Sulfur-Containing Polypeptides. VI. Stability Studies on Unsymmetrical Open-Chain Cystine Derivatives[1-3]

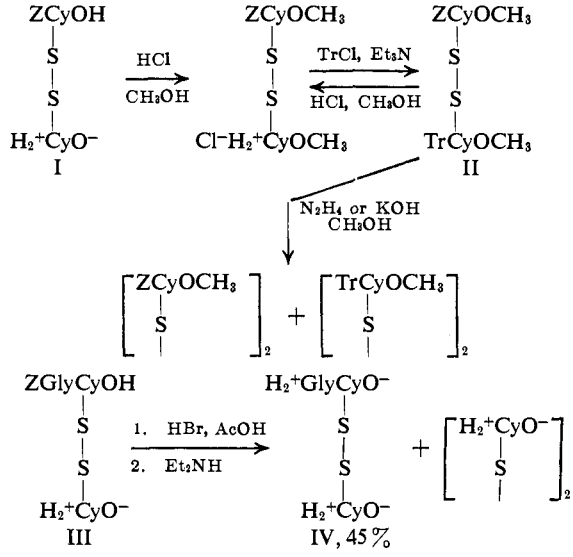
Richard G. Hiskey and Edward L. Smithwick, Jr.[4,5]

Contribution from the Venable Chemical Laboratory, The University of North Carolina, Chapel Hill, North Carolina. *Received August 22, 1966*

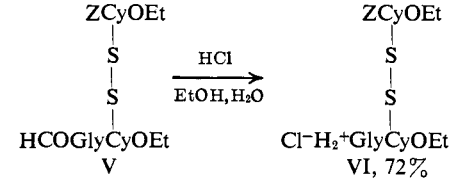
Abstract: The cleavage of amino and carboxyl protecting groups from unsymmetrical open-chain cystine derivatives has been studied. Boron trifluoride in acetic acid has been found to cleave *t*-butyl and benzhydryl esters as well as the N-benzhydryloxycarbonyl group without cleavage of the sulfur–sulfur bond. Using this technique N-benzhydryloxycarbonyl-L-valyl-S-(N′-carbobenzoxy-L-cysteinyl-L-valyl-L-alanylglycine benzhydryl ester)-L-cysteinylglycine benzyl ester has been selectively deblocked and cyclized to S,S′,N-carbobenzoxy-L-hemicystyl-L-valyl-L-alanylglycyl-L-valyl-L-hemicystylglycine benzyl ester. This conversion provides the first example of this synthetic route to cyclic cystine peptides.

The availability of a general method of preparation of unsymmetrical open-chain cystine derivatives[6] permits a study of the chemical properties of these compounds. Of particular importance is information regarding the stability of the sulfur–sulfur bond under conditions required for removal of amino or carboxyl protecting groups elsewhere in the molecule.

Earlier studies have established that unsymmetrical open-chain cystine derivatives are rapidly rearranged under a variety of conditions to equilibrium mixtures of the corresponding symmetrical cystine derivatives. Ryle and Sanger,[7] and subsequently Benesch and Benesch,[8] studied the acid-catalyzed disulfide interchange of mono-N-2,4-dinitrophenyl-L-cystine. In a more detailed investigation, Zervas, *et al.*,[9] examined the stability of mono-N-carbobenzoxy-L-cystine (I) and mono-N-(N-carbobenzoxyglycyl)-L-cystine (III). Esterification of I and tritylation of the resulting ester did not effect the disulfide bond; the N-trityl group could be cleaved with methanolic hydrogen chloride without disulfide interchange. However, attempts to remove the methyl ester of II with either hydrazine or potassium hydroxide in methanol gave only mixtures of the symmetrical disulfides. Treatment of I with aqueous diethylamine also induced disulfide interchange although I appeared to be stable at pH 6.5. Although removal of the N-carbobenzoxy group by treatment of III with 22% hydrogen bromide in acetic acid was reported to provide monoglycyl-L-cystine (IV) in 45% yield, small



amounts of cystine were also isolated, suggesting disulfide interchange. Recently Rydon and dos S. P. Serrão[10] have reported that all attempts to cleave the N-carbobenzoxy group of V using hydrogen bromide in a variety of solvents led to mixtures of disulfides. Treatment of V with ethanolic hydrochloric acid resulted in cleavage of the N-formyl group and provided a 72% yield of the unsymmetrical disulfide, VI. From these results it is apparent that protective groups which require strongly acidic or alkaline conditions for removal cannot be utilized with unsymmetrical open-chain cystine derivatives. Unfortunately, the N-trityl and N-formyl groups, shown to be compatible with unsymmetrical disulfides, are of only limited utility in peptide synthesis; thus a more complete evaluation of other amino and carboxyl protecting groups that could be used in conjunction with unsymmetrical cystine derivatives was required.



(1) Part V of this series: R. G. Hiskey and G. L. Southard, *J. Org. Chem.*, **31**, 3582 (1966).

(2) Supported by Grants A-3416 from the National Institute of Arthritis and Metabolic Diseases and RG-7966 from the National Institute of General Medical Sciences of the National Institutes of Health, U. S. Public Health Service.

(3) The following abbreviations have been incorporated into the text: Z, carbobenzoxy; Tr, trityl; WSC, 1-ethyl-3-(3-N,N-dimethylaminopropyl)carbodiimide hydrochloride; HDEA⁺, N,N-diethylammonium; Bzh, benzhydryl; DCHA⁺, N,N-dicyclohexylammonium; *o*-NPS, *o*-nitrophenylsulfenyl; Bz, benzyl; THF, tetrahydrofuran; DMF, N,N-dimethylformamide; DCC, N,N′-dicyclohexylcarbodiimide; BhOC, benzhydryloxycarbonyl.

(4) U. S. Public Health Predoctoral Fellow, 1962–1965.

(5) Abstracted in part from the dissertation of E. L. Smithwick, Jr. submitted to the University of North Carolina in partial fulfillment of the requirements for the Ph.D. degree, June 1966.

(6) R. G. Hiskey, T. Mizoguchi, and E. L. Smithwick, Jr., *J. Org. Chem.*, **32**, 97 (1967).

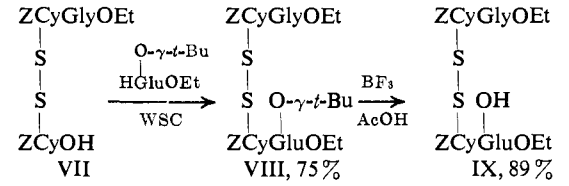
(7) A. P. Ryle and F. Sanger, *Biochem. J.*, **60**, 535 (1955).

(8) R. E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, **80**, 1666 (1958).

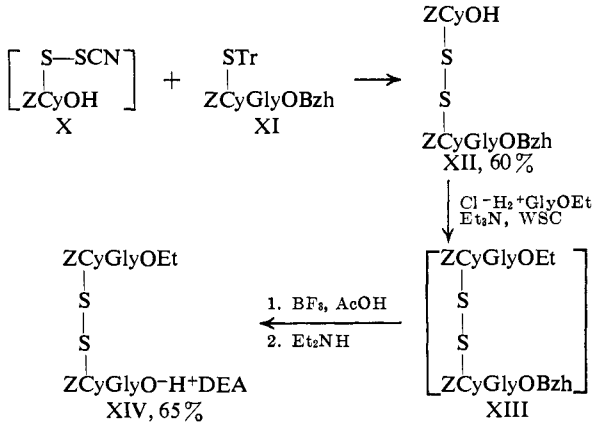
(9) L. Zervas, L. Benoiton, E. Weiss, M. Winitz, and J. P. Greenstein, *ibid.*, **81**, 1729 (1959).

(10) H. N. Rydon and F. O. dos S. P. Serrão, *J. Chem. Soc.*, 3638 (1964).

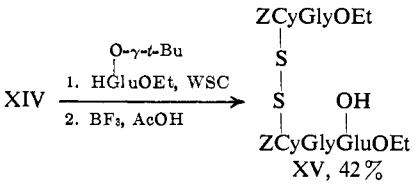
Our initial considerations were centered on the hydrolysis conditions for acid-sensitive esters. Earlier experiments had established that the *t*-butyl and benzhydryl esters were of similar acid lability[11] and, in more recent studies, general conditions for the acid hydrolysis of the *t*-butyl esters of fully protected cysteine derivatives were devised.[12] Although the use of boron trifluoride in glacial acetic acid was without effect on the S-trityl group,[12] no data were available on the effect of these reagents on unsymmetrical disulfides. In order to obtain a substrate which would permit a test of these conditions, N,N-dicarbobenzoxy-L-cystinyl monoglycine ethyl ester (VII) was coupled with L-glutamic acid α-ethyl ester γ-*t*-butyl ester; 1-ethyl-3-(3-N,N-dimethylaminopropyl)carbodiimide hydrochloride (WSC) was the coupling agent of choice. The product of the reaction, N-carbobenzoxy-S-(N′-carbobenzoxy-L-cysteinylglycine ethyl ester)-L-cysteinyl-L-glutamic acid α-ethyl ester γ-*t*-butyl ester (VIII) was obtained in 75% yield. Treatment of VIII with boron trifluoride



in acetic acid provided the acid IX in 89% yield. The structure of the reaction product was demonstrated by the elemental analysis and homogeniety of the reaction product (tlc). No evidence of disulfide interchange could be detected in the crude reaction mixture. A similar evaluation of the benzhydryl ester was obtained from the following sequence. The desired substrate, N-carbobenzoxy-S-(N′-carbobenzoxy-L-cysteine)-L-cysteinylglycine benzhydryl ester (XII) was prepared by the action of the sulfenyl thiocyanate, X, on benzhydryl N-carbobenzoxy-S-trityl-L-cysteinylglycinate (XI). The disulfide, XII, obtained in 60% yield, was coupled with ethyl glycinate to provide the diester, XIII, in 79% crude yield. Treatment of XIII

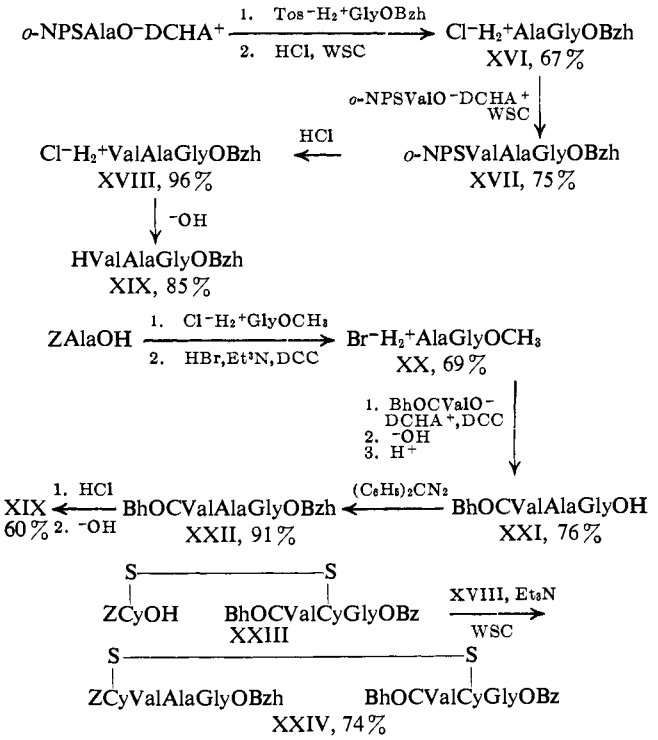


with boron trifluoride in acetic acid provided N-carbobenzoxy-S-(N′-carbobenzoxy-L-cysteinylglycine ethyl ester)-L-cysteinylglycine N,N-diethylamine salt (XIV) in 65% yield. The acid, XIV, was also coupled with L-glutamic acid α-ethyl ester γ-*t*-butyl ester; the resulting crude triester was hydrolyzed under the previous conditions to afford N-carbobenzoxy-S-(N′-carbobenzoxy-L-cysteinylglycine ethyl ester)-L-cysteinylglycyl-L-glutamic acid α-ethyl ester (XV), in 42% yield.



The acid-catalyzed removal of a suitable N-protective group was then considered. When VII was treated with liquid hydrogen bromide[13] or trifluoroacetic acid in the presence or absence of carbonium ion scavengers, mixtures of disulfides were obtained. In view of these results and the data of others on cleavage of the N-carbobenzoxy group, the N-benzhydryloxycarbonyl group[11] was considered for use with unsymmetrical cystine derivatives. In order to test the ease of removal of the N-benzhydryloxycarbonyl group, N-benzhydryloxycarbonyl-L-valyl-S-(N′-carbobenzoxy-L-cysteinyl-L-valyl-L-alanylglycine benzhydryl ester)-L-cysteinylglycine benzyl ester (XXIV) was prepared. The synthesis of XXIV (Chart I) utilized the unsym-

Chart I. Synthesis of XXIV

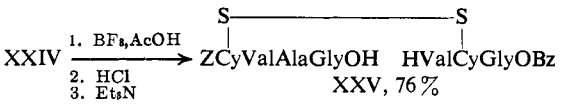


metrical cystine derivative, XXIII, previously prepared.[6] The remaining portion of the molecule, benzhydryl L-valyl-L-alanylglycinate (XIX), was synthesized by two routes. The sequence of choice involved the selective hydrolysis of the *o*-nitrophenylsulfenyl group in the presence of the benzhydryl ester. The alternate route to XIX utilized the selective cleavage of the N-benzhydryloxycarbonyl group from benzhydryl N-benzhydryloxycarbonyl-L-valyl-L-alanylglycinate (XXII). The properties of the tripeptide esters obtained by either

(11) R. G. Hiskey and J. B. Adams, Jr., *J. Am. Chem. Soc.*, 87, 3969 (1965).
(12) R. G. Hiskey and J. B. Adams, Jr., *J. Org. Chem.*, 31, 2178 (1966).
(13) M. Brenner and H. C. Curtius, *Helv. Chim. Acta*, 46, 2126 (1963).

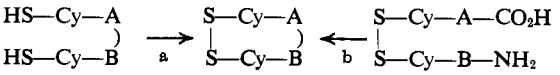
route were identical. The coupling reaction between XIX and XXIII proceeded smoothly using 1-ethyl-3-(3-N,N-dimethylaminopropyl)carbodiimide hydrochloride; the desired heptapeptide, XXIV, was obtained in 74% yield.

Treatment of XXIV with boron trifluoride, under the previous conditions, provided a salt which was converted to the hydrochloride derivative. Neutralization of the hydrochloride afforded a 76% yield of XXV. The substance was homogeneous on paper chromatography and high-voltage paper electrophoresis. Elemental and amino acid analysis were in agreement with the structural assignment of L-valyl-S-(N'-carbobenzoyl-L-cysteinyl-L-valyl-L-alanylglycine)-L-cysteinylglycine benzyl ester (XXV). These data indicate that both the N-benzhydryloxycarbonyl group and the benzhydryl ester can be removed without fission of the

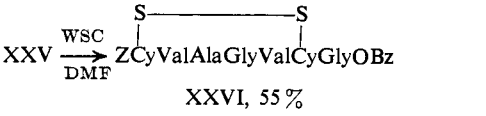


sulfur–sulfur bond using boron trifluoride.

With the availability of XXV another facet of the reactions of unsymmetrical open-chain cystine derivatives was investigated. The usual approach to the synthesis of cyclic cystine peptides, *e.g.*, oxytocin, involves the preparation of a linear peptide containing two S-protected cysteine residues. Removal of the S-protective groups and finally oxidation of the resulting thiols provide the cyclized cystine peptide (path a). An alternate approach (path b) to cyclic cystine derivatives would require the initial formation of the disulfide bond and ring closure by intramolecular peptide bond formation in the penultimate step. The various synthetic approaches to oxytocin have recently been discussed by Photaki[14] who concludes that the route to the hormone involving cyclization of an unsymmetrical open-chain cystine peptide is not feasible.



In order to provide a synthetic precedent for the formation of cystine "loops" by cyclization of suitably deblocked unsymmetrical open-chain cystine derivatives (path b), the conversion of XXV to S,S',N-carbobenzoxy-L-hemicystyl-L-valyl-L-alanylglycyl-L-hemicystylglycine benzyl ester (XXVI) was attempted. Treatment of a suspension of XXV in DMF with 1 equiv of WCS provided the pure cyclic disulfide, XXVI,



in 55% yield. The substance was characterized by elemental and amino acid analysis and molecular weight data. In accounting for the relatively high yields of XXVI (crude yields of 80% were consistantly obtained) it should be noted that, while the maximum concentration of XXV was 0.1 mole/l., the actual concentration was much lower because of the low solubility of XXV. The high dilution undoubtedly favored intramolecular cyclization. In addition, the data of Rydon, *et al.*[15] indicates that "loop" formation is maximized when the cysteine residues are separated by at least four amino acid residues. The cyclization is also probably enhanced by conformational control of this type.

(14) I. Photaki, *J. Am. Chem. Soc.*, 88, 2292 (1966).

(15) H. N. Rydon and J. Savrda, *J. Chem. Soc.*, 4246 (1965), and earlier references cited.

Experimental Section[16]

Attempted Decarbobenzoxylations of N,N-Dicarbobenzoxy-L-cystinemonoglycine Ethyl Ester (VII). A. Liquid Hydrogen Bromide. To 1.0 g (0.0017 mole) of VII in a dry flask immersed in a Dry Ice–acetone bath was added dry bromine-free hydrogen bromide until about 10–15 ml of the gas liquefied. The peptide derivative slowly dissolved; after 30 min the hydrogen bromide was allowed to evaporate under a stream of dry nitrogen. The solid residue was triturated with dry ether and dried *in vacuo* over sodium hydroxide. Paper chromatography of the solid (system A) indicated three ninhydrin-positive spots. Electrophoresis, conducted in pH 6.5 pyridine–acetic acid–water buffer at 1500 v for 3 hr, disclosed two ninhydrin-positive spots migrating toward the cathode and one spot at the origin.

B. Liquid Hydrogen Bromide–Ethyl Mercaptan. A mixture of 0.5 g of VII and 0.2 ml of ethyl mercaptan was treated with liquid hydrogen bromide. Paper chromatography of the residue indicated four ninhydrin-positive components were present.

C. Trifluoroacetic Acid. A 10-mg sample of VII was dissolved in 2 ml of dry, distilled trifluoroacetic acid and incubated at 38°. At various times in separate experiments the trifluoroacetic acid was evaporated and the residue dried and diluted with 1 ml of methanol. After 1 hr, tlc (system A, organic layer) revealed five ninhydrin-positive spots. After 12 hr three ninhydrin-positive spots were present. A paper chromatogram run after 7 hr indicated the presence of three ninhydrin-positive components.

D. Trifluoroacetic Acid–Ethyl Mercaptan. Paper chromatography of the product obtained after 12-hr incubation of VII with a trifluoroacetic acid–ethyl mercaptan mixture indicated the presence of four ninhydrin-positive components.

E. Trifluoroacetic Acid–Catechol. Paper chromatography of the product resulting from 12-hr incubation of VII with trifluoroacetic acid containing catechol indicated the presence of three ninhydrin-positive components.

Hydrolysis of N-Carbobenzoxy-S-(N'-carbobenzoxy-L-cysteinylglycine ethyl ester)-L-cysteinyl-L-glutamic Acid α-Ethyl Ester γ-*t*-Butyl Ester (VIII). To a solution of 1.21 g (0.0015 mole) of VIII[6] in 10 ml of acetic acid was added 2.10 ml (0.015 mole) of boron trifluoride etherate. The reaction mixture was allowed to stand at 40° for 1 hr and was then treated with 3.7 g of sodium acetate and 200 ml of water. The crude product was collected and recrystallized from ethyl acetate–hexane to afford 1.0 g (89%) of IX, mp 151–153°, $[\alpha]^{27}D$ −121.3° (*c* 0.91, DMF).

Anal. Calcd for $C_{33}H_{42}N_4O_{12}S_2$: C, 52.79; H, 5.64; N, 7.48; S, 8.55. Found: C, 53.24; H, 5.86; N, 7.60; S, 8.48.

Preparation of Benzhydryl N-Carbobenzoxy-S-trityl-L-cysteinylglycinate (XI). To a cold stirred suspension of 4.97 g (0.01 mole) of N-carbobenzoxy-S-trityl-L-cysteine and 4.14 g (0.01 mole) of benzhydryl glycinate *p*-toluenesulfonate in 20 ml of methylene chloride was added 1.39 ml (0.01 mole) of triethylamine and 2.25 g (0.011 mole) of DCC. Stirring was continued for 1 hr at ice temperature and for 3 hr at room temperature. After the addition of 10 drops of 50% acetic acid and stirring for 10 min, the reaction mixture was filtered and the filtrate concentrated *in vacuo*. The residue was triturated with ethyl acetate and filtered, and the filtrate was extracted successively with 0.5 *N* sodium bicarbonate, 5% hydrochloric acid, and aqueous, saturated sodum chloride solution. The ethyl acetate solution was dried and concentrated *in vacuo*, and *n*-hexane added to the cloud point. The product, 6.3 g (89%), appeared as a white solid, mp 140.5–142° after one recrystallization from ethyl acetate–hexane; $[\alpha]^{27}D$ +10.86° (*c* 1.1, $CHCl_3$).

(16) Melting points are uncorrected. Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Ill., and Crobaugh Laboratories, Charleston, W. Va. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter using a glass cell.

Ascending paper chromatograms were performed on Whatman No. 1 paper. The solvent systems employed were 1-butanol–acetic acid–water (4:1:5), system A; and 1-butanol–acetic acid–water (12:3:5), system B. Thin layer chromatograms were performed on microscope slides coated with silica gel G or GF_{254}. High-voltage electrophoresis was conducted using an Ensco constant-current apparatus. Amino acids were of C.P. or M.A. quality and were obtained from the Mann Research Laboratories, New York, N. Y.

Anal. Calcd for $C_{45}H_{40}N_2O_5S$: C, 75.00; H, 5.58; N, 3.90; S, 4.45. Found: C, 74.99; H, 5.71; N, 4.14; S, 4.27.

Preparation of N-Carbobenzoxy-S-(N′-carbobenzoxy-L-cysteine)-L-cysteinylglycine Benzhydryl Ester (XII). To a cold, stirred solution of 0.0042 mole of thiocyanogen in 35 ml of methylene chloride was added 1.02 g (0.004 mole) of N-carbobenzoxy-L-cysteine during 2.5 hr, followed by the addition of 2.83 g (0.004 mole) of benzhydryl N-carbobenzoxy-S-trityl-L-cysteinylglycinate (XI) in 25 ml of methylene chloride. Stirring was continued for 5 hr at room temperature, and the reaction mixture was filtered and concentrated *in vacuo*. The concentrate was diluted to 250 ml with ethyl acetate, and the solution was washed with an aqueous solution containing 0.0035 mole of sodium bicarbonate and with aqueous, saturated sodium chloride until the washed liquid gave a negative thiocyanate test with ferric chloride. The ethyl acetate solution was dried, treated with activated carbon, and filtered, and the solvent evaporated *in vacuo*. Trituration of the residue with ether, to remove the trityl thiocyanate, afforded 2.12 g (74%) of material shown by tlc to consist of two compounds. Purification of the material by gradient-elution chromatography on silicic acid (5 to 15% dioxane in methylene chloride) afforded 1.75 g (60%) of XII, mp 136–138°, $[\alpha]^{27}D$ −133.9° (*c* 1.0, DMF).

Anal. Calcd for $C_{37}H_{37}N_3O_9S_2$: C, 60.65; H, 5.10; N, 5.75; S, 8.77. Found: C, 60.35; H, 5.13; N, 5.74; S, 9.10.

Preparation of N-Carbobenzoxy-S-(N′-carbobenzoxy-L-cysteinylglycine ethyl ester)-L-cysteinylglycine N,N-Diethylamine Salt (XIV). To a solution of 4.03 g (0.0055 mole) of XII and 0.80 g (0.00575 mole) of glycine ethyl ester hydrochloride in 15 ml of DMF and 15 ml of methylene chloride was added 0.80 ml (0.00575 mole) of triethylamine. The stirred solution was cooled to − 10° and 1.10 g (0.00575 mole) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride was added. Stirring was continued for 1 hr at −10° and for 12 hr at room temperature. The reaction mixture was diluted to 100 ml with aqueous, saturated sodium chloride and the methylene chloride evaporated *in vacuo*. The crude reaction product was filtered from the DMF–water solution, dissolved in hot methanol, and reprecipitated with cold 2 *N* sulfuric acid. This procedure was repeated until tlc indicated that no N-acylurea remained. The product was dried *in vacuo*, dissolved in ethyl acetate, and reprecipitated with *n*-hexane to afford 3.55 g (79%) of XIII, mp 115–117°. To a solution of 3.02 g (0.0037 mole) of crude XIII in 20 ml of glacial acetic acid was added 2.60 ml (0.0185 mole) of boron trifluoride etherate. The solution was allowed to stand at 40° for 0.5 hr, then 0.60 g (0.0555 mole) of sodium acetate and 400 ml of water were added to the reaction mixture. After standing at 10° for several hours, the precipitate was collected and dissolved in ethyl acetate. The ethyl acetate solution was dried, filtered, and concentrated to 100 ml, and 200 ml of *n*-hexane was added to precipitate the crude product (shown by tlc to contain some starting material). The impure material was dissolved in 30 ml of acetone and treated with 0.27 g (0.0037 mole) of diethylamine. Addition of 30 ml of ether and collection of the precipitate afforded 1.74 g (65%) of pure acid, mp 151–153°, $[\alpha]^{27}D$ −129.1° (*c* 1.1, DMF). Addition of *n*-hexane to the filtrate afforded 0.61 g of material shown by tlc to contain some starting material.

Anal. Calcd for $C_{32}H_{45}N_3O_{10}S_2$: C, 53.10; H, 6.26; N, 9.69; S, 8.89. Found: C, 53.21; H, 6.40; N, 9.65; S, 9.20.

Preparation of N-Carbobenzoxy-S-(N′-carbobenzoxy-L-cysteinylglycine ethyl ester)-L-cysteinylglycyl-L-glutamic Acid α-Ethyl Ester (XV). A suspension of 1.0 g (0.00138 mole) of XIV in 100 ml of ethyl acetate wash was shaken with 2 *N* sulfuric acid until solution occurred. The resulting solution was extracted with water, dried, filtered, and concentrated *in vacuo* to a thick oil. The oil was dissolved in 10 ml of DMF and 0.0014 mole of freshly prepared L-glutamic acid α-ethyl ester γ-*t*-butyl ester in 10 ml of methylene chloride was added. The solution was cooled to 0° and 0.267 g (0.0014 mole) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride added. Stirring was continued for 1 hr at 0° and for 12 hr at room temperature. The reaction mixture was diluted with aqueous, saturated sodium chloride solution, and the methylene chloride was evaporated *in vacuo*. The DMF–water solution was decanted, and the residual oil was dissolved in methanol and reprecipitated with cold 2 *N* sulfuric acid. The solid material was dissolved in ethyl acetate, and the ethyl acetate solution was extracted successively with 2 *N* sulfuric acid, 0.5% sodium bicarbonate, and water. Evaporation *in vacuo* of the dried ethyl acetate solution afforded a lightly yellow product, shown by tlc to consist of two components. The crude product was dissolved in 10 ml of glacial acetic acid and treated with 1.95 ml (0.0138 mole) of boron trifluoride etherate. The reaction mixture was allowed to stand at room temperature for 1 hr and diluted to 200 ml with water, and the resulting solid was dried *in vacuo* over phosphorus pentoxide. The crude acid was dissolved in 3 ml of pyridine and diluted to 100 ml with water. The precipitate was collected, dissolved in ethyl acetate, and reprecipitated with hexane to afford 0.470 g (42%) of XV as a white powder; chromatographically uniform; $[\alpha]^{27}D$ −123.5° (*c* 0.58, DMF).

Anal. Calcd for $C_{35}H_{45}N_3O_{13}S_2$: C, 52.02; H, 5.63; N, 8.68; S, 7.95. Found: C, 52.15; H, 5.77; N, 8.56; S, 7.95.

Preparation of Benzhydryl L-Alanylglycinate Hydrochloride (XVI). A suspension of 6.35 g (0.015 mole) of *o*-nitrophenylsulfenyl-L-alanine N,N-dicyclohexylamine salt[17] and 6.40 g (0.0155 mole) of benzhydryl glycinate toluenesulfonate in 20 ml of methylene chloride was stirred for 10 min and cooled to −10° and 2.96 g (0.0155 mole) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride added. Stirring was continued for 1 hr at −10° and for 12 hr at room temperature. The reaction mixture was filtered, and the filtrate was diluted to 250 ml with ethyl acetate. The resulting solution was extracted successively with cold 2 *N* sulfuric acid, 1 *N* sodium hydroxide, and water, and dried. Evaporation of the solvent *in vacuo* afforded 6.8 g (97% theory) of a yellow oil which could not be crystallized but was homogeneous on tlc. The oil was dissolved in 50 ml of ethyl acetate and 14 ml of 3 *N* hydrogen chloride in ether was added, followed by 200 ml of anhydrous ether. The precipitate was collected and recrystallized several times from ethyl acetate–ether–hexane to afford 4.5 g (67%) of XXI, mp 53–60°, $[\alpha]^{27}D$ −1.5° (*c* 0.6, MeOH).

Anal. Calcd for $C_{18}H_{21}ClN_2O_3$: C, 62.00; H, 6.06; N, 8.06; Cl, 10.10. Found: C, 61.23; H, 6.17; N, 7.76; Cl, 10.15.

Preparation of Benzhydryl N-(*o*-Nitrophenylsulfenyl)-L-valyl-L-alanylglycinate (XVII). To a cold stirred solution of 5.10 g (0.0114 mole) of N-(*o*-nitrophenylsulfenyl)-L-valine N,N-dicyclohexylamine salt[17] and 5.10 g (0.0114 mole) of XVI in 50 ml of methylene chloride was added 2.30 g (0.012 mole) of 1-ethyl-3-(3-N,N-dimethylaminopropyl)carbodiimide hydrochloride. Stirring was continued for 18 hr. Sufficient methylene chloride was added to dissolve the product, and the insoluble N,N-dicyclohexylammonium hydrochloride was removed by filtration. The filtrate was extracted with cold 2 *N* sulfuric acid, water, and aqueous, saturated sodium chloride solution. The methylene chloride was evaporated *in vacuo* and the reaction product was crystallized from tetrahydrofuran–ether to afford 4.85 g (75%) of XVII, mp 191–192°, $[\alpha]^{27}D$ −81.56° (*c* 0.9, $CHCl_3$).

Anal. Calcd for $C_{29}H_{32}N_4O_6S$: C, 61.79; H, 5.27; N, 9.93; S, 5.70. Found: C, 61.97; H, 5.61; N, 9.64; S, 5.97.

Preparation of Benzhydryl L-Valyl-L-alanylglycinate Hydrochloride (XVIII). To a solution of 2.85 g (0.005 mole) of XVII in a mixture of 25 ml of ethyl acetate and 50 ml of THF was added 4.7 ml of 3.2 *N* hydrogen chloride in ether. After standing 2 min at room temperature, the reaction mixture was poured slowly and with vigorous stirring into 300 ml of anhydrous ether. After standing at 10° for 1 hr, the product was collected by filtration. The yield of XVIII was 2.15 g (96%) after two reprecipitations from THF with ether; mp 103–108°; chromatographically uniform.

Anal. Calcd for $C_{23}H_{30}ClN_3O_4$: C, 61.65; H, 6.76; N, 9.39; Cl, 7.92. Found: C, 60.43; H, 6.84; N, 9.03; Cl, 7.70.

Better analytical results were obtained on a sample of the free base, XIX. The substance was prepared by dissolving 0.448 g (0.001 mole) of the hydrochloride salt in 50 ml of 5% sodium bicarbonate solution and then extraction with ethyl acetate. The organic layer was dried and evaporated *in vacuo* to 25 ml, and XIX was precipitated by addition of *n*-hexane to yield 0.350 g (85% conversion) of the free amine, mp 112–114°, $[\alpha]^{27}D$ −73.2° (*c* 0.25, $CHCl_3$).

Anal. Calcd for $C_{23}H_{29}N_3O_4$: C, 67.00; H, 7.10; N, 10.20. Found: C, 67.13; H, 7.28; N, 10.49.

Preparation of Methyl L-Alanylglycinate Hydrobromide (XX). To a suspension of 13.4 g (0.06 mole) of N-carbobenzoxy-L-alanine and 7.65 g (0.61 mole) of methyl glycinate hydrochloride in 50 ml of chloroform was added 8.5 ml (0.061 mole) of triethylamine. The mixture was stirred for 5 min at room temperature, then cooled to −10°, and 12.5 g (0.061 mole) of DCC was added. Stirring was continued for 1 hr at −10° and for 8 hr at 10°. The reaction mixture was filtered, and the filtrate was diluted with 150 ml of ethyl acetate. The organic layer was extracted successively with

(17) L. Zervas, D. Borovas, and E. Gazis, *J. Am. Chem. Soc.*, **85**, 3660 (1963).

water, 2 *N* sulfuric acid, 5% sodium bicarbonate solution, and aqueous, saturated sodium chloride. The solvent was removed *in vacuo* and the residue dissolved in acetone and cooled. A small amount of DCU was removed by filtration and the acetone evaporated *in vacuo*. Crystallization of the residue from ethyl acetate–hexane afforded 12.3 g (79%) of methyl N-carbobenzoxy-L-alanylglycinate; mp 96–97°.

To 8.0 g (0.0272 mole) of methyl N-carbobenzoxy-L-alanylglycinate was added about 15–20 ml of dry, bromine-free, liquid hydrogen bromide. The resulting solution was allowed to stand for 25 min at −70°, and the hydrogen bromide was allowed to evaporate as the reaction vessel was flushed with dry nitrogen. The residue was triturated with ether under a dry nitrogen stream, filtered, and dried *in vacuo* over sodium hydroxide. The yield of XX was 6.50 g (69% over-all), mp 159–161°, $[\alpha]^{27}D$ +7.73° (*c* 1.4, MeOH).

Anal. Calcd for $C_6H_{13}BrN_2O_3$: C, 29.90; H, 5.46; N, 11.62; Br, 32.95. Found: C, 30.21; H, 5.46; N, 11.46; Br, 33.21.

Preparation of Benzhydryl N-Benzhydryloxycarbonyl-L-valyl-L-alanylglycinate (XXII). A stirred suspension of 5.08 g (0.01 mole) of N-benzhydryloxycarbonyl-L-valine N,N-dicyclohexylamine salt[6] and 2.42 g (0.01 mole) of methyl L-alanylglycinate hydrobromide in 20 ml of methylene chloride was cooled to −10° and treated with 2.10 g (0.0104 mole) of DCC. After 12 hr the reaction mixture was filtered, the insoluble residue was extracted with chloroform, and the extractions and the filtrate were combined. About 100 ml of ethyl acetate was added and the resulting solution was extracted successively with 2 *N* sulfuric acid, 1 *N* sodium bicarbonate, and water, and dried. Evaporation of the solvent *in vacuo* afforded a white crystalline residue which was suspended in 15 ml of cold dioxane. To the suspension was added 10.0 ml of 1 *N* sodium hydroxide; solution was complete in 10 min but stirring was continued for an additional 10 min. The hydrolysis mixture was acidified and extracted with chloroform. The chloroform extract was washed with water and dried, and the solvent was evaporated *in vacuo*. Recrystallization of the residue from ethyl acetate–chloroform–hexane afforded 3.45 g (76%) of XXI, mp 180–182°.

To a solution of 2.28 g (0.005 mole) of N-benzhydryloxycarbonyl-L-alanylglycine (XXI) in 100 ml of acetone was added 0.006 mole of diphenyldiazomethane. After gently refluxing overnight, concentrated hydrochloric acid was added dropwise to destroy the excess diphenyldiazomethane. Evaporation of the solvent and recrystallization of the residue afforded 2.8 g (91%) of XXII, mp 188–189°, $[\alpha]^{27}D$ −28.5° (*c* 0.62, $CHCl_3$).

Anal. Calcd for $C_{37}H_{39}N_3O_6$: C, 71.50; H, 6.44; N, 6.75. Found: C, 71.49; H, 6.34; N, 6.75.

Preparation of L-Valyl-L-alanylglycine Benzhydryl Ester (XIX). A mixture of 0.310 g (0.5 mmole) of XXII and 0.5 mole of anhydrous oxalic acid dissolved in 10 ml of 2 *N* HCl in ether–THF was kept at 50° for 1 hr, then added to 200 ml of *n*-hexane. After standing at 0° for several hours, the precipitate was collected and redissolved by partition between ethyl acetate and 5% sodium hydroxide. The ethyl acetate extract was dried and concentrated to about 25 ml. *n*-Hexane was added to the cloud point, and the product was collected to yield 0.123 g (60%) of XIX, mp 112–114°. The preparation was identical with the sample obtained by the alternate route.

Preparation of N-Benzhydryloxycarbonyl-L-valyl-S-(N′-carbobenzoxy-L-cysteinyl-L-valyl-L-alanylglycine benzhydryl ester)-L-cysteinylglycine Benzyl Ester (XXIV). A stirred solution of 1.66 g (0.002 mole) of XXIII, 0.940 g (0.00205 mole) of XVIII, and 0.289 ml (0.00205 mole) of triethylamine in 25 ml of methylene chloride and 5 ml of DMF was cooled to −10° and 0.420 g (0.0022 mole) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride added. After 12 hr the reaction mixture was added to ethyl acetate, and the suspension was extracted with water and 2 *N* sulfuric acid. The suspension was filtered and the residue triturated thoroughly with methanol. The methanol-insoluble residue was dissolved in warm acetic acid and reprecipitated with ether to afford 1.85 g (74%) of XXIV, mp 239–240°, $[\alpha]^{27}D$ −68.2° (*c* 0.47, DMF).

Anal. Calcd for $C_{65}H_{73}N_7O_{13}S_2$: C, 63.55; H, 6.28; N, 7.97; S, 5.23. Found: C, 63.42; H, 6.03; N, 7.87; S, 5.20.

Preparation of L-Valyl-S-(N′-carbobenzoxy-L-cysteinyl-L-valyl-L-alanylglycine)-L-cysteinylglycine Benzyl Ester (XXV). To a suspension of 0.614 g (0.0005 mole) of XXIV in 10 ml of glacial acetic acid was added 0.70 ml (0.005 mole) of boron trifluoride etherate. The reaction mixture was allowed to stand at 40° for 2.5 hr, then 1.26 g (0.0154 mole) of sodium acetate in 50 ml of distilled water was added. The reaction mixture was concentrated at 50° *in vacuo* to a solid residue which was triturated with water, filtered, and dried over P_2O_5 to afford 0.465 g of solid. This material was dissolved in 5 ml of warm acetic acid and 0.001 mole of hydrogen chloride in ether was added. The acetic acid–ether was evaporated *in vacuo* and the residue dried *in vacuo* to afford 0.470 g of solid. To a solution of 0.300 g (0.00034 mole) of the hydrochloride in DMF was added 0.00034 mole of triethylamine. The DMF solution was filtered and added dropwise to aqueous, saturated sodium chloride solution. The fine precipitate was collected, washed thoroughly with water and methanol, and dried over P_2O_5 to afford 0.220 g (76%) of XXV, mp 207–208°, $[\alpha]^{27}D$ −60.7° (*c* 0.46, DMF). Amino acid analysis yielded: Gly (1.94), Val (2.16), Ala (1.00), Cys (0.82).

Anal. Calcd for $C_{38}H_{53}N_7O_{11}S_2$: C, 53.82; H, 6.30; N, 11.55; S, 7.55. Found: C, 53.64; H, 6.59; N, 11.55; S, 7.74.

Preparation of S,S′-N-Carbobenzoxy-L-hemicystyl-L-valyl-L-alanylglycyl-L-valyl-L-hemicystylglycine Benzyl Ester (XXVI). To a stirred suspension of 0.085 g (0.1 mmole) of XXV in 100 ml of freshly distilled DMF and 25 ml of methylene chloride was added 0.020 g (0.105 mmole) of 1-ethyl-3-(3-N,N-dimethylaminopropyl)-carbodiimide hydrochloride. The suspension was stirred for 24 hr, solution being complete after about 7 hr. The reaction mixture was poured into aqueous, saturated sodium chloride solution and allowed to stand at 10° for several hours. The precipitate was collected, extracted with water, and dried *in vacuo*. The crude reaction product was dissolved in 10 ml of warm DMF, treated with 0.10 g of activated carbon, filtered, and reprecipitated with ether to afford, after drying over phosphorus pentoxide, 0.046 g (55%) of XXVI, mp 261–263°, $[\alpha]^{27}D$ −26.0° (*c* 0.5, DMF); mol wt calcd, 830; found, 776 (by the vapor pressure osmometric method in DMF at 100°). Amino acid analysis yielded: Gly (1.92), Val (2.17), Ala (1.00), Cys (0.81).

Anal. Calcd for $C_{38}H_{51}N_7O_{10}S_2$: C, 55.00; H, 6.20; N, 11.90; S, 7.75. Found: C, 54.76; H, 6.19; N, 11.64; S, 7.71.