241–243°; $[\alpha]^{26}D$ −26.4° (*c* 1, acetic acid [lit.[26] mp 230–233; $[\alpha]^{22}D$ −27° (*c* 0.5, formic acid)]; after hydrogenation in methanol containing acetic acid, R_f^1 0.32, R_f^2 2.35 × His; single ninhydrin-positive spot. Amino acid ratios after acid hydrolysis are: $His_{1.0}Asp_{1.0}Glu_{1.0}Val_{1.0}Phe_{1.0}$.

Anal. Calcd for $C_{38}H_{49}N_9O_{10}$: C, 57.6; H, 6.23; N, 15.9. Found: C, 57.8; H, 6.50; N, 15.8.

After catalytic hydrogenation and digestion with leucine amino peptidase,[27] amino acid ratios are: $His_{1.0}Val_{1.1}Phe_{1.1}$. Glutamine and asparagine emerge at the same position and were not determined.

***N*-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidine Hydrazide (XV).** To a suspension of XIV (5 g) in DMF (200 ml) containing hexamethylphosphoramide (10 ml) which was heated to 60° for a few minutes and then cooled to 40° was added hydrazine hydrate (10 ml). The resulting solution was stirred at 40° for 5 hr and at room temperature for 25 hr. During this period the reaction mixture thickened considerably and it was necessary to dilute it with methanol (220 ml). The precipitated hydrazide was subsequently filtered off and washed successively with methanol and water and dried: 4.8 g (96%); mp 245°. A sample for analysis was reprecipitated from dimethyl sulfoxide–methanol: mp 248–250°; $[\alpha]^{27}D$ −18.6° (*c* 1, DMSO) [lit.[26] mp 238–240°; $[\alpha]^{22}D$ −36° (*c* 0.5, formic acid)].

Anal. Calcd for $C_{37}H_{49}N_{11}O_9$: C, 56.1; H, 6.23; N, 19.5. Found: C, 56.0; H, 6.43; N, 19.4.

***N*-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine Methyl Ester (XVI).** To a solution of compound XV (790 mg) in a mixture of DMSO (6 ml) and DMF (6 ml) was added 8.3 *N* HCl in freshly distilled tetrahydrofuran (0.5 ml). To this solution cooled to −20° (Dry Ice–acetone), *tert*-butylnitrite (0.14 ml) was added and the reaction was allowed to proceed for 6 min. To the reaction mixture, cooled to −30°, was then added a solution of L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine methyl ester hydrobromide, prepared from its *N*-benzyloxycarbonyl derivative (1.3 g) as described previously, in the synthesis of compound IX, in DMF (4 ml) containing triethylamine (0.28 ml). After 24 hr at 2° and 24 hr at room temperature the reaction mixture (its pH being kept at about 8.0 by the addition of triethylamine) was diluted with methanol (15 ml) and its pH was adjusted to 6 with acetic acid After 30 min the precipitated material was removed by filtration: 22 mg. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $His_{0.9}Asp_{1.0}Ser_{0.2}Glu_{1.0}Gly_{0.2}Val_{1.0}Leu_{0.2}Phe_{1.0}$*S*-benzylcysteine$_{0.16}$ indicating that this material is predominantly unreacted pentapeptide derivative. The above filtrate was diluted further with methanol (80 ml) and allowed to stand at room temperature overnight. The precipitated product was isolated by filtration and washed with methanol and ether: 737 mg (59%); mp 239–240°. A sample for analysis was reprecipitated from dimethyl sulfoxide–methanol: melting point unchanged; $[\alpha]^{27}D$ −27.7° (*c* 1, DMSO); after HBr in trifluoroacetic acid treatment, R_f^1 0.62, R_f^2 4.2 × His.

Anal. Calcd for $C_{59}H_{79}N_{13}O_{15}S$: C, 57.0; H, 6.41; N, 14.7. Found: C, 57.2; H, 6.32; N, 14.7.

Amino acid analysis after acid hydrolysis showed the following composition expressed in molar ratios: $His_{1.0}Asp_{1.0}Ser_{0.8}Glu_{1.0}Gly_{0.9}Val_{1.0}Leu_{0.9}Phe_{1.0}$*S*-benzylcysteine$_{0.7}$.

***N*-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine Hydrazide (XVII).** The protected nonapeptide ester XVI (20 g) was dissolved in DMF (600 ml) and treated with hydrazine hydrate (40 ml). After 96 hr at room temperature, the reaction mixture was diluted with ice-cold water (4 l.) and the precipitated hydrazide was filtered quickly and washed with water. On reprecipitation from dimethyl sulfoxide–water the product was obtained in analytically pure form: 16 g (80%); mp 250° dec; $[\alpha]^{27}D$ −29° (*c* 1, DMSO).

Anal. Calcd for $C_{58}H_{79}N_{15}O_{14}S$: C, 56.1; H, 6.41; N, 16.9. Found: C, 55.8; H, 6.56; N, 16.5.

Amino acid analysis after acid hydrolysis showed the following composition expressed in molar ratios: $His_{1.0}Asp_{1.0}Ser_{0.8}Glu_{1.0}Gly_{1.0}Val_{1.0}Leu_{1.0}Phe_{1.0}$*S*-benzylcysteine$_{0.9}$. For evaluation of stereochemical homogeneity a sample of the decarbobenzoxylated (HBr in trifluoroacetic acid) derivative was digested with APM. Amino acid analysis of the digest gave the following amino acid composition expressed in molar ratios: $His_{1.0}Gly_{0.8}Val_{1.1}Leu_{0.9}Phe_{1.1}$*S*-benzylcysteine$_{0.7}$. Glutamine, asparagine, and serine emerge at the same position on the chromatogram and were not determined.

Acknowledgment. The authors wish to express their appreciation to Miss Karen Scheibe for the amino acid analyses and the enzymatic analyses.

(27) R. L. Hill and E. L. Smith, *J. Biol. Chem.*, 228, 577 (1957).

Insulin Peptides. XX. The Synthesis of a Heneicosapeptide Derivative Corresponding to the C-Terminal Sequence of the Human Insulin B Chain[1,2]

Panayotis G. Katsoyannis,* James Ginos, and Manohar Tilak

Contribution from the Department of Biochemistry, Mount Sinai School of Medicine, the City University of New York, New York, New York 10029.
Received March 2, 1971

Abstract: The synthesis of a heneicosapeptide derivative embodying the amino acid sequence found at the carboxyl terminus of the human insulin B chain is described. The desired product was obtained by interaction of the C-terminal hexadecapeptide fragment with the azide of the N-terminal nonapeptide fragment.

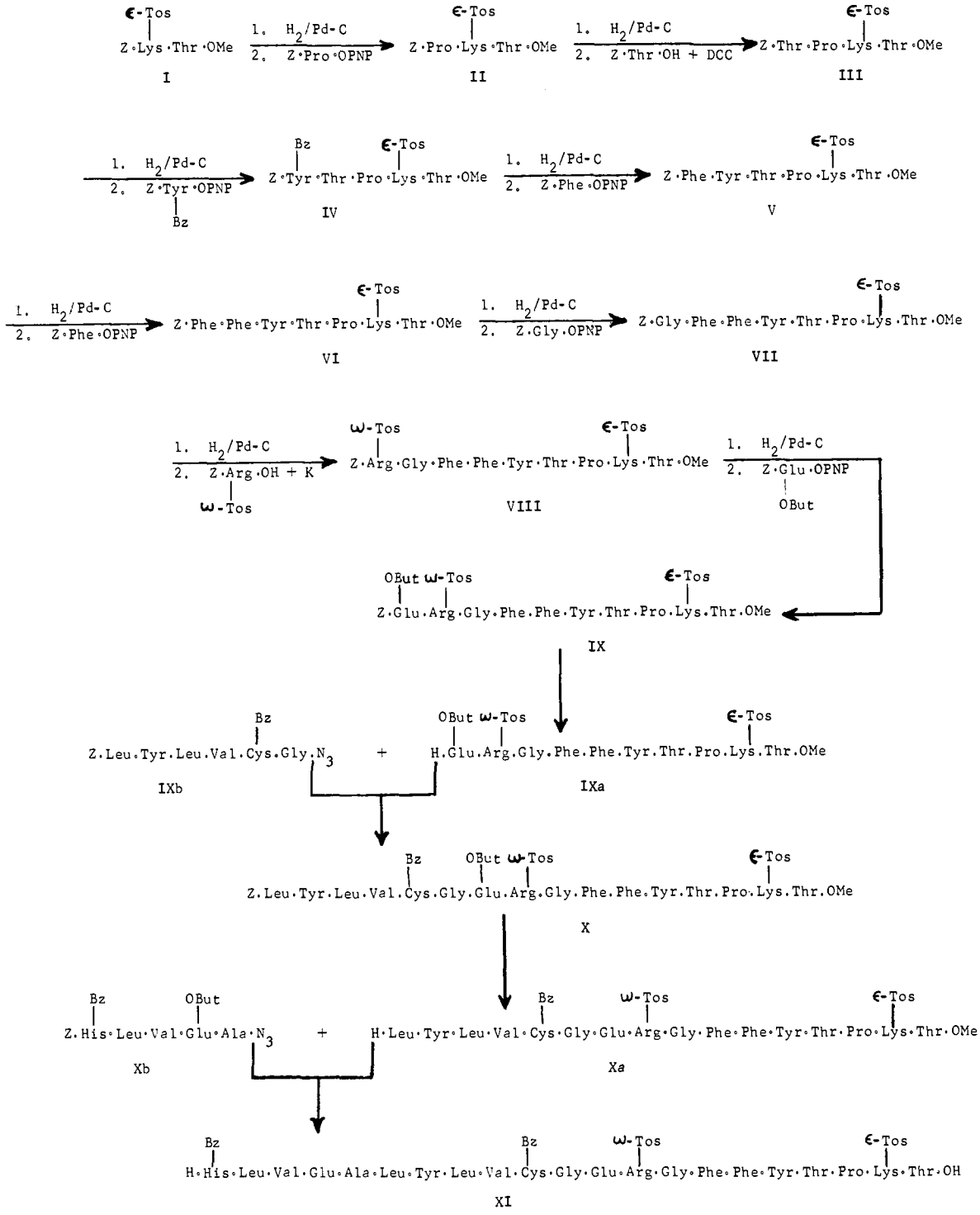
In one of the preceding papers we have reported the synthesis of a partially protected heneicosapeptide embodying the amino acid sequence found at the terminus of the B chain of bovine, sheep, or porcine insulin.[3] In the present paper we describe the synthesis of a heneicosapeptide derivative with the amino acid sequence corresponding to the carboxyl terminal portion of the human insulin B chain. The two heneicosapeptide derivatives differ only in the C-terminal amino acid residue; instead of alanine present in the bovine (sheep

(1) This work was supported by the National Institute for Arthritis and Metabolic Diseases, U. S. Public Health Service (AM-12925).

(2) A preliminary report of part of the results described in this paper has appeared: P. G. Katsoyannis, A. Tometsko, J. Z. Ginos, and M. Tilak, *J. Amer. Chem. Soc.*, 88, 164 (1966).

(3) P. G. Katsoyannis, M. Tilak, and K. Fukuda, *ibid.*, 93, 5857 (1971).

Chart I



or porcine) fragment, the human derivative contains threonine.[4]

For the construction of the desired product we have followed the overall scheme used in the synthesis of the bovine heneicosapeptide derivative.[3] We have thus prepared the C-terminal decapeptide fragment, the adjacent hexapeptide fragment, and the N-terminal pentapeptide fragment. Condensation of the decapeptide and hexapeptide fragments afforded the C-terminal hexadecapeptide fragment, which in turn was coupled with the N-terminal pentapeptide fragment to give eventually the partially protected heneicosapeptide *im*-benzyl-L-histidyl-L-leucyl-L-valyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinyl-glycyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine (XI). This fragment contains the C-terminal amino acid sequence of the B chain of human insulin and it was the key intermediate for the synthesis of that chain.[2,5] The overall procedure for the synthesis of the heneicosapeptide derivative is summarized in Chart I.

(4) D. S. H. W. Nicol and L. F. Smith, *Nature (London)*, 187, 483 (1960).

(5) P. G. Katsoyannis, J. Z. Ginos, C. Zalut, M. Tilak, S. Johnson, and A. C. Trakatellis, *J. Amer. Chem. Soc.*, 93, 5877 (1971).

The C-terminal decapeptide fragment IX was prepared by the stepwise method. Activation of the carbobenzoxylated amino acids used at each synthetic step was brought about in all but two instances by conversion to the corresponding *p*-nitrophenyl esters.[6,7] Activation of benzyloxycarbonyl-L-threonine in the synthesis of the intermediate tetrapeptide derivative III and of N^{α}-benzyloxycarbonyl-N^{ω}-tosyl-L-arginine[8] in the synthesis of the nonapeptide derivative VIII was accomplished by the use of *N,N'*-dicyclohexylcarbodiimide[9] and 2-ethyl-5-phenylisoxazolium 3-sulfonate,[10] respectively. Decarbobenzoxylation of the decapeptide derivative IX and coupling of the resulting product IXa with the hexapeptide azide derivative IXb afforded the protected hexadecapeptide X, which on exposure to hydrogen bromide in trifluoroacetic acid yielded the partially protected derivative Xa. Interaction of the latter compound with the pentapeptide azide Xb resulted in the formation of the protected heneicosapeptide which upon saponification and treatment with hydrogen bromide in trifluoroacetic acid afforded the desired partially protected heneicosapeptide XI.

Elemental analysis, paper chromatography, and, in several instances, amino acid analysis after acid hydrolysis were used to establish the chemical purity of the various synthetic compounds. The final product XI, however, as was the case with the corresponding sheep insulin derivative,[3] was insoluble in all the paper chromatographic systems used in these studies and, hence, paper chromatographic data for this compound are not available. Furthermore, due to the insolubility of the final product XI in the leucine aminopeptidase incubating medium[11] no direct proof of its stereochemical homogeneity could be obtained. However, the fact that this heneicosapeptide fragment was the key intermediate in the synthesis of the human insulin B chain[5] which was completely digested by aminopeptidase M presents an indirect, but convincing, proof regarding its stereochemical homogeneity.

Experimental Section

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

For paper chromatography, the protected peptides were deblocked either with 2 *N* HBr in acetic acid or by catalytic hydrogenation in the presence of HCl. The resulting hydrobromides or hydrochlorides were chromatographed on Whatman No. 1 filter paper at room temperature. R_f^1 values refer to the Partridge system;[12] R_f^2 values refer to the system 1-butanol–pyridine–acetic acid–water,[13] 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 according to the method of Moore, Spackman, and Stein,[14] with a Beckman-Spinco amino acid analyzer, Model 120B, to which a digital readout system, Model CRS-10AB (Infotronics Corporation, Houston, Tex.), has been attached. The infrared spectra were taken as described previously. The following abbreviations are used: Z, carbobenzoxy; Bz, benzyl; But, *tert*-butyl; Tos, *p*-toluenesulfonyl; PNP, *p*-nitrophenyl; Me, methyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; K, 2-ethyl-5-phenylisoxazolium 3'-sulfonate.

N^{α}-Benzyloxycarbonyl-N^{ϵ}-tosyl-L-lysyl-L-threonine Methyl Ester (I). L-Threonine methyl ester hydrochloride (52 g) was dissolved in DMF (280), and triethylamine (43 ml) was added followed by N^{α}-benzyloxycarbonyl-N^{ϵ}-tosyl-L-lysine *p*-nitrophenyl ester[15] (169 g). After standing overnight at room temperature, the solution was diluted with 1 *N* NH_4OH (80 ml), stirred for 0.5 hr, and then mixed with ethyl acetate (1200 ml) and water (500 ml). The organic layer was washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and water. The white solid obtained after removal of the ethyl acetate was dissolved in ethanol (250 ml) from which it crystallized out slowly upon dilution with ether (700 ml) and petroleum ether (200 ml): wt, 107 g; mp 78–82°. Upon concentration of the mother liquor to a small volume and dilution with ether and petroleum ether, a second crop of product was obtained: wt, 39 g; mp 78–82° (overall yield 88%). Material of this purity was used in the preparation of II. A sample for analysis was recrystallized from ethanol–water: mp 99–101°; $[\alpha]^{27}D$ +1.9 (*c* 1.0, DMF); after hydrogenolysis in the presence of HCl, R_f^1 0.71, R_f^2 3.77 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{26}H_{35}N_3O_8S$: C, 56.8; H, 6.42; N, 7.7. Found: C, 57.2; H, 6.52; N, 7.9.

***N*-Benzyloxycarbonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine Methyl Ester (II).** N^{α}-Benzyloxycarbonyl-N^{ϵ}-tosyl-L-lysyl-L-threonine methyl ester (50.4 g) was hydrogenated for 5 hr over 10% palladium/charcoal catalyst (8.6 g) in methanol (370 ml) containing 2 *N* HCl (49 ml). The catalyst was removed by filtration and the solvent was removed by evaporation under reduced pressure. The residue was dried by the addition of methanol followed by evaporation under reduced pressure. The product was dissolved in DMF (240 ml) followed by the addition of triethylamine (13 ml) and *N*-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester[7] (33.6 g). After standing overnight at room temperature, the solution was diluted with 1 *N* NH_4OH (30 ml), stirred for 30 min, dissolved in ethyl acetate (1000 ml), and extracted successively with 1 *N* NH_4OH, 1 *N* HCl, and water. The white solid obtained upon evaporation of the ethyl acetate was dissolved in 95% aqueous ethanol (400 ml) and subsequently precipitated by the addition of water (760 ml): wt, 45 g (75%); mp 122–128°. A sample for analysis was recrystallized from ethanol–water: mp 125–128°; $[\alpha]^{27}D$ −24.9° (*c* 1.0 DMF); after hydrogenolysis in the presence of HCl R_f^1 0.68, R_f^2 4.35 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{31}H_{42}N_4O_9S$: C, 57.6; H, 6.55; N, 8.7. Found: C, 57.6; H, 6.67; N, 8.9.

***N*-Benzyloxycarbonyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine Methyl Ester (III).** *N*-Benzyloxycarbonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine methyl ester (44.8 g) was hydrogenated for 4 hr over 10% palladium/charcoal catalyst (2.3 g) in methanol (190 ml) containing 2 *N* HCl (17 ml). The catalyst was removed by filtration and the solvent was removed by evaporation under reduced pressure. The residue, after it was dried by the repeated addition and evaporation of methanol under reduced pressure, was dissolved in methylene chloride (125 ml) and cooled to 0°. To this solution, triethylamine (9.7 ml) was added followed by *N*-benzyloxycarbonyl-L-threonine[16] (17.7 g) and *N,N'*-dicyclohexylcarbodiimide (15.8 g). After 24 hr, the reaction mixture was diluted with acetic acid (7.0 ml) and stirred for 30 min, and the precipitated *N,N'*-dicyclohexylurea was filtered off. The filtrate was concentrated to dryness under reduced pressure. The residue was dissolved in ethyl acetate (1000 ml) and washed successively with 1 *N* HCl, 1 *N* $NaHCO_3$, and water. The organic phase was dried over Mg_2SO_4, filtered, and then concentrated under reduced pressure to a volume of 200 ml. Addition of petroleum ether caused precipitation of the product: wt, 41.3 g (80%). The amorphous product sintered at 69° and melted over a wide range (75–89%); a sample for analysis was precipitated from ethanol by the addition of ether and petroleum ether: mp 84–92°; $[\alpha]^{27}D$ −32.7° (*c* 1, DMF); after hydrogenolysis in the presence of HCl, R_f^1 0.72, R_f^2 5.07 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{33}H_{39}N_5O_{11}S$: C, 56.2; H, 6.60; N, 9.4. Found: C, 56.3; H, 6.56; N, 9.1.

(6) M. Bodanszky, *Nature (London)*, **175**, 685 (1955).
(7) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959).
(8) J. Ramachandran and C. H. Li, *J. Org. Chem.*, **27**, 4006 (1962).
(9) J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, 77, 1067 (1955).
(10) R. B. Woodward, R. A. Olofson, and H. Mayer, *ibid.*, **83**, 1010 (1961).
(11) R. L. Hill and E. L. Smith, *J. Biol. Chem.*, **228**, 577 (1957).
(12) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).
(13) S. G. Waley and G. Watson, *ibid.*, **55**, 328 (1953).
(14) S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).
(15) M. Bodanszky, J. Meienhofer, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **82**, 3195 (1960).
(16) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. II, Wiley, New York, N. Y., 1961, p 895.

N-Benzyloxycarbonyl-O-benzyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine Methyl Ester (IV). N-Benzyloxycarbonyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine methyl ester (41.3 g) was hydrogenated for 5 hr over 10% palladium/charcoal catalyst (6.1 g) in methanol (400 ml) containing 2 *N* HCl (30 ml). The catalyst was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The residue was dried by repeated addition and evaporation of methanol under reduced pressure, and then dissolved in DMF (240 ml) and cooled to 0°. To this solution, triethylamine (6.1 ml) was added followed by *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester[7] (24.3 g). After 24 hr, the reaction mixture was diluted with 1 *N* NH_4OH (40 ml), stirred for 30 min, and then diluted with ethyl acetate (1000 ml). The organic phase was washed successively with 1 *N* NH_4OH, 1 *N* HCl, and water, and finally filtered after it was dried over Mg_2SO_4. The solid obtained after evaporation of the solvent under reduced pressure was dissolved in methanol (140 ml) and precipitated by the addition of ether (850 ml): wt, 37.8 g (70%). The protected pentapeptide sinters at 95° and melts at 109–113°: $[\alpha]^{27}D$ −32.1° (*c* 2, DMF); after hydrogenolysis in the presence of HCl, R_f^1 0.73, R_f^2 2.99 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{51}H_{64}N_6O_{13}S$: C, 61.2; H, 6.44; N, 8.4. Found: C, 61.0; H, 6.43; N, 8.4.

N-Benzyloxycarbonyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine Methyl Ester (V). Compound IV (40.0 g) was hydrogenated for 4 hr over 10% palladium/charcoal catalyst (8.4 g) in methanol (230 ml) containing 2 *N* HCl (21 ml). The solvent was removed by evaporation under reduced pressure after the catalyst was filtered off. The solid residue, which was dried by repeated addition and evaporation of methanol under reduced pressure, was dissolved in DMF (230 ml) and cooled to 0°. To this solution, triethylamine (5.47 ml) was added followed by *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester[17] (17.6 g). After 24 hr at room temperature, the reaction mixture was poured into cold 1 *N* NH_4OH. The precipitated product was isolated by filtration and washed with water, 1 *N* HCl, and water again. The dried product was dissolved in methanol (240 ml) and precipitated by the addition of water (250 ml): wt, 38 g (90%); melting point undetermined; the peptide sinters at 130° and it is converted to liquid at 141°. A sample for analysis was reprecipitated from methanol–ether: mp 139–141°; $[\alpha]^{27}D$ −40.9° (*c* 1, DMF); after hydrogenolysis in the presence of HCl, R_f^1 0.84, R_f^2 3.96 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{53}H_{67}N_7O_{14}S$: C, 60.2; H, 6.38; N, 9.3. Found: C, 60.3; H, 6.42; N, 9.2.

N-Benzyloxycarbonyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine Methyl Ester (VI). N-Benzyloxycarbonyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine methyl ester (37.0 g) was hydrogenated for 5 hr over 10% palladium/charcoal catalyst (7.6 g) in methanol (400 ml) containing 2 *N* HCl (17.7 ml). The catalyst was filtered off, and the filtrate was concentrated to dryness under reduced pressure. To a solution of the residue in DMF (240 ml), cooled to 0°, triethylamine (4.9 ml) was added followed by *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (14.2 g). The reaction mixture was stirred at room temperature for 24 hr and then poured into cold 1 *N* NH_4OH (1.6 l.). The precipitated product was filtered off and washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and water again. On reprecipitation from ethanol–water, 36.6 g (87%) of product was obtained: mp 152–163°; a sample for analysis was reprecipitated from ethanol–water; mp 163–165°; $[\alpha]^{27}D$ −38.5° (*c* 1, DMF); after hydrogenolysis in the presence of HCl, R_f^1 0.91, R_1^2 5.1 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{62}H_{76}N_8O_{15}S$: C, 61.8; H, 6.36; N, 9.3. Found: C, 61.8; H, 6.40; N, 9.0.

N-Benzyloxycarbonylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine Methyl Ester (VII). Compound VI (34.5 g) was hydrogenated for 4 hr over 10% palladium/charcoal catalyst (8.7 g) in methanol (450 ml) containing 2 *N* HCl (14.4 ml). The catalyst was filtered off, and the filtrate was concentrated to dryness under reduced pressure. The solid residue was dried by the addition of methanol followed by evaporation under reduced pressure. To the solution of this solid in DMF (240 ml), triethylamine (4.0 ml) was added followed by *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester[18] (9.2 g). After stirring at room temperature for 24 hr the solution was poured into cold 1 *N* NH_4OH (2 l.). The precipitated product was filtered off, and washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and water again. The dried product was precipitated twice from methanol (280 ml) by the addition of water (200 ml): wt, 31 g (86%); mp 142–153°; a sample for analysis was reprecipitated from the same solvent system; mp 150–157°; $[\alpha]^{27}D$ −34.3° (*c* 1, DMF); after hydrogenolysis in the presence of HCl, R_f^1 0.89, R_f^2 4.53 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{64}H_{70}N_9O_{10}S$: C, 60.9; H, 6.30; N, 10.0. Found: C, 60.6; H, 6.56; N, 10.0.

N^{α}-Benzyloxycarbonyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine Methyl Ester (VIII). Compound VII (27.0 g) was hydrogenated for 4 hr over 10% palladium/charcoal catalyst (16 g) in methanol (400 ml) containing 2 *N* HCl (12.0 ml). The catalyst was filtered off and the solvent was removed under reduced pressure. The solid residue of the octapeptide ester hydrochloride was dried by the addition of methanol followed by evaporation. The dried product was then used for condensation with N^{α}-benzyloxycarbonyl-N^{ω}-tosyl-L-arginine. A solution of N^{α}-benzyloxycarbonyl-N^{ω}-tosyl-L-arginine[8] (10.4 g) in acetonitrile (300 ml) and DMF (60 ml) was cooled to 0°, and triethylamine (3.1 ml) was added followed by 2-ethyl-5-phenylisoxazolium 3′-sulfonate[10] (5.95 g). After 1 hr at 0°, the reaction mixture was diluted with a solution of the octapeptide ester in DMF and acetonitrile prepared as follows: the hydrochloride salt, which had been prepared as described previously, was dissolved in a mixture of DMF (110 ml) and acetonitrile (110 ml) containing triethylamine (3.3 ml), stirred 5 min, and then added to the reaction mixture prepared as described above. After 24 hr at room temperature, the reaction mixture was poured into ice-cold 0.5 *N* $NaHCO_3$ (500 ml). The precipitated product was filtered off, washed with water, triturated twice with 1 *N* HCl, and then washed again with water. The product was precipitated from a solution in methanol (160 ml) by the addition of ether (700 ml): wt, 26.6 g (80%); mp 156–161°; $[\alpha]^{27}D$ −12.0° (*c* 2, DMF); after hydrogenolysis in the presence of HCl, R_f^1 0.86, R_1^2 4.53 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{77}H_{97}N_{13}O_{19}S_2$: C, 58.8; H, 6.21; N, 11.6. Found: C, 58.8; H, 6.11; N, 11.7.

For evaluation of stereochemical homogeneity, a sample of the protected nonapeptide was deblocked by catalytic hydrogenation and the resulting product was digested by aminopeptidase M[19] (purchased from Henley and Co., New York, N.Y.). Amino acid analysis of the digest showed the following composition expressed in molar ratios: $Thr_{2.0}Pro_{1.0}Gly_{1.0}Tyr_{1.0}Phe_{2.0}$. Tosyllysine and tosylarginine peaks overlap and were not determined.

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-threonine Methyl Ester (IX). The protected nonapeptide ester VIII (20 g) was hydrogenated for 4 hr over 10% palladium/charcoal catalyst (13.0 g) in methanol (260 ml) containing 2 *N* HCl (6.4 ml). The catalyst was filtered off, and the solvent was removed by evaporation under reduced pressure. The solid residue which was dried by repeated addition and evaporation of methanol under reduced pressure was dissolved in DMF (300 ml). To this solution, triethylamine (1.7 ml) was added followed by *N*-benzyloxycarbonyl-L-glutamic acid γ-*tert*-butyl-α-*p*-nitrophenyl ester[20] (6.4 g). After stirring at room temperature for 48 hr, the reaction mixture was poured into 1 *N* NH_4OH (1.5 l.). The precipitated product was filtered off and washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and water again. The product was dissolved in hot methanol (400 ml) and allowed to precipitate as the solution cooled slowly first to room temperature and then to 0° overnight: wt, 16.6 g (74%); mp 200°; $[\alpha]^{28}D$ −22.6° (*c* 2, DMF); after hydrogenolysis in the presence of acetic acid, R_f^1 0.95, R_f^2 4.67 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{86}H_{112}N_{14}O_{22}S_2$: C, 58.7; H, 6.42; N, 11.2. Found: C, 58.6; H, 6.54; N, 11.1.

Amino acid analysis of an acid hydrolysate of the protected decapeptide showed the expected composition expressed in molar ratios: $Lys_{0.9}Arg_{1.0}Thr_{2.0}Glu_{1.2}Pro_{1.0}Gly_{1.1}Tyr_{0.9}Phe_{2.0}$.

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-

(17) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 6072 (1959).

(18) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, 40, 373 (1957).

(19) G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, 340, 552 (1964).

(20) K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaihara, *J. Amer. Chem. Soc.*, 87, 611 (1965).

phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-threonine Methyl Ester (X). The protected decapeptide IX (4.4 g) was hydrogenated for 5 hr over 10% palladium/charcoal catalyst (2.5 g) in a solution of methanol (300 ml) and DMF (60 ml) containing glacial acetic acid (1.0 ml). The catalyst was filtered off and washed with DMF (15 ml) and methanol (50 ml) and the combined filtrates were refiltered through filter-aid (Celite 545). The solvent was removed by evaporation initially under reduced pressure (20 mm) and subsequently under high vacuum (0.5 mm). The solid residue was triturated with absolute ether repeatedly and dried under high vacuum to a constant weight: 4.1 g (98%). This product was dissolved in DMF (60 ml), cooled to 0°, and then neutralized with triethylamine (0.36 ml) just prior to the addition of the hexapeptide azide prepared as follows: *N*-benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycine hydrazide[3] (3.4 g) was dissolved in DMF (60 ml) containing 2 *N* HCl (8.5 ml). After cooling this solution to −10°, $NaNO_2$ (300 mg) dissolved in cold water (0.5 ml) was added. The reaction mixture was stirred for 6 min at −5° and then poured into a saturated aqueous solution of NaCl (150 ml). The white precipitate was filtered off, washed to neutrality with cold 1 *N* $KHCO_3$, and then with water, and dried for 5 hr over P_2O_5 at 0° *in vacuo*. An infrared spectrum[21] of the azide showed the characteristic azide band at 4.75 μ with no evidence of rearrangement as testified by the absence of the isocyanate band at 4.50 μ. The solid azide was then added to the solution of the decarbobenzoxylated decapeptide prepared as described previously. The reaction was allowed to proceed at 0° with stirring over a period of 48 hr during which time it became necessary to add additional DMF (10 ml) to control the increasing viscosity of the reaction solution. The reaction mixture was then poured slowly and with vigorous stirring into 600 ml of ice and water containing 2 *N* HCl (2 ml). The white, semigelatinous solid was isolated by centrifugation (5000 rpm) and washed successively with water, 50% aqueous methanol, and with absolute methanol. On trituration with hot methanol (three 250-ml portions) and reprecipitation from dimethylformamide–ether, 4.9 g (92%) of product was obtained: mp 255–256°; $[\alpha]^{29}D$ −30.7° (*c* 1, DMF); after HBr in trifluoroacetic acid treatment, R_f^1 0.96, R_f^2 2.75 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{124}H_{166}N_{20}O_{29}S_3$: C, 59.7; H, 6.70; N, 11.2. Found: C, 60.1; H, 6.84; N, 11.6.

Amino acid analysis of an acid hydrolysate of the protected hexadecapeptide showed the expected composition expressed in molar ratios: $Lys_{0.9}Arg_{1.0}Thr_{1.8}Glu_{1.0}Pro_{0.9}Gly_{2.1}Val_{1.0}Leu_{2.1}Tyr_{1.8}Phe_{1.8}$*S*-benzylcysteine$_{1.0}$.

***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-threonine Trihydrobromide (XI).** The hexadecapeptide X (2.60 g) was dissolved in trifluoroacetic acid (30 ml) containing 0.6 ml of water, and hydrogen bromide was passed through the solution for 45 min at 10°. After most of the trifluoroacetic acid was removed under reduced pressure, the remaining viscous product was mixed with anhydrous ether. The precipitated hexadecapeptide ester hydrobromide was washed several times with ether and dried over KOH *in vacuo*: wt, 2.63 g (99%). To a solution of this solid in DMF (120 ml) cooled to 0°, triethylamine (0.4 ml) was added followed by the protected pentapeptide azide prepared as follows: a suspension of N^{α}-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-alanine hydrazide[3] (1.77 g) in DMF (30 ml) was cooled to −15° (Dry Ice–acetone) and then diluted with 2 *N* HCl (6.2 ml). To the resulting solution was then added $NaNO_2$ (170 mg) dissolved in cold water (0.8 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 (150 ml) and a saturated solution of $NaHCO_3$ (8 ml). The precipitated pentapeptide azide was filtered off, washed with water, and then dried for 2 hr at 0° over P_2O_5 *in vacuo*. An infrared spectrum of the azide showed the characteristic azide band at 4.75 μ with negligible evidence of rearrangement to the isocyanate derivative.[21] The dry azide was added to the solution of the partially protected hexadecapeptide prepared as described previously, and the reaction was allowed to proceed at 0° with stirring over a period of 48 hr. During this period, it became necessary to add additional amounts of DMF (50 ml) to control the increasing viscosity of the reaction solution. The reaction mixture was then poured into absolute methanol (800 ml) containing acetic acid (1 ml). The precipitated protected heneicosapeptide was isolated by centrifugation and triturated repeatedly with absolute methanol and finally with ether: wt, 3.0 g (91%); mp 260–262°. Amino acid analysis of an acid hydrolysate of the protected heneicosapeptide showed the following composition expressed in molar ratios: $Lys_{0.6}Arg_{1.1}Thr_{2.1}Glu_{2.0}Pro_{1.1}Gly_{2.3}Ala_{0.8}Val_{2.0}Leu_{3.1}Tyr_{1.8}Phe_{2.2}$*S*-benzylcysteine$_{0.8}$ (*im*-benzylhistidine and uncleaved tosyllysine were not determined).

The protected heneicosapeptide (3 g) was subsequently dissolved in hexamethylphosphoramide (80 ml) with stirring and warming to 40°. To this solution, cooled to 2°, was added portionwise and over a period of 25 min 1 *N* NaOH (7.52 ml). After 50 min from the initial addition of NaOH the saponified peptide was precipitated with the addition of water (500 ml) containing 1 *N* HCl (7.9 ml) isolated by centrifugation, and washed twice with water and twice with methanol. After each washing the mixture was centrifuged and the solvent decanted: wt, 1.83 g (61%); mp 258–261°. The methanol washings were combined, concentrated under reduced pressure, and then diluted with ether. The precipitate was isolated by centrifugation: wt, 0.59 g (20%); mp 258–261°. The two fractions were combined.

Amino acid analysis of an acid hydrolysate of the saponified heneicosapeptide showed the following composition expressed in molar ratios: $Lys_{0.7}Arg_{1.0}Thr_{1.8}Glu_{1.9}Pro_{1.0}Gly_{2.2}Ala_{0.8}Val_{2.0}Leu_{2.8}Tyr_{1.8}Phe_{2.2}$*S*-benzylcysteine$_{0.9}$ (*im*-benzylhistidine was not determined).

The saponified heneicosapeptide (830 mg) was decarbobenzoxylated with trifluoroacetic acid and hydrogen bromide as described above. The trihydrobromide salt XI was dried to constant weight (845 mg, 99%) over P_2O_5 and NaOH pellets *in vacuo:* mp 267–269°; $[\alpha]^{28}D$ −22.5° (*c* 1, dimethyl sulfoxide). The peptide failed to move from the origin when the usual paper chromatographic systems were applied; hence, no R_f values are reported.

Anal. Calcd for $C_{147}H_{206}N_{27}O_{34}S_3 3HBr$: C, 54.6; H, 6.41; N, 11.7; Br, 7.4. Found: C, 54.4; H, 6.40; N, 11.1; Br, 6.8.

Amino acid analysis of an acid hydrolysate of the heneicosapeptide XI showed the expected composition expressed in molar ratios: $Lys_{0.8}Arg_{0.9}Thr_{1.9}Glu_{2.1}Pro_{1.0}Gly_{2.1}Ala_{1.0}Val_{2.0}Leu_{2.9}Tyr_{1.8}Phe_{1.9}$*S*-benzylcysteine$_{0.8}$ (*im*-benzylhistidine was not determined).

Acknowledgments. The authors wish to express their appreciation to Miss Karen Scheibe for the amino acid analyses and the enzymatic analyses.

(21) P. G. Katsoyannis, A. Tometsko, C. Zalut, and K. Fukuda, *J. Amer. Chem. Soc.*, 88, 5625 (1966).