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Selective Chemical Cleavage of Threonine Peptides[1)]

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Threonine peptide was converted to an oxazolidone derivative *via* an *O*-chlorocarbonyl derivative by treatment with phosgene and dimethylaniline followed by refluxing in xylene, wherever the threonine residue was located in peptide chain, that is, either at amino or carboxyl terminal, or in the middle of the chain. When the oxazolidone peptide was treated with alkali at room temperature, the peptide bond of the original threonine residue of which the amino group participated was selectively cleaved giving a mixture of the carboxylic acid derived from the *N*-terminal amino acid or peptide, and 5-methyl-2-oxo-oxazolidine-4-carbonyl derivative, whereas other peptide bonds remained unchanged in this reaction. Several threonine peptides were synthesized and were subjected to the cleavage reaction. In all cases the expected cleavage occurred in a satisfactory yield.

Selective cleavage reaction of a peptide bond at the residue of β-hydroxy-α-amino acid by a strong acid such as sulfuric acid[2-5)] or hydrogen fluoride[6)] has been known. This cleavage is based on the N→O acyl migration. However, an entirely different principle can be applied to the same purpose. As already mentioned in the preliminary report,[1)] methyl *N*-benzoylthreoninate was found to be cyclized to *N*-benzoyloxazolidone derivative which was then hydrolyzed to 5-methyl-2-oxo-oxazolidine-4-carboxylic acid by sodium hydroxide under a mild condition accompanied with removal of the benzoyl group. This finding leads to an idea that the peptide bond in which the amino group of the hydroxyamino acid participated would be cleaved by cyclization to the oxazolidone derivative followed by hydrolysis with alkali as shown in Fig. 1, inasmuch as *N*-acyl

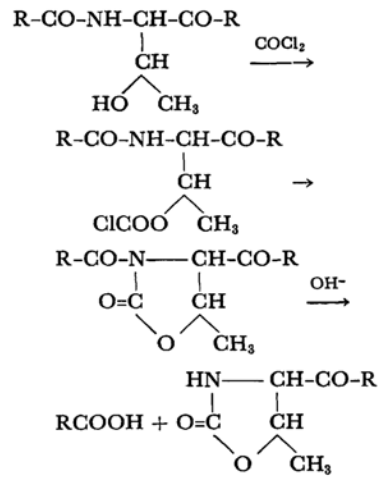

Fig. 1

1) This work was partly reported in a preliminary form: T. Kaneko and T. Inui, This Bulletin, **36**, 1541 (1963), and also presented at the 16th and 17th Annual Meetings of the Chemical Society of Japan, Tokyo, April, 1963 and Tokyo, April, 1964.

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2) D. F. Elliott, "The Chemical Structure of Protein," ed. by G. E. W. Wolstenholme and M. P. Cameron, Little, Brown, Massachusetts (1953), p. 129.

3) D. F. Elliott, *Biochem. J.*, **50**, 542 (1952).

4) G. D. Fasman, *Science*, **131**, 420 (1960).

5) K. Iwai, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **81**, 1302 (1960).

6) K. H. Shin, S. Sakakibara, W. Schneider and G. P. Hess, *Biochem. Biophys. Res. Commun.*, **8**, 288 (1962).

group attached to nitrogen atom in oxazolidone ring might be so labile to basic reagent as to cause the hydrolytic cleavage at the linkage between the acyl group and the ring nitrogen atom.

Actually *N*-protected serine peptide was cleaved selectively by this method to give the *N*-protected amino acid or peptide and the oxazolidone derivative as reported in the previous paper.[7] In this paper, the results obtained when threonine peptides were subjected to the similar reaction were presented.

All threonine peptides used in this study were synthesized mainly through the dicyclohexylcarbodiimide method by the usual precedure. Authentic 5-methyl-2-oxo-oxazolidine-4-carbonyl derivatives were prepared either from threonine itself or from threonyl peptide by treatment with phosgene and the base as shown below in the case of the cleavage reaction.

Di- or tripeptide in which the threonine residue was located at a *C*-terminal position, *e.g.*, *N*-benzyloxycarbonylalanylthreonine methyl ester (I, V), *N*-benzyloxycarbonylglycylthreonine methyl ester (VIII) or *N*-benzyloxycarbonylalanylalanylthreonine methyl ester (XI), was investigated as a first type of a model threonine peptide for the cleavage reaction. *N*-Benzyloxycarbonyl-DL-alanyl-DL-threonine methyl ester (I) or *N*-benzyloxycarbonyl-L-alanyl-L-threonine methyl ester (V) was converted to the *O*-chlorocarbonyl compound in almost quantitative yield according to the procedure by Skinner *et al.*[8] or by Bergel *et al.*[9] The *O*-chlorocarbonyl compound was then cyclized to an oxazolidone derivative by heating in xylene under reflux with evolution of hydrogen chloride. In this reaction, L-L compound V gave an oily oxazolidone derivative (VII) as the sole product, whereas DL-DL compound I afforded a mixture of crystalline and oily substances (IIIa and IIIb respectively). In the latter case, formation of the two substances may be due to a diastereoisomerism. When the compound V was treated with phosgene and pyridine under the condition which was used to prepare cyclic carbonic esters of vicinal glycol of sugars,[10] the oxazolidone derivative VII was obtained directly without isolation of the intermediate, the *O*-chlorocarbonyl compound. An infrared spectrum of this product was identical to that of the product of the stepwise synthesis as mentioned above.

The L-oxazolidone compound VII was treated with potassium hydroxide in methanol at room temperature to afford a mixture of *N*-benzyloxycarbonyl-L-alanine and L-*trans*-5-methyl-2-oxo-oxazolidine-4-carboxylic acid (L-IV) in yields of 71 and 60% respectively, while, in a similar reaction of the crystalline racemic oxazolidone compound IIIa, yields of the corresponding products were 87 and 90% respectively. Under the same condition of alkaline treatment, the original dipeptide, *i.e.*, *N*-benzyloxycarbonylalanylthreonine methyl ester (I or V) was only saponified and no cleavage of the peptide bond took place.

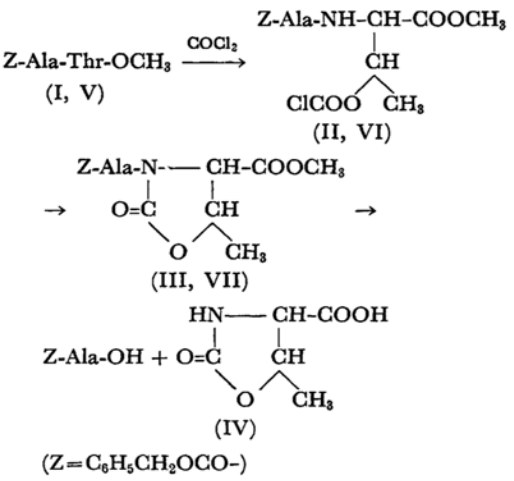

Fig. 2

Similar cleavage reactions also occurred on *N*-benzyloxycarbonylglycyl-DL-threonine methyl ester (VIII) and *N*-benzyloxycarbonyl-L-alanyl-L-alanyl-L-threonine methyl ester (XI), both giving fairly good yields of the corresponding degradation products as shown in Table 1. The reaction of the latter peptide ester XI with phosgene in benzene did not proceed well and only the starting material was recovered, while the reaction in dioxane gave the *O*-chlorocarbonyl compound (XII) in a good yield. From the results obtained above, it is now concluded that the cleavage reaction takes place selectively at the peptide linkage involving the amino group of the threonine residue without any effect on other peptide bonds, and all amino acid residues in the peptide are not subjected to racemization during the reaction.

In order to confirm the conclusion that the cleavage reaction occurs only at the linkage of the amino side of the threonine residue wherever threonine is located in the peptide chain, a reaction of *N*-benzyloxycarbonylglycyl-L-threonyl-L-phenylalanine methyl ester (XIX) was next investigated. In a similar way, the tripeptide XIX was converted to *N*-benzyloxycarbonylglycyl-L-5-methyl-2-oxo-oxazolidine-4-carbonyl-L-phenylalanine methyl ester (XXI) through a corresponding chlorocarbonyl compound (XX) as shown in Fig. 3. This oxazolidone peptide XXI was also cleaved by alkaline hydrolysis to give *N*-benzyloxycarbonylglycine and

7) T. Kaneko, I. Takeuchi and T. Inui, This Bulletin, **41**, 974 (1968).
8) C. G. Skinner, T. J. McCord, J. M. Ravel and W. Shive, *J. Am. Chem. Soc.*, **78**, 2412 (1956).
9) F. Bergel and R. Wade, *J. Chem. Soc.*, **1959**, 941.
10) B. P. Vaterlaus and H. Spiegelberg, *Helv. Chim. Acta*, **47**, 508 (1964).

TABLE 1. RESULTS OF SELECTIVE CLEAVAGE OF THREONINE PEPTIDES

Peptide	Oxazolidone derivative	Product isolated by cleavage reaction (yield)*	
I	IIIa	Z-Ala-OH (DL) (87%)	IV (DL) (90%)
I	IIIb	Z-Ala-OH (DL) (79%)	IV (DL) (67%)
V	VII	Z-Ala-OH (71%)	IV (60%)
VIII	X	Z-Gly-OH (71%)	IV (76%)
XI	XIII	Z-Ala-Ala-OH (86%)	IV (62%)
XIX	XXI	Z-Gly-OH (54%)	XVII (50%)

* Unless otherwise stated, the amino acid residue has an L-configuration.

L-5-methyl-2-oxo-oxazolidine-4-carbonyl-L-phenylalanine (XVII) in 54% and 50% yields respectively. The latter oxazolidone derivative XVII was crystallized as cyclohexylammonium salt and identified by comparison with the authentic compound which was obtained by treatment of L-threonyl-L-phenylalanine with phosgene and potassium carbonate.

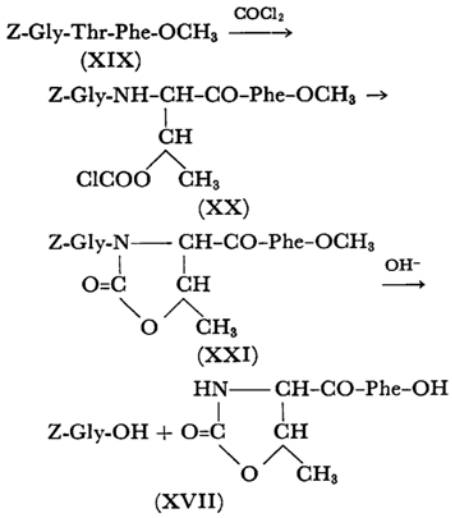

Fig. 3

In conclusion, it is ascertained that the peptide of β-hydroxy-α-amino acid, not only serine but threonine, can be cleaved selectively at the peptide linkage which involves an amino group of the hydroxyamino acid through formation of oxazolidone derivative without any racemization of amino acid residues and any cleavage of other peptide bonds.

Experimental*3

***N*-Benzyloxycarbonyl-DL-alanyl-*O*-chlorocarbonyl-DL-threonine Methyl Ester (II).** Phosgene was passed through a suspension of 6.8 g (0.020 mol) of *N*-benzyloxycarbonyl-DL-alanyl-DL-threonine methyl ester[11] (I) in 80 m*l* of benzene at 8—11°C for 20 min after addition of 2.4 g (0.020 mol) of dimethylaniline until the solid was dissolved. The reaction mixture was stirred below 20°C for 2 hr and then allowed to stand overnight. Through the solution, carbon dioxide was passed to remove excess phosgene. The benzene solution was washed with water and dilute hydrochloric acid, and then dried. Evaporation *in vacuo* gave an oily substance which was readily crystallized; wt 7.8 g (97%). Recrystallization from benzene-petroleum ether gave pure crystals of II; mp 103.5—104.0°C.

Found: C, 51.12; H, 5.26; N, 6.91%. Calcd for $C_{17}H_{21}O_7N_2Cl$: C, 50.94; H, 5.28; N, 6.99%.

In order to confirm a structure of the *O*-chlorocarbonyl compound II, this was converted to *N*-benzyloxycarbonyl-DL-alanyl-*O*-(*N*-phenylcarbamoyl)-DL-threonine methyl ester by a reaction with aniline; mp 155—156°C.

Found: C, 60.38; H, 5.94; N, 9.22%. Calcd for $C_{23}H_{27}O_7N_3$: C, 60.38; H, 5.95; N, 9.19%.

Methyl DL-3-(*N*-Benzyloxycarbonyl-DL-alanyl)-5-methyl-2-oxo-oxazolidine-4-carboxylate (IIIa, b). A solution of 8.6 g of crude II in 80 m*l* of xylene was heated under reflux. An evolution of hydrogen chloride gas ceased after 1 hr. Upon concentration of the reaction mixture to about half a volume, a crystalline solid was formed. It was filtered off; wt 3.8 g (49%), and then recrystallized from ethyl acetate to give crystalline IIIa of mp 129.5—130.0°C; wt 2.8 g.

Found: C, 56.16; H, 5.52; N, 7.60%. Calcd for $C_{17}H_{20}O_7N_2$: C, 56.04; H, 5.53; N, 7.69%.

A mother liquor from the crystals was evaporated *in vacuo* to yield 4.5 g of an oily product IIIb.

Cleavage of IIIa and IIIb by Alkaline Hydrolysis. *a) Hydrolysis of Crystalline IIIa.* To a solution of 2.46 g (6.76 mmol) of IIIa in 38 m*l* of methanol, 1.51 m*l* of 0.90 N aqueous potassium hydroxide solution was added with stirring for 20 min. Stirring at room temperature was continued for an additional 1.5 hr. After evaporation *in vacuo*, water was added to the residue. Undissolved part was extracted with ethyl acetate. The aqueous layer was acidified with concentrated hydrochloric acid to pH 3 to deposit an oily substance which was then extracted with ethyl acetate. Evaporation of the solvent from the latter extract *in vacuo* after drying gave crystals; wt 1.32 g (87%), mp 113—114°C. It was recrystallized from ethyl acetate-petroleum ether; mp 113.5—114.0°C. The melting point and infrared spectrum of this compound were identical to those of authentic *N*-benzyloxycarbonyl-DL-alanine.

The aqueous layer of pH 3 which was separated from the ethyl acetate layer was further acidified to pH 1 by addition of concentrated hydrochloric acid, and evaporated *in vacuo*. The residue obtained was extracted with hot ethyl acetate three times. The ethyl acetate solution

*3 All melting points given are uncorrected.

11) Mp 103.5—105.0°C. This compound had been prepared by Th. Wieland, K. Freter and E. Gross, *Ann.*, **626**, 154 (1959) through the mixed anhydride method. In this study, the same compound was synthesized by the carbodiimide method in a similar way to that of the preparation of L-L-isomer (V) as shown below.

was concentrated after decolorization to give crystals; wt 0.89 g (90%), mp 117—119°C. Recrystallization from ethyl acetate - petroleum ether raised the melting point to 126.5—127.0°C. The melting point and the infrared spectrum of this crystals were identical to those of authentic DL-*trans*-5-methyl-2-oxo-oxazolidine-4-carboxylic acid (DL-IV)[12].

b) *Hydrolysis of Oily IIIb.* In a way similar to the hydrolysis of IIIa, 4.5 g of IIIb was dissolved in 60 m*l* of methanol and treated with 27 m*l* of 1 N potassium hydroxide solution. As reaction products, 2.2 g (79%) of *N*-benzyloxycarbonyl-DL-alanine and 1.2 g (67%) of DL-5-methyl-2-oxo-oxazolidine-4-carboxylic acid (DL-IV) were obtained. Both compounds were identified by comparison with the authentic samples in their melting points and infrared spectra respectively.

N-Benzyloxycarbonyl-L-alanyl-L-threonine Methyl Ester (V). To a mixed solution of 22.3 g (0.10 mol) of *N*-benzyloxycarbonyl-L-alanine and methyl L-threoninate, prepared from 17.0 g (0.10 mol) of the hydrochloride and 10.2 g of triethylamine, in 180 m*l* of chloroform, a solution of 20.6 g (0.10 mol) of *N,N'*-dicyclohexylcarbodiimide in 50 m*l* of chloroform was added with stirring for 30 min. After standing overnight, *N,N'*-dicyclohexylurea formed was removed by filtration. The chloroform solution was washed with water, dilute hydrochloric acid, water, aqueous sodium hydrogen carbonate solution and water successively. Concentration of the solution after drying gave 29.5 g (87%) of a crude product which was then recrystallized from ethyl acetate to yield 22.9 g (68%) of V; mp 132.0—132.8°C, $[\alpha]_D^{16.5}$ −23.6° (*c* 2.88, methanol).

Found: C, 57.15; H, 6,63; N, 8.15%. Calcd for $C_{16}H_{22}O_6N_2$: C, 56.79; H, 6.55; N, 8.28%.

N-Benzyloxycarbonyl-L-alanyl-O-chlorocarbonyl-L-threonine Methyl Ester (VI). Phosgene was passed through a suspension of 6.8 g (0.020 mol) of V in 80 m*l* of benzene in the presence of 2.4 g (0.020 mol) of dimethylaniline at about 8°C for 30 min. The reaction mixture was treated in a manner similar to that in the preparation of DL-compound II to yield 8.0 g (100%) of crude crystals of VI. This was recrystallized from benzene-petroleum ether (1: 1); mp 79.5—81.0°C, $[\alpha]_D^{13.5}$ +30.8° (*c* 3.05, benzene).

Found: C, 51.08; H, 5.15; N, 6.96; Cl. 8.48%. Calcd for $C_{17}H_{21}O_7N_2Cl$: C, 50.94; H, 5.28; N, 6.99; Cl, 8.85%.

Methyl L-3-(N-Benzyloxycarbonyl-L-alanyl)-5-methyl-2-oxo-oxazolidine-4-carboxylate (VII). *a*) *Stepwise Synthesis from VI.* A solution of 7.2 g of the crude VI in 60 m*l* of xylene was heated under reflux. A similar treatment of the reaction mixture to that in the preparation of DL-compound III gave 6.8 g of an oily product VII, $[\alpha]_D^{12}$ −58.8° (*c* 3.0, methanol).

b) *Direct Synthesis from V.* A mixture of 5.0 m*l* (0.062 mol) of pyridine and 12 m*l* of anhydrous benzene was added to 19.8 g of 10% phosgene solution in benzene at 2—4°C for 20 min with stirring. To this sticky half-frozen mixture, a solution of 3.2 g (9.4 mmol) of V in 50 m*l* of anhydrous dioxane was added dropwise at about 0°C for 40 min with stirring. Though the stirring became increasingly difficult, it was continued at this temperature for 1 hr, and then at room temperature for an additional 1 hr. After addition of 80 m*l* of ice-water, the reaction mixture was extracted with ethyl acetate several times. Combined organic layer was washed with 1 N hydrochloric acid, 5% aqueous sodium hydrogen carbonate solution and water successively, and dried with magnesium sulfate. After the solution had been evaporated *in vacuo*, 3.5 g of a pale brownish oil remained which was identified with authentic VII in infrared spectrum. This product seemed to be not so pure, because, on the cleavage reaction, it gave cyclohexylammonium salts of *N*-benzyloxycarbonylalanine and 5-methyl-2-oxo-oxazolidine-4-carboxylic acid in relatively poor yields of 42 and 26% respectively.

Cleavage of VII by Alkaline Hydrolysis. In a way similar to that in the cleavage of DL-compound III, hydrolysis of 3.11 g (8.54 mmol) of the crude VII in 80 m*l* of ethanol and 18 m*l* of 0.95 N potassium hydroxide solution was carried out. Extraction with ethyl acetate from an aqueous solution of pH 3 gave crude crystals of *N*-benzyloxycarbonyl-L-alanine of mp 72—76°C; wt 1.36 g (71%). It was recrystallized from chloroform-petroleum ether (1: 1); wt 0.68 g, mp 86—87°C, $[\alpha]_D^{13.2}$ 13.8° (*c* 3.32, acetic acid). Further acidification of the remaining aqueous solution by hydrochloric acid gave 0.75 g (60%) of crude crystals of IV; mp 129—133°C. Recrystallization from ethyl acetate gave pure crystals of mp 139.0—139.8°C, $[\alpha]_D^{19.8}$ +40.7° (*c* 2.75, water). These physical constants as well as the infrared spectrum of this compound were all identical to those of authentic L-IV.[13]

N-Benzyloxycarbonylglycyl-DL-threonine Methyl Ester (VIII). A solution of 25.1 g (0.12 mol) of *N*-benzyloxycarbonylglycine in 260 m*l* of a mixture of chloroform and dioxane (1: 1) was added to a solution of methyl DL-threoninate which was prepared from 20.4 g (0.12 mol) of the hydrochloride and 12.3 g (0.12 mol) of triethylamine, in 100 m*l* of chloroform. To the reaction mixture, a solution of 24.7 g (0.12 mol) of *N,N'*-dicyclohexylcarbodiimide in 50 m*l* of chloroform was added for 40 min with stirring. After stirring for 2 hr at room temperature, it was allowed to stand overnight. By the usual procedure, 37 g (95 %) of a crude product of VIII was obtained. It was recrystallized from ethyl acetate; wt 26.2 g (67%), mp 110.5—111.8°C.

Found: C, 55.57; H, 6.31; N, 8.87%. Calcd for $C_{15}H_{20}O_6N_2$: C, 55.55; H, 6.22; N, 8.64%.

Cleavage Reaction of VIII. *N*-Benzyloxycarbonylglycyl-*O*-chlorocarbonyl-DL-threonine methyl ester (IX) was obtained from 6.5 g (0.020 mol) of VIII by action of phosgene in the presence of 2.4 g of dimethylaniline in 80 m*l* of benzene at 5—8°C; wt 7.2 g (94%). Whole amount of the crude product IX thus obtained was cyclized by heating in 60 m*l* of xylene under reflux for 1 hr to yield 6.2 g of an oily substance (X) which was then dissolved in 80 m*l* of methanol and hydrolyzed by treatment with 35 m*l* of 1 N potassium hydroxide at room temperature for 1.5 hr. The reaction mixture was acidified to pH 3 by hydrochloric acid, and extracted with ethyl acetate to give crude *N*-benzyloxycarbonylglycine; wt 3.0 g (71% based on VIII). It was recrystallized from ethyl acetate - petroleum ether (4 : 1); mp 121.0—121.8°C. The remaining aqueous solution was acidified to pH 1 and treated by the usual procedure to

12) Lit.: T. Kaneko and T. Inui, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **82**, 1075 (1961); mp 127—128°C.

13) Lit.: T. Kaneko and T. Inui, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **82**, 1078 (1961); mp 139.5—140.5°C, $[\alpha]_D^{25}$ +41.2° (*c* 3.04, water).

give 2.2 g (76% based on VIII) of DL-IV. It was recrystallized from ethyl acetate - petroleum ether (4 : 1); mp 126.5—127.0°C. Both products were identified by comparison with the authentic samples.

***N*-Benzyloxycarbonyl-L-alanyl-L-alanyl-L-threonine Methyl Ester (XI).** *a) Azide Method.* To a cold solution of 6.5 g (0.021 mol) of *N*-benzyloxycarbonyl-L-alanyl-L-alanine hydrazide[14] in a mixture of 43 m*l* of acetic acid, 21.5 m*l* of 5 N hydrochloric acid and 170 m*l* of water, there was added a cold solution of 18.0 m*l* of 10% aqueous sodium nitrite solution at −5°C. An oily product formed solidified immediately. The solid was extracted with a mixture of ethyl acetate and ether (1 : 1). The extract was washed with water, 3% aqueous sodium hydrogen carbonate solution and water successively in the cold, and then dried with sodium sulfate. On the other hand, 3.5 g (0.035 mol) of triethylamine was added to a suspension of 5.8 g (0.034 mol) of methyl L-threoninate hydrochloride in 40 m*l* of chloroform. After addition of 120 m*l* of anhydrous ether, triethylamine hydrochloride formed was filtered off. Free ester solution thus obtained was poured into the azide solution prepared above on ice-cooling. The reaction mixture was stirred for 3 hr and then kept in a refrigerator overnight. A crystalline product of XI was filtered; wt 5.9 g (69%), mp 179—180°C. It was recrystallized from methanol - ethyl acetate (3 : 1); wt 5.0 g, mp 181.0—181.5°C, $[\alpha]_D^{11}$ −56.5° (*c* 1.82, methanol).

Found: C, 55.87; H, 6.68; N, 10.26%. Calcd for $C_{19}H_{27}O_7N_3$: C, 55.73; H, 6.65; N, 10.26%.

b) Carbodiimide Method. A solution of 3.1 g (0.030 mol) of triethylamine and methyl L-threoninate prepared from 5.1 g (0.030 mol) of the hydrochloride, in 30 m*l* of chloroform was added to a solution of 8.8 g (0.030 mol) of *N*-benzyloxycarbonyl-L-alanyl-L-alanine in 40 m*l* of a mixture of dioxane and chloroform (1 : 1). To the mixture, a solution of 6.2 g (0.030 mol) of *N,N'*-dicyclohexylcarbodiimide in 15 m*l* of chloroform was added dropwise. The reaction mixture was stirred at room temperature for 4 hr and then allowed to stand overnight. A mixture of dicyclohexylurea and a condensed product was filtered off. This was heated in 80 m*l* of dioxane under reflux to dissolve the latter only, keeping the former insoluble. To the filtrate, 100 m*l* of petroleum ether was added to crystallize the product; wt 5.3 g (43%), mp 177—179°C. This was recrystallized from methanol-ethyl acetate (3 : 1); wt 3.6 g, mp 181.5—182.8°C, $[\alpha]_D^{12.5}$ −56.3° (*c* 1.83, methanol). All properties of these crystals were quite identical to those of the compound XI obtained by the azide method.

Found: C, 55.79; H, 6.64; N, 10.35%.

The organic layer separated from the solid product was washed with water, dilute hydrochloric acid, aqueous sodium hydrogen carbonate solution and water successively, and then concentrated *in vacuo* to give crystals; wt 3.5 g. It was recrystallized from ethyl acetate - petroleum ether (25 : 10); wt 2.5 g mp 141.5—142.5°C, $[\alpha]_D^{12.5}$ −5.4° (*c* 1.84, methanol).

Found: C, 56.27; H, 6.89; N, 9.91; H_2O, 2.53%. Calcd for $C_{33}H_{43}O_{11}N_5 \cdot H_2O$: C, 56.32; H, 6.45; N, 9.95; H_2O, 2.56%.

From the results of the elementary analysis and an infrared spectrum, a structure of this compound is presumed to be *O,N*-di-(*N*-benzyloxycarbonyl-L-alanyl-L-alanyl)-L-threonine methyl ester hydrate.

***N*-Benzyloxycarbonyl-L-alanyl-L-alanyl-*O*-chlorocarbonyl-L-threonine Methyl Ester (XII).** Through a suspension of 4.1 g (0.01 mol) of XI in 40 m*l* of dioxane, phosgene was passed at 8—10°C for 45 min in the presence of 1.2 g (0.01 mol) of dimethylaniline. After stirring for 3.5 hr, the reaction mixture was allowed to stand overnight. After removal of excess phosgene by passage of carbon dioxide, the solution was concentrated *in vacuo* to give crystals. It was filtered, washed with water and then dried; wt 4.2 g (89%), mp 149—150°C (decomp.). Recrystallization from benzene gave 3.5 g of pure crystals; mp 150.5—151.°C (decomp.), $[\alpha]_D^{15}$ +8.6° (*c* 3.02, benzene).

Found: C, 50.87; H, 5.59; N, 8.81%. Calcd for $C_{20}H_{26}O_8N_3Cl$: C, 50.90; H, 5.55; N, 8.91%.

Methyl L-3-(*N*-Benzyloxycarbonyl-L-alanyl-L-alanyl)-5-methyl-2-oxo-oxazolidine-4-carboxylate (XIII). Heating of 3.1 g (0.066 mol) of XII in 30 m*l* of xylene under reflux for 75 min followed by evaporation *in vacuo* gave a product of a closed ring as a syrup; wt 2.8 g (98%), $[\alpha]_D^{15}$ −84.6° (*c* 3.6, methanol). This was used for the following cleavage reaction without further purification.

Cleavage of XIII by Alkaline Hydrolysis. Hydrolysis of 2.19 g (5.03 mmol) of XIII was carried out in 30 m*l* of methanol by addition of 11.1 m*l* of 0.9 N aqueous potassium hydroxide and keeping the solution at room temperature for 1.5 hr. After methanol had been evaporated *in vacuo*, water was added to the reaction mixture, pH of which was then adjusted to 3 by addition of hydrochloric acid. Extraction with ethyl acetate followed by evaporation of the solvent gave 1.27 g (86%) of crude crystals of mp 135.0—139.5°C. It was recrystallized from chloroform to yield a pure material; mp 149.0—150.0°C, $[\alpha]_D^{13.5}$ −32.5° (*c* 3.26, methanol), which was identified to be *N*-benzyloxycarbonyl-L-alanyl-L-alanine by comparison with the authentic sample.

On the other hand, the aqueous layer separated from the ethyl acetate extract was further acidified and evaporated *in vacuo* to give a residue. This was extracted with hot ethyl acetate and the solvent was removed by evaporation to give L-IV; wt 0.45 g (62%), mp 128—132°C. It was recrystallized from ethyl acetate; mp 138—140°C, $[\alpha]_D^{13.8}$ +41.8° (*c* 2.70, water). These were identical to those of the authentic sample.

***N*-Benzyloxycarbonyl-L-threonyl-L-phenylalanine Methyl Ester (XIV).** To a mixed solution of 51 g (0.20 mol) of *N*-benzyloxycarbonyl-L-threonine in 400 m*l* of acetonitrile and methyl L-phenylalaninate, prepared from 48 g (0.22 mol) of the hydrochloride, in 60 m*l* of acetonitrile, a solution of 42 g (0.20 mol) of *N,N'*-dicyclohexylcarbodiimide in 100 m*l* of acetonitrile was added at room temperature with stirring. Stirring at room temperature was continued for 4 hr. After standing overnight, the urea derivative precipitated was filtered off, and the solvent was removed by evaporation *in vacuo*. Syrupy residue thus obtained was dissolved in ethyl acetate, washed with 5% aqueous potassium hydrogen carbonate solution, 0.5 N hydrochloric acid and water successively, and then dried with sodium sulfate. To a concentrated solution, petroleum ether was added to give

14) This compound was prepared from *N*-benzyloxycarbonyl-L-alanyl-L-alanine methyl ester by the usual procedure; mp 212—213°C, $[\alpha]_D^{13}$ −51.0° (*c* 1.92, methanol), $[\alpha]_D^{18}$ −67.4° (*c* 1.84, 0.5 N hydrochloric acid). Lit.: B. F. Erlanger and E. Brand, *J. Am. Chem. Soc.*, **73**, 3508 (1951).

crystals; wt 74 g (89%), mp 104—106°C. Repeated recrystallization from ethyl acetate - petroleum ether raised the melting point up to 108.5—109.0°C, $[\alpha]_D^{19}$ +3.9° (*c* 1.15, ethanol).

Found: C, 63.72; H, 6.36; N, 6.66%. Calcd for $C_{22}H_{26}O_6N_2$: C, 63.75; H, 6.32; N. 6.76%.

***N*-Benzyloxycarbonyl-L-threonyl-L-phenylalanine (XV).** To a solution of 1.03 g (2.5 mmol) of XIV in a mixture of 35 m*l* of dioxane and 5 m*l* of water, there was added 5.5 m*l* of 0.5 N aqueous sodium hydroxide solution dropwise for 8 hr with stirring. After standing overnight, 5.5 m*l* of 0.5 N hydrochloric acid was added, and the reaction mixture was evaporated *in vacuo* to dryness. The residue obtained was triturated with ethyl acetate to remove insoluble sodium chloride. Ethyl acetate solution was extracted with 5% sodium hydrogen carbonate solution. The aqueous extract was acidified with 2 N hydrochloric acid to yield crystals of XV; wt 0.51 g (51%), mp 152—154°C. It was recrystallized from ethyl acetate - petroleum ether; mp 156—157°C, $[\alpha]_D^{20}$ +23.2° (*c* 1.94, ethanol).

Found: C, 62.74; H, 6.04; N, 6.76%. Calcd for $C_{21}H_{24}O_6N_2$: C, 62.99; H, 6.04; N, 7.00%.

From the mother liquor of the first crystallization of the product, a considerable amount of XVII was obtained.

L-Threonyl-L-phenylalanine (XVI). Hydrogen gas was passed through a solution of 8.0 g (0.020 mol) of XV in a mixture of 150 m*l* of methanol and 20 m*l* of water at room temperature for 6 hr in the presence of palladium black. When a crystalline product was formed during the hydrogenation, water was added to dissolve it. The catalyst was removed by filtration and the filtrate was concentrated to a small volume. On addition of ethanol, crystals of dipeptide XVI were formed; wt 5.9 g (95%). It was recrystallized from water-ethanol; mp 251—253°C (decomp.), $[\alpha]_D^{21}$ +24.8° (*c* 2.02, water).

Found: C, 57.31; H, 7.77; N, 9.16%. Calcd for $C_{13}H_{18}O_4N_2 \cdot C_2H_6O$: C, 57.67; H, 7.74; N, 8.97%.

L-5-Methyl-2-oxo-oxazolidine-4-carbonyl-L-phenylalanine (XVII). To a solution of 3.1 g (0.01 mol) of XVI containing one mole of ethyl alcohol as crystallization solvent in 20 m*l* of water there were added 10 m*l* of 1 N aqueous potassium hydroxide solution and 2.8 g (0.02 mol) of solid potassium carbonate. To the mixture, 9.1 g of toluene solution containing 1.5 g (0.015 mol) of phosgene was added dropwise at 1—5°C for 20 min with vigorous stirring. The reaction mixture was stirred at the same temperature for 1.5 hr and then at room temperature for 40 min. An aqueous layer was separated, washed with ether and then acidified with 2 N hydrochloric acid to give crystals; wt 0.6 g. The filtrate was evaporated *in vacuo* to a residue, which was then triturated with ethyl acetate. To the concentrated ethyl acetate solution, petroleum ether was added to yield a second crop of the crystalline product; wt 2.3 g. Total yield, 2.9 g (99%). This was purified as cyclohexylammonium salt; mp 210.0—210.5°C, $[\alpha]_D^{20}$ +25.2° (*c* 1.27, water).

Found: C, 61.15; H, 7.53; N, 10.56%. Calcd for $C_{14}H_{16}O_5N_2 \cdot C_6H_{13}N$: C, 61.36; H, 7.47; N, 10.74%.

L-Threonyl-L-phenylalanine Methyl Ester Hydrochloride (XVIII). Hydrogen gas was passed through a solution of 8.3 g (0.020 mol) of XIV in 100 m*l* of methanol and 3.5 m*l* of concentrated hydrochloric acid in the presence of palladium black at room temperature for 20 hr. After removal of the catalyst by filtration, the solution was evaporated *in vacuo*. An oily residue thus obtained was dissolved in a small amount of methanol, and on addition of ether needles were obtained; wt 5.2 g (83%). It was recrystallized from methanol - ether; wt 4.4 g, mp 136—138°C.

Found: C, 53.10; H, 6.78; N, 8.67; Cl, 11.06%. Calcd for $C_{14}H_{21}O_4N_2Cl$: C, 53.08; H, 6.68; N, 8.84; Cl, 11.19%.

***N*-Benzyloxycarbonylglycyl-L-threonyl-L-phenylalanine Methyl Ester (XIX).** To a mixed solution of 4.2 g (0.020 mol) of *N*-benzyloxycarbonylglycine in 20 m*l* of tetrahydrofuran and 4.1 g (0.020 mol) of *N,N'*-dicyclohexylcarbodiimide in 20 m*l* of tetrahydrofuran, there was added a solution of a free dipeptide ester, prepared from 6.3 g (0.020 mol) of XVIII and an equivalent amount of triethylamine, in 80 m*l* of tetrahydrofuran. After the reaction mixture had been stirred at room temperature for 4 hr, it was allowed to stand overnight. A precipitate formed upon addition of acetic acid was filtered off and the filtrate was evaporated *in vacuo*. An oily residue obtained was crystallized from ethyl acetate; wt 6.9 g (73%), mp 146.0—148.5°C. It was recrystallized from ethyl acetate; mp 146.5—149.0°C, $[\alpha]_D^{23}$ −14.0° (*c* 2.0, chloroform).

Found: C, 61.25; H, 6.38; N, 8.78%. Calcd for $C_{24}H_{29}O_7N_3$: C, 61.13; H, 6.20; N, 8.91%.

***N*-Benzyloxycarbonylglycyl-*O*-chlorocarbonyl-L-threonyl-L-phenylalanine Methyl Ester (XX).** Phosgene was passed through a solution of 4.7 g (0.010 mol) of XIX and 1.2 g (0.010 mol) of dimethylaniline in 120 m*l* of anhydrous dioxane at 9—11°C 30 min. Stirring at this temperature was continued for 1.5 hr and then at room temperature for 1 hr. After standing overnight, the solvent was removed by evaporation *in vacuo*. A solution of the residue thus obtained in ethyl acetate was washed with 0.3 N hydrochloric acid and then dried with magnesium sulfate. Again, the solution was evaporated *in vacuo* to an oily residue, which was then crystallized from chloroform; wt 4.1 g (62%), mp 90—92°C (decomp.), $[\alpha]_D^{20}$ +36.3° (*c* 0.96, chloroform).

Found: C, 47.70; H, 4.50; N, 6.73; Cl, 21.56%. Calcd for $C_{25}H_{28}O_8N_3Cl \cdot CHCl_3$: C, 47.79; H, 4.48; N, 6.43; Cl, 21.71%.

3-(*N*-Benzyloxycarbonylglycyl)-L-5-methyl-2-oxo-oxazolidine-4-carbonyl-L-phenylalanine Methyl Ester (XXI). A solution of 9.1 g (0.014 mol) of XX in 90 m*l* of xylene was heated under reflux for 30 min. After evaporation *in vacuo*, a crystalline residue obtained was recrystallized from ethyl acetate - petroleum ether; wt 4.2 g (60%), mp 155.0—159.5°C. Repeated recrystallization gave a pure sample; mp 165.0—166.5°C, $[\alpha]_D^{20}$ +4.8° (*c* 1.05, ethanol).

Found: C, 60.05; H, 5.61; N, 8.32%. Calcd for $C_{25}H_{27}O_8N_3$: C, 60.35; H, 5.47; N, 8.45%.

Cleavage of XXI by Alkaline Hydrolysis. To a solution of 3.0 g (6.0 mmol) of XXI in 50 m*l* of methanol, there was added 14.4 m*l* of 1 N sodium hydroxide. After the reaction mixture had been stirred at room temperature for 2.5 hr, 2 N hydrochloric acid was added to pH 3. Methanol was removed by evaporation *in vacuo*. To a residue obtained, 1 N sodium hydroxide solution was added and extracted with ethyl acetate. The aqueous layer was separated and acidified to pH 3.2

with 1 N hydrochloric acid. An oily material formed was separated by decantation and the solution was further acidified to pH 2.5 to yield colorless crystals; wt 0.17 g, mp 122—123°C. Its infrared spectrum was identical to that of *N*-benzyloxycarbonylglycine. More amount of the same compound was obtained when the oily material was dissolved in ethyl acetate, extracted with 0.5 N sodium hydroxide solution and then the aqueous solution was acidified; wt 0.51 g. Total yield; 54%.

The aqueous solution of pH 2.5 was evaporated *in vacuo*. A residue thus obtained was treated with ethyl acetate. An oily residue obtained by evaporation of the solvent was then dissolved in ethanol. An addition of cyclohexylamine and ether to the solution gave colorless crystals of cyclohexylammonium salt; wt 1.17 g (50%), mp 199—201°C (decomp.). Its infrared spectrum was quite identical to that of cyclohexylammonium salt of XVII prepared from XVI by treatment with phosgene. Recrystallization from ethanol - ether gave a pure salt; mp 209—210°C (decomp.), $[\alpha]_D^{13}$ +24.9° (*c* 2.21, water).

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