On Cysteine and Cystine Peptides. III. Synthesis of a Fragment of Insulin Containing the Intrachain Disulfide Bridge[1,2]

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The application of methods developed mostly in this laboratory permitted the synthesis of the fully S,N-protected heptapeptides N-carbobenzoxy-S-trityl-L-cysteinyl-S-diphenylmethyl-L-cysteinyl-L-alanylglycyl-L-valyl-S-trityl-L-cysteinyl-L-serine methyl ester (VIII, Figure 2), N-t-butyloxycarbonyl-S-trityl-L-cysteinyl-S-diphenylmethyl-L-cysteinyl-L-alanylglycyl-L-valyl-S-trityl-L-cysteinyl-L-serine methyl ester (X, Figure 2), and N-o-nitrophenylsulfenyl-S-benzoyl-L-cysteinyl-S-trityl-L-cysteinyl-L-alanylglycyl-L-valyl-S-benzoyl-L-cysteinyl-L-serine methyl ester (XXII, Figure 3). For these syntheses, a variety of N- and S-protecting groups was used; this allowed selective removal of either the N-, or two of three S-protecting groups according to desired aims. Thus, selective removal of the two S-trityl groups from VIII and X and of the two S-benzoyl groups from XXII led to the formation of the corresponding dithiol compounds XXV, XXVI, and XXVII. By oxidation of these thiol compounds a disulfide bridge was established specifically between two of the three cysteine residues incorporated in the above three heptapeptides. The corresponding oxidation products XXVIII, XXIX, and XXX are derivatives of the 6–12 sequence of the A chain of sheep insulin bearing the 6–11 intrachain bridge (Figure 1). Removal of the N-protecting groups from the cyclic peptide esters XXIX and XXX afforded the cyclic peptide ester hydrochlorides XXXI and XXXII from which the remaining S-protecting group can be removed by established methods. The significance of the above cyclic peptides as regards the problem of insulin synthesis is discussed. The possibility of an S→N acyl migration should be taken into account when using S-acylcysteines in peptide synthesis. It was proved that such a migration does not take place, at least in detectable extent, during the course of the synthesis of the S,N-protected heptapeptide XXII.

Introduction

In previous communications from this laboratory[2-4] N-protected S-trityl-, S-diphenylmethyl-, and S-acyl-L-cysteines have been proposed as intermediates for incorporation of cysteine residues into a peptide chain. A further approach to the synthesis of cysteine peptides is offered by incorporation of serine residues into a peptide chain, followed by O-tosylation and subsequent conversion of the resulting O-tosyl moieties to S-protected (S-trityl[3a,5a] or S-acyl[5b]) groups of cysteine derivatives. All S-protecting groups mentioned above do not hinder the further lengthening of the peptide chain provided that, in each case, the amino acid to be incorporated has been properly N-protected. These groups can be selectively removed without affecting sensitive parts of the molecule and especially any already existing S–S bridge.

The above mentioned S-protected cysteines have now been employed in an attempt to overcome the unique difficulties inherent in establishing an S–S bridge, specifically between two of three cysteine residues of a peptide chain. Thus, they have been used to prepare a "fragment" of the A chain of sheep insulin[6] which, up to now, could not have been synthesized otherwise. This fragment (Figure 1) contains the 6–11 intrachain bridge of sheep insulin and consists of a 20-membered disulfide ring, *i.e.*, the same size ring as that found in oxytocin.[7]

Precursors of this fragment, the corresponding fully S,N-protected heptapeptides, *i.e.*, N-carbobenzoxy-S-trityl-L-cysteinyl-S-diphenylmethyl-L-cysteinyl-L-alanylglycyl-L-valyl-S-trityl-L-cysteinyl-L-serine methyl ester (VIII, Figure 2) and a peptide X (Figure 2) of the same size and structure, but with N-*t*-butyloxycarbonyl-S-trityl-L-cysteine as the N-terminal amino acid, were prepared as outlined in Figure 2.

In addition, a third fully protected heptapeptide, N-*o*-nitrophenylsulfenyl-S-benzoyl-L-cysteinyl-S-trityl-L-cysteinyl-L-alanylglycyl-L-valyl-S-benzoyl-L-cysteinyl-L-serine methyl ester (XXII, *cf.* series of reactions in Figure 3) was prepared, containing the same sequence of amino acids. This heptapeptide, however, differed from the other two in the N- and S-protecting groups. All three heptapeptides were prepared in a way which excluded racemization, namely by step-

(1) This investigation was supported by the Royal Hellenic Research Foundation, to which we are greatly indebted.

(2) (a) A summary of a part of this paper was presented at the Fifth European Peptide Symposium, Oxford, Sept. 1962: L. Zervas, I. Photaki, A. Cosmatos, and N. Ghelis, "Peptides: Proceedings of the Fifth European Symposium, Oxford, 1962," G. T. Young, Ed., Pergamon Press, Oxford, 1963, p. 27. (b) A summary of this paper was presented at the Sixth European Peptide Symposium, Athens, Sept. 1963: A. Cosmatos, I. Photaki and L. Zervas, "Peptides: Proceedings of the Sixth European Symposium, Athens, 1963", L. Zervas, Ed., Pergamon Press, Oxford 1965, in press.

(3) (a) L. Zervas and I. Photaki, *Chimia*, **14**, 375 (1960); (b) L. Zervas, *Collection Czech. Chem. Commun.*, **27**, 2242 (1962).

(4) (a) Part I of this series: L. Zervas and I. Photaki, *J. Am. Chem. Soc.*, **84**, 3887 (1962); (b) part II: L. Zervas, I. Photaki, and N. Ghelis, *ibid.*, **85**, 1337 (1963).

(5) (a) I. Photaki, *ibid.*, **85**, 1123 (1963); (b) I. Photaki and V. Bardakos, *ibid.*, **87**, 3489 (1965).

(6) Regarding the structure of sheep insulin *cf.* H. Brown, F. Sanger, and R. Kitai, *Biochem. J.*, **60**, 556 (1955).

(7) (a) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954); C. Ressler, S. Trippett, and V. du Vigneaud, *J. Biol. Chem.*, **204**, 861 (1953). (b) As far as we know, rules for naming compounds such as the cyclic peptide of Figure 1 have not yet been proposed. Following a suggestion of Prof. M. Brenner, University of Basle, this cyclic peptide could be named as S-S′-L-hemicystyl-L-cysteinyl-L-alanylglycyl-L-valyl-L-hemicystyl-L-serine. For another alternative for naming such compounds *cf.* D. G. Large, H. N. Rydon, and J. A. Schofield, *J. Chem. Soc.*, 1749 (1961).

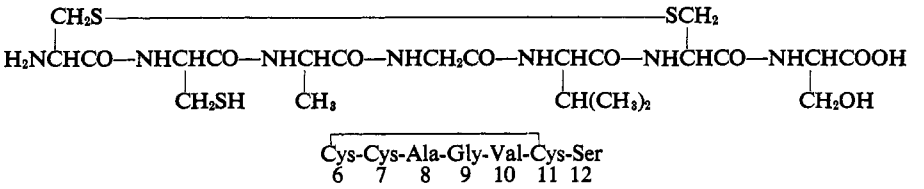


Figure 1.[7b]

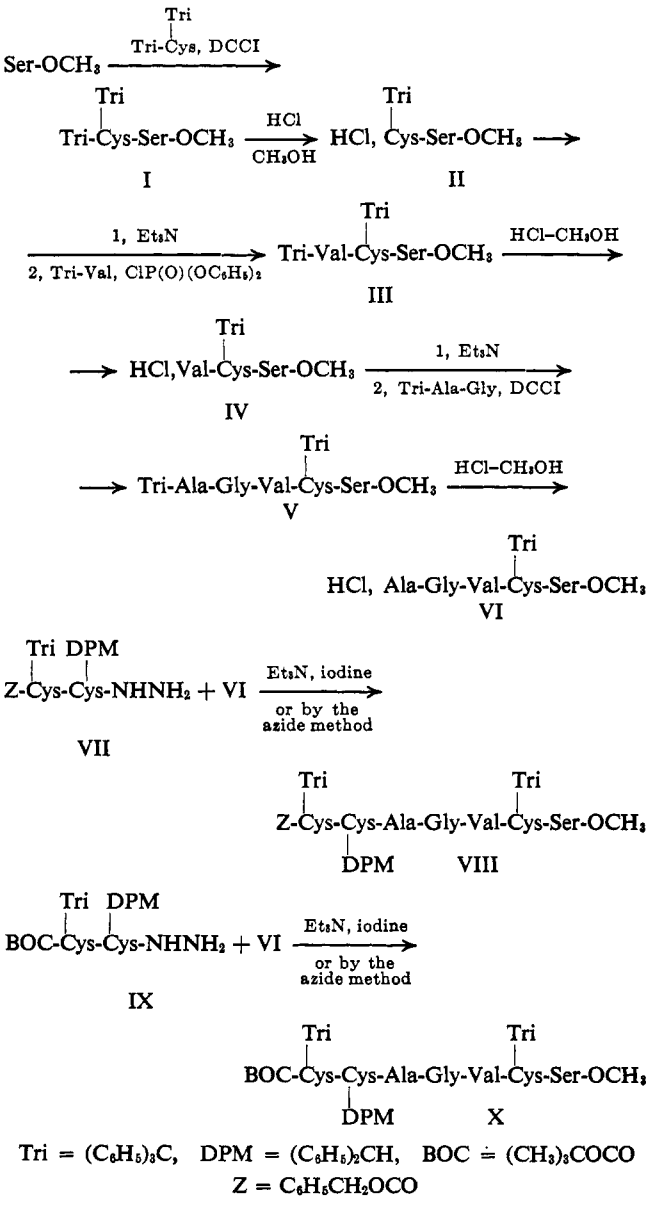


Figure 2. Series of reactions for synthesis of peptides VIII and X.

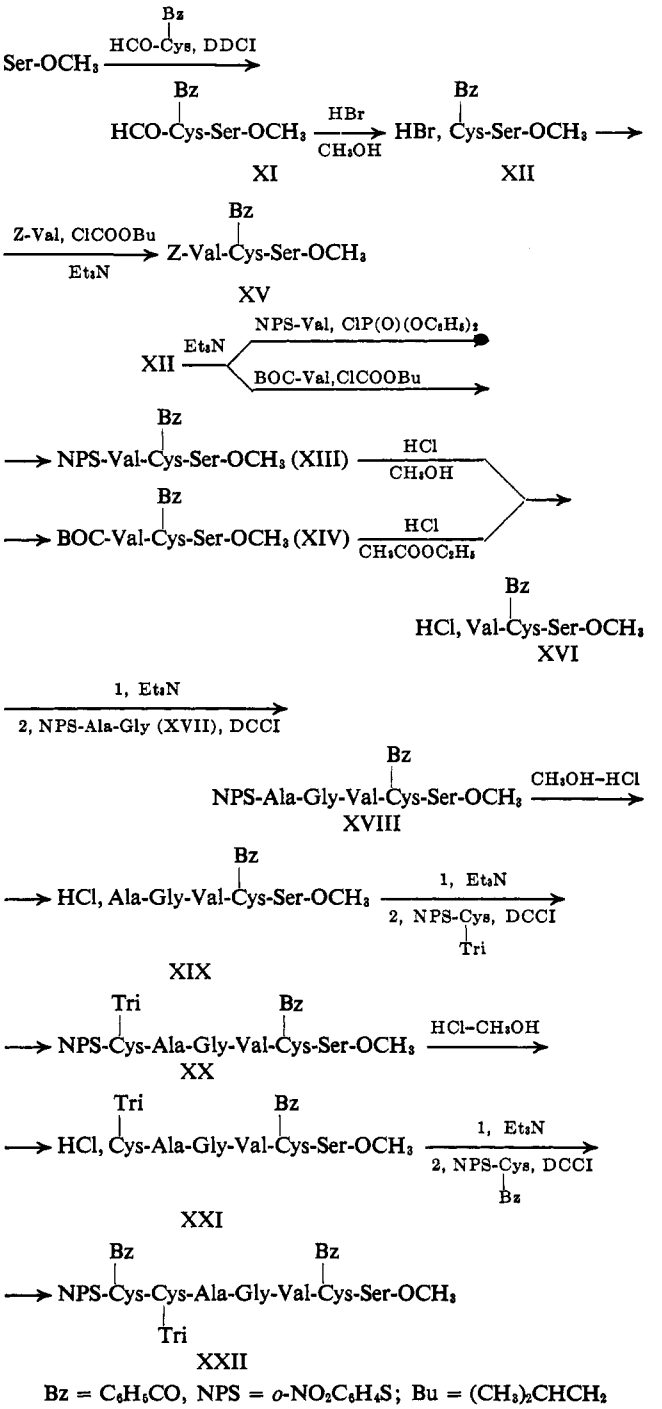
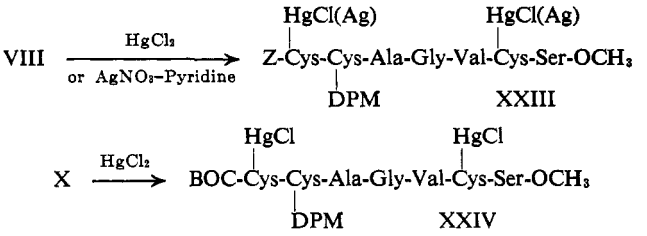


Figure 3. Series of reactions for synthesis of peptide XXII.

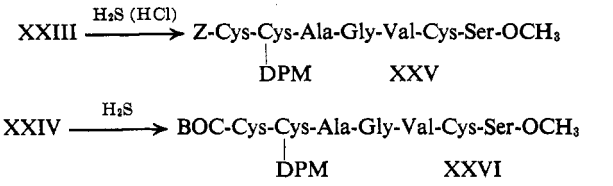
wise lengthening of the peptide chain from its C-terminal amino acid or *via* the azide method of coupling.

In all three fully protected heptapeptides, a variety of N- and S-protecting groups were used which allowed selective removal of either the N- or two of the three S-protecting groups[2-4] according to the desired aims. For example, treatment of the fully protected heptapeptides VIII and X with mercuric chloride in acetic acid or dimethylformamide solution (or silver nitrate–pyridine in dimethylformamide) yielded the corresponding dimercaptides XXIII and XXIV. The diphenylmethyl group was not affected under these conditions.

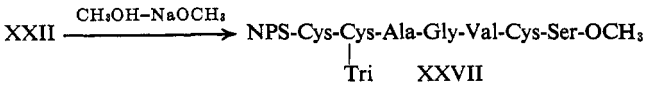
Both heavy metals could be readily removed by means of hydrogen sulfide with the formation of the corresponding peptide derivatives XXV and XXVI,



each bearing two free sulfhydryl groups. In the case of the silver mercaptides, the metal could be displaced also by hydrochloric acid. Again, the S-diphenylmethyl group was stable under these conditions.

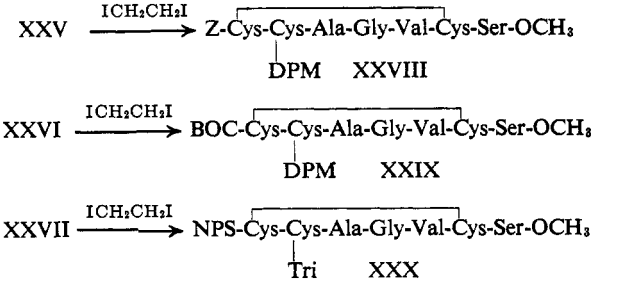


On the other hand, alcoholysis of XXII in the presence of sodium methoxide yielded directly the dithiol peptide XXVII. Under these conditions, the S-trityl group was not affected.



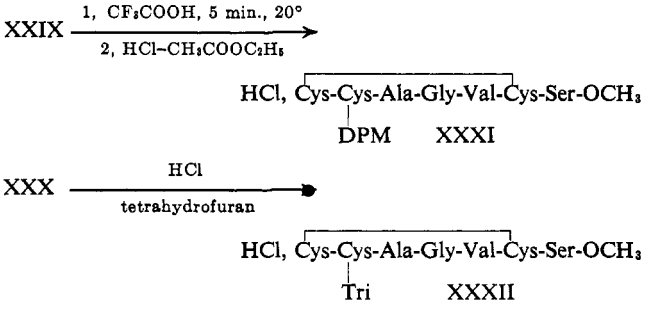
The next step consisted of oxidation of XXV to XXVII to form the S–S bridge between the first and the sixth amino acid residues. This oxidation was accomplished by Weygand's method[8] using 1,2-diiodoethane as the oxidizing agent. The oxidation products XXVIII, XXIX, and XXX thus obtained were derivatives of the cyclic fragment of insulin (Figure 1).

The oxidation products XXVIII–XXX, as well as the N-protected or N-unprotected intermediates III–XXVII, were crystalline substances. The chemical purity of compounds II–XXX was established by elemental analysis. Moreover, all the N-unprotected intermediates were found to be homogeneous by the criteria of thin layer chromatography[9] in two solvent systems and of paper electrophoresis.



Quantitative amino acid analyses of hydrolysates of cyclic compounds XXVIII, XXIX, and XXX, carried out according to the method of Spackman, Stein, and Moore,[10] showed the expected composition for serine, glycine, alanine, and valine. Furthermore, the molar ratio of half-cystine to any one of the other amino acids of compound XXX was found to be approximately 3:1. For XXVIII and XXIX, respective values of 2.1 and 2.3 of half-cystine were found. It has been our experience that hydrochloric acid hydrolysis, carried out according to the method applied for the amino acid analysis,[10] splits the S–Tri bond almost completely, but it does not cleave quantitatively the S–DPM bond. As a matter of fact, S-diphenylmethylcysteine was found by thin layer chromatography to be present in hydrolysates of XXVIII and XXIX.

The above oxidations were performed in dilute solutions and the resulting products were easy to recrystallize. The assumption that these oxidation products were monomeric was unequivocally proved by physical measurements on the heptapeptide ester hydrochloride XXXI. This hydrochloride was obtained by removal of the N-BOC group from XXIX by usual methods.[11]



The molecular weight of the peptide XXXI was kindly determined by Dr. D. Yphantis of the Rockefeller Institute by use of short column equilibrium centrifugation.[12a] At concentrations ranging from 0.125 to 0.5% in 50% (w./w.) acetic acid–water made 0.1 *M* in NaCl the average molecular weight was 960 assuming a partial specific volume of 0.69, or 857 assuming a specific volume of 0.66. A value of 0.69 was estimated[12b] for the partial specific volume of this peptide on the basis of its composition.

Amino acid analysis[10] of a hydrolysate of XXXI gave the same results as those mentioned above for its precursor XXIX. Furthermore, the chemical purity and homogeneity of the hydrochloride XXXI, as well as of hydrochloride XXXII, were established by elemental analysis, thin layer chromatography[8] in two solvent systems, and paper electrophoresis at various conditions. Hydrochloride XXXII was obtained by treatment of the peptide XXX with hydrogen chloride.[13]

The remaining S-protecting groups in XXXI and XXXII might be removed by established methods[2-4] without affecting the already existing peptide bonds and the S–S bridge.

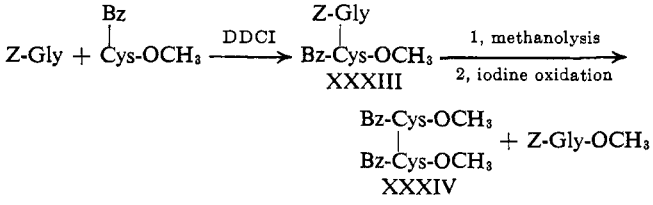
It is suggested that the above S,N-protected cyclic heptapeptides XXVIII to XXX, or at least the most suitable of them, could be used as a starting material for lengthening the peptide chain at the amino and at the carboxyl ends, *e.g.*, for the synthesis of the A

(8) F. Weygand and G. Zumach, *Z. Naturforsch.*, **17b**, 807 (1962).
(9) M. Brenner and A. Niederwieser, *Experientia*, **16**, 378 (1960).
(10) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958); appreciation is expressed to Dr. T. A. Mahowald (Department of Biochemistry, Cornell University, Medical College, New York, N. Y.) for the amino acid analysis of compound XXVIII and to Prof. M. Brenner (Department of Organic Chemistry, University of Basle) for the amino acid analyses of substances XXIX, XXX, and XXXI.
(11) R. Schwyzer, W. Rittel, H. Kappeler, and B. Iselin, *Angew. Chem.*, **72**, 915 (1960); R. Schwyzer, W. Rittel, and A. Costopanagiotis, *Helv. Chim. Acta*, **45**, 2473 (1962).
(12) (a) D. A. Yphantis, *Ann. N. Y. Acad. Sci.*, **88**, 586 (1960); (b) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, pp. 157 ff. and 371 ff.
(13) L. Zervas, D. Borovas, and E. Gazis, *J. Am. Chem. Soc.*, **85**, 3660 (1963).

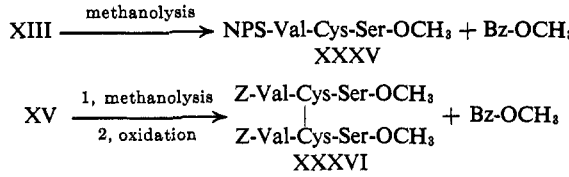
chain of insulin. The lengthening of the peptide chain could be also achieved prior to the removal of the S-protecting groups, and then the establishment of the 6–11 intrachain bridge could follow. Since a variety of selectively removable N- and S-protecting groups are now available,[2-4,13] this lengthening should be rather feasible.

It was reported that when insulin, an unsymmetrical cystine polypeptide with three S–S bridges, was reduced and the chains bearing the sulfhydryl groups thus formed were reoxidized, a small level of insulin activity was regenerated.[14] Hence it was thought that the synthesis of the aforementioned –SH insulin chains and their cooxidation would lead to the formation of the natural product. Indeed, Katsoyannis, *et al.*,[15] and Zahn, *et al.*,[16] succeeded in preparing, in this way, a material with some insulin activity. The synthesis of the cyclic insulin fragment (Figure 1) should now make feasible the synthesis of an A chain already bearing the preformed 6–11 intrachain bridge. It is felt that the combination of such a chain with the B chain of insulin might lead to a product with enhanced insulin activity.[2b,17] However, a completely controlled synthesis of unsymmetrical cystine peptides containing more than one S–S bridge is still the aim[2-4] of our synthetic attempts.

The possibility of an S→N migration in the presence of a base should be taken into account[2b] when using the S-acylcysteine derivatives for peptide synthesis. As a matter of fact, this was the case when N-carbobenzoxyglycine was coupled with S-benzoyl-L-cysteine methyl ester[4b] *via* the carbodiimide method. The resulting compound was a protected S-dipeptide ester, *i.e.*, S-(N-carbobenzoxyglycyl)-N-benzoyl-L-cysteine methyl ester (XXXIII), as has been shown by methanolysis. Upon methanolysis carbobenzoxyglycine was split off and N-benzoyl-L-cysteine methyl ester was formed and was isolated as the corresponding cystine derivative XXXIV.



On the contrary, such an S→N migration was not observed during numerous couplings when an S-acylcysteinyl peptide ester, *e.g.*, XII (for further examples see also ref. 4b), or an N-aminoacyl-S-acylcysteinyl peptide ester, *e.g.*, XVI, XIX, or XXI, was coupled with N-protected amino acid by the mixed anhydride method in a manner already described[2b] or by the carbodiimide method. In all of the latter cases only N-peptides, *e.g.*, N-NPS-derivatives XIII, XVIII, XX, and XXII, were obtained in good yields. This



was again proved by methanolysis. The coupling product, *e.g.*, XIII, XV, etc., was subjected to methanolysis and the sulfur-containing fraction was isolated either as the cysteine derivative, *e.g.*, XXXV, or as the corresponding cystine derivative, *e.g.*, XXXVI, after iodine oxidation. The presence of all the expected amino acids in the hydrolysate of this fraction would support the N-peptide structure. The lack of the amino acid constituents of the N-protected reactant in the coupling reaction would indicate the S-peptide structure. The elemental analysis of XXXV and XXXVI, as well as the presence of valine in their hydrolysates, established beyond any doubt the N-peptide structure in the particular case of XIII and XV.

Experimental Section

For the coupling reactions anhydrous reactants and dry solvents were used; the ether was free from peroxides. Evaporations were carried out *in vacuo* at 35–40°. The melting points are not corrected.

Prior to analysis the compounds were dried at 56° under high vacuum over phosphorus pentoxide. The derivatives of cysteine were determined by titration with 0.1 *N* iodine at pH 5–6.5 as described in a previous communication.[4b]

The R_f values were determined by thin layer chromatography[9] in 1-butanol–acetic acid–water (100:10:30)[18a] (R_{f_A}) and in pyridine–isoamyl alcohol–water–diethylamine (10:10:7:0.3)[18b] (R_{f_B}). Ninhydrin was used for development of both the chromatograms and the paper electropherograms.

S-Trityl-L-cysteinyl-L-serine Methyl Ester Hydrochloride (II). A suspension of 14.9 g. (0.022 mole) of the diethylammonium salt of S,N-ditrityl-L-cysteine[4a,19] in 200 ml. of ethyl acetate was shaken in a separatory funnel with 45 ml. of 0.5 *N* sulfuric acid until it was dissolved. The organic layer was separated and washed repeatedly with water until the aqueous layer was neutral to congo red paper. The ethyl acetate solution of S,N-ditrityl-L-cysteine thus prepared was dried over sodium sulfate, filtered, and added to a solution of 2.1 g. of L-serine methyl ester (this solution was prepared as follows: To a cold solution of 3.1 g. of L-serine methyl ester hydrochloride in absolute methanol, 9 ml. of 2 *N* sodium methoxide was added followed by addition of 150 ml. of anhydrous ether. The mixture was allowed to stand at 0° for a few minutes and the sodium chloride was filtered off) in methanol–ether. The mixture was evaporated *in vacuo* to dryness, the residue was dissolved in 150 ml. of ethyl acetate, and 4.2 g. of N,N′-dicyclohexylcarbodiimide[20] was added. The mixture was then allowed to

(14) G. H. Dixon and A. C. Wardlaw, *Nature*, **188**, 721 (1960); Y. C. Du, Y. S. Zhang, Z. X. Lu, and C. L. Tsou, *Sci. Sinica* (Pekin), **10**, 84 (1961); C. L. Tsou, Y. C. Du, and G. J. Xu, *ibid.*, **10**, 332 (1961); J. L. Bailey and R. D. Cole, *J. Biol. Chem.*, **234**, 1733 (1959).

(15) P. G. Katsoyannis, A. Tometsko, and K. Fukuda, *J. Am. Chem. Soc.*, **85**, 2863 (1963); P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *ibid.*, **86**, 930 (1964).

(16) J. Meienhofer, E. Schnabel, H. Bremer, O. Brinkhoff, R. Zabel, W. Sroka, H. Klostermeyer, D. Brandenburg, T. Okuda, and H. Zahn, *Z. Naturforsch.*, **18**, 1120 (1963).

(17) J. Meienhofer and O. Brinkhoff, *Nature*, **199**, 1095 (1963).

(18) (a) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961); (b) R. J. Block, E. L. Durrum, and G. Zweig, "A Manual of Paper Chromatography," Academic Press Inc., New York, N. Y., 1955, p. 113.

(19) G. Amiard, R. Heymes, and L. Velluz, *Bull. Soc. Chim. France*, 698 (1956).

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

stand at room temperature for 6 hr. A few drops of acetic acid and water were added, the precipitate of N,N′-dicyclohexylurea was filtered off, and the filtrate was concentrated *in vacuo* to dryness. The syrupy residue was dissolved in dry ether, and 2 ml. of diethylamine was added. After standing in the refrigerator for 24 hr. the excess N,S-ditrityl-L-cysteine precipitated as the diethylammonium salt. The filtrate was concentrated *in vacuo* to remove solvent and excess diethylamine. The syrupy residue, consisting of N-trityl-S-trityl-L-cysteinyl-L-serine methyl ester (I), was detritylated by dissolving it in 30 ml. of acetone, containing 2 ml. of concentrated hydrogen chloride. After standing for 2 hr. at room temperature, the solution was evaporated to dryness. Complete removal of acetone and water was ensured by the addition of a few milliliters of methanol and concentration *in vacuo*. Upon trituration of the syrupy residue with dry ether, the amorphous compound II was obtained; it was collected by filtration, washed with dry ether, and dried over calcium chloride and potassium hydroxide. The yield was 8.5 g. (85%), m.p. 108–111° (dec.), $[\alpha]^{20}D$ +38.7° (*c* 4, methanol), R_{f_A} 0.73, R_{f_B} 0.88. On paper electrophoresis in 0.5 *N* acetic acid (pH 2.7, 350 v., 4 hr., Schleicher and Schüll No. 2043B paper) or in pyridine acetate buffer (pH 4, 350 v., 3 hr.), the compound appeared as a single band.

Anal. Calcd. for $C_{26}H_{29}N_2O_4SCl$: N, 5.59; S, 6.40; Cl, 7.08; $(O)CH_3$, 3.0. Found: N, 5.92; S, 6.45; Cl, 7.07; $(O)CH_3$, 3.11.

*N-Trityl-*L*-valyl-S-trityl-*L*-cysteinyl-*L*-serine Methyl Ester (III).* To a solution of 4.32 g. (0.01 mole) of N-trityl-L-valine diethylammonium salt[21] in 30 ml. of tetrahydrofuran precooled to 0° 2.6 g. of diphenylphosphoryl chloride[22] was added. The mixture was left at 0° for 10 min. and then was poured into a solution of 5 g. (0.01 mole) of the above hydrochloride II and 2.8 ml. of triethylamine in 15 ml. of tetrahydrofuran. The mixture was allowed to stand for 6 hr. at room temperature, then filtered to remove the precipitate consisting of salts, and the filtrate was concentrated *in vacuo* to dryness. The residue was dissolved in a mixture of ethyl acetate and water. The ethyl acetate layer was washed once with water, three times with dilute acetic acid, once with water, five times with 5% sodium carbonate, and finally with water. The organic layer was dried over sodium sulfate and filtered, and the solvent was removed *in vacuo*. The syrupy residue was treated with dry ether and the crystalline substance, III, thus obtained was recrystallized from methanol. The yield was 2.6 g. (32%), m.p. 183–184, $[\alpha]^{20}D$ −3° (*c* 6, dimethylformamide).

Anal. Calcd. for $C_{50}H_{51}O_5N_3S$: C, 74.51; H, 6.38; N, 5.21; S, 3.98. Found: C, 74.24; H, 6.46; N, 5.24; S, 3.99.

L-*Valyl-S-trityl-*L*-cysteinyl-*L*-serine Methyl Ester Hydrochloride (IV).* A suspension of 2.4 g. (0.003 mole) of III in 16.5 ml. of 0.2 *N* methanolic hydrogen chloride was boiled for 5 min. and then was allowed to stand at room temperature for 30 min. Any precipitate of trityl ether[21,23] was removed by filtration, and dry ether was added to the filtrate. Upon cooling and scratching, the hydrochloride IV precipitated. The yield was 1.72 g. (96%), m.p. 100° dec., $[\alpha]^{18}D$ +26.6° (*c* 3, methanol), R_{f_A} 0.69, R_{f_B} 0.43. On paper electrophoresis in 0.5 *N* acetic acid (pH 2.7, 350 v., 4 hr., Schleicher and Schüll No. 2043B paper) or in pyridine acetate buffer (pH 4, 350 v., 3 hr., Schleicher and Schüll No. 2043B paper) the compound moved as a single band toward the cathode.

Anal. Calcd. for $C_{31}H_{38}O_5N_3ClS$: C, 62.04; H, 6.38; N, 7.00; Cl, 5.91; S, 5.34. Found: C, 61.88; H, 6.76; N, 7.21, Cl, 5.74; S, 5.40.

*N-Trityl-*L*-alanylglycyl-*L*-valyl-S-trityl-*L*-cysteinyl-*L*-serine Methyl Ester (V).* To a solution of 12 g. (0.02 mole) of the above hydrochloride IV and 8.5 g. (0.02 mole) of N-trityl-L-alanylglycine[23] in 75 ml. of tetrahydrofuran precooled to 0°, 2.8 ml. of triethylamine was added. Triethylamine hydrochloride separated out and was removed by filtration. To the filtrate, 125 ml. of tetrahydrofuran and 4.4 g. of N,N′-dicyclohexylcarbodiimide were added. The solution was allowed to stand for 6 hr. at room temperature and after the addition of 80 ml. of tetrahydrofuran the mixture was heated to 40–50°. The precipitate of N,N′-dicyclohexylurea was removed by filtration. The filtrate was concentrated *in vacuo* to dryness, and the crystalline residue was refluxed for 10 min. with 120 ml. of methanol and then was allowed to stand at room temperature for 12 hr. The crystalline precipitate was collected by filtration and was again refluxed for 5 min. with 70 ml. of methanol. After cooling, the precipitate was filtered off and was washed with cold methanol. The yield was 14 g. (75%), m.p. 223–224°, and 227–228° after recrystallization from a large volume of methanol; $[\alpha]^{18}D$ −34.5° (*c* 2, tetrahydrofuran).

Anal. Calcd. for $C_{55}H_{59}N_5O_7S$: C, 70.72; H, 6.37; N, 7.50; S, 3.43. Found: C, 70.60; H, 6.46; N, 7.37; S, 3.25.

L-*Alanylglycyl-*L*-valyl-S-trityl-*L*-cysteinyl-*L*-serine Methyl Ester Hydrochloride (VI).* A suspension of 20 g. (0.0214 mole) of V in 45 ml. of 0.5 *N* methanolic hydrogen chloride was stirred at room temperature for 5 min. and then was boiled for 3 min. The solvent was removed *in vacuo* and the residue was redissolved in methanol and boiled again for 1 min. The methanol was removed by evaporation and the residue was boiled with 50 ml. of ethyl acetate. Upon cooling the crystalline hydrochloride VI was obtained, and was washed thoroughly with ethyl acetate and finally with ether. The yield was 11 g. (66%), m.p. 204–205°. This melting point was unaltered by recrystallization from a small volume of methanol; $[\alpha]^{18}D$ −32.2° (*c* 2.5, methanol), R_{f_A} 0.45, R_{f_B} 0.80. On paper electrophoresis in 0.5 *N* acetic acid (pH 2.7, 350 v., 4 hr., Schleicher and Schüll No. 2043B paper) or in 50% acetic acid (1500 v., 30 min., Whatman No. 3MM paper) the compound was found to move as a single band toward the cathode.

Anal. Calcd. for $C_{36}H_{46}N_5O_7SCl$: C, 59.37; H, 6.37; N, 9.62; S, 4.40; Cl, 4.87. Found: C, 59.28; H, 6.70; N, 9.63; S, 4.37; Cl, 5.08.

(21) G. C. Stelakatos, D. M. Theodoropoulos, and L. Zervas, *J. Am. Chem. Soc.*, **81**, 2884 (1959).

(22) A. Cosmatos, I. Photaki, and L. Zervas, *Chem. Ber.*, **94**, 2644 (1961).

(23) L. Zervas and D. M. Theodoropoulos, *J. Am. Chem. Soc.*, **78**, 1359 (1956).

N-Carbobenzoxy-S-trityl-L-cysteinyl-S-diphenylmethyl-L-cysteine Hydrazide (VII). A suspension of 12.5 g. (0.022 mole) of N-carbobenzoxy-S-trityl-L-cysteine diethylammonium salt[4a] in ethyl acetate was shaken in a separatory funnel with 50 ml. of 0.5 *N* sulfuric acid until it dissolved. The ethyl acetate layer was separated and washed repeatedly with water until the water extracts were neutral to congo red paper. The ethyl acetate layer was dried over sodium sulfate and evaporated *in vacuo* to dryness. The residue, consisting of free N-carbobenzoxy-S-trityl-L-cysteine, was dissolved in chloroform and to this solution 6.8 g. (0.02 mole) of S-diphenylmethyl-L-cysteine methyl ester hydrochloride,[4a] 2.8 ml. of triethylamine, and 4.3 g. of N,N'-dicyclohexylcarbodiimide were added. After allowing the mixture to stand at room temperature overnight, a few drops of 50% acetic acid was added and the precipitate of dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness and the residue was dissolved in ethyl acetate–water. The organic layer was washed successively with water, dilute hydrochloric acid, potassium hydrogen carbonate solution, and again with water, dried over sodium sulfate, and evaporated *in vacuo* to dryness. To the solution of the syrupy residue in 300 ml. of warm methanol, 12 ml. of hydrazine hydrate was added. The solution was allowed to stand at room temperature overnight, and then in the refrigerator for several hours. The crystalline hydrazide VII was collected by filtration and recrystallized from ethanol. The yield was 11.8 g. (74%), m.p. 178–179°, $[\alpha]^{18}D$ −8.7° (*c* 4.5, dimethylformamide).

Anal. Calcd. for $C_{46}H_{44}N_4O_4S_2$: C, 70.74; H, 5.68; N, 7.17; S, 8.21. Found: C, 70.70; H, 5.77; N, 6.95; S, 8.04.

N-Carbobenzoxy-S-trityl-L-cysteinyl-S-diphenylmethyl-L-cysteinyl-L-alanylglycyl-L-valyl-S-trityl-L-cysteinyl-L-serine Methyl Ester (VIII). *A.* In a precooled mixture of 15 ml. of water and 40 ml. of dioxane 2.91 g. (0.004 mole) of the hydrochloride VI and 3.12 g. (0.004 mole) of compound VII were dissolved. After the addition of triethylamine (2.8 ml.) and of finely powdered iodine[24] (2.03 g.) the mixture was shaken until the evolution of nitrogen ceased. The excess of iodine was removed with a few drops of a concentrated solution of sodium thiosulfate and the heptapeptide derivative VIII was precipitated with 200 ml. of water. After cooling the mixture for a few hours in the refrigerator, the precipitate was collected by filtration and dried on a porous plate. The product was then suspended in a mixture of 30 ml. of methanol and 1.5 ml. of triethylamine and the suspension shaken for 30 min. at room temperature. The solid was filtered off and washed (on the filter) with two 5-ml. portions of methanol. The product, still wet, was suspended in a mixture of 30 ml. of methanol and 2.5 ml. of acetic acid, and the suspension was shaken for 30 min. at room temperature. The solid was filtered off and dried. The yield was 3.1 g. (54%), m.p. 228–228.5° dec., 231–232° after recrystallization from acetic acid (recovery 75%); $[\alpha]^{20}D$ −18.9° (*c* 3.5, dimethylformamide).

(24) Y. Wolman, P. M. Gallop, and A. Patchornik, *J. Am. Chem. Soc.*, **83**, 1263 (1961); Y. Wolman, P. M. Gallop, A. Patchornik, and A. Berger, *ibid.*, **84**, 1889 (1962).

Anal. Calcd. for $C_{82}H_{85}N_7O_{11}S_3$: C, 68.36; H, 5.95; N, 6.81; S, 6.68. Found: C, 68.58; H, 6.17; N, 6.95; S, 6.90.

B. Using substances VII and VI as starting materials, compound VIII was synthesized by the azide method of coupling in the manner described for the preparation of X by method B. The yield was 30%; m.p. 232°, $[\alpha]^{20}D$ −19.0° (*c* 3.5, dimethylformamide).

N-t-Butyloxycarbonyl-S-trityl-L-cysteinyl-S-diphenylmethyl-L-cysteine Hydrazide (IX). A solution of 10.9 g. (0.03 mole) of S-trityl-L-cysteine[4a,19] and 8.5 ml. of *t*-butyloxycarbonyl azide[25] in a mixture of 240 ml. of dioxane and 60 ml. of 1 *N* NaOH was heated with stirring for 20 hr. at 45–50°. The stirring was continued for an additional 20 hr. at room temperature. The dioxane was removed by evaporation *in vacuo*. The solution was diluted with water, acidified with 1 *N* sulfuric acid, and extracted with ethyl acetate–ether (1:1). The organic layer was washed with water, filtered, and repeatedly extracted with 1 *M* $KHCO_3$ and water, alternatively. (Potassium N-*t*-butyloxycarbonyl-S-trityl-L-cysteinate is practically insoluble in potassium bicarbonate solution. For this reason, upon combining the aqueous and the bicarbonate extracts the salt separated as an oily layer.) The combined aqueous extracts were acidified with 5% sulfuric acid and the organic material which separated was extracted with ether. The ethereal layer was repeatedly washed with water until the water layer became neutral to congo red paper. The ethereal layer was dried over sodium sulfate and evaporated to dryness. The syrupy residue (7.1 g.) consisting of N-*t*-butyloxycarbonyl-S-trityl-L-cysteine was coupled with S-diphenylmethylcysteine methyl ester[4a] *via* the carbodiimide method as described above for the preparation of VII. The syrupy dipeptide ester thus obtained was dissolved in 85 ml. of hot methanol, 4 ml. of hydrazine hydrate was added, and the solution was allowed to stand at room temperature for 24 hr. After cooling, the crystalline hydrazide IX was collected by filtration, washed with methanol, and dissolved in 15 ml. of dimethylformamide and 4.5 ml. of methanol, and the solution was filtered. Upon addition of 45 ml. of methanol to the filtrate, the pure crystalline hydrazide IX precipitated. The yield was 7.2 g. (63%); m.p. 195–196° dec., $[\alpha]^{18}D$ −10.6° (*c* 4.5, dimethylformamide).

Anal. Calcd. for $C_{43}H_{46}N_4O_4S_2$: C, 69.13; H, 6.20; N, 7.50; S, 8.58. Found: C, 69.19; H, 6.25; N, 7.61; S, 8.60.

N-t-Butyloxycarbonyl-S-trityl-L-cysteinyl-S-diphenylmethyl-L-cysteinyl-L-alanylglycyl-L-valyl-S-trityl-L-cysteinyl-L-serine Methyl Ester (X). *A.* Compound IX (2.91 g. 0.004 mole) was coupled with the hydrochloride VI (2.9 g., 0.004 mole) in the same manner as described for the coupling of VII with VI. The precipitate consisting of X was collected by filtration and washed with water. The product, still wet, was suspended in methanol, and the solvent was evaporated *in vacuo*. Complete removal of water was ensured by repeated

(25) (a) L. A. Carpino, *ibid.*, **79**, 98 (1957); L. A. Carpino, C. A. Giza, and B. A. Carpino, *ibid.*, **81**, 955 (1959); F. C. McKay and N. F. Albertson, *ibid.*, **79**, 4686 (1957); G. W. Anderson and A. C. McGregor, *ibid.*, **79**, 6180 (1957); (b) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

addition of methanol and evaporation to dryness. The residue was triturated for 5 min. with 20 ml. of boiling methanol. After allowing the mixture to stand for 3 hr. at room temperature and for 1 hr. in the refrigerator the solid was collected by filtration, washed with cold methanol, and dried. Repetition of the trituration with 20 ml. of boiling acetonitrile afforded 3 g. (53%) of material, m.p. 225–226°, unchanged after recrystallization from acetic acid, $[\alpha]^{18}D$ −18.0° (*c* 2.5, dimethylformamide).

Anal. Calcd. for $C_{79}H_{87}N_7O_{11}S_3$: C, 67.44; H, 6.23; N, 6.97; S, 6.84. Found: C, 67.13; H, 6.31; N, 7.14; S, 6.96.

B. To a cold solution of IX (0.75 g., 0.001 mole) in 5 ml. of dimethylformamide, 2 ml. of 1 *N* sulfuric acid and 0.105 g. of sodium nitrite were added. The azide thus formed was extracted into chloroform and the chloroform solution was washed with cold water, saturated sodium chloride, potassium hydrogen carbonate solution, and water, and was dried over potassium carbonate. This solution was added to a cold solution of 0.750 g. of the hydrochloride VI in 4 ml. of dimethylformamide and 0.14 ml. of triethylamine. The mixture was allowed to stand for 2 days in the refrigerator. The precipitate was filtered off and washed with chloroform. The yield of X was 0.26 g., m.p. 214–217°. The product was purified by dissolving it in 1 ml. of dimethylformamide followed by precipitation with 6 ml. of methanol (recovery 60%), m.p. 224–225°, $[\alpha]^{20}D$ −18.9° (*c* 2.5, dimethylformamide).

*N-Formyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester (XI).* L-Serine methyl ester hydrochloride (15.5 g., 0.1 mole) was allowed to react with 46 ml. of 2.1 *N* sodium methylate. Ether was added, the sodium chloride was filtered off, and the filtrate was evaporated to dryness. The residue was dissolved in 125 ml. of dimethylformamide and the solution was cooled to 0°. To this solution was added 27.8 g. (0.11 mole) of N-formyl-S-benzoyl-L-cysteine[4b] and 22 g. of N,N′-dicyclohexylcarbodiimide. The mixture was stirred at 0° for 10 min. and was allowed to stand at room temperature overnight. After addition of a few drops of dilute acetic acid, the N,N′-dicyclohexylurea was filtered off. The filtrate was concentrated *in vacuo* at 55–56° and the crystalline residue was recrystallized from methanol to give 26 g. (73%) of XI, m.p. 168°, $[\alpha]^{25}D$ −13.2° (*c* 2.5, dimethylformamide).

Anal. Calcd. for $C_{15}H_{18}N_2O_6S$: C, 50.83; H, 5.12; N, 7.90; S, 9.04. Found: C, 50.73; H, 5.08; N, 7.93; S, 8.89.

*S-Benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester Hydrobromide (XII).* A solution of 15 g. (0.045 mole) of XI in 105 ml. of absolute methanol and 15 ml. of 2.7 *N* hydrogen bromide in methanol was allowed to stand at room temperature for 3 days. The solvent was evaporated *in vacuo* to dryness. The crystalline residue was triturated with boiling ethyl acetate and the mixture was cooled. The solid was filtered off and was washed with ethyl acetate, followed by ether. The yield was 15 g. (88%), m.p. 170–171°. After recrystallization from hot methanol–ethyl acetate (recovery 80%) the melting point was raised to 172°; $[\alpha]^{25}D$ +7° (*c* 5, methanol), R_{f_A} 0.77, R_{f_B} 0.86. On paper electrophoresis in 0.5 *N* acetic acid (pH 2.7, 350 v., 4 hr., Schleicher and Schüll No. 2043B paper), the compound appeared as a single band.

Anal. Calcd. for $C_{14}H_{19}N_2O_5SBr$: C, 41.28; H, 4.70; N, 6.88; S, 7.87, Br, 19.62. Found: C, 41.57; H, 5.02; N, 7.04; S, 8.05; Br, 19.72.

*N-o-Nitrophenylsulfenyl-*L*-valyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester (XIII).* To a solution of 5.5 g. (0.02 mole) of *o*-nitrophenylsulfenyl-L-valine[13] in 30 ml. of chloroform and 2.7 ml. of triethylamine, precooled to 0°, 5.2 g. of diphenylphosphoryl chloride[22] was added. To this mixed anhydride solution, which was allowed to stand at 0–2° for 15 min., 8 g. (0.02 mole) of XII was added. Then 5.6 ml. of triethylamine was added dropwise[2b] with stirring within a period of 5 min. After the solution was allowed to stand at room temperature overnight, it was evaporated to dryness. The residue was dissolved in 50 ml. of dimethylformamide and compound XIII was precipitated by the addition of 150 ml. of water. The mixture was allowed to stand in the refrigerator, the supernatant liquid was decanted, and the solid material was washed three times with water, each washing being followed by decantation. The residue was then filtered off and washed thoroughly with water, 0.5 *N* sulfuric acid, water, potassium hydrogen carbonate solution, and again with water. The residue was finally filtered off and dried *in vacuo* over P_2O_5. The product was triturated with ethyl acetate, filtered off, and washed with ethyl acetate. The trituration was repeated using ether. The yield was 8.2 g. (71%), m.p. 194°. This substance has been used further without purification. For analysis, a sample was recrystallized from ethyl acetate; m.p. 206°, $[\alpha]^{20}D$ −105.4° (*c* 5, dimethylformamide).

Anal. Calcd. for $C_{25}H_{30}N_4O_8S_2$: C, 51.80; H, 5.22; N, 9.66; S, 11.06. Found: C, 51.59; H, 5.39; N, 9.54; S, 10.85.

Methanolysis.[4b] To a suspension of 0.58 g. (0.001 mole) of XIII in 15 ml. of absolute methanol, 2.2 ml. of methanolic 0.5 *N* sodium methoxide was added in an atmosphere of hydrogen with stirring at *ca.* 20°. The ester dissolved completely during the first few minutes of stirring, which was maintained for 20 min. Upon acidification with 2 ml. of acetic acid and addition of oxygen-free water, *o*-nitrophenylsulfenyl-L-valyl-L-cysteinyl-L-serine methyl ester (XXXV) precipitated. The ester was collected by filtration and washed with water. After recrystallization from ethyl acetate–petroleum ether the yield was 0.33 g. (70%), m.p. 148°, $[\alpha]^{20}D$ −17.2° (*c* 2.5, dimethylformamide).

Anal. Calcd. for $C_{18}H_{26}N_4O_7S_2$: C, 45.55; H, 5.52; N, 11.81; S, 13.51. Found: C, 45.64; H, 5.57; N, 11.70; S, 13.20.

A sample was hydrolyzed in 6 *N* hydrochloric acid at 100° for 10 hr. and the presence of valine, serine, and cysteine (cystine) was confirmed by means of two-dimensional thin layer chromatography (1-propanol–ammonia 33% (67:33) and 1-propanol–water (64:36)[9]).

*N-t-Butyloxycarbonyl-*L*-valyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester (XIV).* Syrupy N-*t*-butyloxycarbonyl-L-valine[25b] (2.7 g. 0.0125 mole) was dissolved in 60 ml. of chloroform and the solution was cooled to −10°. Triethylamine (1.73 ml.) and isobutyl chloroformate[26] (1.65 ml.) were added, and the mixture was

allowed to stand at −10° for 15 min. To the solution of the anhydride, the hydrobromide XII (5.1 g.) was added, followed by the dropwise[2b] addition of 1.73 ml. of triethylamine. The mixture was shaken at −10° until a clear solution was obtained (about 25 min.). Shaking was continued at room temperature for 1 hr. After allowing it to stand overnight, the solution was diluted with chloroform, washed successively with cold dilute sulfuric acid, potassium hydrogen carbonate solution, and water, dried over sodium sulfate, and evaporated to dryness. Methanol was added and evaporated to ensure complete removal of chloroform. The residue was recrystallized twice from methanol, giving 3 g. (50%), m.p. 150–152°, $[\alpha]^{25}D$ −41.5°, (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{24}H_{35}N_3O_8S$: C, 54.84; H, 6.71; N, 7.99. Found: C, 54.63; H, 6.78; N, 8.12.

*N-Carbobenzoxy-*L*-valyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester (XV).* This compound was prepared by coupling carbobenzoxy-L-valine[27] with XII in the same manner as described for the preparation of the corresponding N-*t*-butyloxycarbonyl peptide ester XIV. The crystalline crude product was recrystallized from methanol. The yield was 80%; m.p. 201–202°, $[\alpha]^{18}D$ −46.0° (*c* 3, in dimethylformamide).

Anal. Calcd. for $C_{27}H_{33}N_3O_8S$: C, 57.94; H, 5.94; N, 7.51; S, 5.73. Found: C, 57.72; H, 6.08; N, 7.41; S, 6.10.

Methanolysis of the product and subsequent oxidation with iodine was performed as described earlier.[4b] The iodine consumption was 98.6% of the theoretical amount. The yield of the isolated N′,N″[di-(N-carbobenzoxy-L-valyl)]-L-cystyl-di-L-serine methyl ester (XXXVI) was 92%, m.p. 206–207°, $[\alpha]^{20}D$ −46.6° (*c* 3, dimethylformamide). Recrystallization from methanol led to a recovery of 72%, m.p. 224–225°, $[\alpha]^{18}D$ −49.0° (*c* 3, dimethylformamide).

Anal. Calcd. for $C_{40}H_{56}N_6O_{14}S_2$: C, 52.86; H, 6.21; N, 9.24; S, 7.05. Found: C, 52.41; H, 6.50; N, 9.10; S, 7.14.

An hydrolysate of this compound was analyzed chromatographically as described for XXXV and the presence of valine, serine, and cystine was confirmed.

*L-Valyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester Hydrochloride (XVI).* *A.* A suspension of compound XIII (11.6 g., 0.02 mole, m.p. 194°) in 41 ml. of 2 *N* methanolic hydrogen chloride[13] was shaken at room temperature until the solid went into solution (3–4 min.). Upon addition of ether, the peptide hydrochloride XVI separated out. The mixture was allowed to stand in the refrigerator for 2 hr. and the supernatant liquid was decanted. The precipitate was triturated twice with ether, filtered off, washed with ether, and dried. The yield was 9 g. (98%); m.p. 200–202°. It was recrystallized from hot methanol–ethyl acetate; recovery, 6.8 g. (85%); m.p. 204°, $[\alpha]^{20}D$ +14.9° (*c* 5, methanol), $[\alpha]^{30}D$ −10.8° (*c* 2.5, dimethylformamide), R_{f_A} 0.60, R_{f_B} 0.85. On paper electrophoresis in 0.5 *N* acetic acid (pH 2.7, 350 v., 4 hr., Schleicher and Schüll No. 2043B paper), the compound was found to move as a single band toward the cathode.

Anal. Calcd. for $C_{19}H_{28}N_3O_6SCl$: C, 49.39; H, 6.11; N, 9.07; Cl, 7.67. Found: C, 49.07; H, 6.22; N, 9.09; Cl, 7.95.

B. Compound XIV (0.9 g.) was dissolved in 20 ml. of hot absolute ethyl acetate. After allowing the solution to cool to room temperature, 11 ml. of approximately 9 *N* hydrogen chloride in ether was added. Upon seeding, the hydrochloride XVI began to separate out. After standing at room temperature for 30 min. the mixture was cooled and the solid filtered off to give 0.7 g. of XVI, m.p. 192–193°. After recrystallization from hot methanol–ethyl acetate 0.56 g. was recovered; m.p. 201°, $[\alpha]^{30}D$ −10.8° (*c* 2.5, dimethylformamide), R_{f_A} 0.60, R_{f_B} 0.85.

*N-o-Nitrophenylsulfenyl-*L*-alanylglycine (XVII).* A suspension of 3.1 g. (0.01 mole) of N-*o*-nitrophenylsulfenyl-L-alanylglycine methyl ester[13] in 10 ml. of acetone and 11 ml. of 1 *N* sodium hydroxide was shaken at room temperature until a clear solution was obtained (about 10 min.). Water (50 ml.) was added and the solution was filtered and acidified with 11 ml. of 1 *N* sulfuric acid. After cooling, the crystalline precipitate was collected by filtration, washed with cold water, and dried *in vacuo* over P_2O_5. The yield was 2.4 g. (80%); m.p. 144–145°, $[\alpha]^{20}D$ −44.6° (*c* 5, dimethylformamide).

Anal. Calcd. for $C_{11}H_{13}N_3O_5S$: C, 44.14; H, 4.38; N, 14.04; S, 10.71. Found: C, 44.41; H, 4.06; N, 14.01; S, 10.80.

*N-o-Nitrophenylsulfenyl-*L*-alanylglycyl-*L*-valyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester (XVIII).* *A.* To a suspension of 1.5 g. (0.005 mole) of XVII in 20 ml. of chloroform, precooled to 0°, 0.7 ml. of triethylamine and 0.6 ml. of isobutyl chloroformate were added and the mixture was stirred for 15 min. at −5 to 0°. To the solution of the anhydride, 2.3 g. (0.005 mole) of XVI and 0.7 ml. (0.005 mole) of triethylamine were added at once. After 5–10 min. the peptide began to separate out as a gel. The mixture was shaken for a short time at 0° and afterwards was allowed to stand overnight at room temperature. The mixture was evaporated *in vacuo* to dryness, and traces of chloroform were removed by repeated addition of methanol followed by evaporation to dryness. The residue was dissolved in *ca.* 400 ml. of hot methanol and upon cooling in the refrigerator for 24 hr. compound XVIII crystallized. The yield was 5.45 g. (77%), m.p. 194–200° dec. This substance was used without further purification. An analytical sample was obtained after recrystallization from acetonitrile; m.p. 205–206°, $[\alpha]^{25}D$ −71.3° (*c* 2.5, dimethylformamide).

Anal. Calcd. for $C_{30}H_{38}N_6O_{10}S_2$: C, 50.98; H, 5.42; N, 11.89; S, 9.07. Found: C, 50.59; H, 5.62; N, 11.59; S, 9.35.

B. Compound XVIII was also prepared by coupling N-*o*-nitrophenylsulfenyl-L-alanylglycine hydrazide (m.p. 157–159°) with XVI *via* the azide method; the yield was 20%; m.p. 200–204°, $[\alpha]^{17}D$ −69.8° (*c* 2.5, dimethylformamide).

*L-Alanylglycyl-*L*-valyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester Hydrochloride (XIX).* Compound

(26) J. R. Vaughan and R. L. Osato, *J. Am. Chem. Soc.*, **73**, 5553 (1951).

(27) W. Grassmann and E. Wünsch, *Chem. Ber.*, **91**, 462 (1958).

XVIII (10.7 g., 0.065 mole) was treated with 50 ml. of methanol and 50 ml. of 2 *N* methanolic hydrogen chloride.[13] The mixture was warmed at 60° for a few seconds until the solid dissolved and, after scratching, the hydrochloride XIX separated out in crystalline form. Ether was added and the mixture was left in the refrigerator for 3 hr. The product was filtered, washed with ether, and dried. The yield was 8.45 g. (quantitative), m.p. 202–203°, unaltered after recrystallization from methanol; $[\alpha]^{25}D$ −44.2° (*c* 2.5, dimethylformamide), R_{f_A} 0.94, R_{f_B} 0.97. On paper electrophoresis in 0.5 *N* acetic acid (pH 2.7, 350 v., 4 hr., Schleicher and Schüll No. 2043B paper), or in 50% acetic acid (350 v., 3.5 hr.), the compound appeared as a single band.

Anal. Calcd. for $C_{24}H_{36}N_5O_8SCl$: C, 48.84; H, 6.15; N, 11.87; S, 5.43; Cl, 6.01. Found: C, 48.66; H, 6.30; N, 11.92; S, 5.18; Cl, 6.10.

*N-o-Nitrophenylsulfenyl-S-trityl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester* (*XX*). A solution of 1.04 g. (0.002 mole) of N-*o*-nitrophenylsulfenyl-S-trityl-L-cysteine[13] and 1.18 g. (0.002 mole) of XIX in 7 ml. of dimethylformamide was cooled to 0°. Triethylamine (0.28 ml.) and 0.44 g. of N,N′-dicyclohexylcarbodiimide were added. The mixture was allowed to stand at room temperature overnight. The N,N′-dicyclohexylurea formed was filtered off and washed with a small quantity of cold dimethylformamide. Upon addition of 60 ml. of water to the filtrate the product separated as a syrup which solidified upon scratching. The crystalline product was filtered off, washed with water, dried, and triturated at 0° with 7 ml. of ethyl acetate containing 2–3 drops of triethylamine. The substance was filtered off and washed with ethyl acetate. After recrystallization from methanol (110 ml.) 1 g. (50%) of the above protected hexapeptide was obtained; m.p. 208–209°, $[\alpha]^{20}D$ −48.7° (*c* 2.5, dimethylformamide).

Anal. Calcd. for $C_{52}H_{57}O_{11}N_7S_3$: C, 59.35; H, 5.46; N, 9.32; S, 9.14. Found: C, 59.36; H, 5.67; N, 9.35; S, 9.02.

*S-Trityl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester Hydrochloride* (*XXI*). Compound XX (1.05 g., 0.001 mole) was powdered finely and suspended in 10 ml. of glacial acetic acid and 10 ml. of methanol. Methanolic hydrogen chloride[13] (approximately 0.8 *N*, 2.6 ml.) was added with stirring. The yellow solid dissolved within 5 min., and the hydrochloride XXI started separating out. Ether was added to the solution, and the precipitated substance was filtered off and washed with ether. For purification, the hydrochloride was dissolved in methanol and reprecipitated by addition of ether. The yield was 0.64 g. (68%); m.p. 181–183°. The hydrochloride was recrystallized from a small amount of methanol (recovery 80%), melting point unaltered; $[\alpha]^{20}D$ −36.0° (*c* 2.5, dimethylformamide), R_{f_A} 0.75, R_{f_B} 0.87. On paper electrophoresis in 0.5 *N* acetic acid (pH 2.7, 350 v., 4 hr., Schleicher and Schüll No. 2043B paper) or in pyridine acetate buffer (pH 4, 350 v., 3 hr.), the compound appeared as a single band.

Anal. Calcd. for $C_{46}H_{55}O_9N_6S_2Cl$: C, 59.05; H, 5.90; N, 8.98; S, 6.85; Cl, 3.79. Found: C, 58.86; H, 5.54; N, 9.10; S, 6.78; Cl, 3.93.

*N-o-Nitrophenylsulfenyl-S-benzoyl-*L*-cysteinyl-S-trityl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-S-benzoyl-*L*-cysteinyl-*L*-serine Methyl Ester* (*XXII*). A suspension of 0.62 g. (0.0011 mole) of N-*o*-nitrophenylsulfenyl-S-benzoyl-L-cysteine dicyclohexylammonium salt[28] in 10 ml. of ethyl acetate was shaken in a separatory funnel with 2 ml. of water and 1.5 ml. of 1 *N* sulfuric acid until it dissolved. The ethyl acetate layer was washed with water until the aqueous layer was neutral to congo red paper. The ethyl acetate layer was dried and evaporated to dryness. To a solution of the residue, consisting of N-*o*-nitrophenylsulfenyl-S-benzoyl-L-cysteine in 6 ml. of dimethylformamide, 0.93 g. (0.001 mole) of XXI was added. The solution was cooled to 0°, 0.14 ml. of triethylamine and 0.22 g. of N,N′-dicyclohexylcarbodiimide were added, and the mixture was stirred at room temperature for 1 hr. After allowing the reaction mixture to stand at room temperature for 12 hr. the N,N′-dicyclohexylurea formed was filtered off and washed with cold dimethylformamide. Upon addition of 100 ml. of cold water to the filtrate, a crystalline substance separated out. It was filtered off, washed with water, and dried. The crude product (1.3 g.) was dissolved in hot dimethylformamide (7 ml.) and pure compound XXII precipitated upon addition of methanol. After cooling the product was filtered off and washed with methanol, ethyl acetate, and finally with ether. The yield was 0.9 g. (75%); m.p. 221° dec., $[\alpha]^{22}D$ −25.2° (*c* 2.5, dimethylformamide).

Anal. Calcd. for $C_{62}H_{66}O_{13}N_8S_4$: C, 59.12; H, 5.28; N, 8.90; S, 10.18. Found: C, 58.95; H, 5.07; N, 9.18; S, 9.92.

*N-Carbobenzoxy-S-chloromercuri-*L*-cysteinyl-S-diphenylmethyl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-S-chloromercuri-*L*-cysteinyl-*L*-serine Methyl Ester* (*XXIII*). Compound VIII (4.32 g., 0.003 mole) was dissolved in 100 ml. of acetic acid with gentle heating. The solution was allowed to cool to room temperature and then a solution of 3 g. of mercuric chloride[4a] in 28 ml. of methanol was added, followed by another solution of 0.82 g. of sodium acetate trihydrate in 2 ml. of methanol. The mercaptide XXIII precipitated immediately and the mixture was shaken for 4 hr. at room temperature. The precipitate was separated by centrifugation and washed in the centrifuge tube once with a mixture of acetic acid and methanol (5:1), twice with water, and twice with methanol. Methanol was added and the mixture was evaporated to dryness *in vacuo;* complete removal of water was ensured by repeated addition of methanol and evaporation to dryness. Finally the mercaptide XXIII was washed with ether and dried *in vacuo*. The yield was 2.9 g. (67%), m.p. 210–215° dec.

Anal. Calcd. for $C_{44}H_{55}N_7O_{11}S_3Hg_2Cl_2$: C, 37.05; H, 3.89; N, 6.87; S, 6.74; Hg, 28.13; Cl, 4.97. Found: C, 37.48; H, 4.18; N, 6.84; S, 7.03; Hg, 27.81; Cl, 5.15.

*N-t-Butyloxycarbonyl-S-chloromercuri-*L*-cysteinyl-S-diphenylmethyl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-S-chloromercuri-*L*-cysteinyl-*L*-serine Methyl Ester* (*XXIV*). Compound X (4.2 g., 0.003 mole) was dissolved in a mixture of 100 ml. of dimethylformamide and 30 ml. of methanol with gentle heating. The solu-

(28) L. Zervas and C. Hamalidis, *J. Am. Chem. Soc.*, 87, 99 (1965).

tion was allowed to cool to room temperature and a solution of 3 g. of mercuric chloride[4a] in 20 ml. of methanol was added, followed by a solution of 0.85 g. of sodium acetate trihydrate in 10 ml. of methanol. The mixture was heated gently and 100 ml. of methanol was added. Upon standing in the refrigerator for 3 hr. the mercaptide XXIV precipitated; it was collected by centrifugation, washed successively with acetic acid, methanol–acetic acid (3:1), methanol, water (twice), methanol (three times), and ether, and dried in a desiccator. The yield was 3.1 g. (75%); m.p. 192° dec.

Anal. Calcd. for $C_{41}H_{57}N_7O_{11}S_3Hg_2Cl_2$: C, 35.37; H, 4.13; N, 7.04; S, 6.99; Cl, 5.09; Hg, 28.82. Found: C, 35.27; H, 4.12; N, 7.10; S, 6.76; Cl, 5.12; Hg, 28.98.

*N-Carbobenzoxy-*L*-cysteinyl-S-diphenylmethyl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-cysteinyl-*L*-serine Methyl Ester (XXV).* *A.* Compound XXIII (2.85 g., 0.002 mole) was suspended in 30 ml. of dimethylformamide, and hydrogen sulfide was bubbled in for 5 min. At first, the compound dissolved and then mercuric sulfide precipitated. The mixture was shaken for 4 hr. at room temperature. The mercuric sulfide was removed by centrifugation and extracted first with a few milliliters of dimethylformamide and then with methanol. The extracts were combined with the supernatant liquid and filtered as soon as possible. Upon addition of 200 ml. of cold oxygen-free water compound XXV precipitated. After standing for 1 hr. in the refrigerator the precipitate was collected by centrifugation and was suspended in methanol. The mixture was evaporated *in vacuo* to dryness. Complete removal of water was ensured by repeated addition of absolute methanol and reconcentration *in vacuo*. The residue was boiled in methanol under refluxing. After cooling, the precipitate was collected by filtration or centrifugation and dried in a vacuum desiccator over phosphorus pentoxide. The yield was 1.7 g. (88%), m.p. 225–228° dec. after recrystallization from a thousandfold amount of methanol; $[\alpha]^{18}D$ −26.6° (*c* 3, dimethylformamide); the nitroprusside test for the sulfhydryl group was strongly positive.

Anal. Calcd. for $C_{44}H_{57}N_7O_{11}S_3$: C, 55.27; H, 6.01; N, 10.26; S, 10.06. Found: C, 55.24; H, 6.07; N, 9.99; S, 9.95.

B. Compound VIII (1.08, 0.00075 mole) was dissolved in 7 ml. of dimethylformamide with gentle heating. The solution was allowed to cool to room temperature, and then a solution of 0.51 g. of silver nitrate and 0.24 ml. of pyridine in 8 ml. of methanol[4a] was added. Upon addition of 50 ml. of water silver mercaptide of XXV precipitated, was collected by centrifugation, and washed successively in the centrifuge tube, three times with water, once with methanol, and finally with ether. The silver mercaptide of XXV thus obtained was dissolved in a mixture of 40 ml. of tetrahydrofuran–methanol (1:1) with stirring under hydrogen. Concentrated hydrochloric acid (0.15 ml.) was added and the stirring was continued for 1 hr. The silver chloride was removed by means of centrifugation and extracted several times with a hot mixture of tetrahydrofuran–methanol (1:1). The combined extracts were concentrated *in vacuo* and the crude compound XXV thus obtained was treated as described under method A. The yield was 0.16 g.; m.p. 223–228°. Replacement of the silver by hydrogen was also performed by hydrogen sulfide as described under method A.

*N-t-Butyloxycarbonyl-*L*-cysteinyl-S-diphenylmethyl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-cysteinyl-*L*-serine Methyl Ester (XXVI).* Compound XXIV (2.78 g., 0.002 mole) was suspended in 40 ml. of dimethylformamide and hydrogen sulfide was bubbled in. After 1 min. 0.8 g. of sodium acetate trihydrate dissolved in 10 ml. of methanol was added, and the bubbling of hydrogen sulfide was continued for 10 min. The mixture was allowed to stand at room temperature for 2 hr. and then 2 ml. of glacial acetic acid was added. The mercuric sulfide was removed by centrifugation and was washed with a few milliliters of dimethylformamide. To the combined filtrates 600 ml. of cold oxygen-free water was added. The mixture was allowed to stand in the refrigerator for 1 hr., the precipitate was collected by filtration, and the compound, still wet, was dissolved in boiling methanol. The resulting mixture was filtered and upon keeping the filtrate in the refrigerator for 10 hr. compound XXVI separated. The material was collected by filtration, washed with ether, and dried over phosphorus pentoxide in high vacuum. The yield was 1.5 g. (78%), m.p. 223° dec.; $[\alpha]^{20}D$ −27.1° (*c* 3, dimethylformamide); the nitroprusside test for the sulfhydryl group was strongly positive.

Anal. Calcd. for $C_{41}H_{59}N_7O_{11}S_3$: C, 53.40; H, 6.45; N, 10.63; S, 10.43. Found: C, 53.22; H, 6.35; N, 10.38; S, 10.35.

*N-o-Nitrophenylsulfenyl-*L*-cysteinyl-S-trityl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-cysteinyl-*L*-serine Methyl Ester (XXVII).* To a suspension of 1.25 g. (0.001 mole) of XXII in 16 ml. of absolute methanol and 8 ml. of dimethylformamide, 4 ml. of methanolic 0.55 *N* sodium methoxide[4b] was added with stirring while the mixture was kept under hydrogen. The peptide was almost completely dissolved within 30 min. Some insoluble material was removed by filtration and the filtrate was acidified with acetic acid. Upon addition of cold oxygen-free water compound XXVII precipitated. The solid was collected by centrifugation, washed in the centrifuge tube repeatedly with water, and dried. The product was recrystallized from dimethylformamide–methanol. After cooling, the crystalline compound XXVII was filtered off and washed with methanol and ether. The yield was 1.1 g. (70%), m.p. 195–198° dec.; $[\alpha]^{25}D$ −41.6° (*c* 2.5, dimethylformamide); the nitroprusside test for the sulfhydryl group was strongly positive.

Anal. Calcd. for $C_{48}H_{58}N_8O_{11}S_4$: C, 54.84; H, 5.56; N, 10.66; S, 12.20. Found: C, 54.68; H, 5.63; N, 10.65; S, 12.28.

*S-S′,N-Carbobenzoxy-*L*-hemicystyl-S-diphenylmethyl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-hemicystyl-*L*-serine Methyl Ester (XXVIII).* A solution of 2.3 g. (0.0024 mole) of freshly made XXV in a mixture of 300 ml. of absolute tetrahydrofuran and 10 ml. of dimethylformamide and another solution of 0.752 g. of 1,2-diiodoethane[8] in 300 ml. of absolute tetrahydrofuran were prepared. Both of the above solutions were added simultaneously, dropwise and with stirring, into 300 ml. of absolute tetrahydrofuran under

hydrogen and within a period of an hour. After removal of the tetrahydrofuran *in vacuo* followed by addition of acetone to the residue the oxidation product XXVIII precipitated. The compound was collected by filtration, washed with acetone, redissolved in a few milliliters of dimethylformamide, and reprecipitated by methanol. The yield was 1.5 g. (65%); m.p. 235–240° dec., unchanged after recrystallization from acetic acid, $[\alpha]^{18}D$ +8.5° (*c* 2, dimethylformamide).

A sample was hydrolyzed in 6 *N* hydrochloric acid at 110° for 24 hr. and then analyzed for amino acids.[10] The following molar ratios of amino acids were obtained with the value of glycine taken as 1.0: serine 0.8, glycine 1.0, alanine 1.0, half-cystine 2.1, and valine 0.8.

An hydrolysate of XXVIII prepared as described above was found, by thin layer chromatography,[4a] to contain a considerable amount of S-diphenylmethylcysteine.

Anal. Calcd. for $C_{44}H_{55}N_7O_{11}S_3$: C, 55.39; H, 5.81; N, 10.28; S, 10.08. Found: C, 55.73; H, 5.77; N, 9.98; S, 10.00.

*S-S′,N-t-Butyloxycarbonyl-*L*-hemicystyl-S-diphenylmethyl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-hemicystyl-*L*-serine Methyl Ester (XXIX).* A solution of 1.38 g. (0.0015 mole) of freshly made XXVI in a mixture of 75 ml. of dimethylformamide and 125 ml. of absolute tetrahydrofuran and another solution of 0.465 g. of 1,2-diiodoethane[8] in 200 ml. of absolute tetrahydrofuran were prepared. Both of the above solutions were added simultaneously, dropwise and with stirring, into a mixture of 200 ml. of absolute tetrahydrofuran and 40 ml. of dimethylformamide under hydrogen and within a period of 1 hr. The tetrahydrofuran was evaporated *in vacuo*, and 800 ml. of water was added to the residue. The precipitate was separated by means of centrifugation and triturated with water, and the solid was filtered off and washed successively with water, a few milliliters of a mixture of acetone–ethyl acetate (1:15), and finally with ethyl acetate. The yield was 0.69 g. (50%); m.p. 218° dec., $[\alpha]^{18}D$ +10.5° (*c* 2, dimethylformamide).

A sample was hydrolyzed in 6 *N* hydrochloric acid at 110° for 24 hr. and then analyzed for amino acids.[10] The following molar ratios of amino acids were obtained with the value of glycine taken as 1.0: serine 0.9, glycine 1.0, alanine 1.0, half-cystine 2.3, and valine 1.0.

An hydrolysate of XXIX prepared as described above was found, by thin layer chromatography,[4a] to contain a considerable amount of S-diphenylmethylcysteine.

Anal. Calcd. for $C_{41}H_{57}N_7O_{11}S_3$: C, 53.52; H, 6.24; N, 10.66; S, 10.45. Found: C, 53.54; H, 6.43; N, 10.55; S, 10.27.

*S-S′,N-o-Nitrophenylsulfenyl-*L*-hemicystyl-S-trityl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-hemicystyl-*L*-serine Methyl Ester (XXX).* A solution of 1.05 g. (0.001 mole) of freshly made XXVII in 100 ml. of dimethylformamide and another of 0.32 g. of 1,2-diiodoethane[8] in 100 ml. of methanol were prepared. Both of the above solutions were added simultaneously, dropwise and with stirring, into a mixture of 75 ml. of dimethylformamide, 75 ml. of methanol, and 0.3 ml. of triethylamine under hydrogen and within a period of 1 hr. After evaporation of the methanol *in vacuo* at room temperature 1 l. of cold water was added to the solution. The mixture was allowed to stand in the refrigerator overnight, and compound XXX was collected by means of centrifugation. The solid was washed with water and dried *in vacuo*. For purification the product (0.8 g.) was treated in 10 ml. of boiling methanol under refluxing. After cooling to 0°, compound XXX was collected by filtration and washed with cold methanol. The yield was 0.6 g. (60%); m.p. 215–218° dec., $[\alpha]^{20}D$ −71.1° (*c* 1.2, dimethylformamide), unchanged after recrystallization from dimethylformamide–methanol.

A sample was hydrolyzed in 6 *N* hydrochloric acid at 110° for 24 hr. and then analyzed for amino acids.[10] The following molar ratios of amino acids were obtained with the value of glycine taken as 1.0: serine 0.9, glycine 1.0, alanine 1.0, half-cystine 2.7, and valine 1.0.

Anal. Calcd. for $C_{48}H_{56}N_8O_{11}S_4$: C, 54.94; H, 5.38; N, 10.68; S, 12.22. Found: C, 54.51; H, 5.81; N, 10.64; S, 12.20.

*S-S′,*L*-Hemicystyl-S-diphenylmethyl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-hemicystyl-*L*-serine Methyl Ester Hydrochloride (XXXI).* Compound XXIX (0.092 g., 0.0001 mole) was dissolved in 1 ml. of 90% trifluoroacetic acid,[11] and the resulting solution was allowed to stand for 30 min. at room temperature. A few milliliters of ethyl acetate was then added and the solution was concentrated *in vacuo*. The syrupy residue was dissolved in a small volume of ethyl acetate and a few milliliters of ethyl acetate containing hydrogen chloride was added immediately. The product was purified by dissolving it in methanol, adding ethyl acetate, and removing traces of impurities by filtration. The filtrate was evaporated to dryness and the residue was triturated with ethyl acetate to give 0.06 g. (70%) of pure XXXI, m.p. 205–208° dec.; $[\alpha]^{20}D$ +10.0° (*c* 1, methanol), R_{f_A} 0.63, R_{f_B} 0.87. Paper electrophoresis in 0.5 *N* acetic acid (pH 2.7, 350 v., 4 hr., Schleicher and Schüll No. 2043B paper), in 50% acetic acid (1500 v., 30 min., Whatman No. 3MM paper), or in pyridine acetate buffer (pH 4, 350 v., 3 hr., Schleicher and Schüll No. 2043B paper) revealed a single component migrating toward the cathode.

A sample was hydrolyzed in 6 *N* hydrochloric acid at 110° for 24 hr. and then was analyzed for amino acids.[10] The following molar ratios of amino acids were obtained with the value of glycine taken as 1.0: serine 0.9, glycine 1.0, alanine 1.0, half-cystine 2.3, and valine 1.0.

An hydrolysate of XXXI prepared as described above was found, by thin layer chromatography,[4a] to contain a considerable amount of S-diphenylmethylcysteine.

Anal. Calcd. for $C_{36}H_{50}N_7O_9S_3Cl$: C, 50.48; H, 5.88; N, 11.45; S, 11.23; Cl, 4.14. Found: C, 50.27; H, 6.14; N, 11.28; S, 11.15; Cl, 4.22.

*S-S′,*L*-Hemicystyl-S-trityl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-hemicystyl-*L*-serine Methyl Ester Hydrochloride (XXXII).* Compound XXX (0.105 g., 0.0001 mole) was dissolved in 17 ml. of hot absolute tetrahydrofuran. The solution was filtered and then cooled to room temperature. Absolute ether (17 ml.) containing 30 mg. of trityl chloride, 4 ml. of hydrogen chloride solution[13] in ethyl acetate (*ca.* 1 *N*), and 8 ml. of ether

were added. After allowing the mixture to stand for a short time in the refrigerator the precipitated product was separated by centrifugation, washed in the centrifuge tube with dry ether, and dried *in vacuo*. The yield was 0.063 g. (65%); m.p. 207–210° dec., $[\alpha]^{20}D$ +4.2° (*c* 1.2, dimethylformamide), R_{f_A} 0.68 (one main spot with a faint tail).

Anal. Calcd. for $C_{42}H_{54}N_7O_9S_3Cl$: C, 54.07; H, 5.84; N, 10.52; S, 10.31; Cl, 3.80. Found: C, 53.31; H, 6.04; N, 10.09; S, 10.55; Cl, 3.98.

Purification of XXXII was achieved by the following process: Crude XXXII (0.25 g.) was dissolved in 20 ml. of hot methanol, and 40 ml. of ethyl acetate was added. The material which precipitated (0.08 g.) was removed by means of centrifugation and discarded. Upon addition of ether to the supernatant liquid 0.11 g. of chromatographically pure XXXII was obtained; m.p. 210° dec., $[\alpha]^{20}D$ +4.2° (*c* 1.2, dimethylformamide); R_{f_A} 0.68, R_B 0.84. On paper electrophoresis in 0.5 *N* acetic acid (pH 2.7, 350 v., 4 hr., Schleicher and Schüll No. 2043B paper) or in 50% acetic acid (350 v., 3.5 hr.), the compound moved as a single band toward the cathode.

Anal. Found: C, 53.83; H, 6.14; N, 10.19; S, 10.48; Cl, 3.76.

*S-(N-Carbobenzoxyglycyl)-N-benzoyl-*L*-cysteine Methyl Ester (XXXIII).* To a cold (0°) solution of 2.76 g. (0.01 mole) of S-benzoyl-L-cysteine methyl ester hydrochloride[4b] and 2.5 g. (10% excess) of N-carbobenzoxyglycine in 15 ml. of dimethylformamide, 1.4 ml. of triethylamine was added followed by the immediate addition of 2.2 g. of N,N′-dicyclohexylcarbodiimide. The mixture was stirred at room temperature for 6 hr. and the insoluble precipitate of N,N′-dicyclohexylurea and triethylamine hydrochloride was removed by filtration. The filtrate was diluted with ethyl acetate and washed successively with water, dilute hydrochloric acid, water, saturated solution of potassium hydrogen carbonate, and finally with water. The solution was dried over sodium sulfate and evaporated *in vacuo* to dryness. The crystalline residue was triturated with ether and collected by filtration. The yield was 3.2 g. (75%); m.p. 149°. After recrystallization from methanol, the melting point was raised to 156°; $[\alpha]^{25}D$ −70.2° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{21}H_{22}N_2O_6S$: C, 58.59; H, 5.15; N, 6.51; S, 7.45. Found: C, 58.61; H, 5.06; N, 6.35; S, 7.18.

Methanolysis.[4b] Into a suspension of 0.86 g. (0.002 mole) of XXXIII in 16 ml. of absolute methanol, 4.1 ml. of 0.5 *N* sodium methoxide was added with stirring and under hydrogen. After 2–3 min. the compound dissolved. The stirring was continued for 10 more min. Upon acidification with 2 ml. of acetic acid and titration with 0.1 *N* iodine solution 19 ml. of iodine solution (95% of the theoretical amount) was consumed. The mixture was concentrated *in vacuo* to remove the methanol. The residue was extracted into chloroform; the organic layer was washed successively with potassium hydrogen carbonate solution and water, dried over sodium sulfate, and evaporated *in vacuo* to dryness. The crystalline residue was triturated with petroleum ether, collected by filtration, and washed with a small amount of methanol to give 0.33 g. (70%) of N,N′-bisbenzoyl-L-cystine dimethyl ester (XXXIV); m.p. 174–175°, $[\alpha]^{25}D$ −230° (*c* 1, dimethylformamide); m.p. 178°, $[\alpha]^{25}D$ −231° (*c* 1, dimethylformamide) after recrystallization from ethanol (lit.[4a] m.p. 177–179°, $[\alpha]^{20}D$ −233° (*c* 1, dimethylformamide)).

*N-Carbobenzoxy-S-benzoyl-*L*-cysteine p-Nitrophenyl Ester.* To a cold solution of 1.8 g. (0.005 mole) of N-carbobenzoxy-S-benzoyl-L-cysteine[4b] and 0.7 g. of *p*-nitrophenol in 20 ml. of ethyl acetate, 1.1 g. of N,N′-dicyclohexylcarbodiimide was added.[29] The mixture was stirred for 30 min. at 0° and then allowed to stand overnight at room temperature. The precipitated N,N′-dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The crystalline residue was recrystallized from ethanol. The yield was 2.3 g. (95%); m.p. 128°, $[\alpha]^{22}D$ −55.0° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{24}H_{20}N_2O_7S$: N, 5.83; S, 6.67. Found: N, 5.66; S, 6.45.

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