(100 ml.) and the eluate was evaporated to a small volume *in vacuo* and lyophilized; yield 560 mg. The crude hexapeptide dissolved in water (200 ml.) was applied to a DEAE-cellulose column (15 × 20 cm.) which was eluted with the following solvents: water (200 ml.) and pH 6.9 ammonium acetate buffer 0.001 *M* (300 ml.). Individual fractions of 10 ml. each were collected at a flow rate of 2.5 ml./min. The desired peptide was located in the 0.001 *M* ammonium acetate eluates from which it was isolated in the usual manner. Rechromatography on DEAE-cellulose gave a homogeneous material; yield 380 mg. (18%); $[\alpha]^{27}D$ −13.3° (*c* 0.29, 10% acetic acid); R_f[3] 1.6 × His; ninhydrin-, Pauly-, Sakaguchi-, and methionine-positive spot slightly contaminated with the sulfoxide; R_f[3] 1.1 × His; amino acid ratios in acid hydrolysate $phe_{1.08}glu_{2.02}arg_{1.02}his_{1.00}met_{0.85}$; amino acid ratios in LAP digest $phe_{1.18}glu_{1.43}arg_{1.00}his_{0.93}met_{0.93}$.

Anal. Calcd. for $C_{36}H_{53}O_{11}N_{11}S \cdot CH_3COOH \cdot 2H_2O$: C, 48.3; H, 6.6; N, 16.3; O, 25.4. Found: C, 48.4; H, 7.1; N, 16.1; O, 25.5.

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Studies on Polypeptides. XXXI. Synthetic Peptides Related to the N-Terminus of Bovine Pancreatic Ribonuclease (Positions 12–20)[1,4]

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Syntheses are described of peptides related to positions 12–20 in the amino acid sequence of bovine pancreatic ribonuclease A. In particular, methods are given for preparation of the d-sulfoxides of histidylmethionylaspartylserine and histidylmethionylaspartylserylserylthreonylserylalanylalanine. Evidence is presented for homogeneity of these compounds, one of which served as an intermediate in a synthesis of S-peptide.

In previous communications[3,5] we described syntheses of the protected peptide hydrazides II and III and related compounds whose amino acid sequences correspond to positions 1–7 and 8–11, respectively, of the primary structure of bovine pancreatic ribonuclease A and hence of S-peptide[6] (I). The present study relates synthetic routes to the *d*-sulfoxides of the tetrapeptide histidylmethionylaspartylserine (IV) and the nonapeptide histidylmethionylaspartylserylserylthreonylserylalanylalanine (V).

Potts, *et al.*,[7] digested S-peptide[6] with carboxypeptidase A and observed liberation of 2 moles each of alanine and serine and 1 mole of threonine per mole of peptide. The crude digestion mixture was assayed for its ability to reconstitute active ribonuclease in combination with S-protein. Full activity was restored when an aliquot of the carboxypeptidase digest, corresponding to 1 mole of S-peptide, was added per mole of S-protein. From this result the authors concluded that a peptide corresponding to the N-terminal pentadecapeptide of S-peptide (positions 1–15) was fully as active as S-peptide in restoring enzymic activity with S-protein. Since the pentadecapeptide was neither isolated nor purified it was of importance to produce pure synthetic samples of this compound to verify these conclusions. Histidylmethionylaspartylserine *d*-sulfoxide (IV), a necessary intermediate for a synthesis of this pentadecapeptide, was prepared for this reason.

The nonapeptide *d*-sulfoxide (V) which corresponds to positions 12–20 of the amino acid sequence of pancreatic ribonuclease A provided one of three subunits essential for construction of the entire S-peptide molecule. The previously described compounds (II) and (III) served as the other subunits in this scheme.

In addition to our own studies along these lines Marchiori, *et al.*,[8] have recently reported the preparation of ethyl benzyloxycarbonylserylthreonylserylalanylalaninate, which they obtained by a stepwise process starting with ethyl alanylalaninate.

The section of the S-peptide molecule which is the subject of this study contains the sequence aspartylserine which has attracted considerable attention in view of its occurrence in a number of esteratic and proteolytic enzymes.[9] Peptide derivatives containing

(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 K. Hofmann, W. Haas, M. J. Smithers, R. D. Wells, Y. Wolman, N. Yanaihara, and G. Zanetti, *J. Am. Chem. Soc.*, **87**, 620 (1965), for paper XXX in this series.

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

(5) K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaihara, *ibid.*, **87**, 611 (1965).

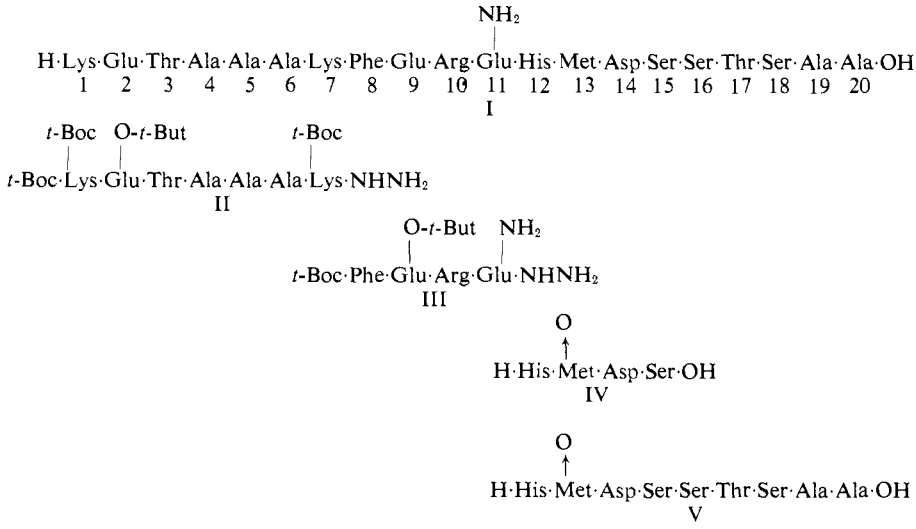
(6) F. M. Richards, *Proc. Natl. Acad. Sci. U. S.*, **44**, 162 (1958).

(7) J. T. Potts, Jr., M. Young, and C. B. Anfinsen, *J. Biol Chem.*, **238**, PC 2593 (1963).

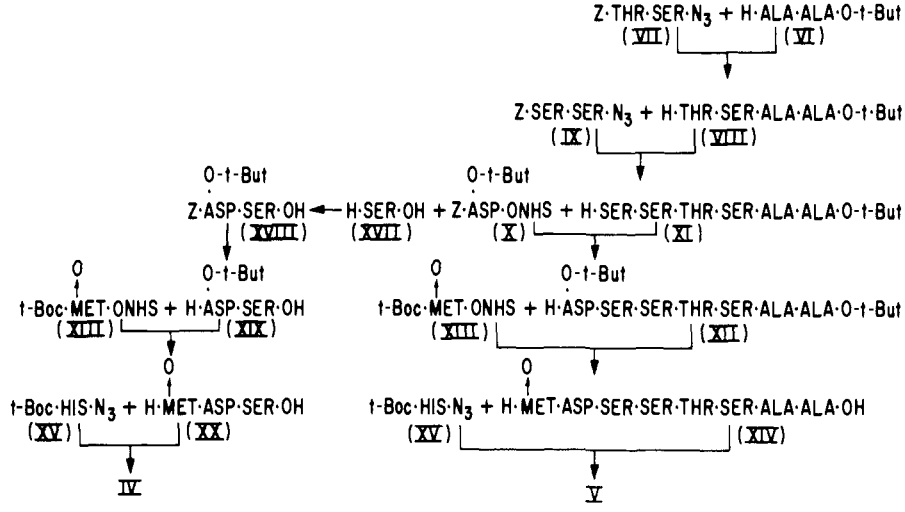
(8) F. Marchiori, R. Rocchi, and E. Scoffone, *Gazz. chim. ital.*, **93**, 834 (1963).

(9) J. A. Cohen, R. A. Oosterbaan, H. S. Jansz, and F. Berends, *J. Cell. Comp. Physiol.*, **54** (Suppl. 1), 231 (1959).

Scheme I



Scheme II



this structural element have been prepared[10] and were shown to exhibit unusual properties. The β-ester group in such compounds as benzyloxycarbonyl-β-benzylaspartylserine amide undergoes hydrolysis under alkaline conditions at unusually high rates and succinimide derivatives are involved intimately as intermediates in the process. Opening of the ring of the cyclic intermediates can occur in two ways resulting in formation of mixtures of α- and β-aspartyl peptides. Thus, in schemes directed toward synthesis of homogeneous aspartyl peptides involving β-ester intermediates the use of alkali must be avoided. The routes which we devised for the synthesis of peptides IV and V (Scheme II) take cognizance of these considerations.

For the synthesis of *t*-butyl serylserylthreonylserylalanylalaninate (XI) the *t*-butyl ester of alanylalanine (VI) was coupled with the azide of benzyloxycarbonylthreonylserine (VII) to give *t*-butyl benzyloxycarbonylthreonylserylalanylalaninate which was decarbobenzoxylated by hydrogenolysis (see Scheme II). The ensuing tetrapeptide ester VIII was then allowed to react with the azide of benzyloxycarbonylserylserine (IX) and the resulting protected hexapeptide ester was subjected to hydrogenolysis. The crystalline α-succiniimido benzyloxycarbonyl-β-*t*-butylaspartate (X) served to introduce the aspartic acid residue. For the preparation of X benzyloxycarbonylaspartic acid anhydride[11] was treated with benzyl alcohol and the resulting mixture of α- and β-esters separated by crystallization of the dicyclohexylammonium salt.[12] The α-ester, liberated from the recrystallized salt, was esterified with isobutylene and the ensuing α-benzyl-β-*t*-butyl ester partially saponified.[13] The resulting oily β-*t*-butyl benzyloxycarbonylaspartate was converted to crystalline X in the usual manner.[14] This "activated" ester was coupled with *t*-butyl serylserylthreonylserylalanylalaninate (XI) to give benzyloxycarbonyl-β-*t*-butylaspartylserylserylthreonylserylalanylalaninate which was decarbobenzoxylated by hydrogenolysis.

In a previous paper[3] we drew attention to the ease of oxidation of the methionine sulfur in peptides corresponding to sections of S-peptide. All the synthetic peptides containing methionine, even following reduction with thioglycolic acid, were more or less contaminated by the respective sulfoxides. In order

(10) S. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, M. Sela, and Y. Shalitin, *J. Am. Chem. Soc.*, **84**, 2421 (1962).

(11) G. L. Miller, O. K. Behrens, and V. du Vigneaud, *J. Biol. Chem.*, **140**, 411 (1941).

(12) E. Wünsch and A. Zwick, *Z. Physiol. Chem.*, **328**, 235 (1962).

(13) R. Schwyzer and H. Dietrich, *Helv. Chim. Acta*, **44**, 2003 (1961).

(14) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

to avoid this difficulty we elected to prepare the *d*-sulfoxides of the methionine peptides IV and V as intermediates for synthesis of S-peptide and the above mentioned pentadecapeptide. The advantages of sulfoxide protection of the methionine sulfur have been discussed by Iselin.[15]

Methionine *d*-sulfoxide was prepared essentially according to Lavine[16] and was converted into the *t*-butyloxycarbonyl derivative. The N-hydroxysuccinimide ester of *t*-butyloxycarbonylmethionine *d*-sulfoxide (XIII) was obtained in the usual manner.[14] The *p*-nitrophenyl ester was also prepared but in our experience proved inferior to the N-hydroxysuccinimide ester as an acylating reagent for XII and XIX.

The partially protected heptapeptide ester (XII) was allowed to react with XIII in aqueous dioxane to give the crystalline sulfoxide of *t*-butyl *t*-butyloxycarbonylmethionyl-β-*t*-butylaspartylserylserylthreonylserylalanylalaninate. This compound was deblocked by exposure to trifluoroacetic acid and the ensuing peptide XIV on reaction with *t*-butyloxycarbonylhistidine azide (XV) afforded the sulfoxide of *t*-butyloxycarbonylhistidylmethionylaspartylserylserylthreonylserylalanylalanine.

Methyl N^{α}-*t*-butyloxycarbonylhistidinate which served as the starting material for preparation of N^{α}-*t*-butyloxycarbonylhistidine hydrazide and the azide XV was prepared according to Schröder and Gibian[17] who obtained the compound in the form of an oil. We have obtained this ester as a sharp melting crystalline compound.

Short exposure to trifluoroacetic acid[18,19] transformed the protected nonapeptide into V.

This same scheme was used to prepare the *d*-sulfoxide of histidylmethionylaspartylserine (IV). To this end sodium serinate (XVII) was coupled with the activated ester X to give benzyloxycarbonyl-β-*t*-butylaspartylserine (XVIII) which was decarbobenzoxylated by hydrogenolysis. The ensuing partially protected dipeptide XIX was then acylated successively with the N-hydroxysuccinimide ester of *t*-butyloxycarbonylmethionine *d*-sulfoxide (XIII) followed by *t*-butyloxycarbonylhistidine azide (XV). Intermediates were deblocked by trifluoroacetic acid. The tetrapeptide IV was obtained in crystalline form (needles) from aqueous acetic acid–methanol.

Peptides IV and V were homogeneous as judged by paper chromatography and their acid hydrolysates gave the theoretically expected amino acid ratios. They were completely digestible by leucine aminopeptidase. The acid hydrolysates of the peptides containing methionine *d*-sulfoxide contained methionine plus traces of homocysteic acid, and not methionine sulfoxide. Elemental analyses verified the expected composition. Peptides XIV and V were homogeneous on paper electrophoresis at various pH values.

Experimental[20]

α-Benzyl Benzyloxycarbonylaspartate. Benzyloxycarbonylaspartic acid anhydride[11] (86.7 g.) was converted to α-benzyl benzyloxycarbonylaspartate according to the method of Bergmann, *et al.*,[21] yielding 74.9 g. of crude product. A solution of dicyclohexylamine (38.0 g.) in ether (60 ml.) was added within 10 min. to a solution of the crude α-benzyl benzyloxycarbonylaspartate (74.9 g.) in ether (200 ml.). The mixture was kept at room temperature for 1 hr. when the ether was removed *in vacuo*. The dicyclohexylammonium salt was recrystallized twice from ethyl acetate; yield 65.6 g. (35%); m.p. 118–119°; $[\alpha]^{30}D$ +2.5° (*c* 1.55, methanol).

Anal. Calcd. for $C_{31}H_{42}O_6N_2$: C, 69.1; H, 7.9; N, 5.2; O, 17.8. Found: C, 69.3; H, 7.9; N, 4.9; O, 18.0.

The dicyclohexylammonium salt (65.6 g.) was suspended in ice-cold 2 *N* sulfuric acid (200 ml.) and the mixture was stirred for 90 min. The α-benzyl benzyloxycarbonylaspartate which precipitated was dissolved in ether (200 ml.) and the sulfuric acid was extracted with three 100-ml. portions of ether. The combined ether fractions were washed with water (three 50-ml. portions) and dried over sodium sulfate. After removal of the ether *in vacuo* the product was recrystallized twice from ether–petroleum ether (b.p. 30–60°); yield 43.0 g. (98%); m.p. 84°; $[\alpha]^{29}D$ −9.3° (*c* 1.2, acetic acid); lit.[11] m.p. 85°; lit[21] m.p. 84–85°; lit.[22] m.p. 84–85°; $[\alpha]^{18}D$ −9.7° (acetic acid).

α-Succinimido β-t-Butyl Benzyloxycarbonylaspartate (X). N,N′-Dicyclohexylcarbodiimide[23] (17.3 g.) was added to an ice-cold solution of N-hydroxysuccinimide[14] (9.7 g.) and crude β-*t*-butyl benzyloxycarbonylaspartate[13] (prepared from 30 g. of α-benzyl benzyloxycarbonylaspartate) in dry dioxane (300 ml.). The mixture was stirred for 6 hr. at 0° and kept in the refrigerator overnight. The N,N′-dicyclohexylurea was removed by filtration and washed with dioxane, and the combined filtrate and washings were evaporated *in vacuo*. The residue was recrystallized twice from ethyl acetate; yield 27.9 g. (78%); m.p. 151–152°; $[\alpha]^{29}D$ −27.4° (*c* 1.1, DMF).

Anal. Calcd. for $C_{20}H_{24}O_8N_2$: C, 57.1; H, 5.8; N, 6.7; O, 30.4. Found: C, 57.0; H, 5.9; N, 7.0; O, 30.3.

Methyl N^{α}-t-Butyloxycarbonylhistidinate. Methyl histidinate dihydrochloride (19.4 g.) was converted into the *t*-butyloxycarbonyl derivative according to the procedure of Schröder and Gibian.[17] The resulting oily material crystallized when kept under petroleum ether in a refrigerator for several days. The substance was recrystallized from a mixture of benzene and ether; yield 16.6 g. (82%); m.p. 127–130°; $[\alpha]^{28}D$ −14.0° (*c* 2.02, methanol).

Anal. Calcd. for $C_{12}H_{19}O_4N_3$: C, 53.5; H, 7.1; N, 15.6. Found: C, 53.4; H, 7.1; N, 15.5.

(15) B. Iselin, *Helv. Chim. Acta*, **44**, 61 (1961).
(16) T. F. Lavine, *J. Biol. Chem.*, **169**, 477 (1947).
(17) E. Schröder and H. Gibian, *Ann.*, **656**, 190 (1962).
(18) L. A. Carpino, *J. Am. Chem. Soc.*, **79**, 98 (1957).
(19) R. Schwyzer, W. Rittel, H. Kappeler, and B. Iselin, *Angew. Chem.*, **72**, 915 (1960).
(20) General experimental and analytical procedures used were those described in paper XXIX of this series.[5] The Lowry reaction was performed as described by O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951). The following abbreviations are used: DMF = dimethylformamide; THF = tetrahydrofuran; O-*t*-But = *t*-butyl ester; *t*-Boc = *t*-butyloxycarbonyl; Z = benzyloxycarbonyl; met$^{\circ}$ = methionine *d*-sulfoxide; ONHS = N-hydroxysuccinimide ester; LAP = leucine aminopeptidase.
(21) M. Bergmann, L. Zervas, and L. Salzmann, *Ber.*, **66**, 1288 (1933).
(22) P. M. Bryant, R. H. Moore, P. J. Pimlott, and G. T. Young, *J. Chem. Soc.*, 3868 (1959).
(23) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

t-Butyloxycarbonylmethionine. Methionine (6.0 g.) was treated with *t*-butyl azidoformate[24] essentially as described.[25] After 20 hr. ice–water (250 ml.) was added to the reaction mixture and the magnesium oxide was removed by filtration. Additional ice–water (150 ml.) was added to the filtrate followed by ice-cold 2 *M* citric acid (100 ml.). The solution was saturated with sodium chloride and extracted with ethyl acetate (four 200-ml. portions), and the combined ethyl acetate layers were washed with two 20-ml. portions of 1 *N* sodium bicarbonate and two 20-ml. portions of water. The combined aqueous extracts were cooled in an ice bath, acidified with 2 *M* citric acid, (approximately 50 ml. needed), and extracted with ethyl acetate (four 300-ml. portions). The organic extracts were washed with water (20 ml.) and saturated sodium chloride (seven 20-ml. portions). The solution was dried over sodium sulfate, the solvent was removed, and the oil was dried *in vacuo*. When kept in a refrigerator for several weeks the material crystallized; yield 2.5 g. (25%). A sample recrystallized from Skellysolve B (b.p. 60–68°) melted at 47–49°; $[\alpha]^{29}D$ −20.0° (*c* 1.3, methanol); lit.[25] oil, $[\alpha]^{32}D$ −21.6 ±1.0° (*c* 0.92, methanol).

Anal. Calcd. for $C_{10}H_{19}O_4NS$: C, 48.2; H, 7.7; N, 5.6. Found: C, 48.2; H, 7.6; N, 5.8.

t-Butyloxycarbonylmethionine d-Sulfoxide.[26] A mixture of methionine *d*-sulfoxide[15] (16.5 g.), water (100 ml.), dioxane (100 ml.), triethylamine (41.7 ml.), and *t*-butyl azidoformate[24] (28.6 g.) was stirred for 48 hr. at 45–50°, then water (100 ml.) was added, and the dioxane was removed *in vacuo*. The basic solution (pH 8) was extracted with three 60-ml. portions of ethyl acetate and the organic extracts were washed with three 30-ml. portions of 0.01 *N* ammonium hydroxide. The organic phase was discarded, the combined aqueous phases were concentrated *in vacuo* to approximately 100 ml., and the concentrate was cooled in an ice bath and slowly acidified with glacial acetic acid. The solution was extracted with six 100-ml. portions of 1-butanol (equilibrated with 2% acetic acid) and the butanol extracts were washed with seven 50-ml. portions of 2% acetic acid (equilibrated with 1-butanol). The combined butanol phases were evaporated *in vacuo* to give an oil which crystallized on drying. Recrystallization from ethyl acetate gave 7.80 g.; m.p. 134–136°. The aqueous phases were combined and concentrated to a small volume *in vacuo* and re-extracted with 1-butanol as above to give additional material; 4.66 g.; m.p. 136–138°. The two fractions were combined and recrystallized from boiling ethyl acetate; yield 11.54 g. (43%); m.p. 142–145°; $[\alpha]^{28}D$ + 41.6° (*c* 2.01, DMF): R_f^1 0.87; R_f^2 0.59; single ninhydrin-negative, chlorine- and methionine-positive spot.

Anal. Calcd. for $C_{10}H_{19}O_5NS$: C, 45.3; H, 7.2; N, 5.3. Found: C, 45.3; H, 7.4; N, 5.3.

t-Butyloxycarbonylmethionine *dl*-sulfoxide[27] was prepared in the manner described above; yield 20%; m.p. 123–125.5°; $[\alpha]^{28}D$ −9.8° (*c* 2.00, DMF); $[\alpha]^{27}D$ +4.3° (*c* 2.00, 95% acetic acid); indistinguishable from the above compound on paper chromatography in solvent systems 1 and 2.

Anal. Calcd. for $C_{10}H_{19}O_5NS$: C, 45.3; H, 7.2; N, 5.3. Found: C, 45.5; H, 7.3; N, 5.4.

p-Nitrophenyl t-Butyloxycarbonylmethioninate. N,N′-Dicyclohexylcarbodiimide[23] (2.16 g.) in ether (2 ml.) was added with stirring to an ice-cold ether solution (15 ml.) containing crystalline *t*-butyloxycarbonylmethionine (2.5 g.) and *p*-nitrophenol (1.55 g.). The mixture was stirred for 15 min., then placed in a refrigerator for 12 hr. Ether (10 ml.) was added, the suspension was filtered, and the filter cake was washed with ethyl acetate. The filter cake was then extracted with a hot mixture of ethyl acetate (25 ml.) and methanol (20 ml.) and the extract was cooled in a refrigerator and filtered. This second filtrate was combined with the first filtrate and washings, and the solution was evaporated *in vacuo*. The residue was recrystallized from methanol; yield 1.69 g. (45%); m.p. 96.5–97.5°; $[\alpha]^{27}D$ −50.6° (*c* 1.2, methanol).

Anal. Calcd. for $C_{16}H_{22}O_6N_2S$: C, 51.9; H, 6.0; N, 7.6. Found: C, 52.0; H, 6.0; N, 7.3.

p-Nitrophenyl t-Butyloxycarbonylmethioninate d-Sulfoxide. N,N′-Dicyclohexylcarbodiimide[23] (6.13 g.) in ice-cold ethyl acetate (20 ml.) was added to an ice-cold DMF solution (50 ml.) containing *t*-butyloxycarbonylmethionine *d*-sulfoxide (7.95 g.) and *p*-nitrophenol (4.16 g.). The mixture was stirred 1 hr. at 0° and overnight at room temperature when a few drops of glacial acetic acid was added. After 1 hr. the suspension was filtered and the residue was washed with ethyl acetate (20 ml.). The combined filtrate and washings were concentrated *in vacuo* to give an oil which was dissolved in ethyl acetate (100 ml.), the extract was washed with three 40-ml. portions of water, and the aqueous phases were extracted with two 70-ml. portions of ethyl acetate. The combined organic phases were dried and concentrated *in vacuo* to give an oil which was dissolved in ethyl acetate (5 ml.) and enough ether was added to bring about crystallization. After 12 hr. at 4° the material was collected and a further recrystallization from ethyl acetate–ether gave 8.0 g. (69%): m.p. 119–121°; $[\alpha]^{28}D$ +4.1° (*c* 2.02, methanol); $[\alpha]^{29}D$ +11.5° (*c* 2.04, 95% acetic acid); R_f^1 0.91: single ninhydrin-negative, chlorine-, and methionine-positive spot.

Anal. Calcd. for $C_{16}H_{22}O_7N_2S$: C, 49.7; H, 5.7; N, 7.2. Found: C, 49.9; H, 5.8; N, 7.1.

p-Nitrophenyl *t*-butyloxycarbonylmethioninate *dl*-sulfoxide was prepared in the manner described above; yield 37%; m.p. 115–117°; $[\alpha]^{28}D$ −1.2° (*c* 2.03, methanol); $[\alpha]^{28}D$ +7.0° (*c* 2.17, 95% acetic acid); indistinguishable from the above compound on paper chromatography in solvent system 1.

Anal. Calcd. for $C_{16}H_{22}O_7N_2S$: C, 49.7; H, 5.7; N, 7.2. Found: C, 49.5; H, 5.8; N, 7.0.

Succinimido t-Butyloxycarbonylmethioninate d-Sulfoxide (XIII). N,N′-Dicyclohexylcarbodiimide[23] (10.3 g.) was added to an ice-cold solution of N-hydroxysuccinimide[14] (5.75 g.) and *t*-butyloxycarbonylmethionine *d*-sulfoxide (11.9 g.) in dry dioxane (200 ml.). The mixture was stirred for 2 hr. at 0° and for 20 hr. at room temperature. The N,N′-dicyclohexylurea

(24) *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.

(25) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

(26) Deblocking of a sample by trifluoroacetic acid at room temperature for 30 min. gave methionine *d*-sulfoxide.

(27) Deblocking of a sample by trifluoroacetic acid at room temperature for 10 min. gave methionine *dl*-sulfoxide.

was removed by filtration and washed with dioxane, and the combined filtrate and washings were evaporated *in vacuo*. The residue was recrystallized twice from benzene; yield 15.6 g. (95%); m.p. 134°; $[\alpha]^{29}D$ +17.9° (*c* 1.68, methanol).

Anal. Calcd. for $C_{14}H_{22}O_7N_2S$: C, 46.4; H, 6.1; N, 7.7: O, 30.9. Found: C, 46.6; H, 6.2; N, 7.6; O, 30.7.

t-Butyl Benzyloxycarbonylalanylalaninate. N,N'-Dicyclohexylcarbodiimide[23] (20.6 g.) in THF (30 ml.) was added to an ice-cold THF solution (70 ml.) containing *t*-butyl alaninate (14.2 g.)[28] and benzyloxycarbonylalanine (22.4 g.).[29] The mixture was kept in a refrigerator for 24 hr., glacial acetic acid (3 ml.) was added, and 15 min. later the suspension was filtered and the filter cake washed with THF (100 ml.). The filtrate and washings were evaporated to dryness *in vacuo*, the residue was dissolved in ethyl acetate (300 ml.), and the solution was washed with 1 *M* citric acid (250 ml.), saturated sodium chloride (75 ml.), saturated sodium bicarbonate (250 ml.), and again with saturated sodium chloride (two 75-ml. portions). The solution was dried over sodium sulfate and concentrated to a small volume *in vacuo*, and petroleum ether (b.p. 37–55°) was added. The mixture was placed in a refrigerator where crystallization occurred; yield 20.6 g. (60%); m.p. 68–71°. A sample for analysis was recrystallized from ethyl acetate; m.p. 69–71°; $[\alpha]^{20}D$ −54.2° (*c* 2.0, methanol).

Anal. Calcd. for $C_{18}H_{26}O_5N_2$: C, 61.7; H, 7.5; N, 8.0. Found: C, 61.6; H, 7.4; N, 8.0.

Benzyloxycarbonylserylserine Hydrazide. Hydrazine hydrate (3.8 ml.) was added to a hot solution of methyl benzyloxycarbonylserylserinate[30] (25.6 g.) in methanol (200 ml.) and the mixture was kept for 4 hr. The crystalline hydrazide was collected, washed with ice-cold methanol, and dried over concentrated sulfuric acid *in vacuo;* the material was recrystallized from water; yield 18 g. (70%); m.p. 221–222° dec.; $[\alpha]^{26}D$ −30.5° (*c* 1.0, 1 *N* hydrochloric acid).

Anal. Calcd. for $C_{14}H_{20}O_6N_4$: C, 49.4; H, 5.9; N, 16.5. Found: C, 49.7; H, 6.1; N, 16.4.

Methyl Benzyloxycarbonylthreonylserinate. To a THF solution (80 ml.) containing methyl serinate (prepared from 17.1 g. of the hydrochloride[31] with 15.3 ml. of triethylamine in methanol in the usual manner) was added benzyloxycarbonylthreonine[32] (25.3 g.) in THF (35 ml.) and the solution was cooled in an ice bath. N,N'-Dicyclohexylcarbodiimide[23] (21.6 g.) in THF (10 ml.) was added and the mixture was kept in a refrigerator for 22 hr. when the N,N'-dicyclohexylurea was removed by filtration. The filter cake was washed with THF (50 ml.) and the combined filtrate and washings were evaporated to dryness. Crystallization occurred when ethyl acetate (65 ml.) was added to the residue. The crystals were collected and washed with cold ethyl acetate (20 ml.) and dried; yield 19.6 g.

Additional ethyl acetate (40 ml.) was added to the original mother liquor and the solution was extracted with 1 *M* citric acid (100 ml.), saturated sodium chloride (50 ml.), saturated sodium bicarbonate (200 ml.), and again with saturated sodium chloride (100 ml.). The solution was dried over sodium sulfate, the solvent was removed *in vacuo*, and ether was added to the residue. The resulting crystals were collected and washed with ether; yield 10.9 g. The two fractions were combined and recrystallized from a mixture of benzene and methanol; yield 22.4 g. (63%); m.p. 135–136°; $[\alpha]^{30}D$ +7.6° (*c* 0.5, DMF).

Anal. Calcd. for $C_{16}H_{22}O_7N_2$: C, 54.2; H, 6.3; N, 7.9. Found: C, 54.0; H, 6.4; N, 7.7.

Benzyloxycarbonylthreonylserine Hydrazide. Hydrazine hydrate (2.8 ml.) was added to a hot solution of methyl benzyloxycarbonylthreonylserinate (20.0 g.) in methanol (160 ml.) and the mixture was kept for 12 hr. at room temperature. The resulting crystals, collected by filtration, were washed with cold methanol (40 ml.) and dried *in vacuo* over concentrated sulfuric acid. The hydrazide was crystallized from water (900 ml.); yield 13.8 g. (69%); m.p. 225–227°. A sample for analysis was recrystallized from water; m.p. 226–228°; $[\alpha]^{29}D$ −34.2° (*c* 1.0, 1 *N* hydrochloric acid).

Anal. Calcd. for $C_{15}H_{22}O_6N_4$: C, 50.8; H, 6.3; N, 15.8. Found: C, 50.9; H, 6.3; N, 15.7.

Benzyloxycarbonyl-β-t-butylaspartylserine (XVIII). To a stirred suspension cooled at −30° of α-succinimido *β-t*-butyl benzyloxycarbonylaspartate (X, 21.0 g.) in ethanol (300 ml.) was added a solution of serine (XVII, 6.3 g.) and sodium bicarbonate (5.0 g.) in water (100 ml.). The mixture was stirred for 2 hr. at −10°, for 2 hr. at 0°, for 16 hr. at 4°, and for 4 hr. at room temperature. The solution was cooled at 0° and acidified with 1 *N* hydrochloric acid (75 ml.), and the ethanol was removed *in vacuo*. The solution was extracted with three 300-ml. portions of ethyl acetate, and the organic phase was washed with water (three 100-ml. portions) and dried over sodium sulfate. The solvent was removed *in vacuo* and the residue was recrystallized twice from ethyl acetate; yield 14.9 g. (73%); m.p. 135°; $[\alpha]^{28}D$ +8.6° (*c* 1.41, methanol); R_f^{III} 0.68; single chlorine-positive spot; amino acid ratios in acid hydrolysate $asp_{1.0}ser_{1.0}$.

Anal. Calcd. for $C_{19}H_{26}O_8N_2$: C, 55.6; H, 6.4; N, 6.8; O, 31.2. Found: C, 55.7; H, 6.5; N, 6.7; O, 31.0.

β-t-Butylaspartylserine (XIX). Benzyloxycarbonyl-*β-t*-butylaspartylserine (XVIII, 10.7 g.) was hydrogenated over palladium in 70% (v./v.) aqueous ethanol (150 ml.). The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The product was recrystallized from ethanol; yield 6.8 g. (94%); m.p. 158–160°, then solidifies and decomposes at 180°; $[\alpha]^{25}D$ +30.3° (*c* 1.45, water): R_f^1 0.62; R_f^2 0.59; single ninhydrin- and chlorine-positive spot.

Anal. Calcd. for $C_{11}H_{20}O_6N_2$: C, 47.8; H, 7.3; N, 10.1; O, 34.7. Found: C, 47.6; H, 7.4; N, 10.3; O, 35.0.

t-Butyl Alanylalaninate Monoacetate (VI). The

(28) *t*-Butyl benzyloxycarbonylalaninate was prepared from benzyloxycarbonylalanine according to G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960). The oily compound was hydrogenated in methanol. The resulting oily *t*-butyl alaninate was used without purification.

(29) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(30) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

(31) St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958).

(32) R. B. Merrifield, *J. Biol. Chem.*, **232**, 43 (1958).

benzyloxycarbonyl derivative (14.0 g.) in methanol (120 ml.) containing glacial acetic acid (2.6 ml.) and water (20 ml.) was hydrogenated in the usual manner over a palladium catalyst. The catalyst was removed by filtration, the solvents were evaporated, and ether (50 ml.) was added to the residue to bring about crystallization. The suspension was kept in a refrigerator for 12 hr., and the crystals were collected, washed with ether (50 ml.), and dried; yield 9.4 g. (85%). A sample for analysis was recrystallized from ethyl acetate; m.p. 106–108°; $[\alpha]^{27}D$ −39.2° (*c* 1.05, methanol).

Anal. Calcd. for $C_{10}H_{20}O_3N_2 \cdot CH_3COOH$: C, 52.2; H, 8.8; N, 10.1. Found: C, 51.9; H, 9.0; N, 10.2.

t-Butyloxycarbonylmethionyl-β-t-butylaspartylserine d-Sulfoxide. To a stirred solution cooled at −20° of succinimido *t*-butyloxycarbonylmethioninate *d*-sulfoxide (XIII, 7.24 g.) in ethanol (150 ml.) was added a solution of *β-t*-butylaspartylserine (XIX, 4.44 g.) and sodium bicarbonate (1.34 g.) in water (100 ml.). The mixture was stirred for 2 hr. at −10°, for 2 hr. at 0°, and for 20 hr. at room temperature. The solution was cooled at 0° and acidified with 1 *N* hydrochloric acid (20 ml.). The ethanol was removed *in vacuo* and the aqueous solution was saturated with sodium chloride and extracted with three 250-ml. portions of ethyl acetate. The ethyl acetate solution was washed with saturated sodium chloride solution (50 ml.), dried over sodium sulfate, and evaporated to dryness. The residue was dissolved in hot ethyl acetate and after cooling the protected tripeptide precipitated as a gel. The material was three times reprecipitated from ethyl acetate; white amorphous solid; yield 6.5 g. (77%); $[\alpha]^{30}D$ +20.1° (*c* 1.79, methanol); R_f^1 0.90; R_f^2 0.77; single chlorine- and methionine-positive spot; amino acid ratios in acid hydrolysate $met^{33}_{0.78}asp_{1.00}ser_{1.00}$.

Anal. Calcd. for $C_{21}H_{37}O_{10}N_3S$: C, 48.2; H, 7.1; N, 8.0; O, 30.6; S, 6.1. Found: C, 48.5; H, 7.3; N, 7.8; O, 30.2; S, 6.3.

Methionylaspartylserine d-Sulfoxide Dihydrate (XX). *t*-Butyloxycarbonylmethionyl-*t*-butylaspartylserine *d*-sulfoxide (6.4 g.) was dissolved in trifluoroacetic acid (15 ml.) and the solution was kept at room temperature for 15 min. Ice-cold ether (500 ml.) was added and the ensuing suspension was kept at −10° for 30 min. when the precipitate was collected and washed with ether. The hydroscopic residue was dissolved in water (300 ml.), Amberlite IRA-400 (acetate cycle) (50 ml. settled in water) was added, and the suspension was stirred for 1 hr. at room temperature. The resin was removed by filtration and washed with three 100-ml. portions of 2% acetic acid. The combined filtrate and washings were evaporated to a small volume, the concentrate was cooled at 0°, and methanol (approximately 150 ml.) was added. The precipitate was collected and reprecipitated twice from water with methanol. The peptide was then dissolved in water and the solution was lyophilized; white amorphous solid; yield 2.7 g. (55%): $[\alpha]^{29}D$ +49.5° (*c* 0.54, water); R_f^1 0.20; R_f^3 0.66 × His; single ninhydrin- and chlorine-positive spot: amino acid ratios in LAP digest $met_{0.94}asp_{0.98}ser_{1.07}$ (84%).

Anal. Calcd. for $C_{12}H_{21}O_8N_3S \cdot 2H_2O$: C, 35.7; H, 6.2; N, 10.4; O, 39.7. Found: C, 35.5; H, 6.0; N, 10.2; O, 39.5.

Nα-t-Butyloxycarbonylhistidylmethionylaspartylserine d-Sulfoxide Dihydrate. *t*-Butyl nitrite (1.61 ml.) was added to a stirred solution cooled at −30° of N^{α}-*t*-butyloxycarbonylhistidine hydrazide[17] (3.76 g.) in DMF (30 ml.) containing 9.2 ml. of 6.1 *N* hydrogen chloride in dioxane. The mixture was stirred at −20° for 30 min., then cooled at −40° and neutralized with triethylamine (7.7 ml.). To this solution containing the azide of N^{α}-*t*-butyloxycarbonylhistidine (XV) was added a solution of methionylaspartylserine *d*-sulfoxide dihydrate (XX, 2.56 g.) in water (20 ml.) containing triethylamine (1.93 ml.), and the mixture was stirred at −10° for 90 min. and at 4° for 24 hr. The solvents were removed *in vacuo*, the residue was dissolved in water (20 ml.), and the solution was added to a Dowex 1-X2 column (2.8 × 47 cm.) which was eluted with water (500 ml.), 0.1 *N* acetic acid (500 ml.), and 0.2 *N* acetic acid (900 ml.). Individual fractions (20 ml. each) were collected at a flow rate of 4 ml./min. The desired peptide was located in the 0.2 *N* eluates by the Pauly reaction. These eluates were combined, the solvent was evaporated *in vacuo*, and the residue was lyophilized twice from water; yield 2.85 g. (70%): $[\alpha]^{28}D$ +5.2° (*c* 0.9, water); R_f^1 0.46; R_f^2 0.26; Pauly- and chlorine-positive, ninhydrin-negative spot; slightly contaminated with the free tripeptide R_f^1 0.20; ninhydrin- and chlorine-positive, Pauly-negative spot; amino acid ratios in acid hydrolysate $his_{0.98}met^{33}_{0.86}asp_{1.09}ser_{1.07}$.

Anal. Calcd. for $C_{23}H_{36}O_{11}N_6S \cdot 2H_2O$: C, 43.1; H, 6.3; N, 13.1; O, 32.5. Found: C, 42.7; H, 6.5; N, 13.2; O, 32.4.

t-Butyl N-Benzyloxycarbonylthreonylserylalanylalaninate. (*a*) *By the Standard Azide Procedure.* This entire operation was conducted in a 5° cold room. Sodium nitrite (3.62 g.) in water (25 ml.) was added slowly to a solution, cooled at −2°, of N-benzyloxycarbonylthreonylserine hydrazide (17.7 g.) in water (125 ml.) and 6 *N* hydrochloric acid (25 ml.). The mixture was kept at −2° for 10 min. when the solid azide was collected by filtration. The azide was then suspended in ice-cold 10% potassium bicarbonate (250 ml.), the suspension filtered, and the filter cake washed with ice-cold 10% potassium bicarbonate (100 ml.), ice-cold saturated sodium chloride (50 ml.), and finally ice-water (40 ml.). The moist azide was then dissolved in ice-cold THF (250 ml.), *t*-butyl alanylalaninate acetate (13.8 g.) and triethylamine (6.85 ml.) were added, and the solution was kept at 5° for 22 hr. The crystalline material which had precipitated was collected, washed with ice-cold THF (40 ml.), and recrystallized from methanol (300 ml.); yield 9.7 g. (36%): this material can also be recrystallized from 80% aqueous acetone: m.p. 209–210°; $[\alpha]^{26}D$ −4.0° (*c* 1.5, DMF).

Anal. Calcd. for $C_{25}H_{38}O_9N_4$: C, 55.8; H, 7.1; N, 10.4. Found: C, 55.9; H, 7.3; N, 10.4.

(*b*) *By the Rudinger modification.*[34] *t*-Butyl nitrite (2.41 ml.) was added to a stirred solution cooled to −30° of N-benzyloxycarbonylthreonylserine hydrazide (7.0 g.) in DMF (75 ml.) containing 13.2 ml. of 6.1 *N* hydrogen chloride in dioxane. The solution, after stirring at

(33) Methionine plus homocysteic acid; values not corrected for destruction.

(34) J. Honzl and J. Rudinger, *Collection Czech. Chem. Commun.*, **26**, 2333 (1961).

$-25°$ for 15 min., was cooled to $-60°$ and triethylamine (12.3 ml.) was added. To this mixture, containing the azide of N-benzyloxycarbonylthreonylserine, was added a solution of *t*-butyl alanylalaninate acetate (5.52 g.) in DMF (50 ml.) containing triethylamine (2.78 ml.). After stirring 1 hr. at $-20°$ and being kept overnight at 4° the crystalline product was collected, triturated with water, and dried; 6.5 g. The filtrate was concentrated *in vacuo*, water was added to the residue, and the amorphous precipitate was collected; 1.9 g. The combined residues were recrystallized from 50% aqueous dioxane; 5.6 g. (52%); m.p. 217–221°; $[\alpha]^{28}D$ $-4.7°$ (*c* 0.51, DMF); R_f^1 0.93; R_f^2 0.90; single ninhydrin-negative and chlorine-positive spot.

Anal. Calcd. for $C_{25}H_{38}O_9N_4$: C, 55.8; H, 7.1; N, 10.4; O, 26.7. Found: C, 55.9; H, 7.3; N, 10.6; O, 26.8.

t-Butyl Threonylserylalanylalaninate Acetate (VIII). *t*-Butyl N-benzyloxycarbonylthreonylserylalanylalaninate (2.69 g.) in acetic acid–methanol–water (3:1:1) (40 ml.) was hydrogenated in the usual manner over palladium catalyst. After 4 hr., when the evolution of carbon dioxide had ceased, the catalyst was removed by filtration through "filter cel" and the filtrate was evaporated to a small volume *in vacuo*. Ether (100 ml.) was added and the mixture was kept at 4° for 12 hr. when the gelatinous precipitate was collected, washed with ether, and dried; yield 2.30 g. Two recrystallizations from absolute ethanol gave 1.74 g. (75%); m.p. 182–184°; $[\alpha]^{27}D$ $-89.1°$ (*c* 1.03, 10% acetic acid); R_f^1 0.65; R_f^2 0.89; single ninhydrin- and chlorine-positive spot; amino acid ratios in acid hydrolysate $thr_{0.92}ser_{0.99}ala_{2.01}$.

Anal. Calcd. for $C_{17}H_{32}O_7N_4 \cdot CH_3COOH$: C, 49.1; H, 7.8; N, 12.1; O, 31.0. Found: C, 49.3; H, 8.0; N, 12.4; O, 31.0.

Histidylmethionylaspartylserine d-Sulfoxide Dihydrate (XXI). N^{α}-*t*-Butyloxycarbonylhistidylmethionylaspartylserine *d*-sulfoxide dihydrate (2.8 g.) was dissolved in anhydrous trifluoroacetic acid (10 ml.) and the solution was kept at room temperature for 15 min. Ice-cold ether (500 ml.) was added and the ensuing suspension was kept at $-10°$ for 30 min. when the precipitate was collected and washed with ether. The hydroscopic residue was dissolved in water (50 ml.) and the solution was added to a Dowex 1-X2 column (1.8 × 27 cm.) which was eluted with water (200 ml.) and 0.05 *N* acetic acid (200 ml.). Individual fractions (20 ml. each) were collected at a flow rate of 2 ml./min. The desired peptide was located in the water and 0.05 *N* acetic acid eluates by the Pauly reaction. These eluates were combined and the solvent was evaporated *in vacuo*. The free tetrapeptide XXI crystallized from dilute acetic acid after addition of methanol; fine needles; yield 1.76 g. (75%); m.p. 184–188° dec.; $[\alpha]^{28}D$ $+28.8°$ (*c* 1.34, 10% acetic acid); R_f^3 0.55 × His; single Pauly-, ninhydrin-, and chlorine-positive spot; amino acid ratios in acid hydrolysate $his_{0.95}met^{\circ}_{0.84}asp_{1.09}ser_{0.96}$; amino acid ratios in LAP digest $his_{1.05}\mathring{m}et_{0.95}asp_{1.04}ser_{0.95}$ (91%).

Anal. Calcd. for $C_{18}H_{28}O_9N_6S \cdot 2H_2O$: C, 40.0; H, 6.0; N, 15.5; O, 32.6. Found: C, 39.8; H, 6.1; N, 15.5; O, 32.6.

t-Butyl Benzyloxycarbonylserylserylthreonylserylalanylalaninate. This entire operation was conducted in a 5° cold room. Sodium nitrite (6.01 g.) in water (50 ml.) was added slowly with stirring to an ice-cold solution of benzyloxycarbonylserylserine hydrazide (28.44 g.) in water (400 ml.) and 6 *N* hydrochloric acid (50 ml.). The solution was kept for 5 min. at 0° when the solid azide was collected by filtration. The azide was suspended in ice-cold potassium bicarbonate (30 g. in 150 ml. of water) and the suspension was filtered. The filter cake was washed with ice-water (50 ml.) and sucked as dry as possible on the funnel. *t*-Butyl threonylserylalanylalaninate acetate (VIII, 23.74 g.) was dissolved in warm DMF (350 ml.), the solution was cooled in an ice bath, and triethylamine (7.1 ml.) was added followed by the above moist azide. The mixture was kept in a refrigerator for 22 hr. when the solvents were removed *in vacuo*. The residue was triturated with water, the suspension filtered, and the filter cake washed with additional quantities of water (total amount 500 ml.). The wet material was then recrystallized from 80% aqueous acetone (2000 ml.); yield 26.4 g. (72%). A sample for analysis was recrystallized from methanol; m.p. 236–238° dec.; $[\alpha]^{29}D$ $-3.4°$ (*c* 0.52, DMF).

Anal. Calcd. for $C_{31}H_{48}O_{13}N_6$: C, 52.2; H, 6.8; N, 11.8; O, 29.2. Found: C, 51.9; H, 6.6; N, 11.7; O, 29.1.

t-Butyl Serylserylthreonylserylalanylalaninate Acetate (XI). *t*-Butyl N-benzyloxycarbonylserylserylthreonylserylalanylalaninate (5.0 g.) was suspended in acetic acid–methanol–water (3:1:1) (100 ml.) and hydrogenated in the usual manner over a palladium catalyst. After 4 hr., when the evolution of carbon dioxide had ceased, the catalyst was removed by filtration through "filter cel" and the filtrate was evaporated to a small volume *in vacuo*. Ether (100 ml.) was added, and the mixture was kept at 4° for 12 hr. when the fine precipitate was collected, washed with ether, and dried; yield 4.4 g.; m.p. 215–217° dec. One recrystallization from 90% aqueous dioxane gave 3.45 g. (77%); m.p. 216–218° dec.; $[\alpha]^{27}D$ $-92.4°$ (*c* 1.05, 10% acetic acid); R_f^1 0.52; R_f^2 0.79; single ninhydrin- and chlorine-positive spot; amino acid ratios in acid hydrolysate $ser_{3.12}thr_{0.92}ala_{1.96}$.

Anal. Calcd. for $C_{23}H_{42}O_{11}N_6 \cdot CH_3COOH$: C, 47.0; H, 7.3; N, 13.2; O, 32.6. Found: C, 47.2; H, 7.4; N, 13.4; O, 32.4.

t-Butyl Benzyloxycarbonyl-β-t-butylaspartylserylserylthreonylserylalanylalaninate. A solution of N-succinimido benzyloxycarbonyl-β-*t*-butylaspartate (X, 5.27 g.) in dioxane (20 ml.) was added to a solution of *t*-butyl serylserylthreonylserylalanylalaninate acetate (6.71 g.) in a mixture of dioxane (150 ml.), water (60 ml.), and triethylamine (1.5 ml.). After a few minutes the crystalline product came out of solution and after keeping the mixture overnight at room temperature it was collected, triturated with 2% acetic acid, washed with water, and dried; 6.74 g.; m.p. 220–222° dec. The filtrate was concentrated *in vacuo* to a small volume, ether was added, and the precipitate was collected and treated as above; 1.86 g.; m.p. 209–211° dec. The combined fractions were recrystallized from 80% aqueous dioxane; yield 7.53 g. (81%); m.p. 230–232° dec.; $[\alpha]^{26}D$ $-4.3°$ (*c* 1.00, DMF); R_f^1 0.90; R_f^2 0.92; single ninhydrin-negative and chlorine-positive

spot; amino acid ratios in acid hydrolysate $asp_{1.03}ser_{3.00}thr_{0.95}ala_{2.00}$.

Anal. Calcd. for $C_{39}H_{61}O_{16}N_7$: C, 53.0; H, 7.0; N, 11.1; O, 29.0. Found: C, 53.2; H, 7.1; N, 10.9; O, 29.0.

t-Butyl β-t-Butyl Aspartylserylserylthreonylseryl-alanylalaninate Acetate (XII). *t*-Butyl benzyloxycarbonyl-β-*t*-butyl aspartylserylserylthreonylserylalanyl-alaninate (3.0 g.) in acetic acid–methanol–water (3:1:1) (80 ml.) was hydrogenated in the usual manner over a palladium catalyst. After 4 hr., when the evolution of carbon dioxide had ceased, the catalyst was removed by filtration through "filter cel," and the filtrate was evaporated to a small volume *in vacuo* and lyophilized. The residue was dissolved in water (750 ml.) and the solution was added to a CMC column (3 × 35 cm.) which was eluted with water (750 ml.) and 0.005 *M* pH 6.9 ammonium acetate buffer (2500 ml.). Individual fractions (20 ml. each) were collected at a flow rate of 4 ml./min. The desired material was located in the 0.005 *M* eluates by the Lowry reaction.[20] These fractions were pooled, concentrated to a small volume *in vacuo*, and lyophilized to constant weight from small volumes of water; colorless fluffy powder; 2.54 g. (92%); $[\alpha]^{28}D$ −69.5° (*c* 0.50; 10% acetic acid); R_f^1 0.72; R_f^2 0.92; single ninhydrin- and chlorine-positive spot; amino acid ratios in acid hydrolysate $asp_{0.97}ser_{3.00}thr_{0.98}ala_{2.05}$.

Anal. Calcd. for $C_{31}H_{55}O_{14}N_7 \cdot CH_3COOH$: C, 48.9; H, 7.3; N, 12.1; O, 31.6. Found: C, 49.1; H, 7.7; N, 12.0; O, 30.8.

Aspartylserylserylthreonylserylalanylalanine Trihydrate. *t*-Butyl β-*t*-butylaspartylserylserylthreonyl-serylalanylalaninate monoacetate (XII, 350 mg.) was dissolved in anhydrous trifluoroacetic acid (2.5 ml.) and the solution was kept for 30 min. at room temperature. Ice-cold ether (100 ml.) was added and, after keeping for 30 min. at −10°, the precipitated trifluoroacetate salt was collected by filtration, washed with ether, and dried *in vacuo* over potassium hydroxide pellets. This material dissolved in water (250 ml.) was added to a Dowex 1-X2 (acetate cycle) column (2 × 20 cm.) which was washed with water (250 ml.) and eluted with the following acetic acid solutions: 0.01 (250 ml.), 0.02 (250 ml.), 0.05 (250 ml.), and 0.10 *M* (500 ml.). Individual fractions (15 ml.) each were collected at a flow rate of approximately 2 ml./min. and the desired peptide was located in the 0.10 *M* eluates by the Lowry reaction.[20] These eluates were pooled and the solvent was evaporated *in vacuo* to a small volume when the peptide precipitated from the concentrated solution.[35] The suspension was lyophilized twice from water, water (20 ml.) was added, and after keeping at 4° overnight the white solid was collected; yield 230 mg. The peptide was dissolved in boiling water (15 ml.) and an equal volume of ethanol added. After 1 week at 4° the gelatinous precipitate was collected, washed with ethanol, and dried; yield 190 mg. (64%); $[\alpha]^{28}D$ −61.2° (*c* 0.431, 0.5 *M* potassium bicarbonate); $[\alpha]^{27}D$ −67.5° (*c* 0.207, water); R_f^1 0.17; R_f^2 0.03; R_f^3 0.85 × His, single ninhydrin- and chlorine-positive component on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0; amino acid ratios in acid hydrolysate $asp_{1.00}ser_{2.90}thr_{0.95}ala_{2.10}$; amino acid ratios in LAP digest $asp_{1.06}ser_{2.86}thr_{0.99}ala_{2.12}$ (89%).

Anal. Calcd. for $C_{23}H_{39}O_{14}N_7 \cdot 3H_2O$: C, 39.9; H, 6.6; N, 14.2; O, 39.3. Found: C, 40.3; H, 6.6; N, 13.5; O, 39.8.

t-Butyl t-Butyloxycarbonylmethionyl-β-t-butyl-aspartylserylserylthreonylserylalanylalaninate d-Sulfoxide. (*a*) *By the N-Hydroxysuccinimide Ester Method.* Succinimido *t*-butyloxycarbonylmethioninate *d*-sulfoxide (1.07 g.) in dioxane (10 ml.) was added to a stirred solution of *t*-butyl β-*t*-butylaspartylserylseryl-threonylserylalanylalaninate acetate (XII, 1.99 g.) in 30% aqueous dioxane (35 ml.) containing triethylamine (0.34 ml.). After 1 hr. a crystalline material started to separate from the solution and, after keeping the mixture overnight at room temperature, it was collected, triturated with water, and dried; 1.42 g.; m.p. 220–222° dec. The filtrate was concentrated *in vacuo* to a small volume, water was added, and the resulting precipitate was collected and treated as above; 0.59 g.; m.p. 210–214° dec. The combined fractions were recrystallized from 70% aqueous dioxane; yield 1.69 g. (67%); m.p. 223–225° dec.; $[\alpha]^{28}D$ −24.5° (*c* 1.07, 90% acetic acid); R_f^1 0.90; R_f^2 0.92; single ninhydrin-negative, chlorine- and methionine-positive spot; amino acid ratios in acid hydrolysate $met^{33}{}_{0.76}asp_{1.00}ser_{3.00}thr_{0.97}ala_{2.09}$.

Anal. Calcd. for $C_{41}H_{72}O_{18}N_8S$: C, 49.4; H, 7.3; N, 11.2; O, 28.9; S, 3.2. Found: C, 49.0; H, 7.2; N, 11.0; O, 28.8; S, 3.3.

(*b*) *By the p-Nitrophenyl Ester Method.* *p*-Nitrophenyl *t*-butyloxycarbonylmethioninate *d*-sulfoxide (841 mg.) in dioxane (6.0 ml.) was added to a stirred solution of *t*-butyl β-*t*-butylaspartylserylserylthreonylserylalanylalaninate acetate (543 mg.) in 50% aqueous dioxane (6.0 ml.) and the resulting solution kept for 2 days at room temperature. The solution was concentrated to a small volume *in vacuo*, ethanol (100 ml.) was added, and after 12 hr. at 4° the gelatinous residue was collected, washed with ether, and dried; yield 455 mg.; m.p. 202–205° dec. Recrystallization from 70% aqueous dioxane gave 360 mg. (54%); m.p. 222–225° dec.,; $[\alpha]^{28}D$ −24.1 (*c* 1.05, 90% acetic acid); chromatographically identical with the above compound in solvent systems 1 and 2; amino acid ratios in acid hydrolysate $met^{33}{}_{0.85}asp_{1.02}thr_{0.97}ser_{3.09}ala_{2.09}$.

Anal. Calcd. for $C_{41}H_{72}O_{18}N_8S$: C, 49.4; H, 7.3; N, 11.2; O, 28.9. Found: C, 49.3; H, 7.6; N, 11.1; O, 29.6.

Methionylaspartylserylserylthreonylserylalanylalanine d-Sulfoxide Tetrahydrate (XIV). *t*-Butyl *t*-butyloxycarbonylmethionyl-β-*t*-butylaspartylserylseryl-threonylserylalanylalaninate *d*-sulfoxide (885 mg.) was dissolved in anhydrous trifluoroacetic acid (5.0 ml.) and the solution was kept for 30 min. at room temperature. Ice-cold ether (200 ml.) was added and after 30 min. at −10° the precipitate was collected, washed with ether, and dried *in vacuo* over KOH at room

(35) A preparation of this peptide was found to be very water soluble and to exhibit a considerably lower rotation in both 0.5 *M* potassium bicarbonate and water. The chromatographic and electrophoretic behavior of this and the above sample was identical. However, although both samples gave the expected amino acids on acid hydrolysis, the LAP digest of this preparation gave low recoveries of aspartic acid, serine, and threonine, and in addition to the expected amino acids there were two unidentifiable peaks at 95 and 132 ml. of effluent from the long column of the amino acid analyzer. Similar behavior has also been observed with a preparation of the octapeptide XIV. We have at present no explanation for these findings.

temperature. The residue, dissolved in water (500 ml.), was added to a Dowex 1-X2 (acetate cycle) column (2 × 20 cm.) which was washed with water (350 ml.) and eluted with the following acetic acid solutions: 0.01 (500 ml.), 0.02 (500 ml.), and 0.05 *M* (1000 ml.) Individual fractions (20 ml. each) were collected at a flow rate of approximately 2 ml./min. and the peptide was located in the 0.02 and 0.05 *M* eluates by the Lowry reaction.[20] These eluates were pooled, the solvent was evaporated *in vacuo*, and the residue was lyophilized from small volumes of water; yield 644 mg. (85%); $[\alpha]^{28}D$ −43.7° (*c* 0.4, 10% acetic acid); R_f^1 0.22; R_f^2 0.18; R_f^3 0.70 × His; single ninhydrin-, chlorine-, and methionine-positive spot; single component on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0; amino acid ratios in acid hydrolysate $met^{33}_{0.86}asp_{1.04}ser_{3.12}thr_{0.96}ala_{2.00}$; amino acid ratios in LAP digest $\check{m}et_{0.98}asp_{1.00}ser_{2.75}thr_{1.08}ala_{2.15}$ (88%); the peptide appeared as a single peak at 72 ml. of effluent when applied to the long column of the amino acid analyzer.

Anal. Calcd. for $C_{28}H_{48}O_{16}N_8S \cdot 4H_2O$: C, 39.2; H, 6.6; N, 13.1; O, 37.3. Found: C, 39.4; H, 6.9; N, 12.8; O, 36.9.

N^{α}-t-Butyloxycarbonylhistidylmethionylaspartylserylserylthreonylserylalanylalanine d-Sulfoxide Trihydrate. A 20% (v./v.) solution of *t*-butyl nitrite in DMF (0.85 ml.) was added to a stirred solution (cooled to −30°) of N^{α}-*t*-butyloxycarbonylhistidine hydrazide[17] (360 mg.) in DMF (4.0 ml.) containing 0.89 ml. of 6.1*N* hydrogen chloride in dioxane. The solution, after stirring at −25° for 15 min., was cooled to −60° and triethylamine (0.75 ml.) was added. This mixture, containing the azide of N^{α}-*t*-butyloxycarbonylhistidine, was added to a solution (cooled to −50°) of methionylaspartylserylserylthreonylserylalanylalanine *d*-sulfoxide tetrahydrate (XIV, 525 mg.) in 50% aqueous DMF (2 ml.) containing a 20% (v./v.) triethylamine in DMF solution (0.94 ml.). After stirring 1 hr. at −25° and 23 hr. at 4°, additional azide (prepared from 180 mg. of the hydrazide) was added, and the solution was stirred for 24 hr. at 4° and 12 hr. at room temperature. The solvent was removed *in vacuo* and the residue was lyophilized twice from a small volume of water. This residue was dissolved in water (1000 ml.) and the solution was added to a Dowex 1-X (acetate cycle) column (3 × 20 cm.) which was washed with water (1000 ml.) and eluted with the following acetic acid solutions: 0.01 (750 ml.), 0.02 (750 ml.), and 0.05 *M* (1500 ml.). Individual fractions of 20 ml. each were collected at a flow rate of approximately 3 ml./min. and the peptide was located in the 0.05 *M* eluates by the Pauly reaction. These eluates were pooled, the solvent was removed *in vacuo*, and the residue was lyophilized from small volumes of water; yield 515 mg. (78%); $[\alpha]^{29}D$ −45.3° (*c* 0.39, 10% acetic acid); R_f^1 0.25; R_f^2 0.06; R_f^3 1.73 × His; single ninhydrin-negative, Pauly-, chlorine-, and methionine-positive spot; amino acid ratios in acid hydrolysate $his_{1.00}met^{33}_{0.80}asp_{1.00}ser_{3.10}thr_{0.96}ala_{2.02}$.

Anal. Calcd. for $C_{39}H_{63}O_{19}N_{11}S \cdot 3H_2O$: C, 43.5; H, 6.5; N, 14.3; O, 32.7; S, 3.0. Found: C, 43.6; H, 6.6; N, 14.3; O, 32.0; S, 3.3.

Histidylmethionylaspartylserylserylthreonylserylalanylalanine d-Sulfoxide Monoacetate Trihydrate (XVI). N^{α}-*t*-Butyloxycarbonylhistidylmethionylaspartylserylserylthreonylserylalanylalanine *d*-sulfoxide trihydrate (385 mg.) was dissolved in anhydrous trifluoroacetic acid (4.0 ml.) and the solution was kept at room temperature for 15 min. Ice-cold ether (150 ml.) was added and after 30 min. at −10° the precipitate was collected, washed with ether, and dried *in vacuo* over KOH at room temperature. The residue was dissolved in water (400 ml.) and the solution was added to a Dowex 1-X2 (acetate cycle) column (2 × 20 cm.) which was washed with water (500 ml.) and eluted with 0.01 *M* acetic acid (500 ml.). Individual fractions (20 ml. each) were collected at a flow rate of approximately 4 ml./min. and the peptide was located in the 0.01 *M* eluates by the Pauly reaction. These eluates were pooled, the solvent was evaporated *in vacuo*, and the residue was lyophilized from small volumes of water; yield 232 mg. (63%); $[\alpha]^{27}D$ −39.5° (*c* 0.40, 10% acetic acid); R_f^1 0.10; R_f^2 0.12; R_f^3 0.62 × His; single ninhydrin-, chlorine-, and Pauly-positive spot; single component on paper electrophoresis at pH 1.9, 3.5, 6.5, and 8.0; amino acid ratios in acid hydrolysate $his_{0.98}met^{33}_{0.77}asp_{1.02}ser_{2.92}thr_{0.94}ala_{2.12}$; amino acid ratios in LAP digest $his_{0.87}met_{0.97}asp_{0.80}ser_{3.00}thr_{1.10}ala_{2.32}$ (96%).

Anal. Calcd. for $C_{34}H_{55}O_{17}N_{11}S \cdot CH_3COOH \cdot 3H_2O$: C, 41.7; H, 6.3; N, 14.9; O, 34.0; S, 3.2. Found: C, 41.8; H, 6.7; N, 14.8; O, 32.7; S, 3.4.

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