Synthesis of a Fully Protected Heptacosapeptide Norleucine Analog Corresponding to Positions 21–47 of the Primary Structure of Staphylococcal Nuclease[1,2]

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Abstract: The synthesis of a heptacosapeptide corresponding to positions 21–47 of the primary structure of a nuclease from *Staphylococcus aureus* is described. Both methionines present in the native sequence at positions 26 and 32 have been substituted with norleucine. Intermediates were isolated and characterized for homogeneity. The final product is fully protected and utilizable for further coupling.

The primary structure[3] (Figure 1) and three-dimensional structure[4] of the major extracellular nuclease of *Staphylococcus aureus* have now been described. Proteolysis of this enzyme by trypsin in the presence of Ca^{2+} and deoxythymidine 3′,5′-diphosphate results in three enzymatically inactive fragments.[5] The two larger fragments called P_2 (residues 6–48 or 49) and P_3 (49 or 50–149) recombine to form nuclease-T, a complex with about 8% of native nuclease activity. Crystals of nuclease-T have now been shown to be isomorphous to those of nuclease.[6]

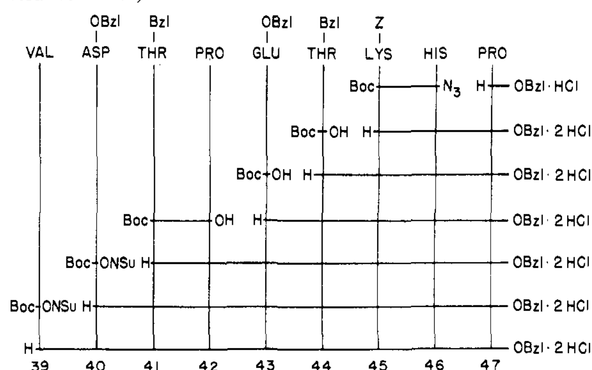
Recently, a semisynthetic nuclease-T has been produced[7] using native P_3 and a product synthesized by the solid phase method[8] corresponding to residues 6–47. Purification of semisynthetic nuclease-T by trypsin digestion in the presence of Ca^{2+} and deoxythymidine 3′,5′-diphosphate yields a complex having up to 90% of the specific activity of native nuclease-T.[9]

A number of analogs of P_2 have also been synthesized by the solid phase method.[10] Among these, the Nle[26],-Nle[32] derivative has been shown to possess activity, although the specific activity was somewhat lower than that of the native analog. The lower specific activity could be attributed to impurities as well as to the amino acid substitutions. This question could be resolved by an independent synthesis of this analog using classical methods of stepwise synthesis and purification of fragments followed by the condensation of these fragments. As a first step in this synthesis, we now report the synthesis of the fully protected Nle[26],Nle[32] heptacosapeptide spanning positions 21–47.

The latter is protected on the α amino terminus by the *tert*-butoxycarbonyl group, on the ϵ amine of lysines by the benzyloxycarbonyl moiety, on α, β, and γ carboxyl groups by the benzyl ester, on the alkyl hydroxyl of threonine and the aryl hydroxyl of tyrosine by the benzyl ether, and on the ω nitrogen of arginine by the nitro group. The imidazole ring of histidine was unprotected because of difficulty encountered in initial attempts to deprotect *im*-benzylhistidine containing peptides.

The overall scheme involves the synthesis of three nonapeptides. Scheme I calls for an essentially stepwise synthesis of sequence 39–47 from monomers with the exception of two dipeptides. The first dipeptide,

Scheme I. Synthesis of Fully Protected Nonapeptide XVI (Residues 39–47)



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(1) For a preliminary report of this work, see A. R. Zeiger and C. B. Anfinsen in "Progress in the Classical Synthesis of Fragment P2 (Residues 6–47) of Staphylococcal Nuclease-T," S. Lande, Ed., Gordon and Breach, New York, N. Y., 1972, p 307.

(2) All optically active amino acids are of the L configuration. The following abbreviations are used: DMF, dimethylformamide; TFA, trifluoroacetic acid; DCC, *N,N′*-dicyclohexylcarbodiimide; OBzl, benzyl ester; Bzl, benzyl ether; Z, benzyloxycarbonyl; Boc, *tert*-butoxycarbonyl; ONSu, *N*-hydroxysuccinimide ester; OMe, methyl ester; OBu^t, *tert*-butyl ester; OEt, ethyl ester; AP-M, aminopeptidase M.

(3) J. L. Cone, C. L. Cusumano, H. Taniuchi, and C. B. Anfinsen, *J. Biol. Chem.*, **246**, 3103 (1971); J. L. Bohnert and H. Taniuchi, *ibid.*, **247**, 4557 (1972).

(4) A. Arnone, C. J. Bier, F. A. Cotton, V. W. Day, E. E. Hazen, Jr., D. C. Richardson, J. S. Richardson, and A. Yonath, *ibid.*, **246**, 2302 (1971); F. A. Cotton and E. E. Hazen, Jr., *Enzymes*, **4**, 153 (1971); F. A. Cotton, C. J. Bier, V. W. Day, E. E. Hazen, Jr., and S. Larsen, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 243 (1972).

(5) H. Taniuchi, C. B. Anfinsen, and A. Sodja, *Proc. Nat. Acad. Sci. U. S.*, **58**, 1235 (1967); H. Taniuchi and C. B. Anfinsen, *J. Biol. Chem.*, **243**, 4778 (1968).

(6) H. Taniuchi, D. Davies, and C. B. Anfinsen, *ibid.*, **247**, 3362 (1972).

(7) D. A. Ontjes and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U. S.*, **64**, 428 (1969).

(8) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963); B. Gutte and R. B. Merrifield, *Science*, **150**, 178 (1965).

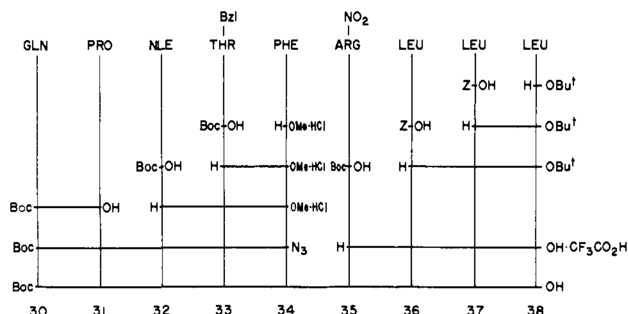
(9) I. M. Chaiken, *J. Biol. Chem.*, **246**, 2948 (1971).

(10) D. A. Ontjes and C. B. Anfinsen, *ibid.*, **244**, 6316 (1969); C. B. Anfinsen, D. A. Ontjes, and I. M. Chaiken in "Proceedings of the Tenth European Peptide Symposium," E. Scoffone, Ed., North Holland Publishing Co., Amsterdam, 1971, pp 121–129; I. M. Chaiken and C. B. Anfinsen, *J. Biol. Chem.*, **245**, 2337 (1970); **246**, 2285 (1971); I. M. Chaiken, *ibid.*, **247**, 1999 (1972).

tert-butoxycarbonyl-ε-benzyloxycarbonyllysylhistidine hydrazide, was added to proline benzyl ester hydrochloride using the azide procedure of Honzl and Rudinger.[11] Product III, obtained in 90% yield, was chromatographically homogeneous but all attempts at crystallization were unsuccessful. The *tert*-butoxycarbonyl group of the powder was removed by HCl in dioxane to give another powder in 100% yield. All subsequent *tert*-butoxycarbonyl-containing intermediates in Scheme I were deprotected with HCl in dioxane because the hydrochloride salts precipitated nicely. Traces of impurity due to further deprotection were removed by washing the ethyl acetate suspension of the neutralized hydrochloride salt with water. The adjacent two monomers were coupled by means of DCC without difficulty. The second dipeptide, *tert*-butoxycarbonyl-*O*-benzylthreonylproline, was added to γ-benzylglutamyl-*O*-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline benzyl ester dihydrochloride to form the *tert*-butoxycarbonyl heptapeptide in 62% yield. A trace of impurity was removed by recrystallization from methanol–ether. The final two monomers were condensed using an excess of *N*-hydroxysuccinimide active esters to give chromatographically pure products in good yield. The *tert*-butoxycarbonyl group of the nonapeptide in HCl and dioxane required more time for complete removal than that of the other *tert*-butoxycarbonyl peptides synthesized in this scheme.

As shown in Scheme II, a pentapeptide and a tetra-

Scheme II. Synthesis of Fully Protected Nonapeptide XXIX (Residues 30–38)



peptide, encompassing residues 30–34 and 35–38, were synthesized by a series of DCC reactions. The synthesis of the tripeptide, norleucyl-*O*-benzylthreonylphenylalanine methyl ester hydrochloride (XXIV), was straightforward. The dipeptide, *tert*-butoxycarbonylglutaminylproline benzyl ester, was obtained either with *tert*-butoxycarbonylglutamine *p*-nitrophenyl ester or *tert*-butoxycarbonylglutamine and DCC. The properties of the products were identical although the yield was about 15% higher with DCC. Hydrogenation of the product resulted in homogeneous *tert*-butoxycarbonylglutaminylproline in 96% yield. This dipeptide was coupled to XXIV to give the pentapeptide XXVII. Yields were generally around 50%, although in one experiment a yield of 70% was achieved. Addition of proline and glutamine separately as the active esters did not result in an improved yield of XXVII. Conversion of XXVII to the hydrazide was accomplished in 85% yield.

Synthesis of the tetrapeptide, *tert*-butoxycarbonylnitroarginylleucylleucylleucine *tert*-butyl ester (XIX),

(11) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

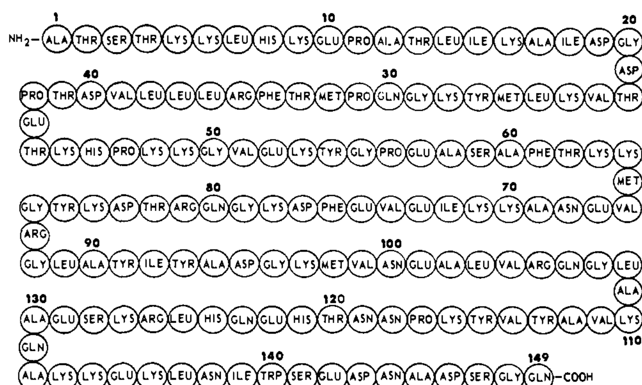
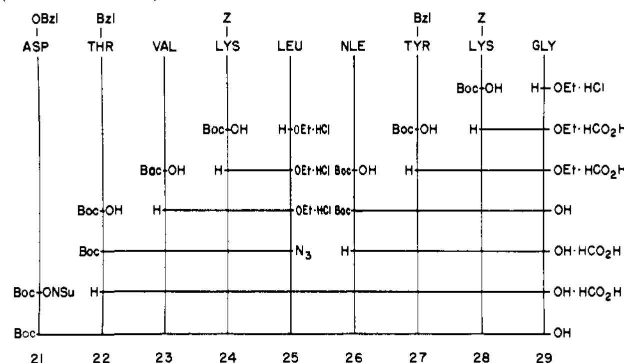


Figure 1. Amino acid sequence of the major extracellular nuclease of *Staphylococcus aureus*, Foggi strain.

proceeded without difficulty. The α amine of the intermediates was protected with the benzyloxycarbonyl group. Deprotection of XIX with trifluoroacetic acid for 3 hr yielded nitroarginylleucylleucylleucine trifluoroacetate (XX). Although XX was chromatographically homogeneous, its melting point and elemental analysis suggested some water to be present. The hydrazide XXVIII and tetrapeptide salt XX were consensed *via* the azide method of Honzl and Rudinger.[11] The pure nonapeptide, XXIX, was isolated in 87% yield.

Scheme III outlines the route used for the synthesis of

Scheme III. Synthesis of Fully Protected Nonapeptide XLVII (Residues 21–29)



the nonapeptide corresponding to positions 21–29. The two amino terminal tetrapeptides were synthesized without difficulty from *tert*-butoxycarbonyl amino acid monomers and DCC. One tetrapeptide, *tert*-butoxycarbonylnorleucyl-*O*-benzyltyrosyl-ε-benzyloxycarbonyllysylglycine ethyl ester, was saponified in 99% yield. The *tert*-butoxycarbonyl group was then removed in +97% formic acid to give the chromatographically pure tetrapeptide in 92% yield. The other tetrapeptide, *tert*-butoxycarbonyl-*O*-benzylthreonylvalyl-ε-benzyloxycarbonyllysylleucine ethyl ester, was converted to the hydrazide. As in Scheme II, an azide coupling was used to condense a completely protected peptide to a tetrapeptide having unprotected amino and carboxyl termini. Removal of the *tert*-butoxycarbonyl group from the octapeptide can be accomplished with either +97% formic acid or HCl in dioxane. The hydrochloride salt was more soluble in DMF and was used for the consensation with the *N*-hydroxysuccinimide ester of *tert*-butoxycarbonyl-β-benzylaspartic acid to form the nonapeptide, XLV, in 79% yield.

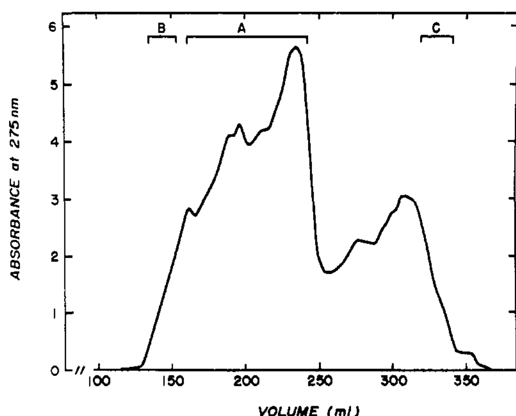


Figure 2. Elution profile of heptacosapeptide reaction mixture on Sephadex LH-60 swelled in DMF.

The two nonapeptides, XVI and XXIX, were coupled in DMF with DCC and *N*-hydroxysuccinimide according to the method of Weygand, Hoffmann, and Wunsch[12] and in the presence of a tenfold excess of imidazole to protect histidine. Gel filtration of the reaction mixture through Sephadex LH-20 with DMF resulted in a chromatographically pure fraction of the octadecapeptide which was subsequently isolated in 36% yield. The octadecapeptide, which was routinely deprotected in HCl and dioxane, was condensed with the nonapeptide, XLVII, using DCC. The reaction mixture was chromatographed on Sephadex LH-60 using DMF as eluent. Elution was monitored at 275 nm (Figure 2) and by amino acid analysis. The fraction with the best amino acid analysis, fraction A, was pooled. Precipitation of this fraction resulted in 36% yield of the heptacosapeptide. Fraction B rechromatographed at essentially the same volume. Amino acid analysis of fraction B indicated that the nonapeptide amino acids were present in a greater proportion than those of the octadecapeptide (Tyr/Phe = 1.32). Upon rechromatography, fraction C eluted at approximately 250 ml (*vs.* 310 ml initially). The peaks for fractions B and C appeared to be Gaussian. These data indicate that the column may have been overloaded.

A small amount of the heptacosapeptide was deblocked with HCl in dioxane and coupled to *N*-hydroxysuccinimido *tert*-butoxycarbonylglycinate. The product, which was obtained in 90% yield, had a glycine content in excellent agreement with theory.

Discussion

In general, the synthesis of peptide fragments proceeded quite smoothly to the nonapeptide stage. In the case of nonapeptides XV, and to a lesser extent XLVII, removal of the *tert*-butoxycarbonyl group became more difficult. Consequently, the decision was reached to use nonapeptide fragments. Application of the azide technique of Honzl and Rudinger to the synthesis of the nonapeptide fragments resulted in high yields and few impurities. Especially useful was the condensation of peptide azides to peptides with free carboxyl groups. Preliminary experiments on the saponification in DMF of nonapeptide esters related to XIV had been totally unsuccessful.

(12) F. Weygand, D. Hoffmann, and E. Wunsch, *Z. Naturforsch.*, 21b, 426 (1966).

The use of unprotected histidine resulted in greater peptide solubility in the organic solvents used. However, these same peptides had a tendency to bind salts and water and thus were somewhat difficult to characterize by elemental analysis and chromatography. Furthermore, during coupling of large fragments, especially in the presence of *N*-hydroxysuccinimide, the histidine content was found to decrease. Although the cause of this decrease in histidine is not known, the addition of imidazole to the reaction mixture served to protect this residue during the reaction, possibly as a trap for free radicals.

Crystallization was generally successful in the purification of the nonapeptide fragments. With respect to the octadecapeptide and the heptacosapeptide, chromatography on Sephadex LH-20 and Sephadex LH-60, respectively, was necessary to effect a product of high purity. Indeed, Sephadex LH-60 appears to offer much promise as a gel for separating high molecular weight, fully protected peptides.

The optical purity of the heptacosapeptide was difficult to ascertain. Pronase and aminopeptidase M digestion were not sufficient to cleave Asp–X and X–Pro bonds. Thus, studies on the heptacosapeptide as well as the octadecapeptide failed to yield much information. However, digestion of the octapeptide XLVI indicated good optical purity. Because the synthesis of XLVI involved synthetic methods used in other fragments, the enzymatic digestion of XLVI could be considered representative of the entire synthesis heretofore.

The final step in the synthesis of the P2 fragment is the coupling of the amino terminal pentadecapeptide to the heptacosapeptide. Deprotection of the heptacosapeptide and coupling to glycine (residue 20) served as a preliminary experiment to test the efficacy of the final reaction.

Experimental Section

All melting points are uncorrected. Reagent grade DMF was redistilled from calcium chloride. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter at the 589-nm sodium line. Elemental analyses were kindly performed by Dr. William C. Alford of the National Institutes of Health. Sephadex LH-60 was kindly furnished by Dr. William Gelb of Pharmacia Fine Chemicals, Inc. Commercially available silica gel plates (Brinkmann) were used for thin layer chromatography.

The following peptide moieties were detected on these plates: free amine, 0.1% ninhydrin spray followed by heating; *tert*-butoxycarbonylamine, hydrochloride vapor for 15 min followed by the procedure to detect free amine; benzyloxycarbonylamine, iodine vapor; peptide bond, 1% sodium hypochlorite spray followed by 1% potassium iodide–1% soluble starch spray; Pauly test for free histidine, 0.1% sulfanilic acid–4% sodium nitrite spray followed by 0.5 *M* sodium carbonate spray. R_f values on these plates refer to the following solvent systems: (1) methanol–ethyl acetate (1:2, v/v); (2) *n*-butyl alcohol–acetic acid–pyridine–water (4:2:1:1, v/v); (3) *n*-butyl alcohol–acetic acid–water (4:1:5, v/v); (4) chloroform–acetic acid–methanol (18:1:1, v/v). Amino acid analyses were carried out on samples which had been hydrolyzed for 16 hr in evacuated, sealed tubes at 110°. Values for threonine and tyrosine are uncorrected. A 10:1 ratio of phenol to tyrosine was used to protect tyrosine during hydrolysis.

The protected amino acid monomers employed in these experiments were purchased from either Fox Chemical Co. or Schwarz-Mann Chemical Co. All materials were subjected to thin-layer chromatography with solvents 1, 2, and 3, and weighed samples were also submitted to quantitative amino acid analysis after hydrolysis in 6 *N* HCl for 20 hr in evacuated, sealed tubes at 110°.

Enzymatic digestions were done as follows: the peptide (2–4 μmol) was dissolved in 0.3 ml of TFA–anisole (5:1, v/v) into which

HBr had been bubbled for 70 min. After stirring overnight, 2 ml of ether was added and the mixture centrifuged. The peptide was washed twice more with ether and dried.

The peptide was dissolved in 200 μl of DMF. An aliquot (10 μl) was dissolved in 0.5 ml of 0.3 *M* phosphate buffer, pH 7.5, and 5 μl of a 1-mg/ml solution of pronase B (Calbiochem) in the phosphate buffer was added. After incubation at 37° for 24 hr, the solution was boiled for 10 min. After cooling, 75 μl of a 2-mg/ml solution of AP-M (Henley and Co.) in the phosphate buffer was added. After 24 hr, the solution was lyophilized.

The standard procedure referred to in the following section to purify the product is as follows: the reaction mixture is filtered and the methylene chloride is evaporated. The residue is dissolved in ethyl acetate and refiltered, if necessary. The organic layer is washed three times with 0.5 *M* citric acid and water, and three times with 1.0 *M* sodium bicarbonate and water. The ethyl acetate solution is dried over anhydrous sodium sulfate, filtered, and either concentrated to a small volume or evaporated to dryness.

***tert*-Butoxycarbonyl-ε-benzyloxycarbonyllysylhistidine Methyl Ester (I).** To a suspension of 7.26 g (30 mmol) of histidine methyl ester dihydrochloride in 110 ml of a mixture of methylene chloride and DMF (10:1, v/v) was added 11.40 g (30 mmol) of *tert*-butoxycarbonyl-ε-benzyloxycarbonyllysine dissolved in 100 ml of methylene chloride. The mixture was cooled to 4° and to this was added 8.4 ml (60 mmol) of triethylamine and 6.18 g (30 mmol) of DCC. The mixture was stirred for 4 hr at 4° and overnight at room temperature. The suspension was filtered and the filtrate was evaporated to an oil. The oil was dissolved in 125 ml of ethyl acetate, and washed once with water, six times with 1.0 *M* sodium bicarbonate, and once with water. The ethyl acetate solution was dried over sodium sulfate, evaporated to a small volume, and crystallized with ether: yield, 9.60 g (60%); mp 127–128; $[\alpha]^{20}D$ −9.4° (*c* 1.0, methanol); R_f 0.82[1]; 0.42[2], 0.77[3].

Anal. Calcd for $C_{26}H_{37}O_7N_5$: C, 58.73; H, 6.95; N, 13.14. Found: C, 59.00; H, 7.25; N, 13.08.

***tert*-Butoxycarbonyl-ε-benzyloxycarbonyllysylhistidine Hydrazide (II).** A solution of 7 ml of 95% (233 mmol) hydrazine dissolved in 15 ml of methanol was added to 13.85 g (26.05 mmol) of the dipeptide dissolved in 100 ml of methanol. After stirring the solution overnight at room temperature, the solvent was concentrated to a small volume and the product was crystallized with ether, washed once with water and once more with ether, and desiccated overnight over P_2O_5: yield, 13.55 g (98%); mp 120–122°; $[\alpha]^{20}D$ −10.0° (*c* 1.0 in methanol); R_f 0.50[1], 0.28[2], 0.71[3].

Anal. Calcd for $C_{25}H_{37}O_6N_7$: C, 56.60; H, 6.97; N, 18.46. Found: C, 56.79; H, 7.04; N, 18.13.

***tert*-Butoxycarbonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester (III).** The dipeptide hydrazide (16.12 g, 30.32 mmol) was almost dissolved in 250 ml of distilled DMF. To this was added, at −25°, 24 ml of 5.03 *N* HCl (120.7 mmol) in dioxane and 3.81 ml (32 mmol) of *tert*-butyl nitrite. After 30 min, the temperature was lowered to −60° and 8.4 g (35 mmol) of proline benzyl ester hydrochloride and 21.8 ml (155.7 mmol) of triethylamine were added. The solution was allowed to stir at −25° for 1 hr, −10° for 1 hr, and at 4° overnight. The reaction mixture was filtered and the filtrate evaporated to dryness. The residue was dissolved in 200 ml of ethyl acetate and washed with 1.0 *M* sodium bicarbonate twice, water, 25 ml of 0.5 *M* citric acid, water, sodium bicarbonate twice, and water. The ethyl acetate was dried over sodium sulfate and evaporated to an oil. The oil was washed and decanted with ether and petroleum ether and desiccated to a powdery residue: yield, 19.28 g (90%); mp 85°; $[\alpha]^{20}D$ −51.6° (*c* 1.0, methanol); R_f 0.82[1], 0.60[2], 0.82[3].

Anal. Calcd for $C_{37}H_{48}O_8N_6$: C, 63.06; H, 6.87; N, 11.92. Found: C, 63.04; H, 6.96; N, 11.82.

ε-Benzyloxycarbonyllysylhistidylproline Benzyl Ester Dihydrochloride (IV). The tripeptide III (18.5 g, 26.13 mmol) was dissolved in 30 ml of 4.5 *N* HCl in dioxane. The peptide precipitated almost immediately. After 10 min, ether was added and the solution was filtered and desiccated over NaOH: yield, 18.3 g (100%); mp 167–169°; $[\alpha]^{20}D$ −31.8° (*c* 1.0, methanol); R_f 0.66[2] (ninhydrin positive trace at 0.46).

Anal. Calcd for $C_{32}H_{42}O_6N_6Cl_2$: C, 56.72; H, 6.25; N, 12.40; Cl, 10.46. Found: C, 56.32; H, 6.44; N, 12.29; Cl, 10.33.

***tert*-Butoxycarbonyl-*O*-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester (V).** A suspension of 18.3 g (27.07 mmol) of IV in 150 ml of ethyl acetate was neutralized with 7.70 ml (55 mmol) of distilled triethylamine. The mixture was shaken with 75 ml of water which removed the impurity. The ethyl acetate layer was dried over sodium sulfate and evaporated to a yellow oil. To the oil dissolved in 125 ml of methylene chloride was added, at 4°, 8.34 g (27.0 mmol) of *tert*-butoxycarbonyl-*O*-benzylthreonine in 75 ml of methylene chloride and 5.59 g (27.0 mmol) of DCC in 50 ml of methylene chloride. After stirring overnight at 4°, the dicyclohexylurea was filtered and the filtrate evaporated to dryness. The semisolid was worked up by the standard procedure. After alternately washing with ether and petroleum ether, 16 g of product precipitated. A second crop of 0.60 g was obtained from the ether layers: yield, 16.60 g (68%); mp 93°; $[\alpha]^{20}D$ −39.1° (*c* 1.0, methanol); R_f 0.94[1], 0.56[2], 0.77[3].

Anal. Calcd for $C_{48}H_{61}O_{10}N_7$: C, 64.34; H, 6.86; N, 10.94. Found: C, 64.39; H, 6.85; N, 10.68.

***O*-Benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester Dihydrochloride Monohydrate (VI).** The tetrapeptide V (16.5 g, 18.60 mmol) was dissolved in 35 ml of 6.1 *N* HCl in dioxane for 15 min. The product was precipitated by ether: yield, 16.1 g (100%); mp 123–125°; $[\alpha]^{20}D$ −43.8° (*c* 1.0, methanol); R_f 0.55[1], 0.81[3] (ninhydrin positive trace at 0.05[1], 0.73[3]).

Anal. Calcd for $C_{43}H_{55}O_8N_7Cl_2 \cdot H_2O$: C, 58.24; H, 6.48; N, 11.06; Cl, 8.00. Found: C, 58.35; H, 6.22; N, 11.03; Cl, 8.07.

***tert*-Butoxycarbonyl-γ-benzylglutamyl-*O*-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester (VII).** To 16.1 g (18.6 mmol) of tetrapeptide VI suspended in 200 ml of ethyl acetate was added 5.2 ml (37.2 mmol) of triethylamine and 100 ml of water. The ethyl acetate layer was separated, dried with anhydrous sodium sulfate, and evaporated to dryness. After the residue was dissolved in 200 ml of methylene chloride 6.74 g (20 mmol) of *tert*-butoxycarbonyl-γ-benzylglutamic acid in 75 ml of methylene chloride was added. The solution was cooled to 4° and 3.83 g (18.5 mmol) of DCC in 25 ml of methylene chloride was added. After 16 hr, the solution was worked up by the standard procedure: yield, 14.8 g (70%). Crystallization was effected from ethyl acetate–ether.

For analysis, 150 mg was recrystallized from ethyl acetate–ether: yield, 112 mg (75%); mp 105–107°; $[\alpha]^{20}D$ −37.9 (*c* 1.0, methanol); R_f 0.85[1], 0.56[3].

Anal. Calcd for $C_{60}H_{74}O_{13}N_8$: C, 64.62; H, 6.69; N, 10.05. Found: C, 64.44; H, 6.62; N, 9.82.

γ-Benzylglutamyl-*O*-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester Dihydrochloride Monohydrate (VIII). The pentapeptide VII (14.5 g, 12.9 mmol) was dissolved in 65 ml of 4.0 *N* HCl in dioxane for 15 min. The product was precipitated by ether: yield, 13.95 g (99%); mp 117–119°; $[\alpha]^{20}D$ −28.5° (*c* 0.66, methanol); R_f 0.62[1].

Anal. Calcd for $C_{55}H_{68}O_{81}N_8Cl_2 \cdot H_2O$: C, 59.72; H, 6.38; N, 10.13. Found: C, 59.24; H, 6.24; N, 9.89.

***tert*-Butoxycarbonyl-*O*-benzylthreonylproline Benzyl Ester (IX).** At 4°, 4.11 g (17 mmol) of proline benzyl ester hydrochloride suspended in 100 ml of methylene chloride was neutralized with 2.38 ml (17 mmol) of triethylamine. Then was added successively 4.64 g (15 mmol) of *tert*-butoxycarbonyl-*O*-benzylthreonine in 150 ml of methylene chloride and 3.11 g (15 mmol) of DCC in 25 ml of methylene chloride. After 16 hr, the solution was filtered and evaporated. The standard procedure was used to yield a chromatographically homogeneous oil: yield, 7.5 g (100%); R_f 0.78[1].

***tert*-Butoxycarbonyl-*O*-benzylthreonylproline (X).** Approximately 2 g (4 mmol) of the dipeptide IX, dissolved in 18 ml of ethanol, was saponified with 4.80 ml (1.2 equiv) of 1 *N* aqueous KOH for 2 hr. Chromatography indicated that some starting material remained, so an additional 4.8 ml of 1 *N* KOH was added. After 1 hr, the solution was acidified to pH 3 with 1 *N* HCl, the ethanol was evaporated, and the product was extracted into ethyl acetate (2 × 50 ml). The ethyl acetate layer was extracted into 1.0 *M* sodium bicarbonate (4 × 30 ml). The solution was again acidified with 1 *N* HCl and extracted into ethyl acetate (2 × 50 ml).

The ethyl acetate was dried with anhydrous sodium sulfate, filtered, and evaporated to a powder: yield, 1.53 g (94%); mp 62–64°, $[\alpha]^{20}D$ −39.8° (*c* 1.08, methanol); R_f 0.48[1], 0.72[2], 0.68[3].

Anal. Calcd for $C_{21}H_{30}O_6N_2$: C, 62.05; H, 7.44; N, 6.89. Found: C, 61.82; H, 7.55; N, 6.94.

***tert*-Butoxycarbonyl-*O*-benzylthreonylprolyl-γ-benzylglutamyl-*O*-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester Monohydrate (XI).** The pentapeptide VIII (13.9 g, 12.8 mmol) was suspended in 150 ml of ethyl acetate, neutralized with 3.60 ml (25.7 mmol) of triethylamine, and washed with water. The ethyl acetate layer (125 ml) was dried with anhydrous sodium sulfate and filtered. The dipeptide X (6.09 g, 15.0 mmol) in 50 ml of ethyl acetate and 2.82 g (13.6 mmol) of DCC in 25 ml of ethyl acetate was added and stirred at room temperature for 16 hr. The solution was worked up by the standard procedure. The product was precipitated from ethyl acetate–ether: yield, 11.16 g (62%). Recrystallization from

methanol–ether yielded 10.21 g (91%); mp 100–102°; $[\alpha]^{20}D$ −48.5° (*c* 1.19, methanol); R_f 0.83[1].

Anal. Calcd for $C_{76}H_{94}O_{16}N_{10}\cdot H_2O$: C, 64.21; H, 6.81; N, 9.85. Found: C, 64.60; H, 7.02; N, 10.28.

O-Benzylthreonylprolyl-γ-benzylglutamyl-O-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester Dihydrochloride Monohydrate (XII). The heptapeptide XI (9.95 g, 7.0 mmol) was dissolved in 60 ml of 3.9 *N* HCl in dioxane for 15 min. Ether was added and the solution was filtered quickly: yield, 9.50 g (97%); mp 122–125°; $[\alpha]^{20}D$ −43.3° (*c* 0.54 methanol); R_f 0.25[1], 0.78[2].

Anal. Calcd for $C_{71}H_{88}O_{14}N_{10}Cl_2\cdot H_2O$: C, 61.16; H, 6.51; N, 10.05; Cl, 5.09. Found: C, 60.79; H, 6.71; N, 10.36; Cl, 4.88.

***tert*-Butoxycarbonyl-β-benzylaspartyl-O-benzylthreonylprolyl-γ-benzylglutamyl-O-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester Monohydrate (XIII).** To a suspension of 9.50 g (6.8 mmol) of XII in 50 ml of distilled DMF at 4° was added 2.0 ml (14.0 mmol) of triethylamine and 3.78 g (9.0 mmol) of the *N*-hydroxysuccinimide ester of *tert*-butoxycarbonyl-β-benzylaspartic acid. After 16 hr, the solvent was removed *in vacuo*. The residue was worked up by the standard procedure. Crystallization from hot ethyl acetate yielded 10.04 g (87%) of product: mp 110–111.5°; $[\alpha]^{20}D$ −45.4° (*c* 1.1, methanol); R_f 0.84[1], 0.83[2], 0.52[3].

Anal. Calcd for $C_{87}H_{105}O_{19}N_{11}\cdot H_2O$: C, 64.24; H, 6.63; N, 9.47. Found: C, 64.29; H, 6.43; N, 9.29.

β-Benzylaspartyl-O-benzylthreonylprolyl-γ-benzylglutamyl-O-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester Dihydrochloride Tetrahydrate (XIV). The octapeptide XIII (5.14 g, 3.16 mmol) was dissolved in 25 ml of 3.6 *N* HCl in dioxane for 10 min. Ether was added and the product was filtered: yield, 4.91 g (99%); mp 111–113°; $[\alpha]^{20}D$ −45.4° (*c* 1.0, methanol); R_f 0.77[1], 0.79[2].

Anal. Calcd for $C_{82}H_{95}O_{17}N_{11}Cl_2\cdot 4H_2O$: C, 59.56; H, 6.52; N, 9.32; Cl, 4.29. Found: C, 59.36; H, 6.29; N, 9.42; Cl, 4.43.

***tert*-Butoxycarbonylvalyl-β-benzylaspartyl-O-benzylthreonylprolyl-γ-benzylglutamyl-O-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester Monohydrate (XV).** To a suspension of 1.24 g (0.79 mmol) of XIV in 50 ml of distilled DMF was added 2.50 ml (1.8 mmol) of triethylamine and 364 mg (1.16 mmol) of *tert*-butoxycarbonylvaline at 4°. After 30 min, the reaction mixture was warmed to room temperature for another 30 min and to 45° for 16 hr. An additional 55 mg (0.17 mmol) of *tert*-butoxycarbonylvaline was added and the reaction kept at 45° for 24 hr more. The solvent was removed *in vacuo* and the mixture was worked up by the standard procedure. The product was precipitated from ethyl acetate–ether: yield, 1.11 g (82%); mp 118.5–120°; $[\alpha]^{20}D$ −48.9° (*c* 1, methanol); R_f 0.88[1], 0.88[2], 0.35[3], 0.24[4]; amino acid ratios in acid hydrolysate $Lys_{0.95}His_{0.94}Asp_{0.95}Glu_{1.12}Pro_{2.11}Val_{1.00}Thr_{1.65}$ (uncorr).

Anal. Calcd for $C_{92}H_{114}O_{20}N_{12}\cdot H_2O$: C, 64.03; H, 6.77; N, 9.74. Found: C, 64.01; H, 6.55; N, 9.83.

Valyl-β-benzylaspartyl-O-benzylthreonylprolyl-γ-benzylglutamyl-O-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Dihydrochloride (XVI). The nonapeptide XV (900 mg, 0.52 mmol) was dissolved in 10 ml of 5.4 *N* HCl in dioxane for 1 hr. The product which was precipitated in ether contained some starting material as well as some multideprotected material: yield, 850 mg (96%); R_f 0.60[1] (major), ninhydrin positive, Pauly positive; R_f 0.86[1] (minor), ninhydrin negative, Pauly positive; and a minor ninhydrin positive, Pauly positive spot at the origin.

Benzyloxycarbonylleucylleucine *tert*-Butyl Ester (XVII). Leucine *tert*-butyl ester hydrochloride (2.24 g, 10 mmol) in 150 ml of methylene chloride was neutralized with 1.40 ml (10 mmol) of triethylamine. At 4°, 2.92 g (11 mmol) of benzyloxycarbonylleucine in 25 ml of methylene chloride and 2.07 g (10 mmol) of DCC in 25 ml of methylene chloride were added. After 45 min, the solution was allowed to warm to room temperature for an additional 2 hr. The solution was worked up by the standard procedure. Three crops of crystals from ethyl ether–petroleum ether yielded 3.55 g (82%) of product: mp 131–132°; $[\alpha]^{20}D$ −42.9°; R_f 0.92[1], 0.87[2], 0.79[3].

Anal. Calcd for $C_{24}H_{38}O_5N_2$: C, 66.34; H, 8.81; N, 6.45. Found: C, 66.26; H, 8.68; N, 6.64.

Benzyloxycarbonylleucylleucylleucine *tert*-Butyl Ester (XVIII). The dipeptide XVII (3.50 g, 8.1 mmol) was dissolved in 50 ml of methanol and 200 mg of 10% Pd on charcoal was added. The solution was treated with hydrogen at 38 lb/in.[2] pressure for 2 hr, filtered, and evaporated to dryness. When the oil (R_f 0.90[1]) was dissolved in 100 ml of methylene chloride at 4°, 2.12 g (8.0 mmol) of benzyloxycarbonylleucine in 25 ml of methylene chloride and 1.66 g (8.0 mmol) of DCC in 25 ml of methylene chloride were added. After 16 hr, the mixture was worked up by the standard procedure. The product precipitated from ethyl acetate–ether–petroleum ether: yield, 3.23 g (74%); mp 132–134°; $[\alpha]^{20}D$ −61.2°; R_f −0.86[2], 0.87[3], 0.97[4].

Anal. Calcd for $C_{30}H_{49}O_6N_3$: C, 65.79; H, 9.02; N, 7.67. Found: C, 65.82; H, 8.99; N, 7.89.

***tert*-Butoxycarbonylnitroarginylleucylleucylleucine *tert*-Butyl Ester (XIX).** To 1.50 g (2.73 mmol) of the tripeptide XVIII dissolved in 25 ml of methanol was added 120 mg of 10% Pd on charcoal and the mixture was hydrogenated at 40 lb/in.[2] pressure. After 1 hr, another 50 mg of 10% Pd on charcoal was added and the mixture rehydrogenated for one more hour. The solution was filtered and evaporated. The residue (R_f 0.69[1], 0.76[2], 0.65[3]) was dissolved in 100 ml of methylene chloride. At 4°, a solution of 0.96 g (3.0 mmol) of *tert*-butoxycarbonylnitroarginine in 10 ml of distilled DMF and 0.56 g (2.7 mmol) of DCC were added. After 16 hr, the mixture was worked up by the standard procedure. The product was crystallized from ethyl acetate–ethyl ether: yield, 1.42 g (73%); mp 154–155°; $[\alpha]^{20}D$ −53.6° (*c* 1.0, methanol); R_f 0.90[1], 0.85[2], 0.79[3], 0.84[4].

Anal. Calcd for $C_{33}H_{62}O_9N_8$: C, 55.45; H, 8.74; N, 15.68. Found: C, 55.64; H, 8.78; N, 15.51.

Nitroarginylleucylleucylleucine Trifluoroacetate Monohydrate (XX). The tetrapeptide XIX (1.32 g, 1.85 mmol) was stirred with 10 ml of trifluoroacetic acid for 1 hr. Precipitation with ethyl ether yielded 1.18 g (93%) of product: mp 147–149°; $[\alpha]^{20}D$ −39.2° (*c* 1.0, glacial acetic acid); R_f 0.70[2], 0.33[3].

Anal. Calcd for $C_{26}H_{47}O_9N_8F_3\cdot H_2O$: C, 45.28; H, 7.16; N, 16.25; F, 8.26. Found: C, 45.99; H, 6.76; N, 16.06; F, 8.18.

***tert*-Butoxycarbonyl-O-benzylthreonylphenylalanine Methyl Ester (XXI).** A suspension of 6.45 g (30 mmol) of phenylalanine methyl ester hydrochloride in 100 ml of methylene chloride was neutralized with 4.2 ml (30 mmol) of triethylamine. At 4°, 9.27 g (30 mmol) of *tert*-butoxycarbonyl-O-benzylthreonine in 100 ml of methylene chloride and 6.18 g (30 mmol) of DCC in 25 ml of methylene chloride were added. After 16 hr, the standard procedure was used to purify the product. The product was crystallized from ethyl acetate–petroleum ether: yield, 10.7 g (76%); mp 85–87.5°; $[\alpha]^{20}D$ +7.8° (*c* 1.0, methanol); R_f 0.02[1], 0.83[2], 0.84[3].

Anal. Calcd for $C_{26}H_{34}O_6N_2$: C, 66.37; H, 7.28; N, 5.95. Found: C, 66.30; H, 7.32; N, 6.05.

O-Benzylthreonylphenylalanine Methyl Ester Hydrochloride (XXII). A solution of 6.80 g (14.4 mmol) of the dipeptide XXI in 50 ml of 4.5 *N* HCl in dioxane was allowed to react for 15 min. The product was precipitated with ether: yield, 5.58 g (95%); mp 174–175.5°; $[\alpha]^{20}D$ +1.1° (*c* 1.0, methanol); R_f 0.84[1], 0.86[2], 0.67[3].

Anal. Calcd for $C_{21}H_{27}N_2O_3Cl$: C, 61.99; H, 6.69; N, 6.88; Cl, 8.11. Found: C, 62.21; H, 6.57; N, 6.76; Cl, 8.38.

***tert*-Butoxycarbonylnorleucyl-O-benzylthreonylphenylalanine Methyl Ester (XXIII).** A suspension of 6.51 g (16 mmol) of XXII in 150 ml of methylene chloride was neutralized with 2.24 ml (16 mmol) of triethylamine. Solutions of 3.70 g (16 mmol) of *tert*-butoxycarbonylnorleucine in 25 ml of methylene chloride and 3.30 g (16 mmol) of DCC in 25 ml of methylene chloride were added at 4°. After 16 hr, the mixture was worked up by the standard procedure. The product was collected in two crops from ethyl acetate: yield, 6.43 g (70%); mp 127–128°; $[\alpha]^{20}D$ −4.6° (*c* 1.1, methanol); R_f 0.92[1], 0.87[2], 0.87[3].

Anal. Calcd for $C_{32}H_{45}O_7N_3$: C, 65.85; H, 7.77; N, 7.20. Found: C, 65.77; H, 7.62; N, 7.29.

Norleucyl-O-benzylthreonylphenylalanine Methyl Ester Hydrochloride (XXIV). The tripeptide XXIII (6.24 g, 10.7 mmol) was dissolved in 20 ml of 5.4 *N* HCl in dioxane for 12 min. The product precipitated upon the addition of ether: yield, 5.53 g (99%); mp 182–183°; $[\alpha]^{20}D$ +27.0° (*c* 1.0, methanol); R_f 0.82[1], 0.77[2], 0.65[3].

Anal. Calcd for $C_{27}H_{38}O_5N_3Cl$: C, 62.36; H, 7.37; N, 8.08; Cl, 6.82. Found: C, 62.50; H, 7.13; N, 7.73; Cl, 6.66.

***tert*-Butoxycarbonylglutaminylproline Benzyl Ester (XXV).** A suspension of 4.82 g (20 mmol) of proline benzyl ester hydrochloride in 40 ml of methylene chloride was neutralized with 2.8 ml (20 mmol) of triethylamine. A solution of 4.92 g (20 mmol) of *tert*-butoxycarbonylglutamine in 20 ml of methylene chloride and 5 ml of distilled DMF was added. After the solution was cooled to 4°, 4.12 g (20 mmol) of DCC was added. After 16 hr, the mixture was worked up by the standard procedure. The first crop was obtained from ethyl acetate alone and a second crop having the same properties was obtained upon the addition of ether: total yield, 6.2 g

(72%); mp 133–134°; $[\alpha]^{20}D$ −81.9° (*c* 1.0, methanol); R_f 0.78[1], 0.82[2], 0.70[3].

Anal. Calcd for $C_{22}H_{31}O_6N_3$: C, 61.01; H, 7.21; N, 9.69. Found: C, 61.04; H, 7.10; N, 9.95.

The product from a synthesis using the *p*-nitrophenyl ester of *tert*-butoxycarbonylglutamine had identical physical properties.

***tert*-Butoxycarbonylglutaminylproline (XXVI).** A solution of 2.68 g (62 mmol) of XXV in 25 ml of methanol was hydrogenated over 120 mg of 10% Pd on charcoal at 30–40 lb of H_2/in^2. After 40 min, an additional 80 mg of 10% Pd on charcoal was added and the mixture was hydrogenated again. After 1 hr, the mixture was filtered and the supernatant was evaporated to dryness. The product was crystallized from ethyl acetate and ether: yield, 2.04 g (96%); mp 146–148°; $[\alpha]^{20}D$ −69.1° (*c* 1.1, methanol); R_f 0.15[1], 0.52[2], 0.36[3].

Anal. Calcd for $C_{15}H_{25}O_6N_3$: C, 52.49; H, 7.34; N, 12.24. Found: C, 52.60; H, 7.24; N, 12.19.

***tert*-Butoxycarbonylglutaminylprolylnorleucyl-*O*-benzylthreonylphenylalanine Methyl Ester (XXVII).** To 5.22 g (10.4 mmol) of XXIV in 150 ml of methylene chloride at 4° was added 1.48 ml (10.4 mmol) of triethylamine, 3.57 g (10.4 mmol) of XXVI in 25 ml of DMF, and 2.15 g (10.4 mmol) of DCC in 25 ml of methylene chloride. After 2 hr, the mixture was filtered, evaporated, shaken with 150 ml of methylene chloride, and refiltered. The supernatant was washed with 0.5 *M* citric acid, water, 1.0 *M* sodium bicarbonate, and water. The methylene chloride layer was dried with anhydrous sodium sulfate and evaporated to dryness. The residue was crystallized from boiling ethyl acetate: yield, 5.90 g (70%); mp 191–192°; $[\alpha]^{20}D$ −27.3° (*c* 1.0, DMF); R_f 0.89[1], 0.85[2], 0.71[3], 0.92[4].

Anal. Calcd for $C_{42}H_{60}O_{10}N_6$: C, 62.36; H, 7.48; N, 10.39. Found: C, 62.50; H, 7.55; N, 10.62.

***tert*-Butoxycarbonylglutaminylprolylnorleucyl-*O*-benzylthreonylphenylalanine Hydrazide (XXVIII).** To a solution of 3.0 g (3.7 mmol) of XXVII in 50 ml of methanol was added a solution of 2 ml (70 mmol) of 95+% hydrazine dissolved in 10 ml of methanol. After 16 hr at 25°, the product was filtered, washed twice with water, and twice with ether, and desiccated over concentrated sulfuric acid: yield, 2.62 g (85%); mp 219–220°; $[\alpha]^{20}D$ −34.5° (*c* 1.0, DMF); R_f 0.82[1], 0.80[4].

Anal. Calcd for $C_{42}H_{60}O_9N_8$: C, 60.88; H, 7.48; N, 13.85. Found: C, 60.64; H, 7.31; N, 13.62.

***tert*-Butoxycarbonylglutaminylprolylnorleucyl-*O*-benzylthreonylphenylalanylnitroarginylleucylleucylleucine (XXIX).** A solution of 825 mg (1.02 mmol) of XXVIII in 10 ml of DMF was cooled to −25°. Then was added 1.0 ml of 4.54 *N* HCl in dioxane and 125 μl (1.05 mmol) of *tert*-butyl nitrite. After 30 min at −25°, the temperature was lowered to −60°, the tetrapeptide XX (7.05 mg, 1.05 mmol) was added, and the solution was neutralized with 765 μl (6.6 mmol) of triethylamine. The mixture was filtered and evaporated. Two crops of product obtained from boiling methanol had similar properties: total yield, 1.15 g (87%); mp 256–258°; $[\alpha]^{20}D$ −34.1° (*c* 1.1, DMF); R_f 0.90[2]; amino acid ratios in acid hydrolysate $Arg_{0.92}Glu_{1.00}Pro_{1.05}Leu_{3.04}Nle_{1.00}Phe_{0.93}Thr_{0.88(uncorr)}$.

Anal. Calcd for $C_{65}H_{102}O_{16}N_{14}$: C, 58.46; H, 7.70; N, 14.68. Found: C, 58.35; H, 7.73; N, 14.47.

***tert*-Butoxycarbonylglutaminylprolylnorleucyl-*O*-benzylthreonylphenylalanylnitroarginylleucylleucylleucylvalyl-β-benzylaspartyl-*O*-benzylthreonylprolyl-γ-benzylglutamyl-*O*-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester (XXX).** To a solution of 218 mg (130 μmol) of XVI and 174 mg (130 μmol) of XXIX in 8 ml of DMF was added 80 mg (1.3 mmol) of imidazole, and 15 mg (130 μmol) of *N*-hydroxysuccinimide. At 4°, 100 μl of a 1.3 *M* solution of DCC in DMF and 45 μl (412 μmol) of *N*-methylmorpholine were added. After 30 min at 4°, the solution was stirred overnight at room temperature and finally for 24 hr at 50°. The solution was applied to a 2-m column of LH-20 equilibrated in spectral grade DMF and eluted at a flow rate of 50 ml/hr. The fractions were monitored by optical density at 275 nm, index of refraction, thin layer chromatography, and amino acid analysis. The purest fractions from two such elutions starting with a total of 0.29 mmol of XVI and XXIX were pooled, concentrated, and precipitated with ether: yield, 300 mg (36%); $[\alpha]^{20}D$ −49.5° (*c* 0.8, DMF); R_f 0.85[1], 0.80[2], 0.63[3]; amino acid ratios in acid hydrolysate $Lys_{0.96}His_{0.88}Arg_{1.02}Asp_{1.06}Thr_{2.45(uncorr)}Glu_{2.10}Pro_{3.10}Val_1Leu_{3.02}Nle_{0.89}Phe_{1.08}$.

Glutaminylprolylnorleucyl-*O*-benzylthreonylphenylalanylnitroarginylleucylleucylleucylvalyl-β-benzylaspartyl-*O*-benzylthreonylprolyl-γ-benzylglutamyl-*O*-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester Dihydrochloride (XXXI). The octadecapeptide (50 mg, 17.1 μmol) was dissolved in 6 *N* HCl in dioxane for 15 min. Ether was added until the product precipitated: yield, 47 mg (95%); $[\alpha]^{20}D$ −35.9° (*c* 1.5, DMF); R_f 0.72[2].

***tert*-Butoxycarbonyl-ε-benzyloxycarbonyllysylglycine Ethyl Ester (XXXII).** To a mixture of 7.6 g (20 mmol) of *tert*-butoxycarbonyl-ε-benzyloxycarbonyllysine and 2.8 g (20 mmol) of glycine ethyl ester hydrochloride in 200 ml of methylene chloride was added 2.8 ml (20 mmol) of triethylamine and 4.12 g (20 mmol) of DCC. The mixture was worked up by the standard procedure. Crystallization was effected from ethyl acetate–petroleum ether: yield, 8.01 g (86%); mp 58–59°; $[\alpha]^{20}D$ −10.7° (*c* 1.0, methanol); R_f 0.86[1], 0.83[2], 0.79[3].

Anal. Calcd for $C_{23}H_{35}O_7N_3$: C, 59.34; H, 7.58; N, 9.03. Found: C, 59.62; H, 7.62; N, 8.88.

ε-Benzyloxycarbonyllysylglycine Ethyl Ester Formate (XXXIII). The dipeptide XXXII (7.97 g, 17.1 mmol) was dissolved in 100 ml of 97+% formic acid for 3 hr. The formic acid was removed *in vacuo* and the product crystallized upon the addition of ether: yield, 6.40 g (91%); mp 116–118°; $[\alpha]^{20}D$ +11.5° (*c* 1.0, methanol); R_f 0.63[2], 0.48[3].

Anal. Calcd for $C_{19}H_{29}O_7N_3$: C, 55.46; H, 7.11; N, 10.21. Found: C, 55.66; H, 7.20; N, 10.26.

***tert*-Butoxycarbonyl-*O*-benzyltyrosyl-ε-benzyloxycarbonyllysylglycine Ethyl Ester (XXXIV).** The dipeptide XXXIII (3.29 g, 8.0 mmol) was suspended in 75 ml of ethyl acetate and allowed to react with 1.19 ml (8.5 mmol) of triethylamine. The ethyl acetate layer was immediately washed with water, dried with anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was dissolved in a solution of 3.25 g (8.0 mmol) of *tert*-butoxycarbonyl-*O*-benzyltyrosine in 150 ml of methylene chloride. At 4°, 1.64 g (8.0 ml) of DCC in 20 ml of methylene chloride was added. After working up the mixture by the standard procedure, the product was crystallized from ethyl acetate–ether: yield, 5.30 g (92%); mp 128–130°; $[\alpha]^{20}D$ −9.1° (*c* 1.0, methanol); R_f 0.91[1], 0.88[2], 0.85[3].

Anal. Calcd for $C_{39}H_{50}O_9N_4$: C, 65.16; H, 7.01; N, 7.79. Found: C, 65.38; H, 6.81; N, 8.03.

***O*-Benzyltyrosyl-ε-benzyloxycarbonyllysylglycine Ethyl Ester Formate (XXXV).** A solution of 4.0 g (5.57 mmol) of XXXIV in 50 ml of 97+% formic acid was allowed to react for 3 hr. The solvent was removed *in vacuo* and the product was obtained from ether: yield, 3.50 g (94%); mp 149–152°; $[\alpha]^{20}D$ −1.7° (*c* 1.0, methanol); R_f 0.67[1], 0.79[2], 0.53[3].

Anal. Calcd for $C_{35}H_{44}O_9N_4$: C, 63.24; H, 6.67; N, 8.43. Found: C, 63.42; H, 6.59; N, 8.47.

***tert*-Butoxycarbonylnorleucyl-*O*-benzyltyrosyl-ε-benzyloxycarbonyllysylglycine Ethyl Ester (XXXVI).** A suspension of 3.32 g (5.0 mmol) of XXXV in 100 ml of ethyl acetate was neutralized with 0.70 ml (5.0 mmol) of triethylamine and washed with water. The organic layer was dried with anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was dissolved in 100 ml of methylene chloride. At 4°, 1.5 g (7.0 mmol) of *tert*-butoxycarbonylnorleucine in 50 ml of methylene chloride and 1.01 g (4.9 mmol) of DCC in 25 ml of methylene chloride were successively added. After 16 hr, the dicyclohexylurea was filtered off and the solvent was removed *in vacuo*. Crystallization occurred from boiling ethyl acetate: yield, 3.13 g (75%); mp 173–174°; $[\alpha]^{20}D$ −18.9° (*c* 1.0, methanol); R_f 0.92[1], 0.96[2], 0.88[3].

Anal. Calcd for $C_{45}H_{61}O_{10}N_5$: C, 64.96; H, 7.39; N, 8.42. Found: C, 65.20; H, 7.65; N, 8.60.

***tert*-Butoxycarbonylnorleucyl-*O*-benzyltyrosyl-ε-benzyloxycarbonyllysylglycine (XXXVII).** A solution of 5.0 g (6.0 mmol) of XXXVI in 150 ml of dioxane was saponified with 12 ml of 1 *N* aqueous potassium hydroxide for 2 hr. An equal volume of water was added and the dioxane was removed *in vacuo*. The solution was acidified with 1 *N* HCl and filtered. A sample was recrystallized from ethyl acetate for analysis: yield, 4.78 g (99%); mp 138–140°; $[\alpha]^{20}D$ −18.9° (*c* 0.8, methanol); R_f 0.50[1], 0.84[2], 0.75[3].

Anal. Calcd for $C_{43}H_{57}O_{10}N_5$: C, 64.25; H, 7.15; N, 8.71. Found: C, 64.34; H, 7.44; N, 8.88.

Norleucyl-*O*-benzyltyrosyl-ε-benzyloxycarbonyllysylglycine Formate (XXXVIII). A solution of 2.16 g (2.69 mmol) of XXXVII was allowed to react in 50 ml of 97+% formic acid for 4 hr. The solvent was removed *in vacuo*, and the product was crystallized with ether: yield, 1.85 g (92%); mp 204° dec; $[\alpha]^{20}D$ +10.3° (*c* 1.93, 98% formic acid); R_f 0.24[1], 0.81[2], 0.71[3].

Anal. Calcd for $C_{39}H_{51}O_{10}N_5$: C, 62.47; H, 6.86; N, 9.34. Found: C, 62.69; H, 7.10; N, 9.23.

***tert*-Butoxycarbonyl-ε-benzyloxycarbonyllysylleucine Ethyl Ester (XXXIX).** To a solution of 6.27 g (16.5 mmol) of *tert*-butoxycarbonyl-ε-benzyloxycarbonyllysine in 250 ml of methylene chlo-

ride at 4° was added 3.91 g (20.0 mmol) of leucine ethyl ester hydrochloride, 2.80 ml (20.0 mmol) of triethylamine, and 3.45 g (16.5 mmol) of DCC in 50 ml of methylene chloride. After working up the mixture by the standard procedure, the product was crystallized from ethyl ether–petroleum ether: yield, 6.37 g (75%); mp 89–91°; $[\alpha]^{20}D$ −28.6° (*c* 1.0, methanol); R_f 0.92[1], 0.85[2], 0.85[3].

Anal. Calcd for $C_{27}H_{43}O_7N_3$: C, 62.16; H, 8.31; N, 8.06. Found: C, 62.39; H, 8.11; N, 7.84.

ε-Benzyloxycarbonyllysylleucine Formate (XL). A solution of 6.15 g (11.8 mmol) of XXXIX was allowed to react in 90 ml of 97+ % formic acid for 4 hr. The formic acid was removed *in vacuo* to yield 5.3 g of an oil (100%); R_f 0.68[1], 0.78[2], 0.73[3].

***tert*-Butoxycarbonylvalyl-ε-benzyloxycarbonyllysylleucine Ethyl Ester (XLI).** The formate salt of XL was removed by washing an ethyl acetate solution (100 ml) of the latter with 50 ml of 1 *M* sodium bicarbonate. The organic layer was washed with water, dried with anhydrous sodium sulfate, filtered, and evaporated to dryness. To a solution of the residue dissolved in 150 ml of methylene chloride at 4° was added 2.61 g (11.8 mmol) of *tert*-butoxycarbonylvaline in 75 ml of methylene chloride and 2.34 g (11.8 mmol) of DCC in 25 ml of methylene chloride. After 16 hr, the dicyclohexylurea was filtered and the solvent was evaporated to dryness. The product was obtained from refluxing ethyl acetate: yield, 5.86 g (80%); mp 148–150°; $[\alpha]^{20}D$ −41.7° (*c* 1.0, methanol); R_f 0.94[1], 0.95[2], 0.93[3].

Anal. Calcd for $C_{32}H_{52}O_8N_4$: C, 61.91; H, 8.49; N, 9.03. Found: C, 62.06; H, 8.54; N, 9.30.

Valyl-ε-benzyloxycarbonyllysylleucine Ethyl Ester Formate (XLII). A solution of 2.4 g (3.87 mmol) of XLI was allowed to react in 50 ml of 97+ % formic acid for 3 hr. The formic acid was removed *in vacuo* and the product was precipitated with ether: yield, 2.06 g (95%); mp 144–147°; $[\alpha]^{20}D$ −18.5° (*c* 1.0, methanol); R_f 0.76[1], 0.75[2], 0.69[3].

Anal. Calcd for $C_{28}H_{44}O_8N_4$: C, 59.56; H, 7.85; N, 9.92. Found: C, 59.84; H, 8.04; N, 9.75.

***tert*-Butoxycarbonyl-*O*-benzylthreonylvalyl-ε-benzyloxycarbonyllysylleucine Ethyl Ester (XLIII).** The formate salt of XLII (2.0 g, 3.5 mmol) was removed from an ethyl acetate suspension (100 ml) by the addition of 0.6 ml (4.3 mmol) of triethylamine and 100 ml of water. The organic layer was dried with anhydrous sodium sulfate, filtered, and evaporated to dryness. To a solution of the residue dissolved in 100 ml of methylene chloride was added successively, at 4°, 1.08 g (3.5 mmol) of *tert*-butoxycarbonyl-*O*-benzylthreonine in 125 ml of methylene chloride and 0.72 g (3.5 mmol) of DCC in 25 ml of methylene chloride. After 16 hr, the dicyclohexylurea was filtered and the filtrate was removed *in vacuo*. The product was crystallized from refluxing ethyl acetate: yield, 2.67 g (94%); mp 185–186°; $[\alpha]^{20}D$ −8.0° (*c* 1.0, DMF); R_f 0.90[1], 0.95[2], 0.80[3], 0.98[4].

Anal. Calcd for $C_{42}H_{67}O_{11}N_5$: C, 63.60; H, 8.07; N, 8.63. Found: C, 63.33; H, 8.15; N, 8.56.

***tert*-Butoxycarbonyl-*O*-benzylthreonylvalyl-ε-benzyloxycarbonylylsylleucine Hydrazide (XLIV).** A solution of 2 ml (70 mmol) of 95% hydrazine in 5 ml of methanol was added to a solution of 2.43 g (3.0 mmol) of XLIII in 40 ml of methanol. After 40 hr, the product was filtered and the filtrate was concentrated for a second crop. Both crops had the same physical characteristics: total yield, 1.80 g (75%); mp 237–238°; $[\alpha]^{20}D$ −7.1° (*c* 1.0, DMF); R_f 0.90[1], 0.95[2], 0.80[3], 0.90[4].

Anal. Calcd for $C_{41}H_{63}O_9N_7$: C, 61.71; H, 7.96; N, 12.30. Found: C, 61.88; H, 8.18; N, 12.15.

***tert*-Butoxycarbonyl *O*-benzylthreonylvalyl-ε-benzyloxycarbonyllysylleucylnorleucyl-*O*-benzyltyrosyl-ε-benzyloxy-carbonyllysylglycine (XLV).** The tetrapeptide hydrazide XLIV (3.88 g, 4.85 mmol) was almost heated into 30 ml of DMF. The mixture was cooled with stirring to −25° and acidified with a pre-cooled solution of 7.0 ml of 2.9 *N* HCl in dioxane and 10 ml of DMF. Then was added 0.60 ml (5.05 mmol) of *tert*-butyl nitrite and the temperature was maintained at −25° for 45 min. The tetrapeptide XXXVIII (3.42 g, 4.56 mmol) was added as a solid. The temperature was lowered to −65° and the solution neutralized with 3.50 ml (25.0 mmol) of triethylamine. The solution was stirred at −25° for 1 hr, −10° for 1 hr, and 4° for 16 hr. Water was added and the solution was filtered and desiccated. The gelatinous residue (9.3 g) was washed with ethyl acetate and refluxed in methanol, Upon cooling, 4.18 g (62%) of pure product was obtained: mp 285 dec; $[\alpha]^{20}D$ −17.7° (*c* 1.0, DMF); R_f 0.46[4]; amino acid ratios in pronase AP-M digest, $Lys_2Thr_{0.95}Gly_{1.07}Val_{0.99}Leu_{1.05}Nle_{1.06}Tyr_{0.91}$.

Anal. Calcd for $C_{70}H_{108}O_{17}N_{10}$: C, 64.56; H, 7.41; N, 9.53. Found: C, 64.80; H, 7.58; N, 9.46.

***O*-Benzylthreonylvalyl-ε-benzyloxycarbonyllysylleucylnorleucyl-*O*-benzyltyrosyl-ε-benzyloxycarbonyllysylglycine Formate (XLVI).** The octapeptide (2.17 g, 1.48 mmol) was dissolved in 50 ml of 97+ % formic acid for 3 hr. The solvent was concentrated to 5 ml and the product was precipitated with ether: yield, 1.70 g (81%); mp 253–255° dec; $[\alpha]^{20}D$ −33.5° (*c* 1.0, 98% formic acid); R_f at origin[4].

Anal. Calcd for $C_{75}H_{102}O_{17}N_{10}$: C, 63.64; H, 7.26; N, 9.89. Found: C, 63.59; H, 7.00; N, 9.59

In order to enhance the solubility in DMF, the formate salt dissolved in formic acid was acidified with HCl in dioxane and precipitated with ether.

***tert*-Butoxycarbonyl-β-benzylaspartyl-*O*-benzylthreonylvalyl-ε-benzyloxycarbonyllysylleucylnorleucyl-*O*-benzyltyrosyl-ε-benzyloxycarbonyllysylglycine (XLVII).** To 412 mg (0.30 mmol) of the hydrochloride salt of XLVI in 15 ml of DMF at 4° was added 380 mg (0.90 mmol) of the *N*-hydroxysuccinimide ester of *tert*-butoxycarbonyl-γ-benzylaspartic acid and 40 μl (0.36 mmol) of *N*-methylmorpholine. The solution was stirred for 16 hr at 4°, 2 hr at room temperature, and 4 hr at 50°. The solution was cooled to room temperature and another 210 mg (0.50 mmol) of the *N*-hydroxysuccinimide ester was added. After 2 hr, the solution was stirred at 50° overnight. The solvent was concentrated and the crude product precipitated with water. The crystals were washed five times with methanol and twice with ether: yield, 390 mg (79%); $[\alpha]^{20}D$ −18.3° (*c* 1.0, DMF); R_f 0.36[4]; amino acid ratios in acid hydrolysate, $Lys_{2.02}Asp_{0.99}Thr_{0.99(uncorr)}Gly_{1.04}Val_1Leu_{1.01}Nle_{1.04}Tyr_{0.96(uncorr)}$.

Anal. Calcd for $C_{90}H_{119}O_{20}N_{11}$: C, 64.54; H, 7.16; N, 9.20. Found: C, 64.24; H, 7.12; N, 9.09.

***tert*-Butoxycarbonyl-β-benzylaspartyl-*O*-benzylthreonylvalyl-ε-benzyloxycarbonyllysylleucylnorleucyl-*O*-benzyltyrosyl-ε-benzyloxycarbonyllysylglycylglutaminylprolylnorleucyl-*O*-benzylthreonylphenylalanylnitroarginylleucylleucylleucylvalyl-β-benzyl-aspartyl-*O*-benzylthreonylprolyl-γ-benzylglutamyl-*O*-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester (XLVIII).** To a solution of 204 mg (70 μmol) of XXXI and 168 mg (100 μmol) of XLVII in 10 ml of DMF at room temperature was added 20 μl (180 μmol) of *N*-methylmorpholine and 500 μl of a DMF solution containing 14.8 mg (72 μmol) of DCC. After 16 hr, the temperature was raised to 45° for 4 hr. The solution was applied to a 1-m column of Sephadex LH-60 swelled in DMF and was eluted with this solvent at 14 ml/hr. The fractions were analyzed by optical density at 275 nm and amino acid analysis. The appropriate fractions were pooled and evaporated: yield, 114 mg (36%); $[\alpha]^{20}D$ −25.3° (*c* 0.45, DMF); amino acid ratios in acid hydrolysate, $Lys_{3.22}His_{0.99}Arg_{0.75}Asp_{1.91}Thr_{3.61(uncorr)}Glu_{1.97}Pro_{2.91}Gly_{1.12}Val_{2.22}Leu_4Nle_{2.08}Tyr_{1.07(uncorr)}Phe_{0.96}$.

***tert*-Butoxycarbonylglycyl-β-benzylaspartyl-*O*-benzylthreonylvalyl-ε-benzyloxycarbonyllysylleucylnorleucyl-*O*-benzyltyrosyl-ε-benzyloxycarbonyllysylglycylglutaminylprolylnorleucyl-*O*-benzylthreonylphenylalanylnitroarginylleucylleucylleucylvalyl-β-benzylaspartyl-*O*-benzylthreonylprolyl-γ-benzylglutamyl-*O*-benzylthreonyl-ε-benzyloxycarbonyllysylhistidylproline Benzyl Ester (XLIX).** The heptacosapeptide (108 mg, 24 μmol) was dissolved in 2 ml of distilled DMF and allowed to react with 10 ml of 4.2 *N* HCl in dioxane. After 30 min, the product was precipitated with ethyl acetate: yield, 89 mg (84%). A solution of 9.2 mg (2.0 μmol) of this product in 1.5 ml of distilled DMF was allowed to react with 1.5 ml of DMF containing 1.5 μl (10 μmol) of triethylamine and 2.71 mg (10 μmol) of *N*-hydroxysuccinimido *tert*-butoxycarbonylglycinate for 16 hr at room temperature and 24 hr at 50°. Two aliquots of active ester in 0.5 ml of DMF totalling 10 μmol were added during the next 30 hr. The solvent was evaporated and the residue washed extensively with ethyl acetate to yield 8.3 mg (90%) of product: amino acid ratios in acid hydrolysate (on long column only), $Asp_{1.97}Glu_{2.18}Pro_{2.76}Gly_{2.00}Val_{(2)}Leu_{4.40}Nle_{2.21}Tyr_{1.23}Phe_{1.22}Thr_{4.00}$.

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