Sulfur-Containing Polypeptides. IX. The Use of the S-Isobutyloxymethyl Protective Group[1-3]

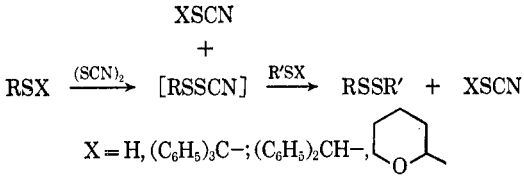
RICHARD G. HISKEY AND JAMES T. SPARROW[4]

Venable Chemical Laboratory, The University of North Carolina, Chapel Hill, North Carolina

Received December 17, 1968

The use of the S-isobutyloxymethyl group in the synthesis of various peptides containing cystine has been studied. The protective group can be used in conjunction with the N-trityl and N-phthaloyl amino protective groups and with *t*-butyl esters. The S-isobutyloxymethyl group is removed by the action of thiocyanogen or sulfenylthiocyanates and is similar to the S-trityl group in lability toward these reagents.

An early paper in this series[5] described the oxidation of S-trityl and S-benzhydryl thio ethers and S-2-tetrahydropyranyl hemithioacetals with thiocyanogen. The product of these oxidations was a sulfenylthiocyanate which could react further with thiols or thio ethers to provide unsymmetrical disulfides. This procedure appeared to hold considerable promise for the synthesis of

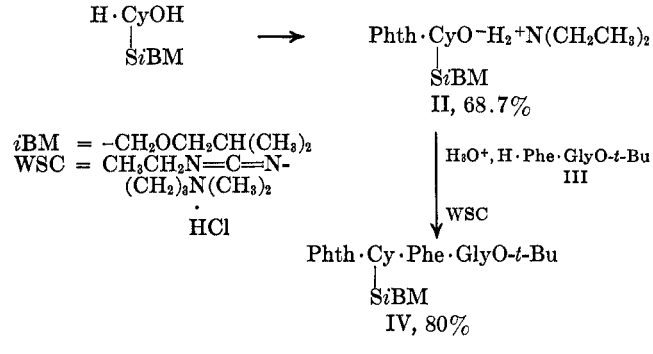


peptides containing several cystine residues. The advantage of this route was that the formation of the intermediate thiol was not required and a disulfide bridge could be produced in the presence of a preformed disulfide bond without the possibility of thiol–disulfide interchange.[6]

Unfortunately, the three protective groups originally considered for the thiocyanogen reaction suffer individual disadvantages. For example, S-trityl-L-cysteine derivatives are readily obtained but poorly crystalline. The S-benzhydryl derivatives are easily accessible and crystalline but severe conditions are required for removal of the group; *i.e.* refluxing trifluoroacetic acid to give the thiol[7] or trifluoroacetic acid–acetic acid (1:1 v/v) for oxidation with thiocyanogen or sulfenylthiocyanates.[6b] The 2-tetrahydropyranyl hemithioacetals of cysteine can be removed by the action of heavy metals[8] or thiocyanogen[5] but in our hands were frequently obtained in a noncrystalline condition or in very low yield, presumably because of the additional asymmetric center in the tetrahydropyran ring.

Several years ago Young, *et al.*,[9] reported the preparation of S-isobutyloxymethyl-L-cysteine (I), a hemithioacetal derivative containing a single asymmetric center. Young, *et al.*, demonstrated that I was stable to 2 *N* hydrochloric acid and 50% acetic acid but decomposed to some extent in 2 *N* sodium hydroxide. Rapid decomposition was also observed when I was treated with 2 *N* hydrogen bromide in acetic acid.

In order to evaluate the properties of the S-isobutyloxymethyl group more fully, we have conducted additional experiments with various derivatives of I. Treatment of I with N-ethoxycarbonylphthalimide in aqueous sodium carbonate–DMF[10] provided N-phthaloyl-S-isobutyloxymethyl-L-cysteine as the N,N-diethylammonium salt (II) in 68.7% yield. Liberation of the free acid from II and coupling with *t*-butyl L-phenylalanylglycinate (III) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide provided *t*-butyl N-phthaloyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (IV) in 80% yield. Qualitative stability studies on IV were conducted using tlc. The S-isobutyloxymethyl group appeared to be stable to warm aqueous acetic acid, 12 *N* hydrochloric acid in acetone (conditions for removal of an N-trityl group) and hydrazine hydrate in refluxing ethanol (the N-phthaloyl group of IV was removed under these conditions). Decomposition was noted and a reaction mixture positive to sodium nitroprusside resulted from treatment of IV with boron trifluoride etherate in acetic acid (conditions for hydrolysis of *t*-butyl esters), silver nitrate in ethanol, or trifluoroacetic acid.



In order to evaluate the reaction of the S-isobutyloxymethyl group with a sulfenylthiocyanate, IV was treated with the sulfenylthiocyanate obtained by the action of thiocyanogen on methyl N-carbobenzoxy-L-cysteinate (V). The resulting unsymmetrical disulfide, VI, was characterized by nmr spectrum and combustion analysis. Various ratios of acetic acid and ethyl ace-

(1) Part VIII of this series: R. G. Hiskey and R. L. Smith, *J. Amer. Chem. Soc.*, **90**, 2677 (1968).

(2) Supported by Grant A-3416 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

(3) The following abbreviations have been employed in the text: Z = carbobenzoxy; Phth = phthaloyl; *t*-Bu = *t*-butyl; *i*-BM = isobutyloxymethyl; Tr = trityl; Bzh = benzhydryl; DCC = N,N-dicyclohexylcarbodiimide; WSC = 1-ethyl-3-(3-*N*,*N*-dimethylaminopropyl)carbodiimide hydrochloride.

(4) Abstracted in part from a dissertation by J. T. Sparrow submitted to the University of North Carolina in partial fulfillment of the requirements for the Ph.D. degree, Aug 1968.

(5) R. G. Hiskey and W. P. Tucker, *J. Amer. Chem. Soc.*, **84**, 4794 (1962).

(6) (a) R. G. Hiskey and D. N. Harpp, *ibid.*, **87**, 3965 (1965); (b) R. G. Hiskey and M. A. Harpold, *Tetrahedron*, **23**, 3923 (1967).

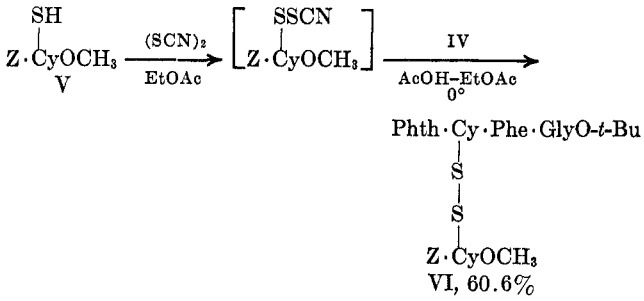
(7) L. Zervas and I. Photaki, *J. Amer. Chem. Soc.*, **84**, 3887 (1962).

(8) G. F. Holland and L. A. Cohen, *ibid.*, **80**, 3765 (1958).

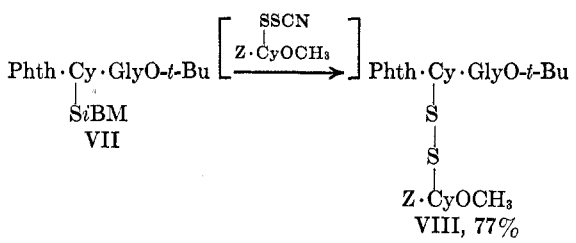
(9) G. T. Young, P. J. E. Brownlee, M. E. Cox, B. O. Handford, and J. C. Marsden, *J. Chem. Soc.*, 3832(1964).

(10) G. H. L. Nefkins, G. J. Tesser, and R. J. F. Nivard, *Rec. Trav. Chim. Pays-Bas*, **79**, 688 (1960).

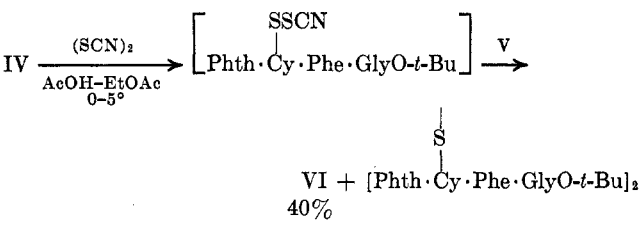
tate were employed; a 1:1 (v/v) mixture of the two solvents provided a 60.6% yield of VI. In these studies recovered IV was obtained from the reaction mixture; the recovery of IV may reflect the fact that optimium concentrations of IV and V were not employed in these



experiments. Indeed when *t*-butyl N-phthaloyl-S-isobutyloxymethyl-L-cysteinylglycinate (VII) was treated with the same sulfenylthiocyanate in more concentrated solution, the unsymmetrical disulfide, VIII, was obtained in 77% yield. No unreacted S-isobutyloxymethyl peptide was present.[11] The unsymmetrical disulfide VI was also prepared by an alternate route involving the action of thiocyanogen on IV. In this experiment VI was obtained in 40% yield, together with



10% of recovered IV and trace quantities of the symmetrical disulfide.

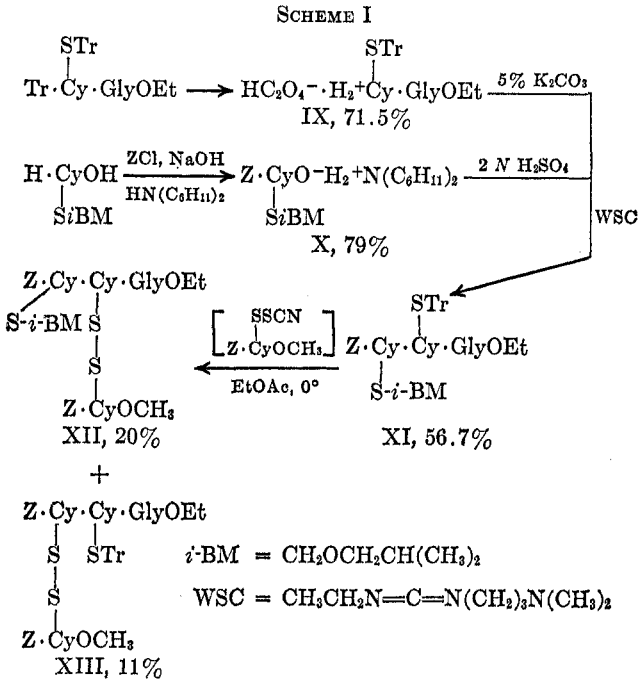


From previous studies[12] the relative reactivities of the thiol, S-trityl and S-benzhydryl groups toward thiocyanogen and sulfenylthiocyanates were found to be thiol = S-trityl > S-benzhydryl. In order to determine approximately the relative reactivity of the S-isobutyloxymethyl group, ethyl N-carbobenzoxy-S-isobutyloxymethyl-L-cysteinyl-S-trityl-L-cysteinylglycinate (XI) was synthesized as shown in Scheme I. Treatment of XI with the sulfenylthiocyanate of methyl L-cysteinate in ethyl acetate (conditions previously shown to lead to incomplete reaction) provided unreacted XI (26%) and two unsymmetrical disulfides, XII and XIII, in 20% and 11% yield. The identity of XII and XIII was confirmed by the nmr spectra and combustion analysis. These results indicate that although the reactivity of the S-trityl is somewhat greater toward thiocyanogen than the S-isobutyloxymethyl, the difference in reactivity is not sufficient to allow selective oxidation of the former in the presence of the latter.

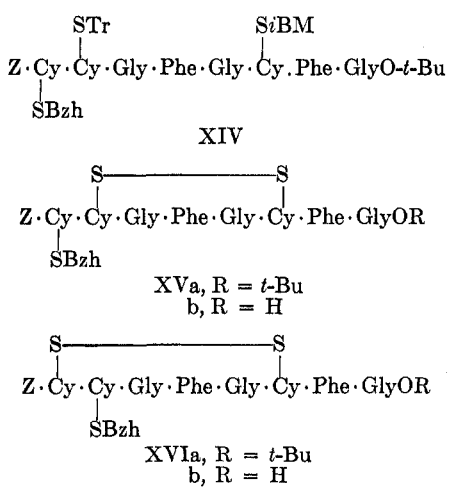
(11) These experiments were performed by Dr. G. M. Beverly.

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

SCHEME I



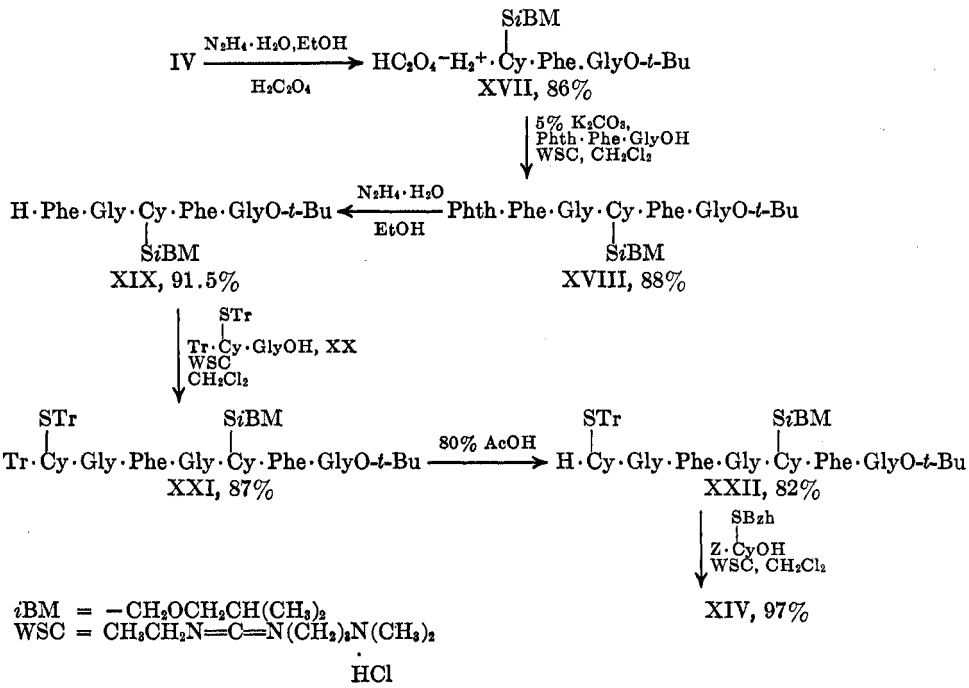
In order to evaluate the utility of the S-isobutyloxymethyl group, the octapeptide derivative, *t*-butyl N-carbobenzoxy-S-benzhydryl-L-cysteinyl-S-trityl-L-cysteinylglycyl-L-phenylalanylglycyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (XIV), was synthesized. The octapeptide derivative, XIV, was also of interest since it presumably could be oxidized to *t*-butyl N-carbobenzoxy-S-benzhydryl-L-cysteinyl-S,S′-hemicystylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycinate (XV) by the action of thiocyanogen. The oxidized peptide, XV, is isomeric with a previously prepared[1,13] octapeptide, XVI, and would allow a comparison of physical properties. The



synthetic route to XIV is outlined in Scheme II. Removal of the N-phthaloyl group from IV with hydrazine hydrate proceeded slowly in refluxing methanol to provide *t*-butyl S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate, isolated as the oxalate salt (XVII). Refluxing ethanol enhanced the rate of hydrazinolysis and was used in the later dephthaloylation of *t*-butyl N-phthaloyl-L-phenylalanylglycyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (XVIII). The

(13) R. G. Hiskey, J. T. Staples, and R. L. Smith, *ibid.*, **32**, 2772 (1967).

SCHEME II



*i*BM = $-CH_2OCH_2CH(CH_3)_2$
WSC = $CH_3CH_2N{=}C{=}N(CH_2)_3N(CH_3)_2 \cdot HCl$

free base, *t*-butyl L-phenylalanylglycyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (XIX), was coupled with N,S-ditrityl-L-cysteinylglycine (XX) using 1-ethyl-3-(3-N,N-dimethylaminopropyl)carbodiimide hydrochloride to provide *t*-butyl N,S-ditrityl-L-cysteinylglycyl-L-phenylalanylglycyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (XXI) in 86.7% yield. The N-trityl group was selectively removed using 80% aqueous acetic acid to provide *t*-butyl S-trityl-L-cysteinylglycyl-L-phenylalanylglycyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (XXII) in 82% yield. Treatment of XXII with N-carbobenzoxy-S-benzhydryl-L-cysteine and 1-ethyl-3(3-N,N-dimethylaminopropyl)carbodiimide hydrochloride afforded XIV. The S-isobutyloxymethyl-L-cysteine derivatives were readily isolable, crystalline solids in contrast to several of the corresponding S-trityl-L-cysteine peptides used in the synthesis of XVIa,b.[13]

The formation of cyclic cystine peptides such as XVI was previously accomplished by the action of thiocyanogen on the di-S-trityl thioether.[1] The availability of XIV allowed the cyclization to be studied using the S-trityl and S-isobutyloxymethyl protective groups. Oxidation of a suspension of XIV in the manner previously described proceeded smoothly and provided the ester XVa in 94% yield (Scheme III). The monomeric nature of XVa was established by a molecular weight determination in *o*-chlorophenol. In contrast to the results obtained in the synthesis of XVIa, the cyclization of XIV provided no detectable quantity of XVb. This result may arise from a alternate method of product work-up used in the case of XIVa, *i.e.* precipitation of XIVa from the cold reaction mixture by the addition of ether.

The acid XVb could be obtained in good yield by the action of boron trifluoride etherate in acetic acid on the ester XVa. The solubility properties of XVa,b were somewhat different from those of XVIa,b; the former peptides being less soluble in acetic acid, pyridine, DMF and DMAc than the latter. The acid, XVb, was coupled with *t*-butyl S-trityl-L-cysteinylglycyl-L-valinate[1] (XXIII), by the pivaloyl chloride method,[14] to provide the undecapeptide derivative, XXIV (Scheme III). The ester, XXIV, contained two different sulfhydryl protective groups and is isomeric with a similar derivative, XXV, prepared from XVIb. The solubility properties of XVb were a critical factor in the formation of XXIV. The isomeric peptide, XXV, had been prepared by the action of DCC in pyridine; however, XVb was virtually insoluble in this solvent. Since the acid was somewhat soluble in DMAc this solvent was employed in a DCC coupling reaction with XXIII. Under these conditions, however, only the N-acylurea derivative, XXVI, was isolated presumably because of marginal solubility and reactant concentrations. Similar results were obtained by treatment of XVb with XXIII in the presence of DCC and N-hydroxysuccinimide in DMAc. The pivaloyl chloride method in DMAc provided XXIV in reasonable yield and appears to be the coupling procedure of choice in this situation.

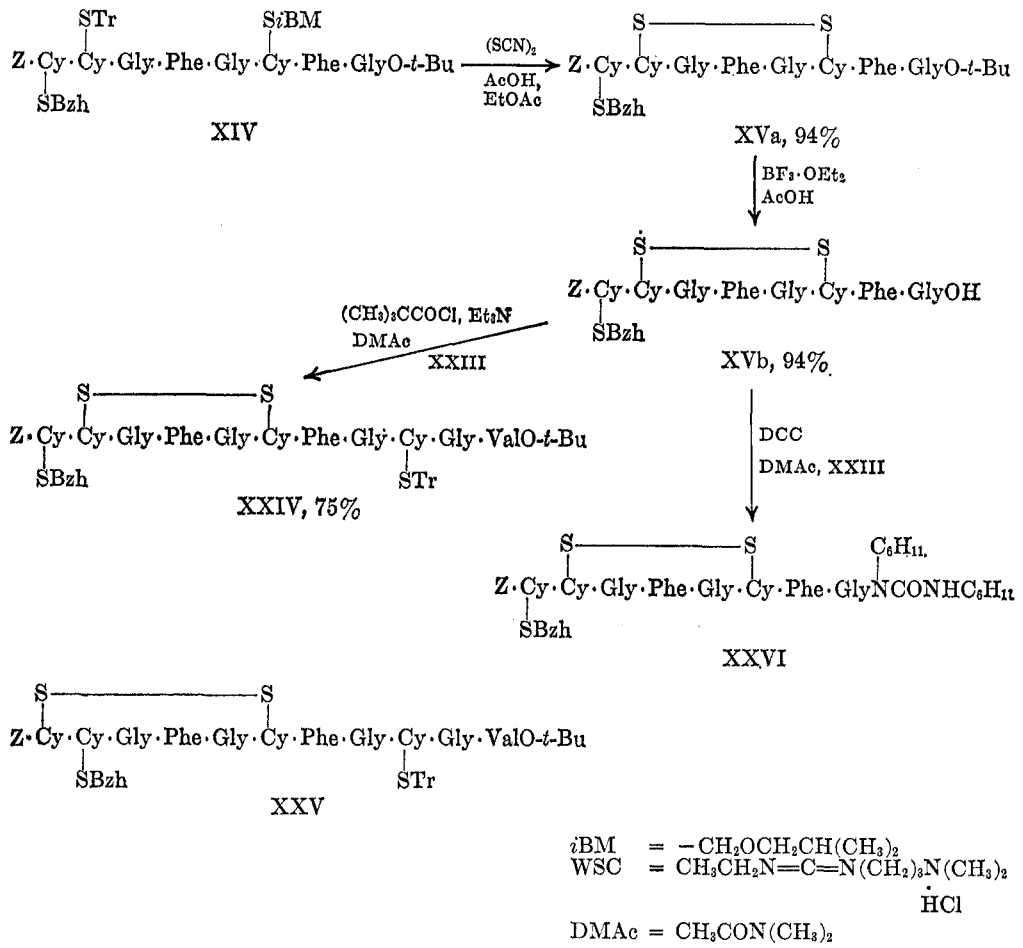
Experimental Section[15]

Preparation of N,N-Diethylammonium N-Phthaloyl-S-isobutyloxymethyl-L-cysteinate (II).—A solution of 21.2 g (0.2 mol) of anhydrous sodium carbonate in 300 ml of water was added to a suspension of 41.4 g (0.2 mol) of S-isobutyloxymethyl-L-cysteine[9] in 300 ml of DMF. N-(Ethyloxycarbonyl)phthalimide (43.8 g, 0.2 mol) was added and the suspension stirred at room temperature for 2 hr. Using 500 ml of water, the thick mass was transferred to 1 l. of water, the pH was adjusted to 2 and the precipitated oil was extracted into ethyl acetate. The dried extract was treated with 20.6 ml (0.2 mol) of N,N-diethylamine, and the precipitated bis(N,N-diethylammonium N-

(14) M. Zaoral, *Collect. Czech. Chem. Commun.*, **27**, 1273 (1962).

(15) Melting points are uncorrected and were taken in unsealed capillary tubes and on a Kofler hot stage. Elemental analyses were performed by Micro-Tech Laboratories, Inc., Skokie, Ill. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were performed with a Beckman Model 116 amino acid analyzer. Thin layer chromatography on microscope slides coated with silica gel G was used as a criterion of purity.

SCHEME III



iBM = $-CH_2OCH_2CH(CH_3)_2$
WSC = $CH_3CH_2N{=}C{=}N(CH_2)_3N(CH_3)_2 \cdot HCl$
DMAc = $CH_3CON(CH_3)_2$

phthaloyl)-L-cystine filtered, washed with warm ethyl acetate and discarded. The combined extract and washings were evaporated to yield a solid which was recrystallized from ethyl acetate–hexane to afford 56.4 g (68.7%) of white solid, mp 119–123. The analytical sample was recrystallized from a methanol–ether–hexane mixture, mp 123–125°; $[\alpha]^{31}D$ −1.02° (*c* 0.7, EtOH).

Anal. Calcd for $C_{20}H_{30}N_2O_5S$: C, 58.51; H, 7.36; N, 6.82; S, 7.80. Found: C, 58.54; H, 7.35; N, 6.64; S, 7.75.

Preparation of *t*-Butyl N-Phthaloyl-S-isobutyloxymethyl-L-phenylalanylglycinate (IV).—*t*-Butyl L-phenylalanylglycinate[13] (13.9 g, 0.05 mol, prepared by neutralization of the oxalate salt with 5% potassium carbonate) and N-phthaloyl-S-isobutyloxymethyl-L-cysteine (16.9 g, 0.05 mol prepared by the neutralization of the N,N-diethylamine salt with 5 *N* sulfuric acid) were dissolved in 50 ml of cold methylene chloride and treated with 10.8 g (0.055 mol) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. After stirring 8 hr, the reaction mixture was diluted with chloroform and extracted with 2 *N* sulfuric acid and water. The organic layer was dried and evaporated to a solid residue. The residue was crystallized from carbon tetrachloride to yield 24.0 g (80.3%) of white solid; mp 136–137.5°, $[\alpha]^{25}D$ −71.2° (*c* 1, $CHCl_3$).

Anal. Calcd for $C_{31}H_{39}N_3O_7S$: C, 62.29; H, 6.57; N, 7.03; S, 5.36. Found: C, 62.21; H, 6.58; N, 7.07; S, 5.48.

Preparation of *t*-Butyl N-Phthaloyl-S-(N′-carbobenzoxyl-L-cysteine methyl ester)-L-cysteinyl-L-phenylalanylglycinate (VI). Method A.—A solution of thiocyanogen was prepared by adding 0.20 g (1.25 mmol) of bromine in 2 ml of dry ethyl acetate to a suspension of 0.24 g (1.39 mmol) of lead thiocyanate in 3 ml of ethyl acetate. After stirring in the dark at room temperature until the color of bromine was no longer visible, the solution was cooled to −10° and a solution of 0.34 g (1.25 mmol) of methyl N-carbobenzoxy-L-cysteinate[6a] in 5 ml of ethyl acetate was added dropwise over a 5-min period. After stirring for 10 min, the solution containing the sulfenylthiocyanate was filtered through glass wool into a solution of 0.75 g (1.25 mmol) of IV in 15 ml of dry acetic acid at 15°. The reaction mixture was immediately cooled to 0° and the lead bromide washed with an additional 5 ml of ethyl acetate. After stirring for 18 hr in the dark, 150 ml of ethyl acetate was added and the organic layer was extracted with water until a negative thiocyanate test was obtained. After drying and evaporating the ethyl acetate, the oily residue was dissolved in 25% ethyl acetate–benzene and applied to a 2.7 × 50 cm silica gel column. Elution with 25% and 50% ethyl acetate–benzene, and repeated crystallization from ethyl acetate–ether–hexane, yielded 0.595 g (60.6%) of VI; mp 96–99°, $[\alpha]^{26.5}D$ −163.0° (*c* 1.02, EtOAc).

Anal. Calcd for $C_{38}H_{42}N_4O_{10}S_2$: C, 58.59; H, 5.44; N, 7.19; S, 8.23. Found: C, 58.75; H, 5.61; N, 7.13; S, 8.19.

Method B.—The sulfenylthiocyanate solution was prepared as in method A and filtered through glass wool into a solution of 0.75 g (1.25 mmol) of IV in 15 ml of ethyl acetate at 0°. After stirring 15 hr in the dark, 100 ml of ethyl acetate was added and the solution extracted with water until a negative thiocyanate test was obtained. After chromatography on silica gel and crystallization from ethyl acetate–ether–hexane, 0.46 g (46.8%) of VI identical with that in method A was obtained, mp 96–99°.

Method C.—The thiocyanogen solution was prepared in ethyl acetate as in method A and was filtered into 10 ml of dry acetic acid at 15°. The solution was immediately cooled to 0–5° and a solution of IV (0.75 g, 1.25 mmol) in 2 ml of 1:1 ethyl acetate–acetic acid was added in one portion. After stirring for 15 min, a solution of methyl N-1-carbobenzoxy-L-cysteinate (0.34 g, 1.25 mmol) in 6 ml of 1:1 ethyl acetate–acetic acid was added dropwise over a 20-min period. After stirring in the dark for 19 hr, the reaction mixture was diluted with 100 ml of ethyl acetate and extracted with water. After chromatography and crystallization as in method A, 0.39 g (39.7%) of VI was obtained, mp 96–99°.

Continued elution with 50% benzene–ethyl acetate gave a small quantity of material identified by the nmr spectrum as the symmetrical disulfide, mp 204–205°.

Anal. Calcd for $C_{52}H_{56}N_6O_{12}S_2$: C, 61.16; H, 5.53; N, 8.23; S, 6.28. Found: C, 60.46; H, 5.57; N, 8.15; S, 5.90.

Method D.—A solution of thiocyanogen, prepared from 0.20 g (1.25 mmol) of bromine and 0.45 g (1.39 mmol) of lead thiocyanate in 5 ml of ethyl acetate, was filtered into a cold suspen-

sion of 0.20 g (1.47 mmol) of zinc chloride in 5 ml of ethyl acetate. A solution of 0.75 g of IV in 10 ml of ethyl acetate was added over a 10-min period. A white precipitate formed 15 min after starting the addition of IV. A solution of methyl N-carbobenzoxy-L-cysteinate (0.34 g, 1.25 mmol) in 5 ml of ethyl acetate was added over a 15-min period. After stirring 5 hr, the reaction mixture was extracted with water and the ethyl acetate dried and evaporated. After chromatography on silica gel and crystallization as in method A, 0.38 g (39%) of VI was obtained, mp 96–99°.

Preparation of *t*-Butyl N-Phthaloyl-S-isobutyloxymethyl-L-cysteinylglycinate. (VII).—N-Phthaloyl-S-isobutyloxymethyl-L-cysteine (1.85 g, 5.5 mmol), prepared by the neutralization of its diethylammonium salt with 2 *N* sulfuric acid, and *t*-butylglycine (0.80 g, 5.5 mmol), prepared by the neutralization of its oxalate salt with saturated aqueous barium hydroxide, were dissolved in 9 ml of methylene chloride at 0°, and treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.15 g, 6.0 mmol). After stirring 10 hr, the reaction mixture was diluted with 100 ml of chloroform and extracted with 2 *N* sulfuric acid and distilled water. After drying over magnesium sulfate, the solvent was removed *in vacuo*, and the resulting oil was crystallized from benzene–hexane, giving 1.55 g (62%) of white solid; mp 94–95°, $[\alpha]^{29}D$ −69.2° (*c* 1.0, $CHCl_3$).

Anal. Calcd for $C_{22}H_{30}N_2O_6S$: C, 58.64; H, 6.72; N, 6.22; S, 7.11. Found: C, 58.69; H, 6.66; N, 7.15.

Preparation of *t*-Butyl N-Phthaloyl-S-(N′-carbobenzoxyl-L-cysteine methyl ester)-L-cysteinylglycinate. (VIII).—Bromine (0.34 g, 2.3 mmol) in 2 ml of dry ethyl acetate was added to a suspension of lead thiocyanate (0.48 g, 2.4 mmol) in 6 ml of dry ethyl acetate. After stirring at room temperature in the dark until the bromine color had disappeared, the solution was cooled to 0°, and methyl N-carbobenzoxy-L-cysteinate (0.54 g, 2.0 mmol) in 12 ml ethyl acetate was added dropwise over a period of 10 min. The mixture was stirred for 1 hr, then VII (0.90 g, 2.0 mmol) in dry acetic acid (30 ml) was added. After stirring in the dark for 15 hr, the reaction mixture was filtered, and the resulting yellowish solid was washed with ethyl acetate. The filtrate was diluted with 300 ml of ethyl acetate, and extracted with water until a negative thiocyanate test was obtained. After drying and stripping the ethyl acetate, the resulting oil was applied to a silica gel (50 g) column. Elution with benzene gave one fraction, believed to be unreacted methyl N-carbobenzoxy-L-cysteinate. Further elution with benzene–ethyl acetate, 6:4 gave 1.0 g (77%), of a white solid, which was homogeneous by tlc. The solid was recrystallized from benzene; mp 126–127°, $[\alpha]^{29}D$ −133.5° (*c* 1.0, EtOAc).

Anal. Calcd for $C_{29}H_{33}N_3O_9S_2$: C, 55.04; H, 5.43; N, 6.64; S, 10.13. Found: C, 55.27; H, 5.19; N, 6.45; S, 10.24.

Ethyl N,S-dityrityl-L-cysteinylglycinate was prepared by the method of Amiard, *et al.*[16] The purification of VI was carried out by elution from a silica gel column with chloroform.

Preparation of Ethyl S-Trityl-L-cysteinylglycinate Oxalate Salt (IX).—Ethyl N,S-dityrityl-L-cysteinylglycinate (5.0 g, 7.25 mmol) was dissolved in 10 ml of warm acetic acid. After water was added to the cloud point, the solution was heated for 10 min on the steam bath. The reaction mixture was poured into 150 ml of ice–water and neutralized with solid potassium carbonate. The basic solution was extracted with ethyl acetate, dried, and evaporated to yield an oily residue. The oil was dissolved in ether and any insoluble material removed by filtration. A solution of anhydrous oxalic acid (0.65 g, 7.2 mmol) in ether was added and the precipitate collected and washed with ether to yield 2.79 g (71.5%) of IX. The oxalate salt was crystallized from ethanol–ether; mp about 85°, $[\alpha]^{25.5}D$ +32.1° (*c* 0.504, EtOH).

Anal. Calcd for $C_{28}H_{30}N_2O_7S$: C, 62.44; H, 5.61; N, 5.20; S, 5.95. Found: C, 62.69; H, 5.65; N, 5.35; S, 5.67.

Preparation of N,N-Dicyclohexylammonium N-Carbobenzoxy-S-isobutyloxymethyl-L-cysteinate (X).—S-Isobutyloxymethyl-L-cysteine (4.14 g, 0.020 mol) was dissolved in 20 ml of 1 *N* sodium hydroxide. Carbobenzoxy chloride (3.81 ml, 0.024 mol) was added dropwise at the same time as 10 ml of 2 *N* sodium hydroxide. The reaction mixture was stirred for 4 hr, and extracted with ether to remove any unreacted carbobenzoxy chloride. After acidifying to approximately pH 2, the precipitated oil was extracted into ethyl acetate and dried. The oil that remained upon evaporation of the ethyl acetate was dissolved in a minimum of warm ethyl acetate and 3.62 g (0.020 mol) of N,N-dicyclohexylamine was added. The N,N-dicyclohexylammonium salt was precipitated with hexane and crystallized several times from ethyl acetate–hexane to yield 8.23 g (79.4%) of white solid; mp 125.5–128.5°, $[\alpha]^{25.5}D$ −2.98° (*c* 1.04, EtOAc).

Anal. Calcd for $C_{28}H_{46}N_2O_5S$: C, 64.33; H, 8.87; N, 5.36; S, 6.13. Found: C, 64.45; H, 8.73; N, 5.42; S, 6.11.

Preparation of Ethyl N-Carbobenzoxy-S-isobutyloxymethyl-L-cysteinyl-S-trityl-L-cysteinylglycinate (XI).—Ethyl S-trityl-L-cysteinylglycinate (2.24 g, 5.0 mmol, prepared by the neutralization of IX with 5% potassium carbonate) and N-carbobenzoxy-S-isobutyloxymethyl-L-cysteine (1.70 g, 5.0 mmol, prepared by the neutralization of X with 2 *N* sulfuric acid) were dissolved in 10 ml of methylene chloride at −10° and treated with 1.1 g (5.6 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. After stirring for 8 hr, the reaction mixture was diluted with chloroform and extracted with 2 *N* sulfuric acid and water. After drying and evaporating the solvent, the oily residue was dissolved in hot ether. Upon cooling 2.19 g (56.7%) of XI precipitated. Repeated crystallization from ethyl acetate–ether–hexane gave fine needles melting from 112–115°, $[\alpha]^{25.5}D$ −19.6° (*c* 0.915, EtOAc).

Anal. Calcd for $C_{42}H_{49}N_3O_7S_2$: C, 65.43; H, 6.41; N, 5.44; S, 8.35; Found: C, 65.55; H, 6.44; N, 5.60; S, 8.27.

The Reaction of the Sulfenylthiocyanate of Methyl N-Carbobenzoxy-L-cysteinate with Ethyl N-Carbobenzoxy-S-isobutyloxymethyl-L-cysteinyl-S-trityl-L-cysteinylglycinate (XI).—The sulfenylthiocyanate was prepared by allowing 0.34 g (1.25 mmol) of methyl N-carbobenzoxy-L-cysteinate to react at 0° with a thiocyanogen solution prepared from 0.20 g (1.25 mmol) of bromine and 0.45 g (1.39 mmol) of lead thiocyanate in 5 ml of ethyl acetate. The sulfenylthiocyanate solution was filtered through glass wool into a solution of 0.965 g (1.25 mmol) of XI in 10 ml of ethyl acetate at 0°. After stirring in the dark for 15 hr, ethyl acetate (100 ml) was added and the solution was extracted with water to obtain a negative thiocyanate test. After drying and evaporating the ethyl acetate, the residue was placed on a 2.7 × 50 cm silica gel column and eluted with 25% ethyl acetate–benzene to remove trityl thiocyanate and unreacted XI (0.25 g, 0.32 mmol, identical with starting XI). Two compounds, XIII [0.13 g, crystallized from ethyl acetate–hexane; mp 110–115°, $[\alpha]^{25.5}D$ −43.2° (*c* 0.40, EtOAc)] and XII [0.20 g, crystallized from ethyl acetate–hexane; mp 92–96°, $[\alpha]^{25.5}D$ −49.05° (*c* 0.42, EtOAc)], were obtained by elution with 40% benzene–ethyl acetate. The nmr spectrum of XIII was consistent with ethyl N-carbobenzoxy-S-(N′-carbobenzoxy-L-cysteine methyl ester)-L-cysteinyl-S-trityl-L-cysteinylglycinate.

Anal. Calcd for $C_{49}H_{52}N_4O_{10}S_3$: C, 61.74; H, 5.50; N, 5.88; S, 10.09. Found: C, 61.76; H, 5.47; N, 5.99; S, 10.09.

The nmr spectrum of the second compound (XII) was consistent with ethyl N-carbobenzoxy-S-isobutyloxymethyl-L-cysteinyl-S-(N′-carbobenzoxy-L-cysteine methyl ester)-L-cysteinylglycinate.

Anal. Calcd for $C_{35}H_{48}N_4O_{11}S_3$: C, 52.74; H, 6.07; N, 7.03; S, 12.07. Found: C, 52.87; H, 6.03; N, 7.05; S, 11.89.

Preparation of *t*-Butyl S-Isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate Oxalate Salt (XVII).—A solution of 5.97 g (0.010 mol) of IV in 50 ml of absolute ethanol containing 0.53 ml (0.0109 mol) of hydrazine hydrate was refluxed for 3 hr. The reaction mixture was transferred to a separatory funnel with 5% potassium carbonate and the amine extracted into ethyl acetate. After drying and evaporating the solvent, the oily residue was dissolved in ether and an ethereal solution of 0.90 g (0.01 mol) of anhydrous oxalic acid was added. The precipitated oxalate salt was collected and crystallized from methanol–ether–hexane to yield 4.79 g (86.2%) of white solid; mp 155–157°, $[\alpha]^{31}D$ −8.37° (*c* 0.515, EtOH).

Anal. Calcd for $C_{25}H_{39}N_3O_9S$: C, 53.94; H, 6.88; N, 7.55; S, 5.76. Found: C, 53.94; H, 7.01; N, 7.39; S, 5.56.

Preparation of *t*-Butyl N-Phthaloyl-L-phenylalanylglycyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (XVIII).—*t*-Butyl S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate [4.68 g, 0.010 mol, prepared by the neutralization of 5.57 g, (0.010 mol) of XVII with 5% potassium carbonate] and N-phthaloyl-L-phenylalanyglycine[18] (3.52 g, 0.010 mol) were dissolved in 50 ml of methylene chloride and cooled to −10°. After adding 2.16 g (0.011 mol) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, the reaction mixture was stirred overnight. After diluting the reaction mixture with 100 ml of chloroform, it was extracted with 2 *N* sulfuric acid and water.

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

After drying and evaporating the solvent, the solid residue crystallized from carbon tetrachloride as 7.04 g (87.8%) of white solid; mp 172–173.5°, $[\alpha]^{31}D$ −88.2° (*c* 0.95, $CHCl_3$).

Anal. Calcd for $C_{42}H_{51}N_5O_9S$: C, 62.90; H, 6.41; N, 8.73; S, 4.00. Found: C, 62.75; H, 6.42; N, 8.87; S, 4.19.

Preparation of *t*-Butyl L-Phenylalanylglycyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (XIX).—A solution of 8.01 g (0.010 mol) of XVIII in 100 ml of absolute ethanol was treated with 0.85 ml (0.0175 mol) of hydrazine hydrate for 3 hr. After transferring the reaction mixture to a separatory funnel with 5% potassium carbonate, the amine was extracted into ethyl acetate. After drying and evaporating the solvent, the residue crystallized from ethyl acetate–hexane as 6.14 g (91.5%) of white solid; mp 129–131° (softens and resolidifies at 98–100°), $[\alpha]^{31}D$ −28.4° (*c* 0.20, EtOH).

Anal. Calcd for $C_{34}H_{49}N_5O_7S$: C, 60.78; H, 7.35; N, 10.12; S, 4.77. Found: C, 60.86; H, 7.53; N, 10.30; S, 4.72.

Preparation of N,S-Dityrityl-L-cysteinylglycine (XX).—A solution of 8.10 g (0.012 mol) of ethyl N,S-dityrityl-L-cysteinylglycinate in 25 ml of dioxane was treated with 15 ml of 1 *N* sodium hydroxide at 0° for 4 hr. The reaction mixture was poured into cold 10% citric acid and the precipitated solid collected. After washing well with water, the solid was dried *in vacuo* over phosphorus pentoxide to yield 6.9 g (88.7%) of XVII. This material showed a small immobile spot by mtlc ($CHCl_3$–MeOH, 9:1) on silica gel. It was used without further purification due to the poor recovery upon crystallization.

Treatment of XX with aqueous hydrochloric acid–acetone converted the material into S-trityl-L-cysteinylglycine; mp 117–120°, $[\alpha]D$ + 15.8° (*c* 2, 0.1 *N* NaOH in MeOH) [lit.[16] mp 120°, $[\alpha]D$ +17.5 ± 1° (*c* 2, 0.1 *N* NaOH in 90% MeOH)].

Preparation of *t*-Butyl N,S-Dityrityl-L-cysteinylglycyl-L-phenylalanylglycyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (XXI).—A solution of 3.36 g (5.0 mmol) of XIX and 3.31 g (5.0 mmol) of XX in 25 ml of methylene chloride was cooled to −10° and treated with 1.1 g (5.6 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. After stirring overnight, 100 ml of hot methanol was added. Upon cooling and collecting the solid precipitate, 5.7 g (86.7%) of XXI was obtained, mp 200–201.5°. Crystallization from methanol–chloroform raised the melting point to 209–210°, $[\alpha]^{25.5}D$ −20.6° (*c* 0.50, $CHCl_3$).

Anal. Calcd for $C_{58}H_{85}N_7O_9S_2$: C, 64.84; H, 6.66; N, 9.13; S, 5.97. Found: C, 64.42; H, 6.61; N, 9.06; S, 5.92.

Preparation of *t*-Butyl S-Trityl-L-cysteinylglycyl-L-phenylalanylglycyl-S-isobutyloxpmethyl-L-cysteinyl-L-phenylalanylglycinate (XXII) —A solution of 5 g (3 8 mmol) of XXI in 10 ml of acetic acid was warmed on the steam bath as 0.5 ml of water was added. The resulting turbid solution was heated for 10 min and poured into 100 ml of ice–water. After neutralization with solid potassium carbonate, the precipitated solid was collected, washed well with water, and air dried. Crystallization from methanol–chloroform–ether yielded 3.23 g (81.6%) of XXII, mp 188.5–190°, $[\alpha]^{25.5}D$ − 20.6° (*c* 0.50, $CHCl_3$).

Anal. Calcd for $C_{58}H_{71}N_7O_9S_2$: C, 64.84; H, 6.66; N, 9.13; S, 5.97. Found: C, 64.42; H, 6.61; N, 9.06; S, 5.92.

Preparation of *t*-Butyl N-Carbobenzoxy-S-benzhydryl-L-cysteinyl-S-trityl-L-cysteinylglycyl-L-phenylalanylglycyl-S-isobutyloxymethyl-L-cysteinyl-L-phenylalanylglycinate (XIV).—A solution of 2.69 g (2.5 mmol) of XXII and 1.10 g (2.6 mmol) of N-carbobenzoxy-S-benzhydryl-L-cysteine in 25 ml of methylene chloride was cooled to −10° and 0.6 g (3.1 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added. After 15 min a gel had formed which was allowed to stand 6 hr at room temperature. The gel was transferred to a beaker with hot methanol and N,N-dimethylformamide was added until solution was effected. Upon cooling 3.58 g (96.7%) of XIV was obtained: mp 240.5–242°, $[\alpha]^{25.5}D$ −22.7° (*c* 1.12, DMF); mol wt 1455.5 (by osmometry in *o*-chlorophenol); theoretical mol wt 1477.81.

Anal. Calcd for $C_{82}H_{92}N_8O_{12}S_3$: C, 66.64; H, 6.28; N, 7.58; S, 6.51. Found: C, 66.53; H, 6.15; N, 7.59; S, 6.40.

Preparation of *t*-Butyl N-Carbobenzoxy-S-benzhydryl-L-cysteinyl-S,S′-L-hemicystylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycinate (XVa).—A solution of 1.48 g (1.0 mmol) of XIV in 400 ml of dry acetic acid was cooled to 16° and 190 ml of dry ethyl acetate added. The suspension of XII was then cooled to 0° and a thiocyanogen solution, prepared from 0.25 g (1.56 mmol) of bromine and 0.55 g (1.70 mmol) of lead thiocyanate in 10 ml of ethyl acetate, was added by filtering through glass wool. After stirring 24 hr at 0° in the dark, the pink, acetic acid–ethyl acetate suspension was poured into 2.5 l. of ice water. The precipitated solid was collected and washed well with water. The solid was dissolved in 150 ml of hot 1:1 (v/v) N,N-dimethylacetamide–methanol and filtered. Ether was added to the warm solution to precipitate 1.08 g (94%) of XVa: mp 246–247.5° dec; $[\alpha]^{25.5}D$ −50.5° (*c* 0.56, DMF); mol wt 1147.5 (by osmometry in *o*-chlorophenol); theoretical mol wt 1147.36.

Anal. Calcd for $C_{58}H_{66}N_8O_{11}S_3$: C, 60.71; H, 5.80; N, 9.77; S, 8.38. Found: C, 60.54; H, 5.87; N, 9.88; S, 8.19.

Preparation of N-Carbobenzoxy-S-benzhydryl-L-cysteinyl-S,S′-L-hemicystylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycine (XVb).—A suspension of 0.58 g (0.5 mmol) of XVa in 20 ml of dry acetic acid was treated for 1 hr at room temperature with 6 ml of boron trifluoride diethyl ether complex. After pouring into 200 ml of ice water, the precipitated solid was collected and washed well with water. The solid was dissolved in hot methanol containing a small amount of N,N-dimethylacetamide and filtered. Ether was added to precipitate 0.51 g (94%) of XVb; mp 242–244° dec; $[\alpha]^{25.5}D$ −52.0° (*c* 0.50, DMF).

Anal. Calcd for $C_{54}H_{58}N_8O_{11}S_3$: C, 59.43; H, 5.36; N, 10.27; S, 8.81. Found: C, 59.48; H, 5.47; N, 10.17; S, 8.58.

Preparation of N-(N″-Carbobenzoxy-S-benzhydryl-L-cysteinyl-S,S′-L-hemicystylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycyl)-N,N′-dicyclohexylurea (XXXVI).—A solution of 0.11 g (0.10 mmol) of XVb and 0.06 g (0.10 mmol) of XXIII in 2 ml of N,N-dimethylacetamide was cooled to −10° and 0.02 g (0.10 mmol) of N,N′-dicyclohexylcarbodiimide was added. After stirring overnight, the reaction mixture was diluted with 20 ml of 50% pyridine–water, filtered, and the solid washed well with water, methanol, and ether. The solid was dissolved in N,N-dimethylacetamide–methanol and precipitated with water. After drying *in vacuo* at 100°, the solid was repeatedly precipitated from N,N-dimethylacetamide–methanol with ether to yield 0.08 g of XXVI; mp 253–255°.

Anal. Calcd for $C_{67}H_{80}N_{10}O_{11}S_3$: C, 62.00; H, 6.21; N, 10.80; S, 7.41. Found: C, 61.63; H, 6.14; N, 10.47; S, 7.82.

Preparation of *t*-Butyl N-Carbobenzoxy-S-benzhydryl-L-cysteinyl-S,S′-L-hemicystylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycyl-S-trityl-L-cysteinylglycyl-L-valinate (XXIV).—A solution of 0.436 g (0.40 mmol) of XVb in 4 ml of N,N-dimethylacetamide was cooled to −10° and 0.056 ml (0.40 mmol) of triethylamine was added. Trimethylacetyl chloride (0.048 ml, 0.40 mmol) was added and the solution stirred for 3 min. A solution of 0.275 g (0.48 mmol) of XXIII in 1 ml of N,N-dimethylacetamide was added. After stirring 15 min at −10°, the ice–acetone bath was removed and stirring was continued overnight at room temperature. The reaction mixture was diluted with 50 ml of ethyl acetate and filtered. The precipitate was washed with warm ethyl acetate–methanol (7:1 vol/vol) and ether. The product was dissolved in methanol–N,N-dimethylacetamide and precipitated with ether to yield 0.492 g (74.8%) of XXIV; mp 240–245° dec; $[\alpha]^{25}D$ −31.9° (*c* 0.52, DMF). Amino acid analysis gave 4.06 μmol of cysteic acid, 1.85 μmol of phenylalanine, 4.00 μmol of glycine, and 0.90 μmol of valine.

Anal. Calcd for $C_{87}H_{97}N_{11}O_{14}S_4$: C, 63.36; H, 5.93; N, 9.34; S, 7.78. Found: C, 63.26; H, 5.96; N, 9.54; S, 7.81.

Registry No.—II, 21996-02-1; IV, 21996-03-2; VI, 21996-04-3; VI (symmetrical disulfide), 21996-05-4; VII, 21996-06-5; VIII, 21996-07-6; IX, 21996-08-7; X, 21996-09-8; XI, 21996-10-1; XII, 21996-11-2; XIII, 21996-12-3; XIV, 17561-37-4; XVa, 17748-97-9; XVb, 21996-15-6; XVII, 21996-16-7; XVIII, 21996-17-8; XIX, 21996-18-9; XXI, 21996-19-0; XXIV, 21996-20-3; XXVI, 21996-21-4.