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Selective Cleavage of Cysteine Peptides

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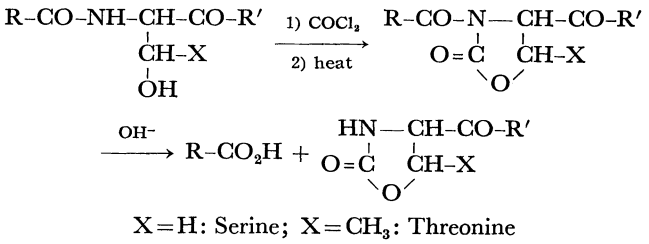
The cysteine peptides such as IV were cyclized to thiazolidone peptides through the corresponding *S*-chlorocarbonyl derivatives. In some peptides such as XXII, however, no cyclized products were isolated because of decomposition of the *S*-chlorocarbonyl intermediates. By the mild alkaline hydrolysis of the thiazolidone peptide the carboxylic acid from the *N*-terminal amino acid or peptide and 2-oxothiazolidine-4-carbonyl derivatives were isolated as a result of the selective cleavage of the peptide bond in which an amino group of cysteine residue participated. This cleavage reaction was applied to several cysteine peptides. The same results were observed whenever the corresponding thiazolidone peptides were formed.

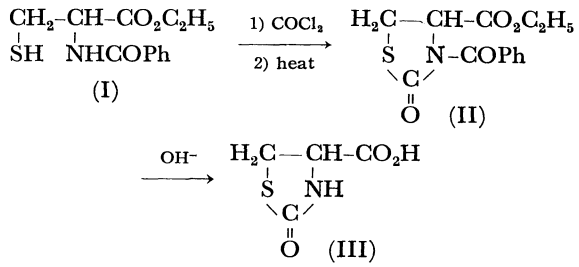
In studying the sequence of amino acid residues in proteins, the selective cleavage of peptide bonds on the special amino acid residue by enzyme or by chemical modification is very valuable in the elucidation of their structure.[1] The use of cyanide ion on cystinyl peptides gives rise to cleavage at the peptide linkage involving the amino group of the cysteine residue.[2] Treatment with base on *S*-acylcysteinyl peptides also afforded a similar cleavage on peptide bond.[3] Modification of cysteinyl residues to *S*-2-aminoethylcysteinyl residues[4] and dehydroalanyl residues[5] can be used to cleave on its residue in proteins.

Protected serine and threonine peptides were cleft selectively by treatment with alkali on their corresponding oxazolidone peptides, which were obtained by the action of phosgene, to give *N*-protected amino acids or



X=H: Serine; X=CH_3: Threonine

peptides and the corresponding oxazolidone derivatives.[6] Cysteine peptides may be expected to cause a similar cleavage because of the reactivity of thiol group. Actually ethyl *N*-benzoyl-L-cysteinate (I) was cyclized to the corresponding thiazolidone derivative (II) by



1) For reviews, *cf.* B. Witkop, *Advan. Protein Chem.*, **16**, 221 (1961); T. F. Spande, B. Witkop, Y. Degani, and A. Patchornik, *ibid.*, **24**, 97 (1970).

2) J. L. Wood and N. Catsimpoolas, *J. Biol. Chem.*, **238**, PC-2887 (1963).

3) Y. Degani, A. Patchornik, and J. A. Maclaren, *J. Amer. Chem. Soc.*, **88**, 3460 (1966).

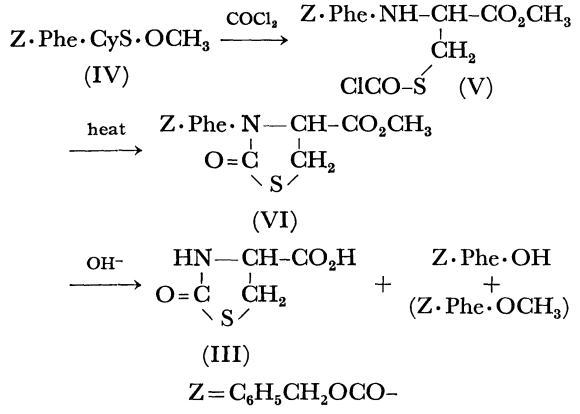
4) H. Lindley, *Nature*, **178**, 647 (1956); M. A. Raftery and R. D. Cole, *Biochem. Biophys. Res. Commun.*, **10**, 467 (1963).

5) M. Sokolovsky, T. Sadeh, and A. Patchornik, *J. Amer. Chem. Soc.*, **86**, 1212 (1964).

6) T. Kaneko, I. Takeuchi, and T. Inui, This Bulletin, **41**, 974 (1968); T. Kaneko, S. Kusumoto, T. Inui, and T. Shiba, *ibid.*, **41**, 2155 (1968).

treatment with phosgene followed by refluxing in xylene. Hydrolysis of II with alkali in 70% ethanol provided L-2-oxothiazolidine-4-carboxylic acid (III) without any racemization.[7] If cysteine peptides are cyclized with phosgene to the corresponding thiazolidone peptides at their cysteine residue, the peptides thus obtained will selectively be cleft in two parts at the amino group of cysteine residue by alkaline hydrolysis.

First, *N*-benzyloxycarbonyl-L-phenylalanyl-L-cysteine methyl ester (IV) in which cysteine residue was located in the *C*-terminal position was studied as a model peptide for the cleavage reaction. Reduction of *N*, *N'*-bis(*N*-benzyloxycarbonyl-L-phenylalanyl)-L-cystine dimethyl ester with zinc and hydrochloric acid in methanol gave IV which was converted with phosgene into *S*-chlorocarbonyl compound (V) by using a method



similar to that in previous papers.[6,7] The *S*-chlorocarbonyl compound V was heated in xylene under reflux to cyclize the thiazolidone peptide VI in good yield. The thiazolidone peptide VI was treated with a mixture of 1 N potassium hydroxide and methanol (1:2.5, v/v) at room temperature to give *N*-benzyloxycarbonyl-L-phenylalanine and L-2-oxothiazolidine-4-carboxylic acid (III, $[\alpha]_D$ −58°)[7] without any racemization in yields of 64 and 82%, respectively. The structures of the products isolated were confirmed by comparison of their properties such as melting point and optical rotation with those of the corresponding authentic specimens. On the other hand, the alkaline hydrolysis of VI with about 0.3N methanolic potassium hydroxide solution afforded *N*-benzyloxycarbonyl-L-phenylalanine and methyl *N*-benzyloxycarbonyl-L-phenylalaninate, which was characterized as *N*-benzyloxycarbonyl-L-phenylalanine hydrazide. Another component, however, 2-oxothiazolidine-4-carboxylic acid was obtained as a racemic form ($[\alpha]_D$ −3.9°). The same cleavage reactions also occurred on *N*-benzyloxycarbonyl-L-alanyl-L-cysteine methyl ester (VII) and *N*-benzyloxycarbonyl-L-phenylalanylglycyl-L-cysteine methyl ester (X), both giving the corresponding cleavage products as shown in Table 1. From the results, we see that the peptides are cleft selectively at the peptide linkage involving the amino group of the cysteine residue though the optical properties of the products vary with experimental conditions.

Next, *N*-benzoyl-L-cysteinyl-L-phenylalanine methyl ester (XV) was chosen as a model compound containing cysteine which was located in the middle of the peptide chain. In a similar way to that reported for the S→N acyl group migration of *S*-acylcysteine derivatives,[8,9] peptide XV was prepared from *S*-benzoyl-L-cysteinyl-L-phenylalanine methyl ester hydrochloride (XIV). The cyclized product, L-3-benzoyl-2-oxothiazolidine-4-carbonyl-L-phenylalanine methyl ester (XVII)

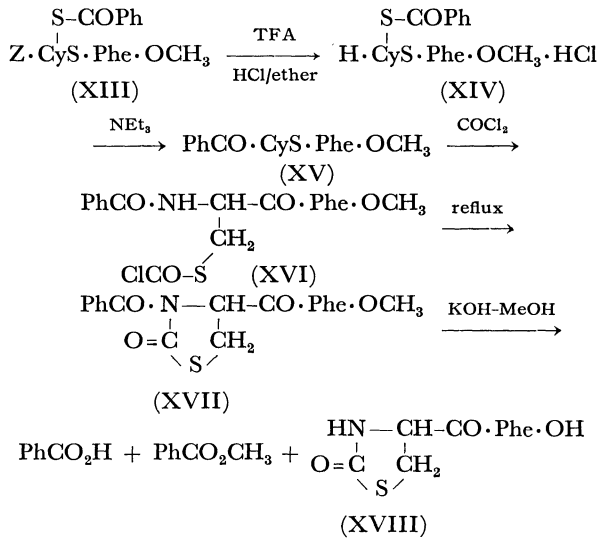


Table 1. Results of cleavage reaction of cysteine peptides

Peptide	Thiazolidone derivative	Condition[a]	Products isolated by cleavage reaction (yields)[b]	
IV	VI	KOH-MeOH	Z·Phe·OH (64%)	III (82%)
IV	VI	KOH/MeOH	Z·Phe·OH (27%) Z·Phe·OCH_3 (34%)	III (74%) (Racemized)
VII	IX	KOH-MeOH	Z·Ala·OH (54%)	III (39%)
X	XII	KOH-MeOH	Z·Phe·Gly·OH (70%)	III (80%)
X	XII	KOH/MeOH	Z·Phe·Gly·OH (32%) (Partially racemized)	III (67%) (Racemized)
XV	XVII	KOH-MeOH	$PhCO_2H$ (10%) $PhCO_2CH_3$ (28%)	XVIII (68%)

a) KOH-MeOH: mixture of 1N potassium hydroxide and methanol (1:2.5, v/v)and KOH/MeOH: 0.3N methanolic potassium hydroxide solution.
b) Unless otherwise stated, the amino acid residue has an L-configuration.

7) T. Kaneko, T. Shimokobe, Y. Ota, E. Toyokawa, T. Inui, and T. Shiba, *ibid.*, **37**, 242 (1964).
8) R. G. Hiskey, T. Mizoguchi, and T. Inui, *J. Org. Chem.*, **31**, 1192 (1966).
9) L. Zervas, I. Photaki, A. Cosmatos, and D. Borovas, *J. Amer. Chem. Soc.*, **87**, 4922 (1965).

was obtained from XV *via* the corresponding *S*-chlorocarbonyl intermediate XVI in a relatively poor yield. Similarly, hydrolysis of XVII with alkali afforded methyl benzoate, benzoic acid and L-2-oxothiazolidine-4-carbonyl-L-phenylalanine (XVIII) which was purified as its cyclohexylammonium salt (Table 1). Similar cyclizations on *N*-benzoyl-L-cysteinylglycine methyl ester (XIX) and *N*-benzyloxycarbonyl-L-phenylalanyl-L-cysteinylglycine methyl ester (XXII) to their thiazolidone derivatives through the corresponding *S*-chlorocarbonyl compounds (XX and XXIII) were studied. XX was cyclized to the thiazolidone derivative (XXI) in only a small yield, the alkaline hydrolysis of which could not be carried out, and was accompanied with formation of a cystine peptide. In the case of XXIII, no cyclized product was obtained since the corresponding *S*-chlorocarbonyl intermediate XXIII, was decomposed by heating in toluene with the evolution of bad smelling gas.

In conclusion, selective cleavage of cysteine peptide takes place at the amino group of cysteine residue in original peptide whenever its *S*-chlorocarbonyl derivative is cyclized to the corresponding thiazolidone derivative.

Experimental[10]

N,N'-Bis(N-benzyloxycarbonyl-L-phenylalanyl)-L-cystine Dimethyl Ester. To a suspension of dimethyl cystinate hydrochloride (8.5 g, 0.025 mol) in chloroform (150 m*l*) were added triethylamine (7.0 m*l*, 0.050 mol) and a solution of *N*-benzyloxycarbonyl-L-phenylalanine (15.0 g, 0.050 mol) in chloroform (70 m*l*). A solution of *N,N'*-dicyclohexylcarbodiimide (11.2 g, 0.054 mol) in chloroform (20 m*l*) was added and the reaction mixture was allowed to stand overnight at room temperature. *N,N'*-dicyclohexylurea thus precipitated was removed by filtration and the filtrate was successively washed with water, dilute hydrochloric acid and dilute aqueous sodium hydrogen carbonate solution. The dried solution was evaporated *in vacuo* to give white crystals which were recrystallized from ethanol. Yield, 15.1 g (72.6%), mp 137—139°C, $[\alpha]_D^{22}$ −88.9° (*c* 3.12, *N,N*-dimethylformamide).

Found: C, 60.98; H, 5.59; N, 6.88%. Calcd for $C_{42}H_{46}N_4O_{10}S_2$: C, 60.71; H, 5.58; N, 6.74%.

N-Benzyloxycarbonyl-L-phenylalanyl-L-cysteine Methyl Ester (IV). According to the method employed in the reduction of cystine derivatives to cysteine derivatives by Zervas,[11] a suspension of the above cystine peptide (15.1 g, 0.018 mol) in methanol (150 m*l*) containing concentrated hydrochloric acid (12.0 m*l*) was treated with zinc dust (6.0 g) in small portions at a time for 30 min at about 50°C. Undissolved zinc was then filtered off and the filtrate was diluted with water to separate IV as white crystals. Yield, 11.6 g (76.8%), mp 126—127°C after recrystallization from ethyl acetate-petroleum ether (50—90°C), $[\alpha]_D^{20}$ −14.4° (*c* 2.19, methanol), lit,[8] mp 125—127°C.

Found: C, 60.32; H, 5.80; N, 6.60; S, 7.48%. Calcd for $C_{21}H_{24}N_2O_5S$: C, 60.56; H, 5.81; N, 6.73; S, 7.70%.

N-Benzyloxycarbonyl-L-phenylalanyl-S-chlorocarbonyl-L-cysteine Methyl Ester (V). Phosgene was passed through a solution of IV (14.6 g, 0.035 mol) and *N,N*-dimethylaniline (4.3 g, 0.035 mol) in dioxane (140 m*l*) for 30 min at 10—12°C. The reaction mixture was stirred for 4 hr below 20°C and bubbled with a stream of carbon dioxide (or nitrogen) to remove excess phosgene. The separated *N,N*-dimethylaniline hydrochloride was filtered off and the crystals obtained by evaporation of the filtrate were washed with benzene and water. Yield, 10.7 g (64%), mp 154—155°C after recrystallization from benzene, $[\alpha]_D^{22}$ −5.6° (*c* 2.15, dioxane).

Found: C, 55.45; H, 5.20; N, 5.92%. Calcd for $C_{22}H_{23}N_2O_6SCl$: C, 55.17; H, 4.84; N, 5.85%.

Treatment of a solution of V in benzene with an etheral solution of aniline gave *N*-benzyloxycarbonyl-L-phenylalanyl-*S*-(*N*-phenylcarbamoyl)-L-cysteine methyl ester; mp 165.3—165.5°C, $[\alpha]_D^{22}$ −51.5° (*c* 2.10, *N,N*-dimethylformamide).

Found: C, 63.06; H, 5.46; N, 7.76; S, 5.52%. Calcd for $C_{28}H_{29}N_3O_6S$: C, 62.79; H, 5.46; N, 7.85; S, 5.99%.

Methyl L-3-(N-Benzyloxycarbonyl-L-phenylalanyl)-2-oxothiazolidine-4-carboxylate (VI). A solution of V (7.2 g, 0.015 mol) in xylene (140 m*l*) was heated under reflux for 5 hr. Evaporation of the solvent afforded an oil which was crystallized by seeding or by scratching on the wall of the vessel. Yield, 5.8 g (88%), mp 103—105°C. Recrystallization from ethyl acetate raised the melting point to 112.0—112.5°C, $[\alpha]_D^{20}$ −75.8° (*c* 1.47, methanol).

Found: C, 59.88; H, 5.07; N, 6.34; S, 7.12%. Calcd for $C_{22}H_{22}N_2O_6S$: C, 59.72; H, 5.01; N, 6.33; S, 7.25%.

Alkaline Hydrolysis of VI. a) With Potassium Hydroxide in Aqueous Methanol: To a suspension of VI (3.10 g, 7 mmol) in methanol (35.0 m*l*) was added 1N potassium hydroxide (14.0 m*l*, 14 mmol) and the reaction mixture was stirred for 1.5 hr at room temperature. After evaporation of the solvent *in vacuo*, the residue was diluted with water and the aqueous solution was acidified with 1N hydrochloric acid (8.0 m*l*) to pH 3. The separated oil was extracted twice with ethyl acetate. Evaporation of the dried solution *in vacuo* provided an oily *N*-benzyloxycarbonyl-L-phenylalanine which was crystallized by seeding. Yield, 1.35 g (64.3%), mp 82—89°C and 88—89°C after recrystallization from ethyl acetate-petroleum ether (50—90°C), lit,[12] mp 88—89°C. A mixed melting point with the authentic sample showed no depression.

It was also identified as its cyclohexylammonium salt,[13] mp 168—169°C, $[\alpha]_D^{19}$ +37.4° (*c* 1.69, ethanol).

An additional 1N hydrochloric acid (8.0 m*l*) was added to the remaining aqueous solution. The residue obtained by evaporation of the acidic solution was extracted three times with hot ethyl acetate. The extract was evaporated *in vacuo* to dryness leaving III as a crystalline residue. Yield, 0.85 g (82.5%), mp 157—164°C and 172—173°C after recrystallization from acetone-petroleum ether (50—90°C), $[\alpha]_D^{22}$ −57.9° (*c* 1.89, water), lit,[7] mp 171—172.5°C, $[\alpha]_D^{16}$ −57.3° (*c* 2.7, water).

Hydrolysis of this sample with 6N hydrochloric acid and subsequent oxidation with air by the usual procedure afforded L-cystine in a yield of 57%, $[\alpha]_D^{19}$ −211° (*c* 1.18, 1N hydrochloric acid), lit,[14] $[\alpha]_D^{25}$ −212° (*c* 1, 1N hydrochloric acid).

b) With Methanolic Potassium Hydroxide Solution: In a manner similar to that described above, a suspension of VI (3.10 g, 7 mmol) in methanol (35.0 m*l*) was treated with 1N methanolic potassium hydroxide solution (14.5 m*l*, 14.5 mmol). After evaporation of the solvent, the residue was diluted with water to give an oil which was extracted twice

10) All melting points are uncorrected.

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

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

13) An authentic sample was prepared from *N*-benzyloxycarbonyl-L-phenylalanine by treatment with cyclohexylamine; mp 169.0—169.5°C, $[\alpha]_D^{22}$ +37.1° (*c* 2.06, ethanol). Found: C, 69.37; H, 7.81; N, 7.10%. Calcd for $C_{23}H_{30}N_2O_4$: C, 69.32; H, 7.59; N, 7.03%.

14) J. P. Greenstein and M. Vinitz, "Chemistry of the Amino Acids" Vol. 3, John Wiley & Sons, New York (1961) p. 1879.

with ethyl acetate. Evaporation of the dried extract provided methyl *N*-benzyloxycarbonyl-L-phenylalaninate as an oil (wt, 1.24 g). Infrared spectra of this sample were identical with those of an authentic sample which was prepared from methyl L-phenylalaninate and benzyloxycarbonyl chloride. Treatment of a solution of the oil in methanol with a solution of an excess of hydrazine hydrate (about 95%) in methanol gave *N*-benzyloxycarbonyl-L-phenylalanine hydrazide. Yield, 0.75 g (34.2%), mp 161—163°C and 170.5—171°C after recrystallization from ethanol, $[\alpha]_D^{24}$ −6.9° (*c* 1.24, *N*, *N*-dimethylformamide), lit, mp 167—168°C[15], mp 168°C.[16] A mixed melting point with the authentic sample [mp 171.0—171.5°C, $[\alpha]_D^{24}$ −7.0° (*c* 1.28, *N*,*N*-dimethylformamide)] showed no depression.

Found: C, 65.34; H, 6.02; N, 13.45%. Calcd for $C_{17}H_{19}N_3O_3$: C, 65.16; H, 6.11; N, 13.41%.

The aqueous solution was acidified to pH 3. From ethyl acetate extract, *N*-benzyloxycarbonyl-L-phenylalanine was isolated as its cyclohexylammonium salt. Yield, 0.74 g (26.6%), mp 160—162°C and 169.5—170.0°C after recrystallization from ethanol-ether, $[\alpha]_D^{21}$ +37.9° (*c* 2.28, ethanol). No melting point depression upon the authentic sample[13] was observed.

As the third product, III was obtained from the acidic solution. Yield, 0.76 g (74.5%), mp 151—153°C and 153.5—154.5°C after recrystallization from acetone-petroleum ether (50—90°C), $[\alpha]_D^{20}$ −3.9° (*c* 2.92, water).

Hydrolysis of this sample with 6N hydrochloric acid and subsequent oxidation gave cystine having $[\alpha]_D^{20}$ −24.8° (*c* 1.39, 1N hydrochloric acid).

N-Benzyloxycarbonyl-L-alanyl-L-cysteine Methyl Ester (VII). In a similar way to that described for the preparation of IV, *N,N'*-bis(*N*-benzyloxycarbonyl-L-alanyl)-L-cystine dimethyl ester[17] (10.2 g, 15 mmol) was reduced to thiol peptide VII with zinc (9.0 g) and concentrated hydrochloric acid (19.5 ml) in methanol (120 ml). Yield, 8.1 g(79.5%), mp 114—116°C and 116.5—118.0°C after recrystalization from ethyl acetate, $[\alpha]_D^{20}$ −26.5° (*c* 1.27, methanol), lit,[8] mp 114—116°C.

Found: C, 53.03; H, 5.99; N, 8.20%. Calcd for $C_{15}H_{20}N_2O_5S$: C, 52.93; H, 5.92; N, 8.23%.

N-Benzyloxycarbonyl-L-alanyl-S-chlorocarbonyl-L-cysteine Methyl Ester (VIII). In the same manner as has been described for the preparation of V, a solution of VII (10.2 g, 0.030 mol) in dioxane (120 ml) was treated with a gentle stream of phosgene in the presence of *N,N*-dimethylaniline (3.6 g,0.030 mol) at 9—11°C for 25 min. After removal of excess phosgene by passage of carbon dioxide, VIII was obtained as a syrup which was crystallized by seeding. Yield, 10.5 g(86.8%), mp 94.5—95.5°C after recrystallization from chloroform-petroleum ether (50—90°C).

Found: C, 48.12; H, 4.66; N, 7.09%. Calcd for $C_{16}H_{19}N_2O_6SCl$: C, 47.70; H, 4.75; N, 6.95%.

It was also identified as *N*-benzyloxycarbonyl-L-alanyl-*S*-(*N*-phenylcarbamoyl)-L-cysteine methyl ester by treating with aniline; mp 158.5—159.5°C after recrystallization from benzene, $[\alpha]_D^{22}$ −30.4° (*c* 1.90, *N*,*N*-dimethylformamide).

Found: C, 57.42; H, 5.22; N, 9.07%. Calcd for $C_{22}H_{25}N_3O_6S$: C, 57.50; H, 5.48; N, 9.14%.

Methyl-L-3-(N-Benzyloxycarbonyl-L-alanyl)-2-oxothiazolidine-4-carboxylaye(IX). Heating of VIII (6.1 g, 15 mmol) in xylene (120 ml) under reflux for 5 hr followed by evaporating *in vacuo* gave IX as a syrup, wt, 5.6 g (101%). It gave a negative Beilstein's test and exhibited a main spot, accompanied by three minor spots, upon thin-layer chromatography with silica gel G(tlc).

This was used for the following cleavage reaction without further purification.

Alkaline Hydrolysis of IX. Hydrolysis of IX (3.68 g,10 mmol) was carried out in methanol (49.0 ml) by adding 1N potassium hydroxide (21.0 ml, 21 mmol) and by keeping the solution at room temperature for 1.5 hr. After methanol had been evaporated *in vacuo*, the residue was diluted with water and acidified with 1N hydrochloric acid (11.0 ml) to pH 3. Extraction with ethyl acetate and subsequent evaporation of the solvent gave *N*-benzyloxycarbonyl-L-alanine as a syrup which was purified as its cyclohexylammonium salt.[18] Yield, 1.85 g (54.4 %), mp 86—87°C after recrystallization from acetone-ether, $[\alpha]_D^{24}$ −10.2° (*c* 3.14, water). A mixed melting point with the authentic sample showed on depression.

Found: C, 59.83; H, 8.41; N, 8.05%. Calcd for $C_{17}H_{26}N_2O_4\cdot H_2O$: C, 59.98; H, 8.29; N, 8.23%.

On the other hand, the aqueous layer separated from the ethyl acetate extract was further acidified with an additional 1 N hydrochloric acid (11.0 ml) and evaporated *in vacuo* to give a residue. From the residue, III was isolated by extracting with hot ethyl acetate. Yield, 0.57 g (39.3%), mp 169—170°C and 172—173°C after recrystallization from acetone-petroleum ether(50—90°C), $[\alpha]_D^{23}$ −58.0° (*c* 2.31, water).

Properties of this sample such as infrared spectra were identical with those of the authentic sample.[8]

N-Benzyloxycarbonyl-L-phenylalanylglycyl-L-cysteine Methyl Ester (X). A mixed solution of *N*-benzyloxycarbonyl-L-phenylalanylglycine[19] (8.9 g, 0.025 mol) and methyl L-cysteinate, prepared from its hydrochloride[11] (4.3 g, 0.025 mol) and triethylamine (3.5 ml, 0.025 mol), in chloroform (90 ml) was treated with a solution of *N,N'*-dicyclohexylcarbodiimide (5.3 g, 0.026 mol) in chloroform (15 ml) at room temperature for 4 hr. After *N,N'*-dicyclohexylurea had been removed by filtration, the filtrate was washed successively with water, dilute hydrochloric acid, dilute aqueous sodium hydrogen carbonate solution and water. Evaporation of the dried solution gave a solid which was dissolved into ethyl acetate. The ethyl acetate solution separated from another *N,N'*-dicyclohexylurea was concentrated *in vacuo* to give X as white crystals. Yield, 9.7 g (82.8%), mp 129—132°C and 135—136°C after recrystallization from ethyl acetate, $[\alpha]_D^{22}$ −25.2° (*c* 1.96, *N*,*N*-dimethylformamide).

Found: C, 58.23; H, 5.78; N, 8.88%. Calcd for $C_{23}H_{27}N_3O_6S$: C, 58.34; H, 5.75; N, 8.87%.

It was oxidized with 1/10 N iodine-potassium iodide solution to the disulfide, *N,N'*-bis(*N*-benzyloxycarbonyl-L-phenylalanylglycyl)-L-cystine dimethyl ester, mp 187—188°C, $[\alpha]_D^{22}$ −49.7° (*c* 1.83, *N*,*N*-dimethylformamide).

Found: C, 58.26; H, 5.39; N, 8.70%. Calcd for $C_{46}H_{52}N_6O_{12}S_2$: C, 58.46; H, 5.55; N, 8.89%.

15) G. W. Anderson and R. W. Young, *J. Amer. Chem. Soc.*, **74**, 5307 (1952).

16) J. I. Harris and J. S. Work, *Biochem. J.*, **46**, 196 (1950).

17) This peptide was synthesized by the carbodiimide method in a similar manner to that for *N,N'*-bis(*N-benzyloxycarbonyl*-L-phenylalanyl)-L-cystine dimethyl ester as shown above. Yield, 75—80%, mp 160—161°C after recrystallization from ethanol, $[\alpha]_D^{22}$ −67.0° (*c* 1.98, *N*,*N*-dimethylformamide). Found: C, 53.20; H, 5.58; N, 8.09%, Calcd for $C_{30}H_{38}N_4O_{10}S_2$: C, 53.08; H, 5.64; N, 8.25%.

18) The authentic sample was prepared as a hydrate from *N*-benzyloxycarbonyl-L-alanine by treatment with cyclohexylamine; mp 85.5—86.0°C, $[\alpha]_D^{23.5}$ −10.2° (*c* 2.78, water). Found: C, 59.79; H, 8.34; N, 8.18%. Calcd for $C_{17}H_{26}N_2O_4\cdot H_2O$: C, 59.98; H, 8.29; N, 8.23%.

19) D. W. Clyaton, J. A. Farrington, G. W. Kenner, and J.M. Turner, *J. Chem. Soc.*, **1957**, 1398.

It was also identified as an *S*-benzoyl derivative, *N*-benzyloxycarbonyl-L-phenylalanylglycyl-*S*-benzoyl-L-cysteine methyl ester, by treating with benzoyl chloride in pyridine; mp 152—153°C, $[\alpha]_D^{22}$ −36.2° (*c* 2.67, *N,N*-dimethylformamide).

Found: C, 62.40; H, 5.41; N, 7.27%. Calcd for $C_{30}H_{31}N_3O_7S$: C, 62.38; H, 5.41; N, 7.27%.

N-Benzyloxycarbonyl-L-phenylalanylglycyl-S-chlorocarbonyl-L-cysteine Methyl Ester (XI). In a way similar to that for the preparation of V phosgene was passed through a solution of X (4.73 g, 10 mmol) in dioxane (50 m*l*) in the presence of *N,N*-dimethylaniline (1.21 g, 10 mmol) at 15°C for 20 min. From the reaction mixture, XI was obtained as crystals. Yield, 3.61 g(67.4%), mp 144—145°C (decomp.).

Found: C, 54.33; H, 4.89; N, 7.71%. Calcd for $C_{24}H_{26}N_3O_7SCl$: C, 53.78; H, 4.89; N, 7.84%.

Methyl L-3-(N-Benzyloxycarbonyl-L-phenylalanylglycyl)-2-oxothiazolidine-4-carboxylate (XII). A solution of XI (5.85 g, 10.9 mmol) in xylene (120 m*l*) was heated for 3 hr under reflux. The reaction mixture was cooled to room temperature to deposit XII as crystals. Yield, 4.85 g(89.0%), mp 175.5—177.0°C and 177.5—178.5°C after recrystallization from ethanol, $[\alpha]_D^{22}$ −81.5° (*c* 1.43, *N,N*-dimethylformamide).

Found: C, 57.60; H, 5.00; N, 8.63%. Calcd for $C_{24}H_{25}N_3O_7S$: C, 57.71; H, 5.04; N, 8.41%.

Alkaline Hydrolysis of XII. a) With Potassium Hydroxide in Aqueous Methanol: XII (3.30 g, 6.6 mmol) was hydrolyzed with a mixture of 1 N potassium hydroxide (14.0 m*l*, 14.0 mmol) and methanol (35.0 m*l*). After evaporation of the solvent, the residue was diluted with water and acidified with 1 N hydrochloric acid to pH 3. From ethyl acetate extract, *N*-benzyloxycarbonyl-L-phenylalanylglycine was isolated; Yield, 1.65 g (70.2%), mp 151.5—153.0°C. Recrystallization from ethyl acetate raised the melting point to 153—154°C, $[\alpha]_D^{20}$ −9.1° (*c* 3.12, acetic acid), lit,[19] mp 154°C, $[\alpha]_D^{18}$ −10.2°C (*c* 4.3, acetic acid)

From the acidic solution separated from ethyl acetate extract, III was isolated as crystals. Yield, 0.78 g (80.4%), mp 164—166°C and 171.5—172.5°C after recrystallization from acetone-petroleum ether (50—90°C), $[\alpha]_D^{22}$ −58.0° (*c* 2.65, water).

b) With Methanolic Potassium Hydroxide Solution: XII (3.20 g, 6.4 mmol) was hydrolyzed with 0.3 N methanolic potassium hydroxide solution (45.0 m*l*, 13.5 mmol). After evaporation of the solvent, the residue was dilute with water and acidified with 1N hydrochloric acid to pH 3. From ethyl acetate extract, *N*-benzyloxycarbonylphenylalanylglycine was isolated; yield, 0.72 g (31.6%), mp 152—153°C, $[\alpha]_D^{24}$ −6.0° (*c* 1.93, acetic acid).

From the aqueous solution, III was isolated; yield, 0.63 g (67.0%), mp 153—154°C, $[\alpha]_D^{24}$ −2.4° (*c* 2.94, water).

N-Benzyloxycarbonyl-S-benzoyl-L-cysteinyl-L-phenylalanine Methyl Ester (XIII). To a mixed solution of *N*-benzyloxycarbonyl-*S*-benzoyl-L-cysteine[20] (36.0 g, 0.10 mol) and methyl L-phenylalaninate prepared from its hydrochloride (22.0 g, 0.10 mol) and triethylamine (14.0 m*l*, 0.10 mol) in chloroform (450 m*l*) a solution of *N,N'*-dicyclohexylcarbodiimide (22.0 g, 0.11 mol) in chloform (50 m*l*) was added, and the reaction mixture was allowed to stand overnight at room temperature. After filtration of *N,N'*-dicyclohexylurea, the filtrate was worked up in the usual way. Yield, 41.4 g (79.6 %), mp 136—137°C after recrystallization from ethanol, $[\alpha]_D^{20}$ −30.7° (*c* 1.01, ethanol).

Found: C, 64.38; H, 5.44; N, 5.39%. Calcd for $C_{28}H_{28}N_2O_6S$: C, 64.60; H, 5.42; N, 5.38%.

20) L. Zervas, I. Photaki, and N. Ghelis, *J. Amer. Chem. Soc.*, **85**, 1337 (1963).

S-Benzoyl-L-cysteinyl-L-phenylalanine Methyl Ester Hydrochloride (XIV). A solution of XIII (5.20 g, 10 mmol) and phenol (2.0 g) in trifluoroacetic acid (15 m*l*) was heated for 30 min under reflux. Evaporation of the solvent followed by addition of ether gave *S*-benzoyl-L-cysteinyl-L-phenylalanine methyl ester trifluoroacetate as crystals. Yield, 3.24 g (64.8%), mp 137°C.

To a solution of the trifluoroacetate (23.5 g, 0.047 mol) in methanol (40 m*l*) was added absolute ether (120 m*l*) saturated with hydrogen chloride. The reaction mixture was diluted with ether (400 m*l*) to separate XIV as crystals. Yield, 16.4 g (82.4%), mp 151—152°C (decomp.) and 153.5—154.0°C (decomp.) after recrystallization from methanol-ether, $[\alpha]_D^{24}$ +1.5° (*c* 2.61, ethanol); +3.1° (*c* 4.55, ethanol).

Found: C, 56.67; H, 5.39; N, 6.61%. Calcd for $C_{20}H_{22}N_2O_4S\cdot HCl$: C, 56.80; H, 5.48; N, 6.62%.

N-Benzoyl-L-cysteinyl-L-phenylalanine Methyl Ester (XV). A suspension of XIV (1.51 g, 3.57 mmol) in chloroform (20 m*l*) was treated with triethylamine (0.50 m*l*, 3.57 mmol) and the reaction mixture was stirred for 4 hr at 25—35°C. After evaporation of the solvent, the residue was dissolved into ethyl acetate and the ethyl acetate solution was washed successively with water, dilute hydrochloric acid, dilute aqueous sodium hydrogen carbonate solution and water. Evaporation of the dried solution provided XV as white crystals. Yield, 1.16 g (84.0%), mp 154—155°C after recrystallization from ethyl acetate, $[\alpha]_D^{20}$ −32.1° (*c* 1.32, ethanol).

Found: C, 62.31; H, 5.64; N, 7.48%. Calcd for $C_{20}H_{22}N_2O_4S$: C, 62.16; H, 5.74; N, 7.25%.

When the reaction was carried out below 25°C, pure XV was not isolated.

N-Benzoyl-S-chlorocarbonyl-L-cysteinyl-L-phenylalanine Methyl Ester (XVI). In a similar way to that for the preparation of V, phosgene was passed through a slution of XV (5.80 g, 15 mmol) in dioxane (60 m*l*) in the presence of *N,N*-diemthylaniline (1.82 g, 15 mmol) at 10—15°C for 20 min. From the reaction mixture, XV was isolated as crystals melted at 133—134°C (decomp.). Yield, 5.58 g (82.8%). Recrystallization from benzene raised the melting point to 137.0—137.5° C (decomp.), $[\alpha]_D^{25}$ −40.0° (*c* 2.06, dioxane).

Found: C, 56.36; H, 4.66; N, 5.72%. Calcd for $C_{21}H_{21}N_2O_5SCl$: C, 56.18; H, 4.72; N, 6.24%.

Methyl L-3-Benzoyl-2-oxothiazolidine-4-carbonyl-L-phenylalaninate (XVII). A solution of XVI (3.00 g, 6.68 mmol) in xylene (60 m*l*) was heated for 3.5 hr under reflux. The crude product XVII precipitated when the reaction mixture was cooled to room temperature. Yield, 1.06 g (38.5%), mp 213.5—214.5°C. It gave a negative Beilstein's test. Recrystallization from ethyl acetate raised the melting point to 216—217°C, $[\alpha]_D^{23.5}$ +34.2° (*c* 1.29, *N,N*-dimethylformamide).

Found: C, 61.45; H, 4.88; N, 6.59%. Calcd for $C_{21}H_{20}N_2O_5S$: C, 61.15; H, 4.89; N, 6.79%.

Alkaline Hydrolysis of XVII. A suspension of XVII (2.06 g, 5.0 mmol) in methanol (30 m*l*) was treated with 1 N potassium hydroxide (11.0 m*l*, 11.0 mmol) for 1.5 hr at room temperature with stirring. After evaporation of the solvent, the residue was diluted with water to separate an oil which was extracted with ethyl acetate. Methyl benzoate was isolated as a sweet-smelling oil from the extract; yield, 0.19 g (27.9%). Properties of this sample such as infrared spectra were identical with those of the authentic sample.

The aqueous solution separated from the ethyl acetate extract was acidified with 1 N hydrochloric acid to pH 1 to give an oily product which was extracted twice with ethyl acetate. Evaporation of the dried extract left a syrup (wt, 1.54 g) which was extracted three times with hot petroleum ether (50—90°C). The extract was evaporated *in vacuo* to

give crystals which were identified as benzoic acid by its infrared spectra; yield, 0.06 g (10.0%).

From the remaining syrup (wt, 1.50 g), XVIII was isolated as its cyclohexylammonium salt by treating with cyclohexylamine. Yield, 1.34 g (68.0%), mp 204.5—205.5°C (decomp.) and 207.5—208.0°C (decomp.) after recrystallization from ethanol-ether, $[\alpha]_D^{22}$ −6.0° (*c* 1.66, ethanol).

Found: C, 57.98; H, 7.02; N, 10.48%. Calcd for $C_{19}H_{27}N_3O_4S$: C, 57.99; H, 6.92; N, 10.68%.

N-Benzoyl-L-cysteinylglycine Methyl Ester (XIX). In a way similar to that described for the preparation of XV, a solution of *S*-benzoyl-L-cysteinylglycine methyl ester hydrochloride[20] (6.65 g, 20 mmol) and triethylamine (2.80 m*l*, 20 mmol) in chloroform (80 m*l*) was stirred for 4 hr at room temperature. From the reaction mixture, pure XIX was isolated. Yield, 4.49 g (75.7%), mp 114—115°C, $[\alpha]_D^{20}$ −32.3° (*c* 2.69, methanol).

Found: C, 52.64; H, 5.40; N, 9.35%. Calcd for $C_{13}H_{16}N_2O_4S$: C, 52.69; H, 5.44; N, 9.45%.

Oxidation of XIX by 1/10 N iodine-potassium iodide solution gave a disulfide, *N,N'*-bisbenzoyl-L-cystinyldiglycine dimethyl ester; mp 226—227°C, $[\alpha]_D^{20}$ −211° (*c* 1.65, *N,N*-dimethylformamide).

Found: C, 52.80; H, 4.68; N, 9.56%. Calcd for $C_{26}H_{30}N_4O_8S_2$: C, 52.87; H, 5.12; N, 9.49%.

N-Benzoyl-S-chlorocarbonyl-L-cysteinylglycine Methyl Ester (XX). In a manner similar to that for the preparation of V, XX was obtained from a solution of XIX (5.50 g, 18.6 mmol) in dioxane (75 m*l*) by treatment with an excess of phosgene in the presence of *N,N*-dimethylaniline (2.25 g, 18.6 mmol). Yield, 3.35 g(50.3%), mp 109—110°C (decomp.).

Methyl L-3-Benzoyl-2-oxothiazolidine-4-carbonylglycinate (XXI) and N,N'-Bisbenzoyl-L-cystinylglycine Dimethyl Ester. A solution of XX (2.51 g, 7.0 mmol) in xylene (50 m*l*) was heated for 2 hr under reflux and cooled to room temperature to separate out crystals (wt, 0.49 g, mp 189—192°C). It gave a negative Beilstein's test and exhibited two spots upon tlc.

The crude product was dissolved into ethanol (50 m*l*) and kept for a short time at room temperature to separate crystals. Yield, 0.14 g (6.8%), mp 205—208°C and 224°C after recrystallization from ethanol, $[\alpha]_D^{20}$ −206° (*c* 1.06, *N,N*-dimethylformamide). Its structure was confirmed to be *N,N'*-bisbenzoyl-L-cystinyldiglycine dimethyl ester by comparison of the properties with those of the oxidized product of XIX.

The mother liquor separated from crystals was allowed to stand overnight at room temperature. XXI was separated as the second product. Yield, 0.14 g (6.2%), mp 203—204°C. Recrystallization from ethanol changed the melting point to 202.5—203.0°C, $[\alpha]_D^{20}$ −36.7° (*c* 0.98, *N,N*-dimethylformamide).

Found: C, 52.08; H, 4.21; N, 8.45%. Calcd for $C_{14}H_{14}N_2O_5S$: C, 52.16; H, 4.38; N, 8.69%.

N-Benzyloxycarbonyl-L-phenylalanyl-S-trityl-L-cysteinylglycine Methyl Ester. A solution containing *N*-benzyloxycarbonyl-L-phenylalanine (7.93 g, 26.5 mmol), *S*-trityl-L-cysteinylglycine methyl ester hydrochloride[21] (12.50 g, 26.5 mmol), and triethylamine (3.70 m*l*, 26.5 mmol) in methylene chloride (100 m*l*) was treated with a solution of *N,N'*-dicyclohexylcarbodiimide (5.47 g, 26.5 mmol) in methylene chloride (10 m*l*). The crystalline peptide was obtained by the usual procedure; yield, 15.10 g (79.6%), mp 165—167°C. Recrystallization from ethanol raised the melting point to 176.0—176.5°C, $[\alpha]_D^{20}$ −12.6° (*c* 2.23, *N,N*-dimethylformamide).

Found: C, 70.40; H, 5.83; N, 6.11; S, 4.39%. Calcd for $C_{42}H_{41}N_3O_6S$: C, 70.47; H, 5.77; N, 5.87; S, 4.48%.

N-Benzyloxycarbonyl-L-phenylalanyl-L-cysteinylglycine Methyl Ester (XXII). A solution of the above *S*-trityl peptide (7.16 g, 10 mmol) in acetic acid (200 m*l*) was treated with a solution of mercuric chloride (5.43 g, 20 mmol) in methanol (20 m*l*) followed by a solution of crystalline sodium acetate (1.37 g, 10 mmol) in methanol (25 m*l*). After standing for 4 hr at room temperature, the precipitates were collected and washed with aqueous acetic acid, water, and ether. Yield, 5.85 g (82.6%).

A stream of hydrogen sulfide was passed through a solution of the above *S*-chloromercury compound in a mixture of *N,N*-dimethylformamide (40 m*l*) and methanol (20 m*l*) for 15 min, and the precipitated mercuric sulfide was filtered off. The filtrate was diluted with water (250 m*l*) to give a thiol peptide XXII as crystals. Yield, 2.43 g (75.7%), mp 164—165°C. After recrystallization from ethyl acetate, the melting point was raised to 171—172°C, $[\alpha]_D^{20}$ −15.9° (*c* 2.26, *N,N*-dimethylformamide).

Found: C, 58.36; H, 5.84; N, 8.61; S, 6.72%. Calcd for $C_{23}H_{27}N_3O_6S$: C, 58.34; H, 5.75; N, 8.87; S, 6.77%.

It was oxidized to *N,N'*-bis(*N*-benzyloxycarbonyl-L-phenylalanyl)-L-cystinyldiglycine dimethyl ester by the treatment with 1/10 N iodine-potassium iodide solution; mp 220—223°C, $[\alpha]_D^{20}$ −86.4° (*c* 2.20, *N,N*-dimethylformamide), lit,[20] mp 223—224°C, $[\alpha]_D^{25}$ −87° (*c* 1, *N,N*-dimethylformamide).

Found: C, 58.54; H, 5.52; N, 8.88%. Calcd for $C_{46}H_{52}N_6O_{12}S_2$: C, 58.46; H, 5.55; N, 8.89%.

N-Benzyloxycarbonyl-L-phenylalanyl-S-chlorocarbonyl-L-cysteinylglycine Methyl Ester (XXIII). In a way similar to that for the preparation of V, XXIII was obtained from a suspension of XXII (4.73 g, 10 mmol) in dioxane (50 m*l*) by treatment with excess phosgene in the presence of *N,N*-dimethylaniline (1.21 g, 10 mmol). Yield, 3.65 g (68.1%), mp 142—144°C (decomp.) and 153—154°C (decomp.) after recrystallization from benzene, $[\alpha]_D^{20}$ −33.4°C (*c* 2.87, dioxane).

Found: C, 53.95; H, 4.87; N, 7.86%. Calcd for $C_{24}H_{26}N_3O_7SCl$: C, 53.78; H, 4.89; N, 7.84%.

In order to obtain a cyclized product, a solution of XXIII (2.5 g, 4.7 mmol) in toluene (50 m*l*) was heated for 3 hr under reflux and then concentrated to dryness to give a brownish syrup, from which no detectable substance was isolated.

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