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Selective Cleavage of Serine Peptides*1

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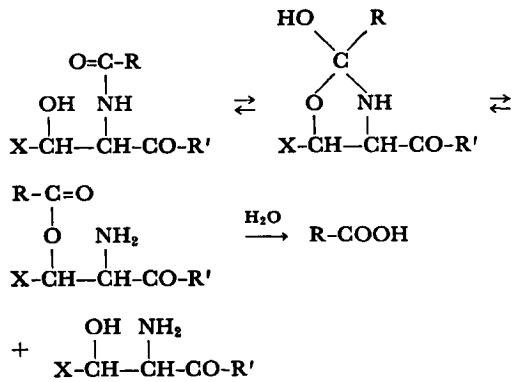
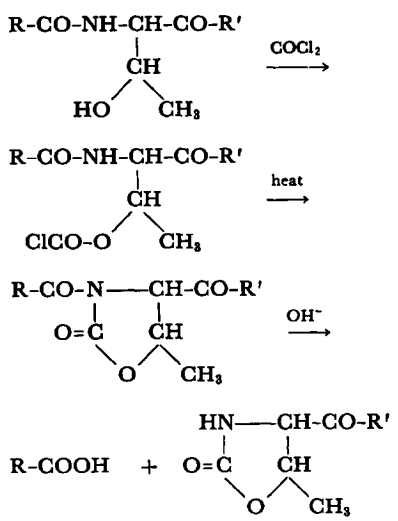
The treatment of *N*-benzyloxycarbonyl-L-alanyl-L-serine methyl ester (I) with phosgene afforded the corresponding *O*-chlorocarbonyl derivative (II), which was then cyclized to methyl L-3-(*N*-benzyloxycarbonyl-L-alanyl)-2-oxo-oxazolidine-4-carboxylate (III) accompanied with *N*-benzyloxycarbonyl-L-alanyl-3-chloro-L-alanine methyl ester (IV) by refluxing in xylene. By the mild alkaline hydrolysis of III, *N*-benzyloxycarbonyl-L-alanine and L-2-oxo-oxazolidine-4-carboxylic acid (V) were isolated as a result of the selective cleavage of the peptide bond in which an amino group of the serine residue participated. This method was also applied to three other serine peptides; the same results were obtained.

The method of the selective cleavage of proteins by enzymes is very valuable in the elucidation of their structure.[1,2] On the other hand, the use of the selective cleavage of peptide bonds on the special amino acid residue in proteins by chemical reagents is also important in achieving fragments different from those obtained by enzymes and so in determining their sequences. Recently, the techniques for the selective chemical cleavage on tyrosyl, tryptophyl, histidyl and methionyl peptides have been developed by Witkop and others.[1,3-6] N→O Acyl group migration with acid and subsequent hydrolysis induced selective cleavage on the serine or threonine residue in proteins.[7] Threonine peptides were cyclized with phosgene to their oxazolidone derivatives, the hydrolysis of which with a dilute alkali also afforded selective cleavage at the peptide linkage involving the amino group of the threonine residue.[8]



X=H: Serine, X=CH_3: Threonine

Another hydroxy-amino acid, serine, was also cyclized with phosgene to an oxazolidone derivative.[9]



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1) B. Witkop, "Advances in Protein Chemistry," Vol. 16, Academic Press, New York (1961), p. 221.

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3) G. L. Schmir and L. A. Cohen, *J. Am. Chem. Soc.*, **83**, 723 (1961).

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8) Short communication: T. Kaneko and T. Inui, This Bulletin, **36**, 1541 (1963). The details will be reported in this Bulletin later.

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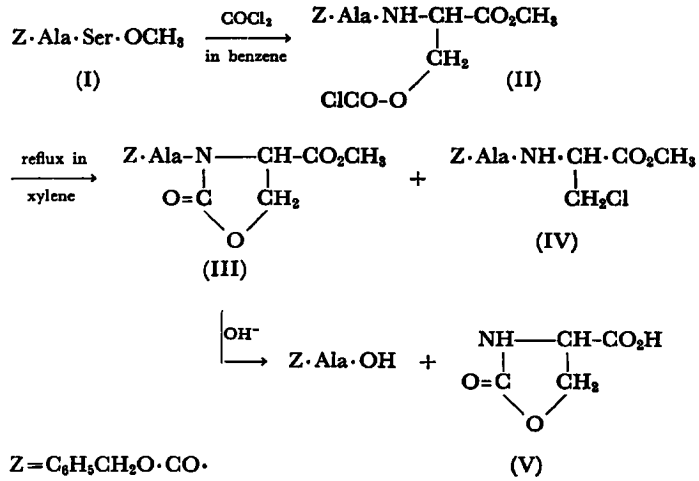
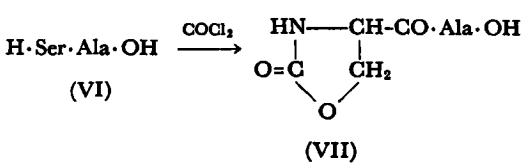


In a similar fashion, if serine peptides involving a peptide linkage between the amino group of the serine residue and the carboxyl group of another amino acid residue are cyclized with phosgene to the corresponding oxazolidone peptides at their serine residue, the peptides thus obtained will selectively be cleft in two parts at the amino group of the serine residue by alkaline hydrolysis. In order to investigate the selective cleavage of this type on serine peptides, *N*-benzyloxycarbonyl- L -alanyl- L -serine methyl ester (I), L-seryl-L-alanine (VI), *N*-benzyloxycarbonylglycyl-L-seryl-L-alanine methyl ester (VIII), and *N*-benzyloxycarbonyl- L -alanyl-L-alanyl-L-serine methyl ester (IX) were used as the model peptides in the present study.

I,[10] obtained from *N*-benzyloxycarbonyl-L-alanine and methyl L-serinate hydrochloride by using the *N,N'*-dicyclohexylcarbodiimide method, was converted with phosgene to the *O*-chlorocarbonyl derivative (II) by using the method of Bergel.[11] In threonine peptides, their *O*-chlorocarbonyl compounds were easily cyclized, under reflux in xylene, to the corresponding oxazolidone derivatives in good yields.[8] Under the same experimental conditions, II provided a crystal melting at 93—109°C; this was clarified as being a mixture of methyl L-3-(*N*-benzyloxycarbonyl-L-alanyl)-2-oxo-oxazolidine-4-carboxylate (II) and *N*-benzyloxycarbonyl-L- alanyl - 3 - chloro-L-alanine methyl ester (IV) by thin-layer chromatography with silica gel G (TLC) and by means of a study of the infrared spectra. The ratio of III to IV in the reaction mixture was established to be about two-to-one by an analysis of its infrared spectra. The separation of III from IV was achieved by using a column with silica gel and by eluting it with a mixture of benzene and methanol (19 : 1, v/v). The structure of III was confirmed by elemental analysis and by a study of its infrared spectra. On the other hand, the minor component, exhibiting a strongly positive Beilstein's test, was also confirmed to have IV by a study of its nuclear magnetic resonance spectra. Various conditions for cyclizing of II were then studied. The yield of the mixture which was obtained by the reflux of II in toluene was decreased to about two-thirds of that obtained under the above conditions, but the ratio of III to IV was increased to about eight-to-one. In another experiment, II was treated with an equivalent amount of pyridine in benzene; the product isolated was identified by TLC as IV, accompanied by a small amount of I.

The alkaline hydrolysis of III afforded *N*-benzyloxycarbonyl-L-alanine and L-2-oxo-oxazolidine-4-carboxylic acid (V) in yields of 89% and 68% respectively. The structures of the products isolated were confirmed by comparisons of their melting points, specific rotations, and infrared spectra with those of the corresponding authentic specimens. For comparison, the authentic V was prepared from L-serine by the method described for the cyclization of L-threonine.[12]

A peptide containing the serine residue as the amino terminal unit should be cyclized to an oxazolidone peptide without any change in the peptide bond. VI[13] was readily cyclized with phosgene to L-2-oxo-oxazolidine-4-carbonyl-L-alanine (VII) in an alkaline solution.



10) J. I. Harris and J. S. Fruton, *J. Biol. Chem.*, **191**, 143 (1951).
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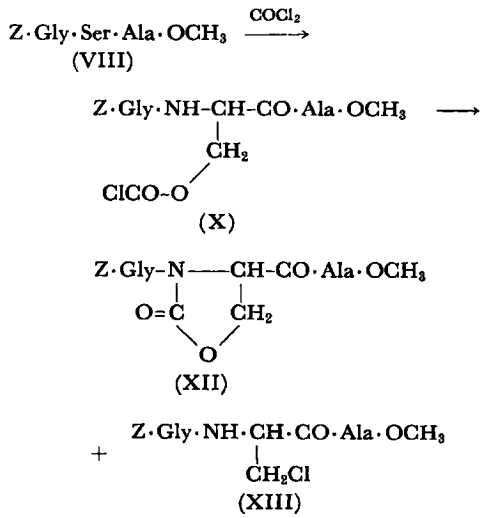
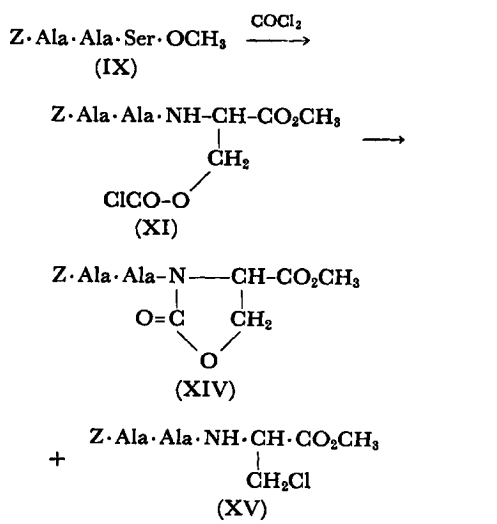
12) T. Inui and T. Kaneko, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **83**, 1078 (1961).
13) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

TABLE 1. RESULTS OF THE SELECTIVE CLEAVAGE OF SERINE PEPTIDES

Peptide	Oxazolidone derivative	Products isolated by alkaline hydrolysis (yields)	
I	III	Z·Ala·OH (89%)	V (68%)
VIII	XII	Z·Gly·OH (81%)	VII (40%)
IX	XIV	Z·Ala·Ala·OH (60%)	V (46%)



Similarly, the treatment of tripeptides, VIII and IX,[*4] with phosgene provided the corresponding *O*-chlorocarbonyl derivatives, X and XI respectively. A product obtained by heating on X was also a mixture of the desired oxazolidone derivative, XII, and the chlorinated compound, XIII. The two components, XII and XIII, in the mixture were separated from each other by fractional crystallization. The same treatment on XI provided the oxazolidone peptide XIV as an amorphous solid (probably contaminated with a small amount of the chlorinated compound XV, for it gave a slightly positive Beilstein's test), which was used for the next step without further purification. The cleavage reactions of XII and XIV were also studied; the results were summarized in Table 1, plus those for III.



In conclusion, this selective cleavage of serine peptide occurs on the peptide linkage involving an amino group of the serine residue through its oxazolidone derivative without racemization.

Experimental[*5]

***N*-Benzyloxycarbonyl-L-alanyl-L-serine Methyl Ester (I).** A solution containing 22.3 g (0.10 mol) of *N*-benzyloxycarbonyl-L-alanine, 15.6 g (0.10 mol) of methyl L-serinate hydrochloride, and 14.0 m*l* (0.10 mol) of triethylamine in 225 m*l* of chloroform was treated with a solution of 20.6 g (0.10 mol) of *N,N'*-dicyclohexylcarbodiimide in 50 m*l* of chloroform. After the mixture had been stirred for 3 hr and then stood overnight at room temperature, the *N,N'*-dicyclohexylurea which precipitated was filtered off, 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*, thus providing white crystals which were then recrystallized from ethyl acetate - petroleum ether. Yield, 22.5 g (70%), mp 134.5—135.5°C, $[\alpha]_D^{23.5}$ −17.7° (*c* 3.43, methanol), lit.,[10] mp 134—135°C.

Found: C, 55.80; H, 6.32; N, 8.64%. Calcd for $C_{15}H_{20}O_6N_2$: C, 55.55; H, 6.22; N, 8.64%.

***N*-Benzyloxycarbonyl-L-alanyl-*O*-chlorocarbonyl-L-serine Methyl Ester (II).** Phosgene was passed through a stirred suspension of 8.1 g (0.025 mol) of I and 3.0 g (0.025 mol) of *N,N*-dimethylaniline in 100 m*l* of benzene for 30 min at 5—10°C. After it had been stirred for 3 hr and then left to stand overnight below 20°C, the reaction mixture was bubbled with a stream of nitrogen (or carbon dioxide) until the excess phosgene had been removed, and then washed with dilute hydrochloric acid and water. The dried solution was evaporated *in vacuo*, thus providing white crystals which were then recrystallized by precipitation with the addition of petroleum ether to a solution of the product in a minimum amount of benzene. Yield, 7.6 g (79%), mp 93.5—94°C, $[\alpha]_D^{23}$ +20.1° (*c* 3.58, benzene).

Found: C, 49.76; H, 5.16; N, 7.63; Cl, 8.30%. Calcd for $C_{16}H_{19}O_7N_2Cl$: C, 49.68; H, 4.95; N, 7.24; Cl, 9.17%.

The treatment of a solution of II in benzene with an

[*4] The preparations of these peptides, VIII and IX, will be described in Experimental section below.

[*5] All melting points are uncorrected.

etheral solution of aniline gave the *N*-benzyloxycarbonyl-L-alanyl-*O*-(*N*-phenylcarbamoyl)-L-serine methyl ester; mp 146.3—146.8°C, $[\alpha]_D^{23.5}$ −7.8° (*c* 2.31, methanol).

Found: C, 60.02; H, 5.70; N, 9.40%. Calcd for $C_{22}H_{25}O_7N_3$: C, 59.58; H, 5.68; N, 9.48%.

Methyl L-3-(*N*-Benzyloxycarbonyl-L-alanyl)-2-oxo-oxazolidine-4-carboxylate (III) and *N*-Benzyloxycarbonyl-L-alanyl-3-chloro-L-alanine Methyl Ester (IV). A solution of 7.0 g (0.018 mol) of II in 70 m*l* of xylene was refluxed for 6 hr. The solution was then evaporated *in vacuo*, leaving an oil which was crystallized by seeding. Yield, 4.3—5.0 g, mp 93—109°C. It gave a positive Beilstein's test and exhibited two spots on TLC. The ratio of III to IV in the crystals was found to be 1.9—2.1 by an analysis of its infrared spectra.

A solution of 3.0 g of the crude product in 10 m*l* of hot benzene was adsorbed on a column (1.6×30 cm) with 30 g of silica gel, eluted with a mixture of benzene and methanol (19 : 1, v/v), and divided into four fractions by TLC.

The evaporation of the first fraction (170 m*l*) left no residue.

The second fraction (25 m*l*), exhibiting a single spot on TLC, was evaporated *in vacuo*, leaving III as white crystals. Yield, 1.1 g, mp 101.5—103°C. For analysis, it was also recrystallized from benzene - petroleum ether (3 : 1); mp 102.5—103.5°C, $[\alpha]_D^{23.5}$ −126.5° (*c* 3.09, methanol).

IR (Nujol mull): 1820, 1785 (C=O group in the oxazolidone ring); 1755 (C=O group in the ester); 1710 cm^{-1} (C=O group in the benzyloxycarbonyl group).

NMR (Chloroform solution): 1.46 (doublet, J=6.8 cps, CH_3 peak in the alanine residue); 3.82 ppm (singlet, CH_3 peak in the ester group).

Found: C, 54.99; H, 5.26; N, 8.02%. Calcd for $C_{16}H_{18}O_7N_2$: C, 54.85; H, 5.18; N, 8.00%.

The evaporation of the third fraction (20 m*l*) provided 0.8 g of a mixture of III and IV which melted at 95—108°C.

The evaporation of the fourth fraction (35 m*l*) provided 0.3 g of IV contaminated with a minor amount of III, as indicated by TLC. The recrystallization of the crude product, which melted at 120—134°C, from methanol and then from benzene raised the melting point to 150.5—151.3°C, $[\alpha]_D^{21.5}$ +14.1° (*c* 1.46, methanol).

IR (Nujol mull): 1655, 1530 (-CONH- group in the peptide bond); 1750 (C=O group in the ester); 1690 cm^{-1} (C=O group in the benzyloxycarbonyl group).

NMR (Chloroform solution): 1.26 (doublet, J= 7.2 cps, CH_3 peak in the alanine residue); 3.80 (singlet, CH_3 peak in the ester group); 3.91 ppm (doublet, J= 3.0 cps, $>CH_2$ peak in the $Cl{\cdot}CH_2{\cdot}CH<$ group).

Found: C, 52.73; H, 5.60; N, 8.31; Cl, 10.24%. Calcd for $C_{15}H_{19}O_5N_2Cl$: C, 52.56; H, 5.59; N, 8.17; Cl, 10.34%.

The refluxing of a solution of 3.7 g (0.0096 mol) of II in 40 m*l* of toluene for 6 hr provided another mixture of III and IV, in which the ratio of III to IV was 8.4. Yield, 1.7 g, mp 99—102°C.

L-2-Oxo-oxazolidine-4-carboxylic Acid (V). A solution of 4.2 g (0.04 mol) of L-serine in 80 m*l* (0.04 mol) of 0.5 N potassium hydroxide was treated with a solution of 4.8 g (0.048 mol) of phosgene in 20 m*l* of toluene in the presence of 8.3 g (0.06 mol) of anhydrous potassium carbonate for 3 hr at 0—5°C. The aqueous layer which separated from the toluene layer was acidified with 6 N hydrochloric acid to pH 1 and evaporated to dryness *in vacuo*. The residue was then extracted five times with hot ethyl acetate, and the dried extract was evaporated *in vacuo* to give crystals which melted at 116—117°C. Yield, 2.8 g (54%). Recrystallization from ethyl acetate - petroleum ether raised the melting point to 118.5—120°C, $[\alpha]_D^{22.5}$ −17.7° (*c* 3.44, water), lit.,[14] mp 118°C, $[\alpha]_D^{15}$ −10.95° (*c* 4.20, water).

Found: C, 36.80; H, 3.96; N, 11.03%. Calcd for $C_4H_5O_4N$: C, 36.65; H, 3.85; N, 10.69%.

Cyclohexylammonium salt; mp 155.0—155.8°C (decomp.), $[\alpha]_D^{19}$ −19.3° (*c* 3.11, water).

Found: C, 51.90; H, 8.12; N, 12.09%. Calcd for $C_{10}H_{19}O_4N_2$: C, 52.16; H, 7.88; N, 12.17%.

The hydrolysis of the sample of V with 6 N hydrochloric acid gave the L-serine; mp 214—215°C (decomp.), $[\alpha]_D^{21.5}$ +14.7° (*c* 2.91, 1 N hydrochloric acid).

Alkaline Hydrolysis of III. To a solution of 2.80 g (0.008 mol) of III in 40 m*l* of methanol 16.0 m*l* (0.016 mol) of 1 N potassium hydroxide were added, after which the reaction mixture was stirred for 1.5 hr at room temperature. After the evaporation of the methanol *in vacuo*, the residue was diluted with water and the aqueous solution was acidified with 8.5 m*l* of 1 N hydrochloric acid to pH 3.5. The separated oil was extracted with ethyl acetate. The dried extract was then evaporated to dryness *in vacuo*, leaving a crystalline *N*-benzyloxycarbonyl-L-alanine. Yield, 1.59 g (89%), mp 80—82.7°C and 86.0—87.3°C after recrystallization from chloroform - petroleum ether, $[\alpha]_D^{20.5}$ −13.0° (*c* 3.15, acetic acid); lit.,[15] mp 87°C, $[\alpha]_D^{27}$ −13.9° (*c* 2, acetic acid).

A mixed melting point with an authentic sample was not depressed.

An additional 8.5 m*l* of 1 N hydrochloric acid was added to the remaining aqueous solution. The residue, obtained by the evaporation of the acidic solution, was extracted three times with 20-m*l* portions of hot ethyl acetate; the evaporation of the combined extracts provided V as an oil which was crystallized by seeding. Yield, 0.71 g (68%), mp 95—103°C and 117—119°C after recrystallization from ethyl acetate - petroleum $[\alpha]_D^{19.5}$ −17.5° (*c* 3.43, water).

All the properties of the sample were identical with those of the cyclized product of L-serine.

L-Seryl-L-alanine (VI). This compound was prepared by the catalytic hydrogenation of 4.5 g (0.014 mol) of *N*-benzyloxycarbonyl-L-seryl-L-alanine [mp 157—158°C, $[\alpha]_D^{22.5}$ −12.5° (*c* 3, methanol)], which had been obtained from its methyl ester by hydrolysis with an equivalent amount of 1 N sodium hydroxide in methanol, with 0.5 g of 10% palladium on carbon in a mixture of 120 m*l* of methanol and 50 m*l* of water, according to the directions of Fruton.[13] Yield, 2.2 g (89%), mp 223—225°C (decomp.) after recrystallization from water-acetone, $[\alpha]_D^{24.5}$ −31.8° (*c* 6, 1 N hydrochloric acid), lit.,[13] $[\alpha]_D^{26}$ −30.4° (*c* 6, 1 N hydrochloric acid).

14) T. Saito, This Bulletin, **37**, 624 (1964).
15) M. Hunt and V. du Vigneaud, *J. Biol. Chem.*, **124**, 699 (1938).

Found: C, 40.99; H, 6.86; N, 15.85%. Calcd for $C_6H_{12}O_4N_2$: C, 40.90; H, 6.87; N, 15.90%.

L-2-Oxo-oxazolidine-4-carbonyl-L-alanine (VII). In the way similar to that described for the preparation of V, a solution of 3.7 g (0.021 mol) of VI in 44 m*l* (0.022 mol) of 0.5 N potassium hydroxide was treated with 25 g (0.025 mol) of a 10% solution of phosgene in toluene in the presence of 3.5 g (0.025 mol) of anhydrous potassium carbonate. The crude product which separated from the salt was dissolved in water, the aqueous solution was passed through a column of Dowex 50 (H^+ form), and the column was thoroughly washed with water. The combined aqueous solution was then evaporated to dryness *in vacuo* to give a syrup which was crystallized by the addition of petroleum ether to a methanol solution of the product. Yield, 2.5 g (60%), mp 178—181°C (decomp.). Recrystallization from ethanol - petroleum ether raised the melting point to 188—191°C (decomp.), $[\alpha]_D^{18}$ −28.3° (*c* 3, water).

Found: C, 41.43; H, 5.15; N, 13.96%. Calcd for $C_7H_{10}O_5N_2$: C, 41.58; H, 4.99; N, 13.86%.

Cyclohexylammonium salt; mp 196—197.5°C (decomp.) after recrystallization from methanol-ether.

Found: C, 51.83; H, 7.83; N, 14.12%. Calcd for $C_{13}H_{23}O_5N_3$: C, 51.81; H, 7.69; N, 13.95%.

***N*-Benzyloxycarbonylglycyl - L - seryl - L - alanine Methyl Ester (VIII).** A solution of 1.4 g (0.005 mol) of *N*-benzyloxycarbonylglycyl-L-serine methyl ester[10] [mp 96.5—98.5°C, $[\alpha]_D^{14.5}$ −4.0° (*c* 6, methanol)] and 0.5 m*l* of 85% hydrazine hydrate in 30 m*l* of ethanol was allowed to stand overnight at room temperature. The crystal, *N*-benzyloxycarbonylglycyl-L-serine hydrazide, which separated from the solution were collected by filtration. Yield, 1.3 g (93%), mp 220—221°C (decomp.) after recrystallization from ethanol-water, $[\alpha]_D^{13}$ −9° (*c* 1, acetic acid).

Found: C, 50.61; H, 5.97; N, 18.12%. Calcd for $C_{13}H_{18}O_5N_4$: C, 50.31; H, 5.85; N, 18.06%.

In a mixture of 50 m*l* of water, 5 m*l* of acetic acid, and 20 m*l* of 6 N hydrochloric acid, 3.1 g (0.010 mol) of the above hydrazide was dissolved and the resultant solution was cooled to −5°C. To the solution a cold 7-m*l* portion (0.010 mol) of a 10% aqueous sodium nitrite solution was added, and the mixture was stirred for 20 min at 0°C. The azide which separated from the reaction mixture was extracted with 250 m*l* of ethyl acetate, and the extract was washed with ice water, a 3% aqueous sodium hydrogen carbonate solution, and finally ice water below −5°C. To the cold, dried solution there was then added another cold, dried solution of methyl L-alaninate equivalent to 1.4 g (0.010 mol) of its hydrochloride in 20 m*l* of chloroform at 0°C. The reaction mixture was stirred for 2 hr below 5°C and allowed to stand overnight in a refrigerator. After washing with water, a 3% aqueous sodium hydrogen carbonate solution, 1 N hydrochloric acid, and water, the evaporation of the dried organic layer *in vacuo* provided 1.9 g (50%) of VIII. Mp 135.5—137°C after recrystallization from water, $[\alpha]_D^{13}$ −38.2° (*c* 3, methanol).

Found: C, 51.29; H, 6.33; N, 10.67%. Calcd for $C_{17}H_{23}O_7N_3 \cdot H_2O$: C, 51.12; H, 6.31; N, 10.52%.

***N*-Benzyloxycarbonylglycyl-*O*-chlorocarbonyl-L-seryl-L-alanine Methyl Ester (X).** In the same manner as has been described for the preparation of II, a solution of 3.9 g (0.01 mol) of VIII in 50 m*l* of anhydrous dioxane was treated with an excess of phosgene in the presence of 2.0 g (0.016 mol) of *N*,*N*-dimethylaniline below 10°C. After the excess phosgene had been removed, the reaction mixture was evaporated *in vacuo* and the residue was taken up with ethyl acetate. The ethyl acetate solution was washed with 0.1 N hydrochloric acid and water, and the dried solution was evaporated *in vacuo* to give an oil which was crystallized by adding petroleum ether. Yield, 3.2 g (73%), mp 126.5—127°C after recrystallization from acetone-petroleum ether, $[\alpha]_D^{18}$ −4.5° (*c* 0.7, dioxane).

Found: C, 49.39; H, 5.23; N, 8.93; Cl, 7.79%. Calcd for $C_{18}H_{22}O_8N_3Cl \cdot \frac{1}{2}C_3H_6O$: C, 49.25; H, 5.34; N, 8.89; Cl, 7.50%.

L-3-(*N*-Benzyloxycarbonylglycyl)-2-oxo-oxazolidine-4-carbonyl-L-alanine Methyl Ester (XII) and *N*-Benzyloxycarbonylglycyl-3-chloro-L-alanyl-L-alanine Methyl Ester (XIII). A solution of 4.8 g (0.010 mol) of X in 250 m*l* of toluene was refluxed for 4 hr under a stream of nitrogen and then evaporated to dryness *in vacuo*. The residue was extracted with chloroform, and the dried extract was again evaporated *in vacuo* to provide an oil which exhibited two spots upon TLC. From this oil, XII was separated as crystals by adding a small amount of petroleum ether and by scratching on the wall of the vessel. Yield, 1.2 g (27%), mp 155—160°C. Recrystallization from chloroform-petroleum ether and then from ethyl acetate - petroleum ether raised the melting point to 172.5—173°C, $[\alpha]_D^{14}$ −113.8° (*c* 0.9, methanol).

Found: C, 52.84; H, 5.17; N, 10.18%. Calcd for $C_{18}H_{21}O_8N_3$: C, 53.07; H, 5.20; N, 10.32%.

The mother liquor remaining after the filtration of XII was evaporated to dryness *in vacuo*, and to the residue there was added a large amount of petroleum ether in order to obtain XIII as another crystal, which was collected by filtration. Yield, 0.2 g, mp 137—139°C. Recrystallization from ethyl acetate - petroleum ether raised the melting point to 140.5—141°C. It gave a positive Beilstein's test.

Found: C, 51.19; H, 5.55%. Calcd for $C_{17}H_{22}O_6N_3Cl$: C, 51.06; H, 5.56%.

Alkaline Hydrolysis of XII. In a manner similar to that described for the hydrolysis of III, 1.0 g (0.0025 mol) of XII was hydrolyzed with a mixture of 5 m*l* (0.005 mol) of 1 N potassium hydroxide and 50 m*l* of methanol. After the evaporation of the solvent, the residue was diluted with water and acidified with 1 N hydrochloric acid to pH 3. From the ethyl acetate extract, 0.42 g (81%) of *N*-benzyloxycarbonylglycine was isolated, all the properties of which were identical with those of an authentic sample.

On the other hand, 0.2 g (40%) of VII was isolated from the aqueous solution as an oil which was purified as its cyclohexylammonium salt. No melting-point depression upon mixture with an authentic specimen obtained from the cyclized product of VI was observed.

***N*-Benzyloxycarbonyl-L-alanyl-L-alanyl-L-serine Methyl Ester (IX).** Seven and seven-tenths grams (0.025 mol) of *N*-benzyloxycarbonyl-L-alanyl-L-alanine hydrazide [mp 212—213°C, $[\alpha]_D^{18}$ −67.4° (*c* 1.84, 0.5 N hydrochloric acid)] were converted into its azide according to the directions of Erlanger.[16] To the cold,

16) B. F. Erlanger and E. Brand, *J. Am. Chem. Soc.*, **73**, 3508 (1951); E. Brand, B. F. Erlanger, H. Sachs and J. Polantnick, *ibid.*, **73**, 3510 (1951).

dried solution of the above azide in 200 ml of ethyl acetate - ether (1 : 1), there was added another cold, dried solution of methyl L-serinate equivalent to 6.1 g (0.039 mol) of its hydrochloride in a mixture of 45 ml of chloroform and 100 ml of ethyl acetate below 5°C. The reaction mixture was then stirred for 3 hr below 5°C and allowed to stand for 20 hr in a refrigerator. The crystals which separated were collected by filtration and washed with acetone, acetone-water (1 : 1), and finally acetone again. Yield, 6.4 g (65%), mp 197—198°C (sintered at 164°C). The melting point was unchanged after recrystallization from acetone. $[\alpha]_D^{16.5}$ −50.1° (*c* 3.53, methanol).

Found: C, 54.73; H, 6.54; N, 10.52%. Calcd for $C_{18}H_{25}O_7N_3$: C, 54.67; H, 6.37; N, 10.63%.

***N*-Benzyloxycarbonyl-L-alanyl-L-alanyl-*O*-chlorocarbonyl-L-serine Methyl Ester (XI).** In a manner similar to that described for the preparation of II, 8.0 g (0.02 mol) of IX was converted to XI with phosgene in the presence of 2.4 g (0.02 mol) of *N*,*N*-dimethylaniline in 100 ml of anhydrous dioxane. Yield, 7.7 g (83%), mp 143.5—144.0°C (decomp.) after recrystallization from benzene, $[\alpha]_D^{23.5}$ −3.7° (*c* 2.72, dioxane).

Found: C, 50.34; H, 5.44; N, 9.24; Cl, 7.31%. Calcd for $C_{19}H_{24}O_8N_3Cl$: C, 49.84; H, 5.28; N, 9.18; Cl, 7.74%.

Methyl L-3-(*N*-Benzyloxycarbonyl-L-alanyl-L-alanyl)-2-oxo-oxazolidine-4-carboxylate (XIV). A solution of 10.0 g (0.022 mol) of XI in 130 ml of xylene was refluxed for 6 hr. The solvent was then evaporated *in vacuo*, leaving a syrup which was dissolved in 130 ml of ethyl acetate. The solution was treated with 3 ml of pyridine for 1.5 hr at room temperature under occasional shaking and then washed with dilute hydrochloric acid, a dilute aqueous sodium hydrogen carbonate solution, and water. The evaporation of the dried solution *in vacuo* provided a syrup, which was taken up with 50 ml of acetone. The decolorized solution was evaporated again to dryness *in vacuo* to give an amorphous solid. Yield, 6.2 g (67%). It gave a slightly positive Beilstein's test and exhibited a main spot, accompanied with two minor spots, upon TLC.

Alkaline Hydrolysis of XIV. In a way similar to that described for the hydrolysis of III, 4.67 g (0.011 mol) of the amorphous XIV was hydrolyzed with a mixture of 23.0 ml (0.023 mol) of 1 N potassium hydroxide and 75 ml of methanol. After the evaporation of the solvent, the residue was diluted with water and acidified with 1 N hydrochloric acid to pH 3. From the ethyl acetate extract, 1.96 g (60%) of crude *N*-benzyloxycarbonyl-L-alanyl-L-alanine were isolated, found to be contaminated with minor components upon TLC. It was purified and identified as its cyclohexylammonium salt*6 (0.73 g); mp 195—197°C (decomp.), $[\alpha]_D^{23}$ −34.7° (*c* 3.14, water).

V was isolated from the aqueous solution. Yield, 0.67 g (46%), mp 116—118°C after recrystallization from ethyl acetate - petroleum ether, $[\alpha]_D^{23}$ −17.8° (*c* 2.97, water). It was also identified as its cyclohexylammonium salt; mp 154—155°C (decomp.), $[\alpha]_D^{23.5}$ −19.8° (*c* 3.43, water).

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*6 An authentic sample was prepared from *N*-benzyloxycarbonyl-L-alanyl-L-alanine[17] by the treatment of cyclohexylamine; mp 197—198°C (decomp.), $[\alpha]_D^{23}$ −35.0° (*c* 3.32, water). Found: C, 61.10; H, 8.00; N, 10.50%. Calcd for $C_{20}H_{31}O_5N_3$: C, 61.05; H, 7.94; N, 10.68%.

17) W. H. Stein, S. Moore and M. Bergmann, *J. Biol. Chem.*, **154**, 191 (1944).