Sulfur-Containing Polypeptides. VIII. Formation of Cyclic Cystine Peptides with Thiocyanogen[1-3]

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Abstract: The sulfenyl thiocyanate method of disulfide synthesis has been applied to the preparation of S,S′-N-carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycine (II). Chain elongation of II afforded the undecapeptide, S,S′-N-carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycyl-S-trityl-L-cysteinylglycyl-L-valine (VI).

In the preceding report of this series[1] the synthesis of the protected octapeptide derivative, *t*-butyl N-carbobenzoxy-S-trityl-L-cysteinyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-S-trityl-L-cysteinyl-L-phenylalanylglycinate (I) was described. This substance was desired as a subunit for the construction of a molecule shown schematically as IV. The projected synthesis of molecules related to IV allows the stepwise introduction of the three disulfide bonds; oxidation of I and ester hydrolysis would provide the intrachain disulfide loop in the form of S,S′-N-carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycine (II). Chain elongation of II using an appropriate S-trityl-L-cysteine residue would yield an A chain, III, containing a preformed intrachain cystine residue and two protected cysteine units; selective oxidation of the cysteine residues and combination with the B chain would allow the formation of IV by a specific and highly selective route. The present report concerns the preparation of II and the successful synthesis of an undecapeptide, S,S′-N-carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycyl-S-trityl-L-cysteinylglycyl-L-valine (VI) which is suitable for use as an A chain in the eventual synthesis of molecules related to IV.

The cyclization of I has more than ample precedent. In the years since the classical synthesis of oxytocin by du Vigneaud, *et al.*,[4] a number of alternate routes to the hormone have been described.[5] The earlier syntheses employed the S-benzyl group to protect cysteine residues 1 and 6 during the chain-elongation steps. Removal of the S-benzyl groups with sodium in liquid ammonia provided the dithiol which was oxidized in a separate operation.[6] Despite the fact that more recent routes have employed other S-protective groups, *e.g.*, S-trityl,[7] S-*p*-methoxybenzyl,[8] S-4,4′-dimethoxydiphenylmethyl,[9] S-carbamoyl,[10] and S-benzoyl[5] and various oxidizing agents (air, hydrogen peroxide, 1,2-diiodoethane, and ferricyanide ion), the approach to loop formation has remained the same, *i.e.*, S-protective group removal, isolation, and subsequent oxidation of the dithiol.

Application of this general procedure to a peptide such as I which contains both S-trityl and S-benzhydryl-L-cysteine residues appeared to present no particular problem since the former protective group could be removed in the presence of the latter with mercury(II) or silver ion.[11] However, the reaction of I with either metal ion in a pyridine–methanol solvent provided only recovered starting material and gave none of the desired dimercaptide. The lack of reactivity exhibited by I was believed to result from the low solubility of I in these solvents; indeed, treatment of I with silver nitrate in a pyridine–methanol–DMF solvent afforded the disilver mercaptide and trityl methyl ether. Purification of the dimercaptide was difficult due to the insolubility of the substance; thus, the crude solid was treated with hydrogen sulfide in DMF to afford the crude dithiol. Oxidation of the dithiol was carried out in a methanol–DMF mixture using iodine. The crude oxidation product was contaminated with unreacted I, again indicating incomplete removal of the S-trityl groups by silver ion, apparently the result of coprecipitation of I with the disilver mercaptide. Purification of the crude oxidation product provided the ester of II, *t*-butyl S,S′-N-carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycinate (V), in 29% over-all yield (Scheme I). The ester V was smoothly converted into the desired acid II by the action of boron trifluoride in acetic acid; the ester and acid were readily distinguished by their mobilities in tlc.

Although acid II could be obtained in pure condition by oxidation of the crude dithiol, this procedure suffered from several disadvantages. Among these were the low over-all yield of II and the difficult work-up at the dithiol stage which resulted in prolonged exposure

(1) Part VII of this series: R. G. Hiskey, J. T. Staples, and R. L. Smith, *J. Org. Chem.*, 32, 2772 (1967).

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

(3) The following abbreviations have been employed in the text: Z = carbobenzoxy; Tr = trityl; Bzh = benzhydryl; ^{t}Bu = *t*-butyl; DCC = N,N′-dicyclohexylcarbodiimide; WSC = 1-ethyl-3-(3′-N,N-dimethylaminopropyl)carbodiimide hydrochloride; Phth = phthaloyl; DMF = N,N-dimethylformamide; Ox^- = oxalate; $(AA)_n$ = amino acid residues.

(4) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Am. Chem. Soc.*, 75, 4879 (1953).

(5) I. Photaki, *ibid.*, 88, 2292 (1966), has reviewed the various alternatives.

(6) The cystine residues in insulin have also been introduced by this route; for example, see P. G. Katsoyannis, A. Tometsko, and K. Fukuda, *ibid.*, 85, 2863 (1963).

(7) L. Velluz, G. Amiard, J. Bartos, B. Goffinet, and R. Heymes, *Bull. Soc. Chim. France*, 1464 (1956).

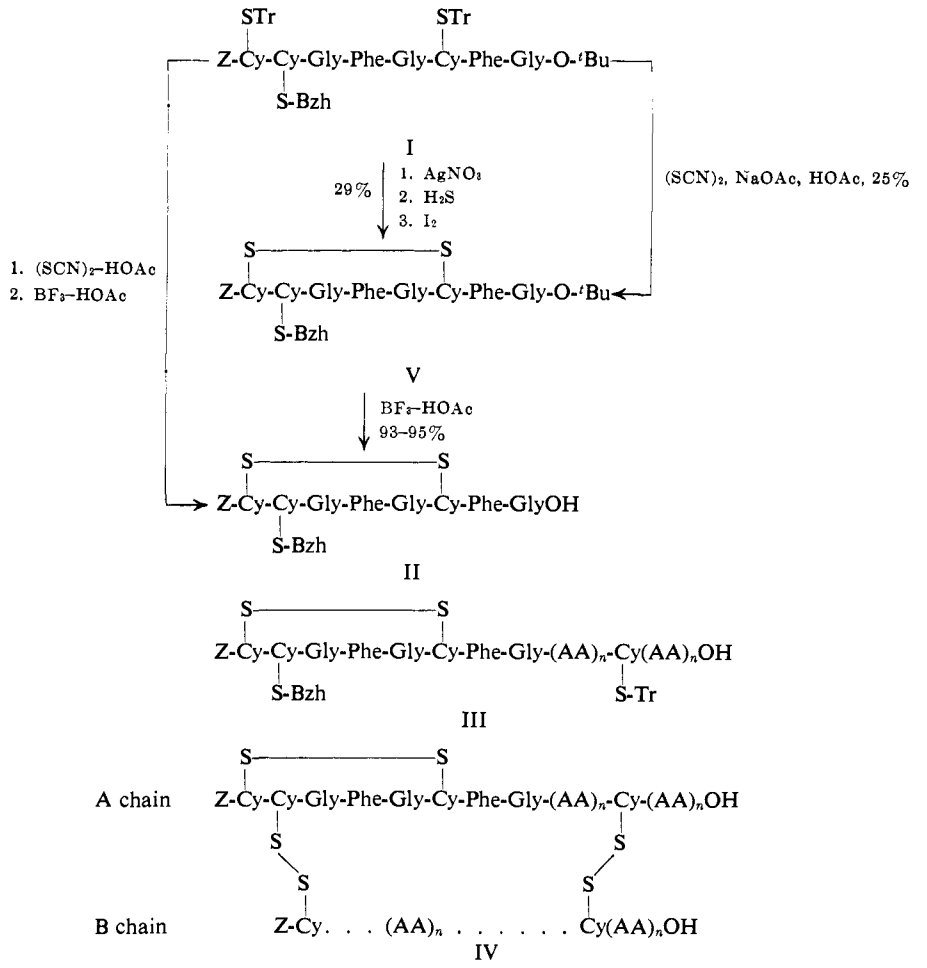
(8) S. Sakakibara, Y. Nobuhara, Y. Shimonishi, and R. Kiyoi, *Bull. Chem. Soc. Japan*, 38, 120 (1965).

(9) R. W. Hanson and H. D. Law, *J. Chem. Soc.*, 7285 (1965).

(10) S. Guttmann, *Helv. Chim. Acta*, 49, 83 (1966).

(11) R. G. Hiskey, T. Mizoguchi, and H. Igeta, *J. Org. Chem.*, 31, 1188 (1966), using the procedure of L. Zervas and I. Photaki, *J. Am. Chem. Soc.*, 84, 3887 (1962).

Scheme I



of the dimercaptide and dithiol. In addition, a method for the direct cyclization of two S-trityl-L-cysteine residues would preclude the possibility of thiol–disulfide interchange in a molecule containing two such thioethers and a preformed disulfide bond.

Previous studies with the sulfenyl thiocyanate method have established that unsymmetrical open-chain cystine derivatives can be obtained *via* either an S-trityl[12] or an S-benzhydryl[12] thioether, the former being more reactive toward thiocyanogen or a sulfenyl thiocyanate than the latter.[13,14] Accordingly a suspension of I in acetic acid was treated with thiocyanogen. Analysis of the reaction mixture by tlc indicated the formation of trityl thiocyanate ester V, and a significant quantity of acid II. Since V is known to be unaffected by acetic acid the formation of II was unexpected. A possible explanation for this result would involve the production of equilibrium concentrations of thiocyanic acid by the reaction of trityl thiocyanate and acetic acid. Thiocyanic acid in acetic acid would quite likely catalyze the hydrolysis of the *t*-butyl ester. Support for this possibility was obtained by treatment of I with a thiocyanogen solution containing 2 equiv of dry sodium acetate. Under these conditions II was not produced and only V, together with some unreacted I, resulted. Purification of the ester fraction provided 25% of V, with physical properties similar to those of the sample obtained by iodine oxidation of the dithiol. A molecular weight determination on the sample of V obtained *via* the thiocyanogen reaction indicated intramolecular cyclization had occurred. Hydrolysis of this material with boron trifluoride in acetic acid produced II in 93% yield. The presence of unreacted octapeptide in the thiocyanogen–sodium acetate reaction with I suggested that the sodium acetate probably consumed thiocyanogen; hence the salt was omitted. Treatment of I with thiocyanogen (30 hr, 0°) in acetic acid followed by addition of boron trifluoride to the mixture of V and II provided pure II in 76% over-all yield. When the reaction time for the cyclization step was reduced to 14 hr a 60% over-all yield of II resulted. The direct addition of boron trifluoride to the oxidized peptide proved to provide a better yield of II (76%) than when the ester fraction was isolated and hydrolyzed in a separate step (55%).[15]

With the availability of a reasonable synthetic route to II, the synthesis of VI was considered. Preparation of the C-terminal tripeptide, *t*-butyl S-trityl-L-cysteinyl-

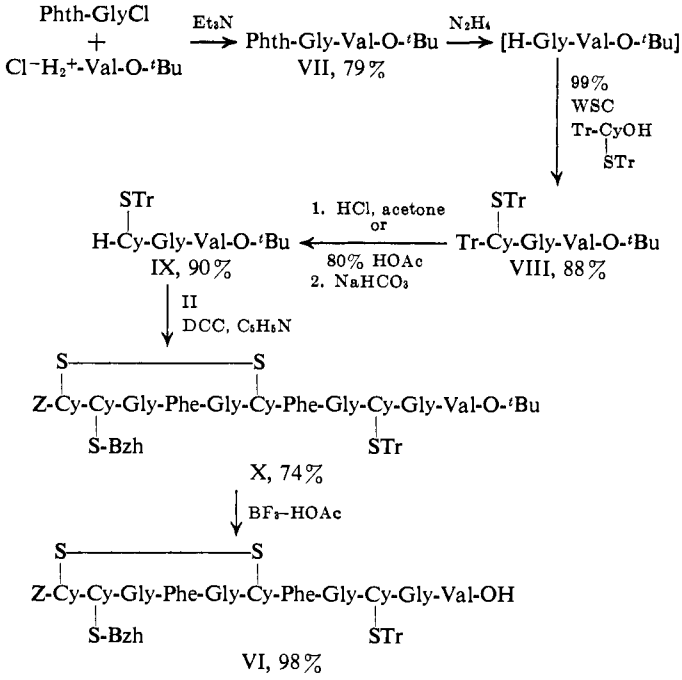
(12) R. G. Hiskey and D. N. Harpp, *J. Am. Chem. Soc.*, 87, 3965 (1965).

(13) R. G. Hiskey and M. A. Harpold, *Tetrahedron*, 23, 3923 (1967).

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

(15) The specific rotations of the esters V obtained by the classical and thiocyanogen method were +32.4 and +30.5°, respectively. Boron trifluoride hydrolysis of V obtained by the classical route gave a sample of the anhydrous acid II with a specific rotation of +32.4° whereas similar treatment of the ester obtained by the thiocyanogen cyclization, provided a substance which analyzed as a monohydrate with a specific rotation of +22.0°. Since we have not encountered racemization in our previous experiments with boron trifluoride in acetic acid, we tentatively ascribe the difference in specific rotation of the samples to the difference in degree of hydration.

Scheme II



glycyl-L-valinate (IX, Scheme II), proceeded *via* *t*-butyl N,S-dityrityl-L-cysteinylglycyl-L-valinate (VIII), a peptide derivative containing amino-, thiol-, and carboxyl-protective groups of differing acid lability.[1] The fully protected tripeptide, VIII, was obtained in good yield coupling N,S-dityrityl-L-cysteine with *t*-butyl glycyl-L-valinate produced by hydrazinolysis of *t*-butyl N-phthaloylglycyl-L-valinate (VII). Initially the condensation of II with IX was performed with WSC in DMF. The major product was isolated by preparative tlc and extracted from the support with a chloroform–methanol–pyridine mixture. Combustion analysis indicated the substance to be the methyl ester of VI rather than the desired *t*-butyl ester X. When N-ethyl-5-phenylisoxazolium-3′-sulfonate in DMF was employed the desired undecapeptide, X, was formed but was contaminated with significant amounts of a component of similar tlc mobility. The contaminant is assumed to be the N,N-dimethylamide derivative of II. The most satisfactory coupling system proved to be DCC in anhydrous pyridine; the solubility of II permitted optimum concentrations of the reactants to be utilized and pure X could be readily obtained in 74% yield. The molecular weight of X, obtained using a sample of II prepared *via* the thiocyanogen reaction, was consistant with the monomeric structure. Treatment of X with boron trifluoride in acetic acid provided VI in 98% yield.

A sample of X was deblocked by the action of trifluoroacetic acid saturated with hydrogen bromide and the resulting residue oxidized to the cysteic acid with performic acid. Enzymic hydrolysis of the oxidized undecapeptide with aminopeptidase M provided essentially theoretical quantities of the component amino acids; thus little if any racemization appears to have occurred in the synthesis of X.

Experimental Section[16]

***t*-Butyl S,S′-N-Carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycinate (V). A. *Via* Dithiol.** A solution containing 320 mg (1.88 mmoles) of silver nitrate and 149 mg (1.88 mmoles) of dry pyridine in 25 ml of dry methanol was added to 1.536 g (0.94 mmole) of I in 50 ml of dry DMF. The reaction mixture was stirred in the dark at 20° for 6 hr. The methanol was removed *in vacuo* and the resulting solution was treated with 300 ml of cold water. The precipitated disilver mercaptide was washed with ether and dried to a constant weight (1.4 g).

The disilver mercaptide was dissolved in 50 ml of dry DMF and treated with a stream of dry hydrogen sulfide for 20 min. The silver sulfide was filtered and washed with DMF, and the filtrate was diluted with 300 ml of cold water. The resulting white solid was dried to a constant weight (1.05 g).

The dithiol (1.05 g) was dissolved in 400 ml of a dry methanol–DMF mixture (3:1, v/v) and the solution was added dropwise to a stirred solution of 249 mg (0.94 mmole) of iodine in 400 ml of dry methanol. The addition required 2.5 hr. The solution was allowed to stand at 20° for 0.5 hr, the methanol was removed *in vacuo*, and the remaining solution was diluted with 200 ml of cold water. The resulting white suspension was treated with 17 ml of a 1% aqueous sodium thiosulfate solution, 200 ml of cold water, and 100 ml of saturated aqueous sodium chloride solution. The solid was collected and dried to yield 0.980 g of white powder. The solid was crystallized from 30 ml of a chloroform–*n*-hexane mixture (1:2, v/v). The tlc of this material exhibited a spot due to the original octapeptide as well as a new spot at lower R_f (system A). The crystallized solid and filtrate were combined and the solvent was removed in *vacuo*; the resulting gum was washed with hot chloroform (15 ml) and the insoluble material was collected. The solid was washed with additional hot chloroform to provide 0.314 g (29% over-all) of V: mp 234–238° dec; $[\alpha]^{30}D$ +30.5° (*c* 0.94, DMF). *Anal.* Calcd for $C_{58}H_{66}N_8O_{11}S_3$: C, 60.71; H, 5.80; N, 9.77; S, 8.38; mol wt, 1147. Found: C, 60.40; H, 5.78; N, 9.53; S, 8.16; mol wt, 1090.

(16) Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Ill. Optical rotations were determined with a Perkin-Elmer Model 141 polarimeter (glass cell). Molecular weights were determined using a Mechrolab vapor pressure osmometer with *o*-chlorophenol as solvent. Thin-layer chromatography was used as a criterion of purity. Microscope slides were used as support for the adsorbents, silica gel G and silica gel G_{254}. Iodine vapor, ninhydrin, and ultraviolet light were used to develop the chromatograms. The following solvent systems were employed: system A, chloroform–methanol (9:1, v/v); system B, chloroform–methanol–acetic acid (8:1:1, v/v); and system C, benzene–ethyl acetate (1:1, v/v). Melting points were taken in unsealed capillary tubes and on a Kofler hot stage and are uncorrected. Solvents were dried and distilled prior to use. Solvent removal was effected with rotary evaporators; bath temperatures were maintained at or below 40°.

B. *Via* **Thiocyanogen–Sodium Acetate.** Thiocyanogen was generated by the dropwise addition of a solution of bromine (0.1 g, 0.625 mmole) in 15 ml of ethyl acetate to a suspension of 0.243 g (0.75 mmole) of lead(II) thiocyanate in 15 ml of ethyl acetate. The colorless thiocyanogen solution was added to a cold suspension (0–5°) of 0.817 g (0.5 mmole) of I in 100 ml of dry acetic acid containing 8.2 mg (1.0 mmole) of dry sodium acetate. Stirring was continued in the dark at 0–5° for 20 hr, the suspension was filtered, and the filtrate and ethyl acetate washings were concentrated *in vacuo* to 150 ml. The solution was poured into ice water and the solid was filtered, washed with water, and dried. The collected solid (0.25 g) was dissolved in hot chloroform and filtered through Celite, and the filtrate was concentrated to 15 ml and diluted with an equal volume of hexane. The resulting solid (0.20 g) was collected, washed with ether and chloroform, and dried. The residue was recrystallized from a chloroform–methanol–hexane mixture (5:1:10, v/v) to provide 0.141 g (25%) of V: mp 227–228°, $[\alpha]^{24}D$ +33.5° (*c* 0.54, DMF). The substance was homogeneous (system A) and exhibited an R_f identical with the ester obtained in A. *Anal.* Calcd for $C_{58}H_{66}N_8O_{11}S_3$: C, 60.71; H, 5.80; N, 9.77; S, 8.38; mol wt, 1147. Found: C, 60.57; H, 5.90; N, 9.90; S, 8.56; mol wt, 1150.

S,S′-N-Carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycine (II). A. *Via* **V.** To a solution containing 0.238 g (0.2 mmole) of V (obtained *via* iodine oxidation of the dithiol) in 5 ml of glacial acetic acid was added 0.25 ml (2.0 mmole) of boron trifluoride–diethyl etherate complex. The yellow solution was stirred at 20° for 1 hr, poured into ice water, and filtered, and the solid was washed with water. The solid was dried to yield 0.208 g (95%) of II: mp 243–246° dec; single spot on tlc, system B. The analytical sample was prepared by recrystallization from a DMF–ether mixture: mp 240–241.5°, $[\alpha]^{30}D$ +32.4° (*c* 1.01, DMF). *Anal.* Calcd for $C_{54}H_{58}N_8O_{11}S_3$: C, 59.44; H, 5.36; N, 10.27; S, 8.80. Found: C, 59.55; H, 5.61; N, 10.32; S, 9.08.

B. *Via t*-**Butyl N-Carbobenzoxy-S-trityl-L-cysteinyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-S-trityl-L-cysteinyl-L-phenylalanylglycinate (I).** Thiocyanogen was generated by the dropwise addition of a solution of bromine (0.2 g, 1.25 mmoles) in 30 ml of ethyl acetate to a rapidly stirred suspension of 0.486 g (1.5 mmoles) of lead thiocyanate in 30 ml of ethyl acetate. The solution was filtered after 10 min and the clear thiocyanogen solution was added dropwise (5 min) to a slurry containing 1.634 g (1.0 mmole) of I in 200 ml of glacial acetic acid. The reaction was carried out at 0°. The resulting suspension was stirred in the dark for 36 hr and filtered to provide a yellow solid. The material was washed with ethyl acetate and the combined washings and filtrate were evaporated *in vacuo* to neat acetic acid solution. The solution was treated with 1.27 ml (10.0 mmole) of boron trifluoride–diethyl ether complex and stirred at 20° for 1.5 hr. The solution was poured into ice water and the solid was filtered and washed with water. The dried solid (1.23 g) was washed with hot chloroform and ether to yield 0.83 g (76%) of II: mp 236–240° dec; homogeneous (system B) and identical in R_f with the sample obtained by procedure A. Recrystallization from a DMF–ether–hexane mixture (8:75:2.5, v/v) raised the melting point to 240–241°, $[\alpha]^{21}D$ +21.9° (*c* 1.02, DMF).

C. *Via t*-**Butyl S,S′-N-Carbobenzoxy-L-hemicystyl-S-benzhydryl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycinate Obtained by the Thiocyanogen Reaction.** Boron trifluoride–diethyl ether complex (0.076 ml, 0.61 mmole) was added to a solution of 0.07 g (0.061 mmole) of V (obtained *via* the thiocyanogen reaction using sodium acetate) in 1.5 ml of dry acetic acid. Work-up in the manner previously described provided 0.062 g (93%) of II: mp 239–241; homogeneous (system B) and identical in R_f with the previous preparations. The analytical sample was prepared by recrystallization from a DMF–ether mixture (1:10, v/v): mp 249–250° dec, $[\alpha]^{24}D$ +22.0° (*c* 0.50, DMF). *Anal.* Calcd for $C_{54}H_{58}N_8O_{11}S_3 \cdot H_2O$: C, 58.44; H, 5.45; N, 10.11; S, 8.67. Found: C, 58.58, 58.65; H, 5.44, 5.38; N, 10.22; S, 8.84.

t-**Butyl N-Phthaloylglycyl-L-valinate (VII).** A solution of 22.36 g (0.1 mole) of N-phthaloylglycyl chloride[17] in 100 ml of benzene was added dropwise in 1 hr to a suspension of 20.97 g (0.1 mole) of *t*-butyl L-valinate hydrochloride[18] in 500 ml of benzene containing 28 ml (0.2 mole) of triethylamine. The suspension was refluxed for 4 hr and diluted with ethyl acetate, and the organic layer was extracted with 5% hydrochloric acid, water, 5% sodium bicarbonate, water, and saturated sodium chloride solution. The dried organic layer was evaporated and the residue was recrystallized from a methylene chloride–hexane mixture to yield 28.6 g (79%) of VII: mp 150–151°, $[\alpha]^{31}D$ +4.7° (*c* 1.0, CH_2Cl_2). *Anal.* Calcd for $C_{19}H_{24}N_2O_5$: C, 63.32; H, 6.71; N, 7.77. Found: C, 63.14; H, 6.57; N, 7.86.

t-**Butyl Glycyl-L-valinate Oxalate Salt.** A solution of 10.8 g (0.03 mole) of VII in 100 ml of methanol was treated with 14.6 ml (0.0301 mole) of 99% hydrazine hydrate. The solution was refluxed for 30 min and treated with excess 5% potassium carbonate solution. The solution was extracted with ether; the organic extract was dried and evaporated to yield 6.84 g (99%) of crude amine. This material was normally used in the subsequent coupling step without further purification. The amine was converted into the oxalate salt by the usual method: mp 161–162° (from methanol–ether), $[\alpha]^{31}D$ −17.0° (*c* 1, EtOH). *Anal.* Calcd for $C_{13}H_{24}N_2O_7$: C, 48.74; H, 7.55; N, 8.75. Found: C, 48.74; H, 7.74; N, 9.01.

t-**Butyl N,S-Ditrityl-L-cysteinylglycyl-L-valinate (VIII).** To a solution of 20.7 g (0.03 mole) of N,S-ditrityl-L-cysteine[19] and 6.8 g (0.03 mole) of the crude amine in methylene chloride at −10° was added 6.5 g (0.033 mole) of WSC. The reaction mixture was stirred for 2 hr at −10° and 6 hr at 25°. The mixture was extracted with cold 2 *N* sulfuric acid, water, and saturated sodium chloride solution. The dried organic layer was decolorized with charcoal and evaporated; a tlc of the residue (system C) indicated two spots (iodine). The residue was dissolved in chloroform and filtered through Florisil to yield 21.6 g (88%) of product, homogeneous (system C), mp 100–110°. Recrystallization from a chloroform–pentane mixture raised the melting point to 105–110°, $[\alpha]^{32}D$ +68.9° (*c* 1, EtOH). *Anal.* Calcd for $C_{52}H_{55}N_3O_4S$: C, 76.34; H, 6.78; N, 5.14; S, 3.92. Found: C, 76.65; H, 6.67; N, 5.18; S, 4.21.

t-**Butyl S-Trityl-L-cysteinylglycyl-L-valinate (IX). Method A.** A solution of 2.04 g (2.5 mmoles) of the N,S-ditrityl ester VIII in 4.1 ml of acetone was treated with 0.27 ml of concentrated hydrochloric acid. The reaction mixture was kept at 25° for 3 hr and poured into water, and the pH of the solution was adjusted to 8 with sodium bicarbonate. The solution was extracted with ethyl acetate, dried, and evaporated to an oil which crystallized to yield 1.03 g (72%), mp 155–159°, homogeneous (system A). The properties of this material were identical with those of the preparation obtained by method B.

Method B. A solution containing 2.04 g (2.5 mmoles) of the N,S-ditrityl ester VIII in 10 ml of 80% acetic acid was heated on a steam bath for 5 min and poured into water, and the pH was adjusted to 8 with sodium bicarbonate. The solution was extracted with ethyl acetate; the extract was dried and evaporated to an oil which was crystallized from ether, mp 158–160°, 1.07 g (74%). The analytical sample was prepared by recrystallization from a methanol–ether–hexane solvent; mp 162–163°, $[\alpha]^{31}D$ +0.6° (*c* 1, EtOH), $[\alpha]^{26}D$ +2.4° (*c* 1, DMF). *Anal.* Calcd for $C_{33}H_{41}N_3O_4S$: C, 68.84; H, 7.18; N, 7.30; S, 5.57. Found: C, 69.14; H, 7.23; N, 7.32; S, 5.48.

Methyl S,S′-N-Carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycyl-S-trityl-L-cysteinylglycyl-L-valinate. To a cold solution containing 0.218 g (0.2 mmole) of II and 0.115 g (0.2 mmole) of IX in 5 ml of dry DMF was added 0.038 g (0.2 mmole) of WSC. The solution was stirred at −10° for 1.5 hr and at 20° for 40 hr. The solution was poured into cold, dilute acetic acid (20 ml) and the resulting solid (0.026 g) was collected, washed with water, methanol, and ether, and dried.

The filtrate was freed of ether and diluted with water to yield a solid which was washed with water and dried. The material (0.260 g) exhibited four spots on tlc (system A) and was not further purified by recrystallization from DMF–ether (1:40, v/v). A 0.057-g sample was adsorbed on a preparative tlc plate and eluted with system A. The more mobile component was extracted with chloroform–methanol–pyridine (8:1:1, v/v) and slowly recrystallized from methanol to provide 0.021 g of white solid, mp 232–236°. *Anal.* Calcd for $C_{84}H_{91}N_{11}O_{14}S_4$: C, 62.77; H, 5.71; N, 9.59; S, 7.99. Found: C, 62.48; H, 6.01; N, 9.63; S, 8.08.

Stability of *t*-**Butyl S-Trityl-L-cysteinylglycylvalinate (IX) in Pyridine–DMF.** A solution containing 0.100 g of IX in 2 ml of a

(17) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1961, p 968.

(18) Prepared in 81% yield by the method of R. W. Roeske, *J. Org. Chem.*, **28**, 1251 (1963), using a chloroform solvent: mp 146–147°, lit. mp 147–149°.

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

pyridine–DMF mixture (1:1, v/v) was heated to 100° for 15 min. The pale yellow solution was diluted with water and the suspension was stirred at 20° for 1 hr. The solid was collected, washed with water, and dried. The material (0.095 g, 95% recovery) was demonstrated to be homogeneous (system A) with the same R_f as starting material, mp 151–153°.

t-Butyl **S,S′-N-Carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-L-hemicystyl-L-phenylalanylglycyl-S-trityl-L-cysteinylglycyl-L-valinate (X).** A cold suspension of 0.437 g (0.4 mmole) of II (obtained *via* the thiocyanogen reaction) and 0.231 g (0.4 mmole) of IX in a pyridine–DMF mixture (3:2, v/v) was treated with 0.083 g of DCC. The viscous reaction mixture was stirred at −10° for 2 hr and at 25° for 16 hr. Dilution with water afforded a solid which was collected, washed with water, and dissolved in methanol (20 ml). The clear solution was cooled and the small amount of precipitated solid was filtered. The filtrate was evaporated to a residue which crystallized from an acetone–hexane mixture (4:3, v/v, 25 ml) to yield (after drying to constant weight) 0.490 g (74%) of X, homogeneous (system A): mp 228°, $[\alpha]^{30}D$ +17.3° (*c* 0.52, DMF). The amino acid analysis was $CySO_3H_{3.9}$, $Gly_{4.2}$, $Val_{1.0}$, and $Phe_{2.0}$. *Anal.* Calcd for $C_{87}H_{97}N_{11}O_{14}S_4$: C, 63.37; H, 5.93; N, 9.34; S, 7.78; mol wt, 1649. Found: C, 63.29; H, 6.10; N, 9.26; S, 7.56; mol wt, 1642.

S,S′-N-Carbobenzoxy-L-hemicystyl-S-benzhydryl-L-cysteinylglycyl-L-phenylalanylglycyl-S-trityl-L-cysteinylglycyl-L-valine (VI). To a solution of 0.137 g (0.083 mmole) of X in 2.5 ml of dry acetic acid was added 0.127 ml (1.0 mmole) of boron trifluoride–diethyl ether. The solution was stirred for 1 hr, poured into 30 ml of ice water, and filtered. The precipitate was washed with water and dried to yield 0.129 (98%) of VI, homogeneous (systems A and B). The analytical sample was prepared by recrystallization from chloroform–hexane (1:2, v/v, 5 ml): 168°, $[\alpha]^{27}D$ +9.1° (*c* 0.5, DMF). *Anal.* Calcd for $C_{88}H_{89}N_{11}O_{14}S_4 \cdot 0.5CHCl_3$: C, 60.68; H, 5.46; N, 9.32; S, 7.76. Found: C, 60.30; 60.50; H, 5.68, 5.75; N, 9.64, 9.76; S, 7.97.

Enzymic Hydrolysis of X. To 6.34 mg (3.845 μmoles) of X was added 2.0 ml of freshly prepared trifluoroacetic acid saturated with hydrogen bromide. The reaction mixture was kept to 40° for 40 min and evaporated *in vacuo* to a yellow residue. The residue was washed by decantation with ether, dried, and dissolved in 1.1 ml of 88% formic acid–methanol. The solution was cooled to −10° and treated with 3.2 ml of a solution of 88% formic acid and 30% hydrogen peroxide (9:1, v/v). The solution was kept at −10° for 4 hr, treated with 0.48 ml of 48% hydrobromic acid, and evaporated *in vacuo* at 40°. The dried residue was dissolved in 4.0 ml of water and aliquots of this solution were used as described below.

A 1.0-ml aliquot was treated with 1.0 ml of concentrated hydrochloric acid and hydrolyzed in a sealed tube at 100° for 24 hr. Automatic amino acid analysis of the residue indicated the following amino acid ratios (corrected for destruction during hydrolysis): $CySO_3H_{3.9}$, $Gly_{4.2}$, $Phe_{2.0}$, and $Val_{1.0}$.

A second 2.0-ml aliquot of the stock solution of deblocked X was incubated with 0.25 ml of aminopeptidase M[20,21] solution (10,000 mEU/ml of water). The pH of the solution was adjusted to 7.75 by the addition of Tris buffer and the solution was covered with a layer of toluene. The reaction mixture was incubated at 40° for 40 hr; the enzyme was denatured by heating for 10 min and the mixture was filtered. The combined filtrate and washings were evaporated to dryness *in vacuo* and the residue was dissolved in 4.0 ml of 0.2 *N* citrate buffer (pH 2.2). Automatic amino acid analysis of a 1.0-ml aliquot of this solution indicated the following ratios of amino acids: $CySO_3H_{4.4}$, $Gly_{4.1}$, $Phe_{2.8}$, and $Val_{1.0}$. No peaks due to cysteic acid peptides were observed in the elution curve.

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Nuclear Magnetic Resonance Studies of the Interaction of Tryptophan with α-Chymotrypsin

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Abstract: The Michaelis complex formed by the interaction of tryptophan with α-chymotrypsin has been investigated by high-resolution nmr techniques. Appreciable line broadening is observed with both the D and L isomers of the amino acid. The D form appears to be more tightly bound to the enzyme than the L isomer. It is concluded that the binding is tight enough to imbue the bound tryptophan molecule with the same over-all rotational characteristics that characterize the motion of the enzyme in solution. Experiments with chemically modified enzymes suggest that tryptophan interacts with only one site on the enzyme and that this site is at or near the active site.

As a result of a great deal of experimental effort the mechanism of α-chymotrypsin-catalyzed reactions is largely understood.[1] Minimally, the reaction (eq 1) involves a preequilibrium between substrate (S) and enzyme (E) to give an enzyme–substrate complex (ES) which reacts to give an acylated enzyme (ES′) that is derived by transfer of the acyl portion of the substrate to a serine hydroxyl on the enzyme. Deacylation of the enzyme essentially completes the reaction although a third equilibrium involving the complexation of the product acyl group with the enzyme in a manner analogous to the formation of ES may intervene between the deacylation step and the regeneration of the enzyme.[2]

A large number of substrates and inhibitors of α-chymotrypsin-catalyzed reactions have been examined and the factors responsible for the specificity of the

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \xrightarrow{k_3} \text{products} \qquad (1)$$

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