Studies on Polypeptides. XXIX. Synthetic Peptides Related to the N-Terminus of Bovine Pancreatic Ribonuclease A (Positions 1–7)[1-4]

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Syntheses are described of a series of peptides and peptide derivatives which correspond to the sequence of the seven N-terminal positions in the primary structure of bovine pancreatic ribonuclease A. The hydrazides of N^{α},N^{ϵ}-di-t-butyloxycarbonyllysyl-γ-t-butylglutamylthreonylalanylalanylalanyl-N^{ϵ}-t-butyloxycarbonyllysine, N-t-butyloxycarbonylthreonylalanylalanylalanyl-N^{ϵ}-t-butyloxycarbonyllysine, and t-butyloxycarbonylalanylalanylalanyl-N^{ϵ}-t-butyloxycarbonyllysine whose preparation is described served to introduce the N-terminal section into more complex peptides related to ribonuclease.

This is the first of a series of articles dealing with syntheses of peptides corresponding to sections of the amino acid sequence of the N-terminal eicosapeptide portion (S-peptide) of the enzyme bovine pancreatic ribonuclease A. Based on the studies of Richards,[5] this segment of the enzyme molecule has become a focal point in ribonuclease chemistry. Richards exposed ribonuclease to the action of the bacterial protease subtilisin and observed that this treatment, which does not impair enzymic activity, brings about severance of a single peptide bond located between amino acid residues 20 and 21 in the enzyme. The N-terminal eicosapeptide (S-peptide) remains attached to the rest of the molecule (S-protein) by noncovalent linkages but can be removed by precipitation of the protein with trichloroacetic acid[5] or on Sephadex G-75 columns.[6] Neither S-peptide nor S-protein exhibits enzymic activity but their 1:1 combination brings about full regeneration of enzyme (ribonuclease S′). These events are schematized in Figure 1.

The S-peptide–S-protein system offers intriguing possibilities for study of the structural elements which endow S-peptide with the ability to generate ribonuclease activity with S-protein and, in addition, provides a particularly attractive model for exploration of those features of S-peptide which are responsible for its remarkably strong noncovalent association with S-protein.

A number of studies have appeared[7] dealing with the ability of chemically modified S-peptides to regenerate active enzyme with S-protein. However, this approach to structure–function studies is limited and suffers from the disadvantage that contamination of the modified peptide by unreacted S-peptide may lead to erroneous conclusions. This becomes particularly crucial in situations where high peptide to protein ratios are necessary to bring about activation. Thus, at a molar peptide:protein ratio of 5000:1 contamination of an inactive peptide by 0.02% of S-peptide would result in full activation of S-protein. This degree of purity is not likely to be achieved when chemically altering S-peptide. Activation experiments with pure synthetic peptides eliminate these difficulties. Based on these considerations we have initiated a program aimed at the preparation of S-peptide and related compounds.

In this communication we describe the preparation of a number of peptides and peptide derivatives which are related to the seven N-terminal positions of ribonuclease A.

The synthesis of ethyl N^{α},N^{ϵ}-dibenzyloxycarbonyllysylglutamylthreonylalanylalanylalaninate, a partially protected peptide corresponding to the N-terminal hexapeptide portion of the ribonuclease molecule, has been described by Rocchi, *et al.*[8] The presently accepted amino acid sequence of S-peptide[6,9] and the structures of some of the compounds whose synthesis is described are shown in Scheme I. Scheme II provides orientation regarding the various synthetic routes that were employed to produce peptide XIII and the three hydrazides XIV, XX, and XXI.

Interaction of the azide of benzyloxycarbonylalanylalanine (I) with the triethylammonium salt of N^{ϵ}-t-butyloxycarbonyllysine[10] (II) gave benzyloxycarbonylalanylalanyl-N^{ϵ}-t-butyloxycarbonyllysine (III) which was partially deblocked by hydrogenolysis with formation of alanylalanyl-N^{ϵ}-t-butyloxycarbonyllysine (IV). This material was then treated with benzyloxycarbonylthreonylalanine azide (V) to give the protected pentapeptide VI. This compound was transformed into the methyl ester VII with diazomethane and the latter was partially deblocked by hydrogenolysis to afford VIII. The elegant *p*-nitrophenyl ester method of Bodanszky[11] served to introduce the glutamic acid

(1) The authors wish to express their appreciation to the U. S. Public Health Service, the National Science Foundation, and the American Cancer Society for generous support of this investigation.

(2) The peptides and peptide derivatives mentioned are of the L-configuration. In the interest of space conservation the customary L-designation for individual amino acid residues is omitted.

(3) See *J. Am. Chem. Soc.*, **86**, 4991 (1964), for paper XXVIII in this series.

(4) A preliminary communication describing some of the results presented in this paper has appeared: *ibid.*, **85**, 833 (1963).

(5) F. M. Richards and P. J. Vithayathil, *J. Biol. Chem.*, **234**, 1459 (1959).

(6) D. G. Smyth, W. H. Stein, and S. Moore, *ibid.*, **237**, 1845 (1962).

(7) (a) P. J. Vithayathil and F. M. Richards, *ibid.*, **235**, 1029 (1960); (b) P. J. Vithayathil and F. M. Richards, *ibid.*, **235**, 2343 (1960); (c) P. J. Vithayathil and F. M. Richards, *ibid.*, **236**, 1380 (1961); (d) J. M. Parks, M. B. Barancik and F. Wold, *J. Am. Chem. Soc.*, **85**, 3521 (1963).

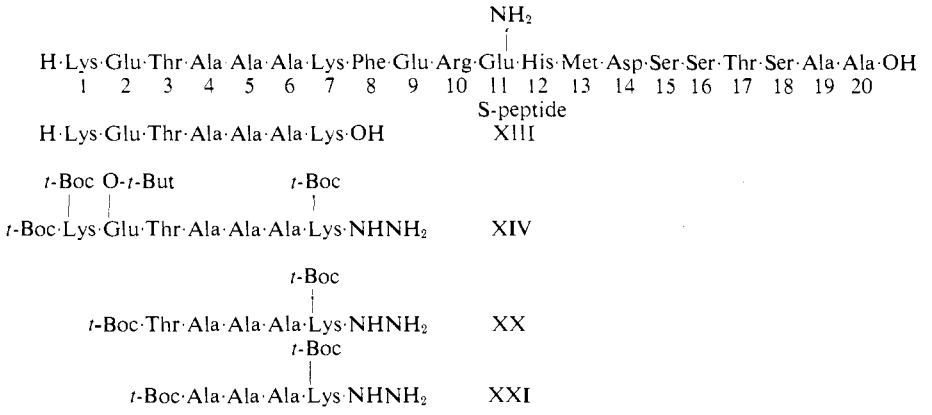
(8) R. Rocchi, F. Marchiori, and E. Scoffone, *Gazz. chim. ital.*, **93**, 823 (1963).

(9) (a) J. T. Potts, A. Berger, J. Cooke, and C. B. Anfinsen, *J. Biol. Chem.*, **237**, 1851 (1962); (b) E. Gross and B. Witkop, *ibid.*, **237**, 1856 (1962).

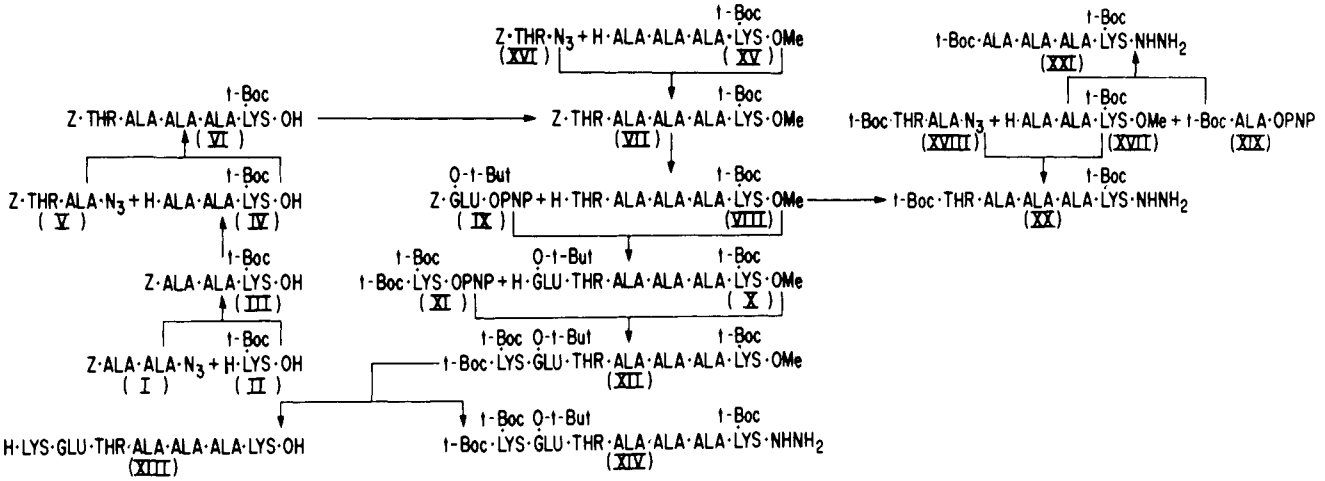
(10) R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, **44**, 159 (1961).

(11) M. Bodanszky, *Nature*, **175**, 685 (1955).

Scheme I



Scheme II



residue into VIII in the form of α-*p*-nitrophenyl N-benzyloxycarbonyl-γ-*t*-butylglutamate (IX). The ensuing protected hexapeptide ester was partially deblocked to give compound X which was coupled with *p*-nitrophenyl N^{α},N^{ϵ}-di-*t*-butyloxycarbonyllysinate (XI).[12] For assessment of stereochemical homogeneity

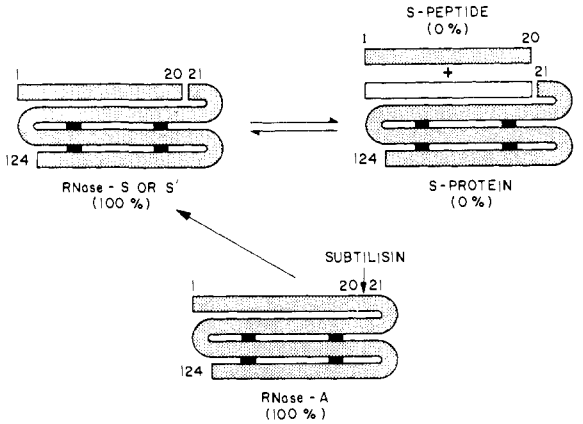


Figure 1. Schematic representation of the relation of various ribonucleases and fragments. The figures in parentheses represent relative enzymic activity.

the resulting fully protected heptapeptide ester XII was saponified and completely deblocked by short exposure to trifluoroacetic acid.[13]

(12) Ed. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1637 (1963).

Trifluoroacetate ions were exchanged for acetate ions with Amberlite IRA-400 and the heptapeptide XIII was obtained by lyophilization in the form of its diacetate tetrahydrate. Elemental analysis, including oxygen determination, supports this composition of the heptapeptide. Paper chromatography and paper electrophoresis at three pH values showed the presence of a single component. The amino acid ratios in an acid hydrolysate of XIII were those theoretically expected. Complete digestibility of the peptide by leucine aminopeptidase (LAP) with a theoretical average recovery of the constituent amino acids in the digest supports the stereochemical homogeneity of this heptapeptide. Exposure of the protected methyl ester XII to hydrazine hydrate gave the hydrazide XIV in the form of a gelatinous amorphous material. The corresponding azide served to introduce the N-terminal heptapeptide sequence into a variety of S-peptide derivatives.

A number of intermediates used in this synthesis were prepared by alternate routes. For example, the fully protected pentapeptide methyl ester VII was also obtained by a stepwise process from methyl N^{ϵ}-*t*-butyloxycarbonyllysinate.[11] The three alanyl residues were introduced by the repeated use of *p*-nitrophenyl benzyloxycarbonylalaninate[14] as the acylating component. The intermediate methyl benzyloxycarbonylalanylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysinate exhibited properties (melting point, rotation, and R_f values)

(13) (a) L. A. Carpino, *J. Am. Chem. Soc.*, **79**, 98 (1957); (b) R. Schwyzer, W. Rittel, H. Kappeler, and B. Iselin, *Angew. Chem.*, **72**, 915 (1960).

(14) F. Marchiori, R. Rocchi, and E. Scoffone, *Ric. Sci. Rend. Sez. A*, (6) 2, 647 (1962).

in good agreement with those of the same compound prepared from III with diazomethane. Hydrogenolysis of methyl benzyloxycarbonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysinate afforded compound XV which was transformed into VII by reaction with benzyloxycarbonylthreonine azide (XVI). Benzyloxycarbonylthreonine hydrazide was prepared according to Schröder and Gibian.[15] The properties (melting point, rotation, and R_f values) of VII prepared according to this route were in satisfactory agreement with those of the same compound obtained by esterification of VI.

For the synthesis of S-peptide analogs lacking two and three of the N-terminal amino acid residues we required the hydrazides of N-*t*-butyloxycarbonylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysine (XX) and of *t*-butyloxycarbonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysine (XXI), respectively. Methyl N-*t*-butyloxycarbonylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysinate was prepared either by treating the pentapeptide methyl ester VIII with *t*-butyl azidoformate[16] or from the methyl ester XVII with N-*t*-butyloxycarbonylthreonylalanine azide (XVIII). *p*-Nitrophenyl *t*-butyloxycarbonylalaninate (XIX)[12] was treated with methyl alanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysinate (XVII) for preparing methyl *t*-butyloxycarbonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysinate. Conversion of the protected peptide esters into the hydrazides XX and XXI was carried out in the usual manner. In addition to the various compounds which served directly as intermediates in the preparation of the compounds listed in Scheme I we prepared a series of lysine derivatives which were required for future studies. The oily N$^{\alpha}$,N$^{\epsilon}$-di-*t*-butyloxycarbonyllysine *via* the crystalline methyl ester was converted into the hydrazide. The hydrazide of N$^{\alpha}$-*t*-butyloxycarbonyl-N$^{\epsilon}$-formyllysine was obtained by the same route from the acylated amino acid. Using the procedure of Anderson[17] N$^{\alpha}$-*t*-butyloxycarbonyl-N$^{\epsilon}$-formyllysine was converted into the crystalline N-hydroxysuccinimide ester.

Experimental[18]

t-Butyloxycarbonylthreonine. *t*-Butyl azidoformate (11.4 g.) in dioxane (150 ml.) was added with stirring to a solution of threonine (7.2 g.) in water (120 ml.) containing suspended magnesium oxide (4.8 g.), and the mixture was stirred for 40 hr. at room temperature. The bulk of the dioxane was removed *in vacuo*, the suspension was filtered, and the yellow filtrate was extracted with ethyl acetate. The aqueous layer was cooled at 0°, saturated with sodium chloride, and acidified by addition of solid citric acid. The solution was extracted with eight 100-ml. portions of ice-cold ethyl acetate, the extracts were washed with saturated sodium chloride and dried over sodium sulfate, and the solvent was evaporated. The resulting oil crystallized when kept under petroleum ether; yield 10.0 g. (75%); m.p. 76–80°. A sample for analysis was recrystallized from a mixture of ether and petroleum ether; m.p. 76–80°; $[\alpha]^{26}D$ −2.52° (*c* 0.98, methanol); R_f^{I} 0.68.

Anal. Calcd. for $C_9H_{17}O_5N$: C, 49.3; H, 7.8; N, 6.4. Found: C, 49.0; H, 7.8; N, 6.4.

The dicyclohexylammonium salt was obtained when an equimolar portion of dicyclohexylamine was added to an ether solution of the acylamino acid. The salt was recrystallized from acetone; m.p. 154–155°; $[\alpha]^{27}D$ +11.37° (*c* 0.99, methanol); R_f^{I} 0.68.

Anal. Calcd. for $C_{21}H_{40}O_5N_2$: C, 63.0; H, 10.1; N, 7.0. Found: C, 62.9; H, 9.9; N, 7.1.

N$^{\alpha}$,N$^{\epsilon}$-Di-t-butyloxycarbonyllysine. *t*-Butyl azidoformate (43 g.) in dioxane (150 ml.) was added to a solution of lysine monohydrochloride (18.2 g.) in water (150 ml.) containing sodium carbonate (42 g.). The mixture was stirred at 50° for 40 hr. when the dioxane was removed *in vacuo*. Water (150 ml.) was added and the solution was extracted with three 75-ml. portions of ether. The aqueous layer was cooled at 0°, acidified with solid citric acid, and extracted with three 150-ml. portions of ethyl acetate which were dried over sodium sulfate. Upon removal of the solvent *in vacuo* 27 g. (78%) of an oil was obtained; $[\alpha]^{30}D$ −3.1° (*c* 1.05, methanol).

N$^{\alpha}$-t-Butyloxycarbonyl-N$^{\epsilon}$-formyllysine. *t*-Butyl azidoformate (26 g.) in dioxane (200 ml.) was added with stirring to a solution of N$^{\epsilon}$-formyllysine (17.5 g.) in water (200 ml.) containing triethylamine (28.8 ml.) and the mixture was stirred for 48 hr. at 45°. The dioxane was removed *in vacuo* and the aqueous solution was extracted with three 100-ml. portions of ethyl acetate. The aqueous layer was cooled at 0° and acidified by the addition of solid citric acid. The solution was extracted with three 150-ml. portions of ethyl acetate, the combined organic solutions were

(15) E. Schröder and H. Gibian, *Ann.*, **656**, 190 (1962).

(16) *t*-Butyl azidoformate was prepared from the hydrazide as described by L. A. Carpino, C. A. Giza, and B. A. Carpino, *J. Am. Chem. Soc.*, 81, 955 (1959), and was used directly without distillation.

(17) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *ibid.*, **86**, 1839 (1964).

(18) The organic solvents were freshly distilled. Doubly distilled (from glass) water from which a sizable forerun was removed was employed. The melting points were determined in an aluminum block and are uncorrected. Rotations were determined with a Rudolph Model 80 precision polarimeter with a Model 200 photoelectric attachment. Elemental analyses were by Schwarzkopf Microanalytical Laboratory, New York, N. Y. The amino acid composition of acid and enzymic hydrolysates was determined with a Beckman-Spinco Model 120 amino acid analyzer according to the method of S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958). Paper chromatography was carried out on Whatman No. 1 filter paper by the descending technique with the following solvent systems: R_f^{1}, Partridge system (S. M. Partridge, *Biochem. J.*, **42**, 238 (1948)); R_f^{2}, 2-butanol–ammonia system (J. F. Roland, Jr., and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954)); R_f^{3}, pyridine system (S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953)). With the latter system R_f values are expressed as multiples of the distance traveled by a histidine marker. Ascending thin layer chromatography was performed on silica gel G (E. Merck and Co., Darmstadt, Germany) with the following solvent systems: R_f^{I}, methanol–chloroform, 1:1; R_f^{II}, dioxane–water, 9:1; R_f^{III}, methanol; R_f^{IV}, chloroform; R_f^{V}, 96% ethanol–ammonium hydroxide, 100:27; R_f^{VI}, 1-butanol–acetic acid–water, 60:20:20; R_f^{VII}, 1-butanol–pyridine–acetic acid–water, 30:20:6:24; R_f^{VIII}, ethyl acetate–cyclohexane, 1:1; R_f^{IX}, chloroform–acetone, 1:1; R_f^{X}, methanol–chloroform 2:1; R_f^{XI}, methanol–chloroform–ammonium hydroxide–water, 20:20:6.6:3.4. Unless stated otherwise, solvents were evaporated at a bath temperature of 40–50° in a rotary evaporator. Leucine aminopeptidase digests were prepared in the manner described in *J. Am. Chem. Soc.*, **84**, 4481 (1962). Figures in parentheses following the amino acid ratios refer to the average recovery of amino acids from the digest. Color tests used to identify the peptides and peptide derivatives on papers or on thin layer plates were essentially those described in "Composition, Structure and Reactivity of Proteins" Vol. II of a Laboratory Manual on Analytical Methods of Protein Chemistry, P. Alexander and R. J. Block, Ed., Pergamon Press, New York, N. Y., 1960. The chlorine test was carried out as described by J. Barrollier, *Naturwiss.*, **48**, 554 (1961). For the hydrazine test the papers were sprayed with a 1:1 (v./v.) mixture of 0.3 *M* $FeCl_3$ in 0.1 *N* acetic acid and 0.2 *M* $K_3[Fe(CN)_6]$ in 0.1 *N* acetic acid. The following abbreviations are used: LAP = leucine aminopeptidase; Z = benzyloxycarbonyl; *t*-Boc = *t*-butyloxycarbonyl; O-*t*-But = *t*-butyl ester; ONP = *p*-nitrophenyl ester; DMF = dimethylformamide.

dried over sodium sulfate, and the solvent was removed *in vacuo*. The resulting oil crystallized on addition of petroleum ether, and the compound was recrystallized from ethyl acetate; yield 20 g. (73%); m.p. 131–132°; $[\alpha]^{27}D$ −3.1° (*c* 1.65, methanol); R_f^{I} 0.71.

Anal. Calcd. for $C_{12}H_{22}O_5N_2$: C, 52.5; H, 8.1; N, 10.2. Found: C, 52.8; H, 8.2; N, 9.5.

Methyl N^{α},N^{ϵ}-Di-t-butyloxycarbonyllysinate. N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysine (4.38 g.) was dissolved in methanol (50 ml.), ethereal diazomethane in excess was added, and the mixture was kept at room temperature for 45 min. After destroying the excess of diazomethane by addition of a few drops of acetic acid, the solvents were removed *in vacuo* and the residue was crystallized from diisopropyl ether; yield 3.6 g. (79%); m.p. 83–85°; $[\alpha]^{25}D$ −15.4° (*c* 1.78, methanol); R_f^{I} 0.84; R_f^{II} 0.77; R_f^{VI} 0.86.

Anal. Calcd. for $C_{17}H_{32}O_6N_2$: C, 56.6; H, 8.9; N, 7.8. Found: C, 56.4; H, 8.8; N, 7.8.

p-Nitrophenyl N^{α},N^{ϵ}-Di-t-butyloxycarbonyllysinate (XI). N,N′-Dicyclohexylcarbodiimide[19] (4.12 g.) was added to an ice-cold solution of N^{α},N^{ϵ}-di-*t*-butyloxycarbonyllysine (6.92 g.) and *p*-nitrophenol (2.92 g.) in ethyl acetate (150 ml.). The mixture was kept at 0–3° for 30 min. and at room temperature for 1 hr. when a few drops of glacial acetic acid was added. The N,N′-dicyclohexylurea was removed by filtration and washed with ethyl acetate (20 ml.), and the combined filtrate and washings were evaporated *in vacuo*. The residue was redissolved in ethyl acetate (20 ml.) and crystallization occurred when petroleum ether (50 ml.) was added to the solution. The crystals (needles) were collected and dried; yield 6.82 g. (71%); m.p. 120–121°. A sample for analysis was recrystallized from ethanol; m.p. 122–123°; $[\alpha]^{30}D$ −29.7° (*c* 1.06, methanol); $[\alpha]^{27}D$ −27.2° (*c* 0.89, DMF); lit.[12] m.p. 127°, $[\alpha]^{22}D$ −26.0° (in DMF).

Anal. Calcd. for $C_{22}H_{33}O_8N_3$: C, 56.5; H, 7.1; N, 9.0. Found: C, 56.1; H, 7.1; N, 9.2.

Succinimido N^{α}-t-Butyloxycarbonyl-N^{ϵ}-formyllysinate. N,N′-Dicyclohexylcarbodiimide (2.06 g.) was added to an ice-cold solution of N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-formyllysine (2.75 g.) and N-hydroxysuccinimide (1.15 g.) in methylene chloride (100 ml.). The mixture was kept in an ice bath for 30 min. and at room temperature for 1 hr. when a few drops of glacial acetic acid were added. The N,N′-dicyclohexylurea was removed by filtration and washed with methylene chloride, and the combined filtrate and washings were evaporated *in vacuo*. The residue was dissolved in ethyl acetate (30 ml.) and crystallization occurred when petroleum ether (60 ml.) was added. The crystals were collected and dried; yield 3.2 g. (86%); m.p. 81–82°; $[\alpha]^{28}D$ −25.1° (*c* 4.04, methanol).

Anal. Calcd. for $C_{16}H_{23}O_7N_3$: C, 51.7; H, 6.8; N, 11.3. Found: C, 52.0; H, 7.0; N, 11.3.

N^{α},N^{ϵ}-Di-t-butyloxycarbonyllysine Hydrazide. Methyl N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysinate (1.08 g.) was dissolved in methanol (2 ml.) and 0.44 ml. of 95% hydrazine was added. The mixture was kept at room temperature for 115 hr. when the solvent was evaporated and the residue dried over concentrated sulfuric acid and KOH pellets. The amorphous product was crystallized twice from a mixture of 1 ml. of methanol and 10 ml. of diisopropyl ether; yield 0.68 g. (63%); m.p. 102–103°; $[\alpha]^{27}D$ −7.27° (*c* 1.70, methanol); R_f^{I} 0.72.

Anal. Calcd. for $C_{16}H_{32}O_5N_4$: C, 53.3; H, 9.0; N, 15.5. Found: C, 53.1; H, 9.1; N, 15.5.

N^{α}-t-Butyloxycarbonyl-N^{ϵ}-formyllysine Hydrazide. N-$^{\alpha}$*t*-Butyloxycarbonyl-N^{ϵ}-formyllysine (5.0 g.) was dissolved in methanol (50 ml.), an excess of ethereal diazomethane was added, and the mixture was kept at room temperature for 10 min. After destroying the excess of diazomethane by the addition of a few drops of acetic acid, the solvent was removed *in vacuo*. The resulting oil was dissolved in methanol (30 ml.) and 95% hydrazine (1 ml.) was added. The mixture was kept at room temperature for 24 hr., the solvent was removed *in vacuo*, and the residue was dried over concentrated sulfuric acid and KOH pellets. The amorphous product was crystallized twice from a mixture of 2 ml. of ethanol and 20 ml. of diisopropyl ether; yield 3 g. (57%); m.p. 87–91°; R_f^{I} 0.58; ninhydrin-negative, chlorine- and hydrazine-positive spot.

Anal. Calcd. for $C_{12}H_{24}O_4N_4$: C, 50.0; H, 8.4; N, 19.4. Found: C, 49.9; H, 8.5; N, 19.2.

p-Nitrophenyl t-Butyloxycarbonylalaninate (XIX). N,N′-Dicyclohexylcarbodiimide[19] (4.53 g.) in ethyl acetate (10 ml.) was added to a solution cooled at −70° of *t*-butyloxycarbonylalanine (3.78 g.) and *p*-nitrophenol (3.06 g.) in ethyl acetate (40 ml.). The mixture was kept at −70° for 5 min. and then was allowed to reach room temperature. It was stirred at room temperature for 4 hr. and cooled at 0°, and the N,N′-dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness and the oily residue crystallized when kept under petroleum ether. The compound was recrystallized first from a mixture of ether and petroleum ether (3:4, v./v.), then from aqueous methanol; yield 3.0 g. (48%); m.p. 82–83°; $[\alpha]^{27}D$ −65.5° (*c* 1.53, methanol); R_f^{I} 0.71; R_f^{II} 0.77; lit.[12] m.p. 83°, $[\alpha]^{22}D$ −63.5 ± 1° (in methanol).

Anal. Calcd. for $C_{14}H_{18}O_6N_2$: C, 54.2; H, 5.8; N, 9.0. Found: C, 54.4; H, 6.0; N, 8.9.

p-Nitrophenyl Benzyloxycarbonyl-γ-t-butylglutamate (IX). N,N′-Dicyclohexylcarbodiimide[19] (2.37 g.) was added to an ice-cold solution of benzyloxycarbonyl-γ-*t*-butylglutamic acid[20] (3.90 g.) and *p*-nitrophenol (1.60 g.) in ethyl acetate (70 ml.). The mixture was kept at 0–3° for 30 min. and at room temperature for 30 min. when a few drops of glacial acetic acid was added. The N,N′-dicyclohexylurea was removed by filtration and washed with ethyl acetate (20 ml.), and the combined filtrate and washings were washed with four 50-ml. portions of saturated sodium bicarbonate and four 50-ml. portions of saturated sodium chloride. The solution was dried over sodium sulfate and evaporated, and the ensuing oil was dissolved in a small volume of ether. Petroleum ether was added to bring about crystallization; yield 3.9 g. (74%); m.p. 45–47°. A sample for analysis was recrystallized from a mixture of ether and petroleum ether (1:5, v./v.); m.p. 48–50°; $[\alpha]^{27}D$ −33.4° (*c* 0.40, methanol).

Anal. Calcd. for $C_{23}H_{26}O_8N_2$: C, 60.3; H, 5.7; N, 6.1. Found: C, 60.3; H, 6.0; N, 6.3.

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

(20) E. Klieger and H. Gibian, *Ann.*, **655**, 195 (1962).

Methyl Benzyloxycarbonylalanylalaninate. A mixed anhydride prepared in the usual manner from benzyloxycarbonylalanine[21] (13.7 g.) in dioxane (60 ml.) with tri-*n*-butylamine (14.7 ml.) and ethyl chloroformate (5.7 ml.) was added slowly, with stirring, to a chilled solution of methyl alaninate (obtained from 8.4 g. of the hydrochloride and 8.6 ml. of triethylamine) in dioxane (50 ml.). The mixture was stirred at ice-bath temperature for 1 hr. and at room temperature for 2 hr. The solvent was removed *in vacuo* and the residue was suspended in ethyl acetate (200 ml.). The suspension was washed with three 100-ml. portions of 2 *N* citric acid, three 100-ml. portions of saturated sodium bicarbonate, and three 100-ml. portions of saturated sodium chloride. The combined organic solutions were dried over sodium sulfate and concentrated to a small volume *in vacuo*. Upon the addition of petroleum ether crystallization occurred, and the compound was collected and washed with petroleum ether; yield 11 g. (59%); m.p. 104–105°; $[\alpha]^{27}D$ −52.9° (*c* 0.72, methanol).

Anal. Calcd. for $C_{15}H_{20}O_5N_2$: C, 58.4; H, 6.5; N, 9.1. Found: C, 58.7; H, 6.5; N, 8.9.

Benzyloxycarbonylalanylalanine Hydrazide. Hydrazine hydrate (2 ml.) was added to a solution of the above methyl ester (10.5 g.) in methanol (100 ml.) and the mixture was kept at room temperature for 12 hr. at which time the crystalline hydrazide was collected. The compound was washed with 25 ml. of ice-cold methanol and 50 ml. of ether and dried *in vacuo* over concentrated sulfuric acid. The compound was recrystallized from methanol (100 ml.); yield 8.5 g. (81%); m.p. 210–211°; lit.[22] m.p. 209°.

Anal. Calcd. for $C_{14}H_{20}O_4N_4$: C, 54.5; H, 6.6; N, 18.2. Found: C, 54.3; H, 6.8; N, 18.2.

Methyl Benzyloxycarbonylthreonylalaninate. N,N′-Dicyclohexylcarbodiimide[19] (12.36 g.) was added at room temperature to a methylene chloride solution (600 ml.) containing methyl alaninate hydrochloride (8.38 g.), benzyloxycarbonylthreonine[23] (15.20 g.), and triethylamine (8.34 ml.). The mixture was kept at room temperature for 5 hr. when a few drops of glacial acetic acid was added. The N,N′-dicyclohexylurea (13.0 g.) was removed by filtration and washed with methylene chloride, and filtrate and washings were combined and evaporated to dryness. The residue was dissolved in ethyl acetate (250 ml.), and the solution was washed in the usual manner and dried over sodium sulfate. The solvent was evaporated and the residue recrystallized from a small volume of ethyl acetate; yield 16.6 g. (81%); m.p. 127–129°; $[\alpha]^{27}D$ −29.0° (*c* 1.02, methanol).

Anal. Calcd. for $C_{16}H_{22}O_6N_2$: C, 56.8; H, 6.6; N, 8.3. Found: C, 56.9; H, 6.9; N, 8.4.

Benzyloxycarbonylthreonylalanine Hydrazide. Hydrazine hydrate (4.0 ml.) was added to a solution of methyl benzyloxycarbonylthreonylalaninate (13.5 g.) in methanol (200 ml.) and the mixture was kept at room temperature for 6 hr. when the crystalline hydrazide was collected. The hydrazide was washed with ice-cold methanol (50 ml.) and dried *in vacuo* over concentrated sulfuric acid and potassium hydroxide pellets; yield 12.5 g. (93%); m.p. 210–212°. A sample for analysis was recrystallized from methanol; m.p. 213–214°.

Anal. Calcd. for $C_{15}H_{22}O_5N_4$: C, 53.2; H, 6.6; N, 16.6. Found: C, 52.9; H, 6.6; N, 16.6.

t-Butyloxycarbonylthreonylalanine Hydrazide. N,N′-Dicyclohexylcarbodiimide[19] (1.44 g.) in methylene chloride (10 ml.) was added with stirring to a methylene chloride solution (40 ml.) of *t*-butyloxycarbonylthreonine (1.39 g.), methyl alaninate hydrochloride (0.98 g.), and triethylamine (0.97 ml.) cooled at −40°. The mixture was stirred at 0° for 2 hr. and was then placed in a refrigerator for 12 hr. when a few drops of glacial acetic acid was added. After standing for 30 min. the N,N′-dicyclohexylurea was removed by filtration and washed with methylene chloride, and filtrate and washings were evaporated to dryness. The residue was dissolved in ethyl acetate, the solution was washed with ice-cold 1 *N* citric acid, saturated sodium bicarbonate, and saturated sodium chloride, and dried over sodium sulfate. Evaporation of the solvent gave an oil; yield 1.44 g. (67%); R_f^{I} 0.79; R_f^{II} 0.77. This compound was also prepared by the mixed anhydride procedure in comparable yield. Hydrazine hydrate (0.7 ml.) was added to a methanol solution (10 ml.) containing methyl *t*-butyloxycarbonylthreonylalaninate (1.43 g.) and the mixture was kept at room temperature for 12 hr. when it was evaporated to dryness *in vacuo*. The residue was dried over concentrated sulfuric acid and potassium hydroxide pellets and was recrystallized from ethanol; yield 0.95 g. (66%); m.p. 173–174° dec.; $[\alpha]^{28}D$ −34.6° (*c* 1.05, methanol); R_f^{I} 0.54; R_f^{II} 0.66; R_f^{V} 0.72. This hydrazide is water soluble.

Anal. Calcd. for $C_{12}H_{24}O_5N_4$: C, 47.4; H, 8.0; N, 18.4. Found: C, 47.2; H, 8.0; N, 18.7.

Methyl Benzyloxycarbonylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate. (*a*) *By the p-Nitrophenyl Ester Procedure.* *p*-Nitrophenyl benzyloxycarbonylalaninate[14] (1.14 g.) was added to an ethyl acetate solution (20 ml.) containing methyl N$^{\epsilon}$-*t*-butyloxycarbonyllysinate acetate[10] (0.96 g.) and triethylamine (0.41 ml.) and the mixture was kept at room temperature for 2 hr. Ethyl acetate (100 ml.) was then added and the solution was washed with ten 30-ml. portions of 1 *N* ammonium hydroxide, three 30-ml. portions of 2 *N* citric acid, and three 30-ml. portions of saturated sodium chloride, and dried over sodium sulfate. Evaporation of the solvent gave an oil which crystallized on addition of ether–petroleum ether (1:1, v./v.); yield 1.14 g. (82%); m.p. 88–89°. A sample for analysis was recrystallized from ether; m.p. 89–91°; $[\alpha]^{30}D$ −18.2° (*c* 0.88, methanol).

Anal. Calcd. for $C_{23}H_{35}O_7N_3$: C, 59.3; H, 7.6; N, 9.0. Found: C, 59.4; H, 7.6; N, 8.8.

(*b*) *By the Mixed Anhydride Procedure.* A mixed anhydride was prepared in the usual manner from benzyloxycarbonylalanine[20] (0.74 g.) in freezing dioxane (10 ml.) with tri-*n*-butylamine (0.74 ml.) and ethyl chloroformate (0.29 ml.). This preparation was added to a solution of methyl N$^{\epsilon}$-*t*-butyloxycarbonyllysinate acetate[10] (0.96 g.) and triethylamine (0.41 ml.) in dioxane (10 ml.). The mixture was stirred at ice-bath temperature for 30 min. and at room

(21) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).
(22) B. F. Erlanger and E. Brandt, *J. Am. Chem. Soc.*, **73**, 3508 (1951).
(23) R. B. Merrifield, *J. Biol. Chem.*, **232**, 43 (1958).

temperature for 1 hr. when the solvents were evaporated. The residue was dissolved in ethyl acetate (90 ml.), the solution was washed successively with three 30-ml. portions each of saturated sodium bicarbonate, 2 *N* citric acid, and saturated sodium chloride, and was dried over sodium sulfate. The oil which ensued when the solvent was evaporated crystallized on addition of a mixture of ether and petroleum ether (1:1, v./v.). The compound was recrystallized from ether; 1.03 g. (74%); m.p. 90–91°; $[\alpha]^{27}D$ −18.7° (*c* 1.06, methanol).

Anal. Calcd. for $C_{23}H_{35}O_7N_3$: C, 59.3; H, 7.6; N, 9.0. Found: C, 59.4; H, 7.8; N, 9.1.

Methyl Alanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate Acetate. The above benzyloxycarbonyldipeptide ester (2.16 g.) was hydrogenated in the usual manner over palladium in 70% v./v. aqueous methanol (100 ml.) containing glacial acetic acid (1.8 ml.). The catalyst was removed by filtration, the filtrate was evaporated to dryness *in vacuo*, and the residue was washed by decantation with several portions of a 1:1 (v./v.) mixture of ether and petroleum ether and dried over P_2O_5; yield 1.76 g. (97%); R_f^1 0.76; R_f^2 0.86; ninhydrin positive. This material was used immediately for coupling as the corresponding diketopiperazine forms rapidly on standing; diketopiperazine, m.p. 174–176°; R_f^1 0.81; ninhydrin negative.

Anal. Calcd. for $C_{14}H_{25}O_4N_3$: C, 56.2; H, 8.4; N, 14.0. Found: C, 56.0; H, 8.3; N, 13.3.

Methyl Benzyloxycarbonylalanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate. (*a*) *From p-Nitrophenyl Benzyloxycarbonylalaninate and Methyl Alanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate.* *p*-Nitrophenyl benzyloxycarbonylalaninate[14] (3.43 g.) in ethyl acetate (20 ml.) was added to a DMF solution (20 ml.) containing methyl alanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysinate acetate (3.57 g.) and triethylamine (0.62 ml.) and the mixture was kept at 5° for 12 hr. The solvents were removed, the residue was distributed between 1 *N* ammonium hydroxide (50 ml.) and ethyl acetate (150 ml.), and the organic layer was extracted with ten 50-ml. portions of 1 *N* ammonium hydroxide, three 50-ml. portions of 2 *N* citric acid, and four 50-ml. portions of saturated sodium chloride. Evaporation of the sodium sulfate dried organic layer gave a gelatinous mass which was collected and purified by precipitation from ethyl acetate with petroleum ether; yield 3.42 g. (70%); m.p. 125–126°; $[\alpha]^{26}D$ −41.1° (*c* 0.43, methanol); R_f^1 0.91; ninhydrin negative, chlorine positive.

Anal. Calcd. for $C_{26}H_{40}O_8N_4$: C, 58.2; H, 7.5; N, 10.4. Found: C, 58.2; H, 7.6; N, 10.4.

(*b*) *From the Benzyloxycarbonyltripeptide (III) with Diazomethane.* Ethereal diazomethane was added to an ice-cold solution of the benzyloxycarbonyltripeptide (III) (0.50 g.) in methanol (50 ml.) until the yellow color remained. The solution was kept for 5 min. when a few drops of glacial acetic acid was added, then the solvents were removed. The residue was precipitated from ethanol with ether; yield 0.38 g. (74%); m.p. 125–126°; mixture melting point with material prepared according to method a, 125–126°; $[\alpha]^{28}D$ −41.7° (*c* 0.9, methanol); R_f^1 0.91; ninhydrin negative, chlorine positive.

Anal. Calcd. for $C_{26}H_{40}O_8N_4$: C, 58.2; H, 7.5; N, 10.4. Found: C, 58.1; H, 7.6; N, 10.4.

Methyl Alanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate Monoacetate (XVII). The above benzyloxycarbonyltripeptide ester (4.91 g.) was hydrogenated in the usual manner over palladium in methanol (200 ml.) plus 50% acetic acid (0.57 ml.). The catalyst was removed by filtration, the solvents were removed *in vacuo*, and the sirupy residue was lyophilized; yield 3.90 g. (94%); $[\alpha]^{26}D$ −33.1° (*c* 0.59, 10% acetic acid); R_f^1 0.69; ninhydrin positive. On repeated lyophilization from dioxane the free base was obtained in the form of a hydroscopic powder which was dried over P_2O_5.

Anal. Calcd. for $C_{18}H_{34}O_6N_4$: C, 53.7; H, 8.5; N, 13.9. Found: C, 53.4; H, 8.2; N, 13.8.

Benzyloxycarbonylalanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysine (III). Sodium nitrite (6.2 g.) dissolved in ice-water (60 ml.) was added to an ice-cold solution of benzyloxycarbonylalanylalanine hydrazide (27.0 g.) in 0.5 *N* hydrochloric acid (640 ml.). The oily azide (I) was extracted with one 350-ml. and two 150-ml. portions of ice-cold ethyl acetate and the extracts were washed with three 150-ml. portions each of ice-cold saturated sodium bicarbonate and saturated sodium chloride. The combined ethyl acetate extracts were added to an ice-cold solution of N$^{\epsilon}$-*t*-butyloxycarbonyllysine[10] (II, 19.0 g.) and triethylamine (12 ml.) in tetrahydrofuran (210 ml.) and water (320 ml.). The mixture was stirred for 20 hr. at 5°, filtered, and extracted with one 250-ml. and three 150-ml. portions of 1 *N* ammonium hydroxide. The combined aqueous phases were cooled in an ice bath, acidified with solid citric acid, and extracted with one 250-ml. and three 200-ml. portions of ethyl acetate which were washed with three 150-ml. portions of saturated sodium chloride and dried over sodium sulfate. The solvent was removed *in vacuo* and petroleum ether was added to the residue to bring about crystallization. The product was recrystallized from ethyl acetate–petroleum ether; needles; yield 28.5 g. (67%); m.p. 103–104°; $[\alpha]^{26}D$ −33.3° (*c* 1.02, methanol); R_f^1 0.88; R_f^2 0.71; ninhydrin negative.

Anal. Calcd. for $C_{25}H_{38}O_8N_4$: C, 57.5; H, 7.3; N, 10.7; O, 24.5. Found: C, 57.5; H, 7.6; N, 10.4; O, 24.2.

Alanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysine (IV). The benzyloxycarbonyltripeptide (III, 28.5 g.) was hydrogenated over palladium in 70% aqueous methanol (500 ml.) containing glacial acetic acid (5 ml.). The catalyst was removed by filtration, the solvents were removed *in vacuo*, and the residue was washed with ice-cold methanol and dried; yield 16.5 g. (78%); m.p. 206–208° dec.; $[\alpha]^{27}D$ −26.5° (*c* 1.00, water); R_f^1 0.67; single ninhydrin-positive spot.

Anal. Calcd. for $C_{17}H_{32}O_6N_4$: C, 52.6; H, 8.3; N, 14.4. Found: C, 52.3; H, 8.3; N, 13.9.

Methyl Benzyloxycarbonylalanylalanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate. *p*-Nitrophenyl benzyloxycarbonylalaninate[14] (3.39 g.) in ethyl acetate (25 ml.) was added to a DMF solution (30 ml.) containing methyl alanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysinate acetate (XVII, 4.2 g.) and triethylamine (0.61 ml.) and the mixture was kept at room temperature for 12 hr. The solvents were removed *in vacuo*, the residue was dissolved in ethyl acetate (100 ml.), and the solution was washed successively with ten 100-ml. portions of

1 *N* ammonium hydroxide and three 100-ml. portions of 2 *N* citric acid and saturated sodium chloride. The solution was dried over sodium sulfate and concentrated to a small volume, and the resulting gelatinous mass was collected. The material was dissolved in hot ethyl acetate (200 ml.) and the solution was filtered and kept at 5° for 12 hr. when the gelatinous material was collected and dried; yield 5.02 g. (91%); m.p. 177–178°; $[\alpha]^{26}D$ −43.7° (*c* 0.62, methanol).

Anal. Calcd. for $C_{29}H_{43}O_9N_5$: C, 57.3; H, 7.5; N, 11.5; O, 23.7. Found: C, 57.1; H, 7.6; N, 11.5; O, 24.0.

Methyl Alanylalanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate Acetate (XV). The above benzyloxycarbonyltetrapeptide ester (4.9 g.) was hydrogenated over palladium in methanol (100 ml.) containing 50% acetic acid (1 ml.). The catalyst was removed by filtration and the filtrate evaporated to dryness *in vacuo*. The resulting oil was dissolved in a small volume of water and lyophilized to give an amorphous white powder; yield 3.6 g. (84%); m.p. 109–112°; $[\alpha]^{26}D$ −43.7° (*c* 0.49, methanol); R_f^1 0.70; single ninhydrin-positive spot.

Anal. Calcd. for $C_{23}H_{43}O_9N_5$: C, 51.8; H, 8.1; N, 13.1; O, 27.0. Found: C, 51.6; H, 8.2; N, 13.4; O, 27.0.

Methyl t-Butyloxycarbonylalanylalanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate. A solution of methyl alanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysinate (XVII, 1.32 g.) and *p*-nitrophenyl *t*-butyloxycarbonylalaninate (XIX, 0.93 g.) in ethyl acetate (30 ml.) and tetrahydrofuran (20 ml.) was kept at 40° for 17 hr. The solvents were removed *in vacuo*, the residue was dissolved in ethyl acetate (150 ml.), and the solution was washed successively with eight 15-ml. portions of 2 *N* ammonium hydroxide, one 2-ml. portion of saturated sodium chloride, one portion of 1 *N* citric acid, and finally with saturated sodium chloride. The solution was dried over sodium sulfate and the solvent was evaporated; yield 1.39 g. (74%); m.p. 181–183°. The material was recrystallized from 30% aqueous methanol; m.p. 187–188°; $[\alpha]^{28}D$ −50.9 (*c* 1.10, methanol); R_f^I 0.80; R_f^{IX} 0.42.

Anal. Calcd. for $C_{26}H_{47}O_9N_5$: C, 54.4; H, 8.3; N, 12.2. Found: C, 54.0; H, 8.0; N, 12.1.

t-Butyloxycarbonylalanylalanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysine Hydrazide (XXI). Hydrazine hydrate (0.16 ml.) was added to a methanol solution (15 ml.) containing the above protected peptide methyl ester (0.57 g.) and the mixture was kept at room temperature for 24 hr. when the solution was evaporated to dryness and the residue dried *in vacuo* over P_2O_5 and sulfuric acid. The residue was dissolved in methanol (15 ml.), hydrazine hydrate (0.16 ml.) was added, and the solution was kept at room temperature for an additional 24 hr. The hydrazine treatment was repeated a third time. The resulting residue was dissolved in hot ethanol (30 ml.) and the solution was filtered and slowly cooled. The gelatinous product was collected, washed with ice-cold ethanol (5 ml.), and dried over P_2O_5 and sulfuric acid; yield 0.41 g. (71%); m.p. 226–228°; R_f^{III} 0.60; chlorine- and hydrazide-positive, ninhydrin-negative spot.

A sample for analysis was purified by dissolving the material in boiling ethanol and allowing the peptide to precipitate slowly; m.p. 233–234°.

Anal. Calcd. for $C_{25}H_{47}O_8N_7$: C, 52.3; H, 8.3; N, 17.1; O, 22.3. Found: C, 52.4; H, 8.5; N, 17.0; O, 22.5.

Methyl Benzyloxycarbonylthreonylalanylalanyl-alanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate (VII). (a) From Benzyloxycarbonylthreonine Azide (XVI) and Methyl Alanylalanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysinate (XV). Sodium nitrite (0.33 g.) in ice–water (1 ml.) was added to an ice-cold solution of benzyloxycarbonylthreonine hydrazide[15] (1.20 g.) in 0.5 *N* hydrochloric acid (18 ml.). The oily azide (XVI) was extracted into ice-cold ethyl acetate (50 ml.), the extract was washed with two 20-ml. portions each of ice-cold saturated sodium bicarbonate and saturated sodium chloride, and dried over sodium sulfate. The solution containing the azide of benzyloxycarbonylthreonine (XVI) was then added to an ice-cold solution of methyl alanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysinate acetate (XV, 1.61 g.) in DMF (10 ml.) containing triethylamine (0.41 ml.) and the mixture was kept at 5° for 18 hr. when ethyl acetate (300 ml.) was added. The solution was extracted successively with three 150-ml. portions of ice-cold 2 *N* citric acid and eight 100-ml. portions of ice–water and was dried over sodium sulfate. Evaporation of the solvent gave a gelatinous mass which was collected, dissolved in boiling ethanol, and precipitated on slow cooling; yield 1.7 g. (78%); m.p. 222–224° dec.; $[\alpha]^{28}D$ −40.6° (*c* 1.01, methanol); R_f^1 0.81; amino acid ratios in acid hydrolysate $lys_{1.00}thr_{0.97}ala_{3.06}$; single chlorine-positive spot.

Anal. Calcd. for $C_{33}H_{52}O_{11}N_6$: C, 55.9; H, 7.4; N, 11.9; O, 24.8. Found: C, 55.6; H, 7.4; N, 11.4; O, 25.0.

For evaluation of stereochemical homogeneity a sample of the protected peptide methyl ester was deblocked by hydrogenation and the reaction product was digested with LAP; amino acid ratios in digest $thr_{1.00}ala_{3.06}$; N$^{\epsilon}$-*t*-butyloxycarbonyllysine was not determined.[24]

(b) From the Benzyloxycarbonylpentapeptide (VI) with Diazomethane. Ethereal diazomethane was added to an ice-cold solution of the benzyloxycarbonylpentapeptide (VI, 1.56 g.) in methanol (70 ml.) until the yellow color remained. The solution was kept for 5 min., then a few drops of glacial acetic acid was added and the solvents were removed. The residue was dissolved in hot ethanol and precipitated on cooling; yield 1.34 g. (84%); m.p. 221–222° dec.; $[\alpha]^{28}D$ −40.9° (*c* 0.89, methanol); R_f^1 0.91; R_f^2 0.91; single chlorine-positive spot.

Anal. Calcd. for $C_{33}H_{52}O_{11}N_6$: C, 55.9; H, 7.4; N, 11.9. Found: C, 55.7; H, 7.6; N, 11.8.

Benzyloxycarbonylthreonylalanylalanylalanyl-N$^{\epsilon}$-t-butyloxycarbonyllysine (VI). Sodium nitrite (4.5 g.) in water (30 ml.) was added to an ice-cold solution of benzyloxycarbonylthreonylalanine hydrazide (20.0 g.) in 0.5 *N* hydrochloric acid (240 ml.). The azide (V) was extracted with one 150-ml. and three 100-ml. portions of ice-cold ethyl acetate and the extract was washed with three 100-ml. portions of ice-cold saturated sodium bicarbonate. This solution containing the

(24) We have observed that *t*-butyloxycarbonyl amino acids are cleaved on Dowex 50 columns under the conditions used for standard amino acid analysis.

azide (V) was added to an ice-cold solution of alanyl-alanyl-N^{ϵ}-*t*-butyloxycarbonyllysine (IV, 14.2 g.) in water (120 ml.), tetrahydrofuran (100 ml.), and triethylamine (5.8 ml.). The mixture was stirred at 5° for 20 hr., filtered, and extracted with three 150-ml. portions of 1 *N* ammonium hydroxide. The aqueous phases were cooled at 0°, acidified with 10% acetic acid, and extracted with six 100-ml. portions of 1-butanol (equilibrated with 10% acetic acid). The butanol layer was washed with six 50-ml. portions of 10% acetic acid and six 50-ml. portions of water (equilibrated with 1-butanol) and was evaporated. The resulting solid was dissolved in hot ethanol (500 ml.) and the solution was cooled at room temperature to give a gelatinous precipitate which was collected, washed with ice-cold ethanol, and dried to constant weight *in vacuo* over P_2O_5; yield 17.5 g. (69%); m.p. 193–196° dec.; $[\alpha]^{20}D$ −40.2° (*c* 1.39, methanol); R_f^1 0.89; R_f^2 0.60; ninhydrin negative, chlorine positive; amino acid ratios in acid hydrolysate $thr_{0.96}$-$ala_{2.98}lys_{1.02}$.

Anal. Calcd. for $C_{32}H_{50}O_{11}N_6$: C, 55.3; H, 7.3; N, 12.1. Found: C, 55.1; H, 7.5; N, 12.2.

Methyl t-Butyloxycarbonylthreonylalanylalanyl-alanyl-N^{ϵ}-t-butyloxycarbonyllysinate. (a) From t-Butyl Azidoformate and Methyl Threonylalanylalanyl-alanyl-N^{ϵ}-t-butyloxycarbonyllysinate (VIII). *t*-Butyl azidoformate (0.57 g.) was added to a pyridine solution (12 ml.) containing the acetate salt of VIII (1.27 g.) and the solution was kept at room temperature for 22 hr. The solvent was removed, the residue was distributed between ethyl acetate and water, and the organic phase was washed with 1 *N* citric acid, saturated sodium bicarbonate, and saturated sodium chloride solutions. The sodium sulfate dried solution was evaporated and the residue was dried over P_2O_5 and potassium hydroxide pellets; yield 0.96 g. (71%). For purification the compound was dissolved in acetone and precipitated with petroleum ether; m.p. 173–174° dec.; $[\alpha]^{26}D$ −46.9° (*c* 1.49, methanol); R_f^I 0.82; R_f^{II} 0.79.

Anal. Calcd. for $C_{30}H_{54}O_{11}N_6$: C, 53.4; H, 8.1; N, 12.5. Found: C, 53.6; H, 8.2; N, 12.1.

(b) From t-Butyloxycarbonylthreonylalanine Azide (XVIII) and Methyl Alanylalanyl-N^{ϵ}-t-butyloxycarbonyllysinate (XVII). *t*-Butyloxycarbonylthreonylalanine hydrazide (0.91 g.) was dissolved in 4 ml. of 1.88 *N* hydrogen chloride in tetrahydrofuran and the solution was cooled at −40°. *t*-Butyl nitrite (0.38 ml.) was added with stirring and the solution was allowed to warm to −20° and diluted with ethyl acetate (12 ml.) pre-cooled at −20°. A solution saturated at room temperature with both potassium chloride and potassium bicarbonate was cooled at −20° and the supernatant was used to extract the above solution. The organic layer was dried over sodium sulfate and added to a pre-cooled solution of methyl alanylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysinate acetate (XVII, 1.30 g.). A heavy gel formed and the solution was diluted with tetrahydrofuran (45 ml.) and stirred for 24 hr. at 4° and 48 hr. at room temperature. The solvents were evaporated, the residue was dissolved in 1-butanol, and the extract was washed with 10% acetic acid (equilibrated with 1-butanol) and then with water. The solvent was removed *in vacuo*, the residue was dissolved in acetone, and the product was precipitated by addition of petroleum ether; yield 1.50 g. (69%); m.p. 172–174° dec.; $[\alpha]^{26}D$ −48.0° (*c* 1.48, methanol); R_f^I 0.82; R_f^{II} 0.80.

t-Butyloxycarbonylthreonylalanylalanylalanyl-N^{ϵ}-t-butyloxycarbonyllysine Hydrazide (XX). Hydrazine hydrate (0.48 ml.) was added to a methanol solution (10 ml.) containing methyl *t*-butyloxycarbonylthreonyl-alanylalanylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysinate (0.37 g.) (prepared according to method a), and the mixture was kept at room temperature for 20 hr. when a heavy gelatinous precipitate formed. The material was collected, dried over concentrated sulfuric acid and potassium hydroxide pellets, and recrystallized from DMF–water (1:4, v./v.); yield 0.28 g. (75%); m.p. 230–231° dec.; $[\alpha]^{28}D$ −47.9° (*c* 0.64, acetic acid); R_f^I 0.55. Hydrazide obtained from ester, prepared according to method b, had m.p. 226–228°; $[\alpha]^{28}D$ −47.1° (*c* 0.62, acetic acid); R_f^I 0.56; chlorine and hydrazine positive.

Anal. Calcd. for $C_{29}H_{54}O_{10}N_8$: C, 51.6; H, 8.1; N, 16.6. Found: C, 51.2; H, 8.0; N, 16.2.

Methyl Threonylalanylalanylalanyl-N^{ϵ}-t-butyloxycarbonyllysinate Acetate (VIII). The benzyloxycarbonyl derivative (VII, 2.37 g.) was hydrogenated over palladium in methanol (70 ml.) containing 50% (v./v.) aqueous acetic acid (1.0 ml.). The catalyst was removed by filtration, the solvent was removed *in vacuo*, and ether was added to the residue. The amorphous solid was collected, washed with ether, and dried; yield 2.00 g. (90%); $[\alpha]^{26}D$ −78.6° (*c* 1.5, 10% acetic acid); R_f^1 0.66; single ninhydrin-positive spot; amino acid ratios in LAP digest $thr_{0.97}$-$ala_{3.03}$ (N^{ϵ}-*t*-butyloxycarbonyllysine not determined).[24]

Anal. Calcd. for $C_{27}H_{49}O_{11}N_6$: C, 51.2; H, 7.8; N, 13.3. Found: C, 51.0; H, 8.1; N, 13.4.

Methyl Benzyloxycarbonyl-γ-t-butylglutamylthreonylalanylalanylalanyl-N^{ϵ}-t-butyloxycarbonyllysinate. *p*-Nitrophenyl benzyloxycarbonyl-γ-*t*-butylglutamate (IX, 8.3 g.) was added to a DMF solution (60 ml.) containing methyl threonylalanylalanylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysinate acetate (VIII, 10.0 g.) and triethylamine (1.7 ml.) and the mixture was kept at room temperature for 15 hr. Addition of 1 *N* ammonium hydroxide (300 ml.) to the ice-cold solution afforded a precipitate which was collected, resuspended in 150 ml. of 1 *N* ammonium hydroxide, and filtered. The material was washed four additional times by suspension in 1 *N* ammonium hydroxide and was then washed on a filter with 150 ml. of water, 150 ml. of 1 *N* citric acid, and finally again with 150 ml. of water. The dried compound was dissolved in hot ethanol (50 ml.) and the solution was left to cool slowly. The resulting gelatinous mass was collected and dried; yield 10.5 g. (74%); m.p. 204–205° dec.; $[\alpha]^{27}D$ −11.7° (*c* 0.54, DMF); R_f^1 0.92; R_f^2 0.85; ninhydrin negative, chlorine positive; amino acid ratios in acid hydrolysate $glu_{0.99}thr_{0.96}ala_{3.08}lys_{1.04}$.

Anal. Calcd. for $C_{42}H_{67}O_{14}N_7$: C, 56.4; H, 7.6; N, 11.0. Found: C, 56.9; H, 7.9; N, 11.1.

Methyl γ-t-Butylglutamylthreonylalanylalanyl-alanyl-N^{ϵ}-t-butyloxycarbonyllysinate Acetate (X). The above benzyloxycarbonylhexapeptide ester (7.63 g.) was hydrogenated in the usual manner over pal-

ladium in methanol (200 ml.) containing glacial acetic acid (1.0 ml.). The catalyst was removed by filtration, the filtrate was evaporated, and the amorphous residue was washed with ether and dried; yield 6.0 g. (86%); m.p. 235–236° dec.; $[\alpha]^{27}D$ −66.7° (*c* 1.46, 10% acetic acid); R_f^1 0.82; R_f^2 0.88; sharp single ninhydrin-positive spot; amino acid ratios in LAP digest $thr_{0.96}ala_{3.07}$γ-*t*-butylglu$_{0.96}$ (95%) (N$^\epsilon$-*t*-butyloxycarbonyllysine not determined).[25]

Anal. Calcd. for $C_{36}H_{65}O_{14}N_7$: C, 52.7; H, 8.0; N, 12.0. Found: C, 52.7; H, 7.8; N, 12.2.

Methyl N$^\alpha$,N$^\epsilon$-Di-t-butyloxycarbonyllysyl-γ-t-butylglutamylthreonylalanylalanylalanyl-N$^\epsilon$-t-butyloxycarbonyllysinate (XII). *p*-Nitrophenyl N$^\alpha$,N$^\epsilon$-di-*t*-butyloxycarbonyllysinate (XI, 2.9 g.) was added at room temperature to a DMF solution (100 ml.) containing methyl γ-*t*-butylglutamylthreonylalanylalanylalanyl-N$^\epsilon$-*t*-butyloxycarbonyllysinate acetate (X, 6.0 g.) and triethylamine (1.0 ml.) and the mixture was kept at room temperature for 15 hr. Addition of 1 *N* ammonium hydroxide (300 ml.) to the ice-cold solution afforded a precipitate which was collected, resuspended in 1 *N* ammonium hydroxide (150 ml.), and filtered. The material was washed four additional times by suspension in 1 *N* ammonium hydroxide, was then washed on the filter with water (100 ml.), 1 *N* citric acid (200 ml.), and water (200 ml.), and was dried. The compound was dissolved in tetrahydrofuran (80 ml.) and precipitated by addition of water (100 ml.); yield 6.6 g. (83%); m.p. 203–209° dec.; $[\alpha]^{26}D$ −15.6° (*c* 0.55, DMF); R_f^1 0.88; R_f^2 0.88; ninhydrin negative, chlorine positive; amino acid ratios in acid hydrolysate $lys_{2.08}glu_{1.00}thr_{0.95}ala_{3.05}$.

Anal. Calcd. for $C_{50}H_{89}O_{17}N_9$: C, 55.2; H, 8.2; N, 11.6. Found: C, 55.2; H, 8.4; N, 11.6.

N$^\alpha$,N$^\epsilon$-Di-t-butyloxycarbonyllysyl-γ-t-butylglutamylthreonylalanylalanylalanyl-N$^\epsilon$-t-butyloxycarbonyllysine Hydrazide (XIV). Hydrazine hydrate (1.0 ml.) was added to a methanol solution (100 ml.) containing the protected heptapeptide methyl ester (XII, 1.50 g.) and the mixture was kept at room temperature for 20 hr. and at 5° for an additional 50 hr. The gelatinous precipitate which had formed was collected, washed with ice-cold methanol, and dried over concentrated sulfuric acid and potassium hydroxide pellets; yield 1.20 g. (80%); m.p. 229–231° dec. A sample for analysis was dissolved in methanol and precipitated by addition of water; amino acid ratios in acid hydrolysate $lys_{1.95}glu_{0.99}thr_{0.98}ala_{3.07}$.

(25) The γ-*t*-butyl ester of glutamic acid was determined by standard amino acid analysis; it evolves from the long column at 350 ml. at the position of methionine; small amounts of free glutamic acid are formed.

Anal. Calcd. for $C_{49}H_{89}O_{16}N_{11}$: C, 54.1; H, 8.2; N, 14.2. Found: C, 53.9; H, 8.5; N, 14.0.

N$^\alpha$,N$^\epsilon$-Di-t-butyloxycarbonyllysyl-γ-t-butylglutamylthreonylalanylalanylalanyl-N$^\epsilon$-t-butyloxycarbonyllysine. The protected heptapeptide ester (XII, 330 mg.) was dissolved in dioxane (25 ml.), 1 *N* sodium hydroxide (1.2 ml.) was added, and the solution was kept at room temperature for 2 hr. The bulk of the dioxane was removed *in vacuo*, 1 *N* ammonium hydroxide (30 ml.) was added to the residue, and insoluble material was removed by filtration. The filtrate was cooled at 0° and acidified by addition of ice-cold 2 *N* citric acid, and the precipitate was collected, washed with water (50 ml.), and dried. The material was dissolved in tetrahydrofuran (25 ml.), the solution was filtered, and the compound was precipitated from the concentrated tetrahydrofuran solution by addition of water (30 ml.). The amorphous solid was collected and dried over P_2O_5; yield 171 mg. (52%); m.p. 190–198° dec.; $[\alpha]^{26}D$ −14.6° (*c* 0.30, DMF); R_f^1 0.90; R_f^2 0.80; ninhydrin negative, chlorine positive; amino acid ratios in acid hydrolysate $lys_{2.04}glu_{1.00}thr_{0.92}ala_{3.00}$.

Anal. Calcd. for $C_{49}H_{87}O_{17}N_9$: C, 54.8; H, 8.2; N, 11.7. Found: C, 54.5; H, 8.2; N, 11.4.

Lysylglutamylthreonylalanylalanylalanyllysine Diacetate Tetrahydrate (XIII). The above protected heptapeptide (80 mg.) was dissolved in anhydrous trifluoroacetic acid (1.0 ml.) and the solution was kept at room temperature for 45 min. when water (15 ml.) was added. The solution was extracted with a mixture of benzene and ether (1:1, v./v.) containing a few drops of 1 *N* hydrochloric acid, and Amberlite IRA-400 (acetate cycle) was added to the aqueous phase until the supernatant was free of chloride ions. The resin was removed and washed with water (15 ml.), the combined filtrate and washings were concentrated to a small volume *in vacuo*, and the residue was lyophilized; yield 56 mg. (82%); $[\alpha]^{28}D$ −60.8° (*c* 0.17, 10% acetic acid); R_f^3 0.45 × His; single ninhydrin-positive component on paper electrophoresis at pH 1.9, 3.9, and 6.5; amino acid ratios in acid hydrolysate $lys_{2.03}glu_{0.97}thr_{0.93}ala_{3.07}$; amino acid ratios in LAP digest $lys_{2.07}glu_{0.97}thr_{0.93}ala_{3.00}$ (100%).

Anal. Calcd. for $C_{30}H_{56}O_{11}N_9 \cdot 2CH_3COOH \cdot 4H_2O$: C, 44.8; H, 8.0; N, 13.8; O, 33.4. Found: C, 45.3; H, 8.4; N, 13.7; O, 33.8.

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