooled to 0°, 1 *N* NaOH (2.85 ml) was added dropwise over a period of 0.5 hr, and the reaction mixture was further stirred at 0° for 1 hr and subsequently diluted with water (400 ml) and 1 *N* HCl (3 ml). The precipitated partially protected heneicosapeptide was isolated by centrifugation, washed successively with water, methanol, and ether, and dried: 0.83 g (61% based on III); mp 254–259° dec. This heneicosapeptide derivative was dissolved in trifluoroacetic acid (15 ml) containing water (0.5 ml) and HBr was passed through the solution at 0°. After 1 hr the reaction mixture was poured into ether (150 ml) and the precipitated heneicosapeptide hydrobromide was isolated by filtration, washed with ether, dried, and reprecipitated from dimethyl sulfoxide–ethyl acetate: 0.78 g; mp 265–268°; $[\alpha]^{27}D$ −24.4° (*c* 0.9, DMF). Amino acid analysis of an acid hydrolysate of the heneicosapeptide hydrobromide by the automatic analyzer showed the following composition expressed in molar ratios: *S*-benzylcysteine$_{0.7}$$Lys_{1.1}Arg_{1.2}Thr_{1.0}Glu_{2.0}Pro_{1.0}Gly_{2.3}Ala_{2.0}Val_{1.8}Leu_{2.9}Tyr_{2.0}Phe_{2.2}$; *im*-benzylhistidine was not determined.

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