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Synthetic Studies on Bacitracin. VII.[1] Isomerization of Amino Acid Components of Thiazoline Peptides*1

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Ethyl 2-(1-benzyloxycarbonylaminoethyl)-R-Δ^2-thiazoline-4-carboxylate (III), ethyl 2-(1-benzyloxycarbonylamino-2-methyl-*n*-butyl)-R-Δ^2-thiazoline-4-carboxylate (VI) and their acid amide derivatives (V, VII and VIII) were prepared by either the iminoether coupling method or the dehydration method. As a general character of thiazoline peptide, it was found that the amino acid residue connected to thiazoline ring at *N*-terminal side is completely racemized at the stage of thiazoline ring formation, and further epimerized, in the case of isoleucine, by treatment with the basic reagent.

In earlier works on an amino acid composition of bacitracin A, the ambiguity of isoleucine content made the final conclusion of the molecular formula difficult.[2-6] Amino acid analysis of the hydrolyzate of somewhat impure bacitracin A showed often less than one mole of D-alloisoleucine which compensates the loss of L-isoleucine from three mole value in pure peptide.[2,5,6] The inactivation of the antibiotic activity of bacitracin A is closely related to isomerization of *N*-terminal isoleucine residue of the peptide.[7,8] Konigsberg *et al.* separated the high and low potency forms of bacitracin A by carbomethoxy cellulose chromatography after exposure in acidic medium, and showed that the high potency forms has L-isoleucine as *N*-terminal residue which is changed to D-alloisoleucine in the low potency one.[8]

In the present investigation, optical properties of the amino acid components of thiazoline ring of the various model peptides were studied in order to clarify the basic character of the isomerization of *N*-terminal amino acid of bacitracin A and to gain fundamental knowledge of its total synthesis.

General procedure used for the syntheses of thiazoline peptides were described previously.[9-10] Measurement of optical purity of the amino acid was performed by gaschromatography after conversion into trifluoroacetyl amino acid menthyl ester[11] for alanine and by amino acid analysis for isoleucine.

Acid hydrolysis of ethyl 2-(1-benzyloxycarbonylaminoethyl)-R-Δ^2-thiazoline-4-carboxylate (III) which was obtained by imino ether coupling method[9] from L-2-benzyloxycarbonylamino-propioimino ethyl ether (IV) and ethyl L-cysteinate hydrochloride gave completely racemized DL-alanine and L-cystine, while optically pure L-alanine was recovered from an acid hydrolyzate of IV. This fact indicates that racemization occurred during ring closure of thiazoline ring through the imino ether procedure. In order to know whether racemization depends on the method of ring closure or is an essential nature characteristic to thiazoline ring, the same thiazoline peptide III was prepared by dehydration method[10] as follows.

Benzyloxycarbonyl-L-alanyl-L-cysteine ethyl ester (II) was synthesized by coupling of benzyloxycarbonyl-L-alanine with diethyl L-cystinate dihydro chloride[12] using *N,N'*-dicyclohexylcarbodiimide followed by reduction of the product, *N,N'*-bisbenzyloxycarbonyl-L-alanyl-L-cystine diethyl ester (I), with zinc dust and hydrochloric acid. Cyclization of II to the thiazoline derivative was carried out with hydrogen chloride in chloroform. Neutralization

*1 This paper is dedicated to Emeritus Professor Munio Kotake in commemoration of his 75th birthday, November 30, 1969.

1) Part VI of this series: This Bulletin, **43**, 1564 (1970).

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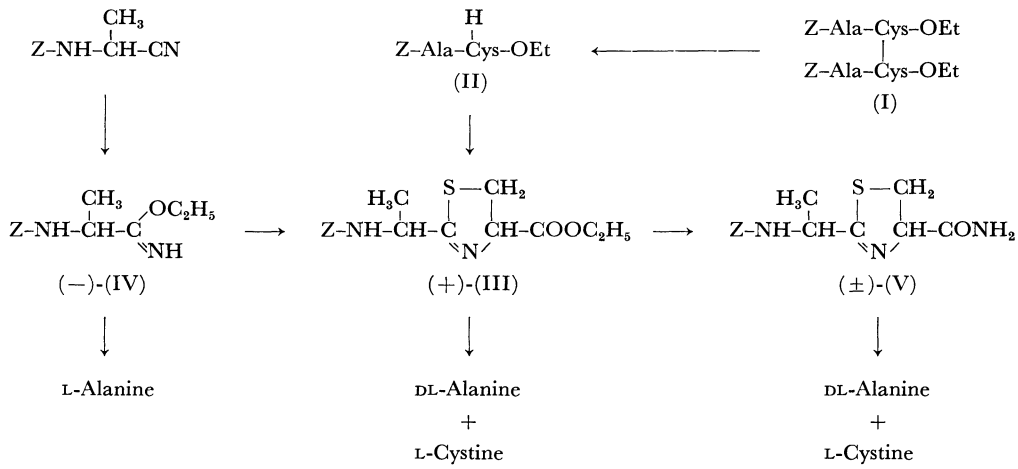

Fig. 1

$Z = C_6H_5CH_2OCO$

Cystine and alanine used are of L-configurations.

of the product with aqueous potassium carbonate afforded compound III. Thiazoline peptide III thus obtained was hydrolyzed with hydrochloric acid to give DL-alanine as well as L-cystine. Acid amide derivative V derived from compound III by treatment with ammonia in ethyl alcohol gave DL-alanine and DL-cystine on acid hydrolysis.[9]

From the results, it is concluded that *N*-terminal amino acid of the thiazoline peptide III is completely racemized during the stage of ring closure regardless of the method employed for the ring formation. Cysteine residue does not change in optical property at the ring closure but is also racemized under basic condition.

A similar result was observed in hydrolyzate of the other thiazoline peptide in which isoleucine is linked at the *N*-terminal side. 2-(1-Benzyloxycarbonylamino-2-methyl-*n*-butyl)-R-Δ^2-thiazoline-4-carboxamide (VIII) was synthesized by coupling of L-2-benzyloxycarbonylamino-3-methylvaleroimino

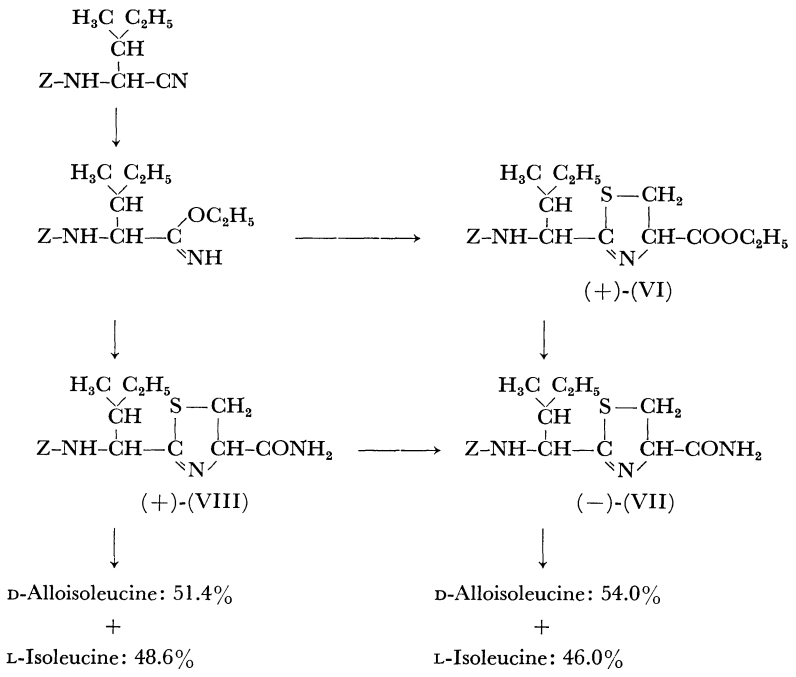

Fig. 2

$Z = C_6H_5CH_2OCO$

Cystine and isoleucine used are of L-configurations.

ethyl ether[9] and L-cysteinamide hydrobromide[1] as shown in Fig. 2. On amino acid analysis, hydrolysis of this thiazoline compound with hydrochloric acid gave a mixture of exactly the same amounts of L-isoleucine and D-alloisoleucine. This indicates that the racemization of the amino acid residue at *N*-terminal side of thiazoline ring is not limited to alanine derivative but is probably a general character based on thiazoline ring structure.

However, after a basic treatment of the thiazoline peptide, for instance, conversion of ethyl 2-(1-benzyloxycarbonylamino-2-methyl-*n*-butyl)-R-Δ^3-thiazoline-4-carboxylate (VI)[9] into carboxamide derivative (VII) with ammonia in ethyl alcohol or keeping of VIII in ethyl alcohol saturated with ammonia at room temperature for 3 days, a ratio of L-isoleucine and D-alloisoleucine in acid hydrolyzate changed to 46 : 54. Deviation from the ratio 50 : 50 seems to be significant in consideration of the accuracy of the analytical method. This means that the isoleucine residue connected to thiazoline ring at *N*-terminal side is racemized instantly on ring closure and further epimerized under basic condition. Epimerization of α-asymmetric carbon of isoleucine might progress under the steric influence of β-asymmetric carbon atom of isoleucine.

Konigsberg *et al.* demonstrated that the high potency form of bacitracin A isomerized to a low potency form on standing in 3% acetic acid, and the amount of the two forms becomes nearly equal after two days. Interconversion of the two isomers occurs even at pH 5.45. This isomerization can be well understood as the general character of thiazoline peptide. However, it is supposed that in comparison with rapid racemization of the model thiazoline peptide, the rate of isomerization of natural bacitracin A slows down because of a peculiar structural factor like the cyclol structure in the intact molecule of bacitracin A.

For the total synthesis of bacitracin A, it is expected that the racemization of the *N*-terminal isoleucine residue can not be avoided whatever method is applied for closure of the thiazoline ring, and the product must be purified by the fractional method based on the principle of separation of diasteromers to secure pure bacitracin A.

Experimental

All melting points are uncorrected. Ultraviolet spectra were obtained in 95% ethanol and 12N hydrochloric acid - 95% ethanol (1 : 1) with a Hitachi EPS-3 spectrophotometer. For gas chromatography a Shimazu GC-4APF was used. Amino acid analysis was carried out by a Hitachi Amino Acid Analyzer LKA-3.

N,N'-Bisbenzyloxycarbonyl-L-alanyl-L-cystine Diethyl Ester (I). To a suspension of 11.0 g (0.03 mol) of diethyl L-cystinate dihydrochloride[12] in 100 m*l* of chloroform was added 6.0 g (0.06 mole) of triethylamine. After the mixture was stirred for 1 hr, 100 m*l* of ether was added. The precipitate formed was filtered off, and the filtrate was concentrated *in vacuo*. To a solution of the residue thus obtained in 150 m*l* of dioxane, 13.4 g (0.060 mol) of benzyloxycarbonyl-L-alanine and 13.6 g (0.066 mol) of *N,N'*-dicyclohexylcarbodiimide were added. After the reaction mixture had been stirred for several hours, 1 m*l* of acetic acid was added. *N,N'*-Dicyclohexylurea formed was filtered off, and the filtrate was diluted with water. The precipitate formed was filtered off and washed with water. Recrystallization from ethanol gave 18.6 g (82%) of I; mp 165—167°C, $[\alpha]_D^{15}$ −74.0° (*c* 2.00, dimethylformamide).

Found: C, 54.48; H, 6.02; N, 7.88; S, 9.05%. Calcd for $C_{32}H_{42}O_{10}N_4S_2$: C, 54.38; H, 5.99; N, 7.93; S, 9.07%.

Benzyloxycarbonyl-L-alanyl-L-cysteine Ethyl Ester (II). To a suspension of 17.7 g (0.025 mol) of I in 200 m*l* of dioxane and 16.5 m*l* of concentrated hydrochloric acid was added 7.5 g of zinc dust in many portions over a period of 15 min with vigorous stirring at 30—40°C. The mixture was stirred for additional 30 min, and then filtered. The filtrate was concentrated *in vacuo*. An oily product separated out on addition of water was extracted with ethyl acetate. The extract was washed with water, dried with magnesium sulfate and then evaporated *in vacuo*. Recrystallization of the residual solid from ethyl acetate-petroleum ether gave 11.5 g (65%) of II; mp 106—109°C, $[\alpha]_D^{15}$ −14.1° (*c* 2.18, dimethylformamide).

Found: C, 54.17; H, 6.26; N, 7.91; S, 8.86%. Calcd for $C_{10}H_{22}O_5N_2S$: C, 54.22; H, 6.26; N, 7.91; S, 9.05%.

Ethyl 2-(1-Benzyloxycarbonylaminoethyl)-R-Δ^2-thiazoline-4-carboxylate (III). A solution of 10.6 g (0.03 mol) of II in 500 m*l* of chloroform was saturated with dry hydrogen chloride gas on cooling in an ice bath. The mixture was allowed to stand at room temperature overnight and then concentrated *in vacuo*. To the residue was added 300 m*l* of ether, and the mixture was treated with a concentrated aqueous potassium carbonate. The ether layer was washed with water, dried with sodium sulfate and then concentrated *in vacuo* to give a crude oil of 7.5 g: this was dissolved in a small amount of ether and subjected to silica gel column chromatography (Mallincrodt Chemical Worker, 100 mesh, 100 g). Elution with petroleum ether - anhydrous ether gave 5.8 g (57%) of III, $[\alpha]_{25}^{D}$ +59.4° (*c* 3.26, ethanol).

Found: C, 57.06; H, 5.97; N, 8.29; S, 9.40%. Calcd for $C_{16}H_{20}O_4N_2S$: C, 57.12; H, 5.99; N, 8.33; S, 9.53%.

2-(1-Benzyloxycarbonylamino-2-methyl-*n*-butyl)-Δ^2-thiazoline-4-carboxamide (VII). A solution of 1.9 g (0.005 mole) of ethyl 2-(1-benzyloxycarbonylamino-2-methyl-*n*-butyl)-R-Δ^2-thiazoline-4-carboxylate (VI)[9] in 20 m*l* of anhydrous ethanol was saturated with ammonia at 0°C, and the mixture was kept at room temperature for 3 days. The solution was concentrated *in vacuo* below 40°C. The residue thus obtained was recrystallized from ethanol-water to give 1.4 g (82%) of VII, mp 131—133°C, $[\alpha]_D^{25}$ −5.8° (*c* 1.25, dimethylformamide), λ_{max}^{EtOH} 235 mμ (ε 2300), 252 mμ (ε 2800), $\lambda_{max}^{EtOH\text{-}HCl}$ 269 mμ (ε 4800).

Found: C, 58.36; H, 6.63; N, 12.00; S, 8.94%. Calcd for $C_{17}H_{23}O_3N_3S$: C, 58.43; H, 6.63; N, 12.03; S, 9.18%.

2-(1-Benzyloxycarbonylamino-2-methyl-*n*-butyl)-R-Δ^2-thiazoline-4-carboxamide (VIII). To a solution of 5.7 g (0.026 mol) of L-cysteinamide hydrobromide[1]

in tetrahydrofuran and 10 m*l* of anhydrous methanol was added 7.5 g (0.026 mole) of L-2-benzyloxy-carbonylamino-3-methylvaleroimino ethyl ether.[9] After the reaction mixture had been kept at room temperature for 3 hr, ammonium bromide was deposited. The reaction mixture was concentrated *in vacuo*, and the residue obtained was dissolved in ethyl acetate. The ethyl acetate solution was washed with water, dried with sodium sulfate, and then evaporated *in vacuo*. The residue obtained was recrystallized from ethyl acetate - petroleum ether gave 2.8 g (35%) of VIII; mp; 138—140°C, $[\alpha]_D^{24}$ +26.7° (*c* 2.20, dimethylformamide).

Found: C, 58.40; H, 6.69; N, 11.82; S, 8.89%.

Isolation of L-Alanine from Hydrolyzate of IV. Hydrolysis of 5.0 g (0.02 mole) of IV was carried out by refluxing in 100 m*l* of 6N hydrochloric acid for 3 hr. After cooling, the reaction mixture was extracted with ether. An aqueous layer was evaporated *in vacuo*. The residue obtained was dissolved in 30 m*l* of water, subjected to Dowex-50 column (W×8, H^+ form, 200—400 mesh, 30 m*l*) and eluted with 50 m*l* of N ammonium hydroxide. The eluate was evaporated *in vacuo* and the residue obtained was then crystallized from water - ethyl alcohol; wt. 1.45 g (81%), $[\alpha]_D^{25}$ +14.3° (*c* 2.58, N HCl).

Found: C, 40.42; H, 7.97; N, 15.61%. Calcd for $C_3H_7O_2N_2$: C, 40.44; H, 7.92; N, 15.72%.

Gaschromatography of Menthyl Trifluoroacetylalaninate obtained from Hydrolyzates of III and V. Hydrolysis of 0.3 g each of III, which was synthesized by either the imino ether or the dehydration method, and of V, was carried out by refluxing in 20 m*l* of 6N hydrochloric acid with 0.3 g of anisole for 3 hr. After cooling, the reaction mixture was extracted with ether. An aqueous layer was evaporated *in vacuo*. The residue was dissolved in 10 m*l* of water and evaporation was repeated. The residue thus obtained was dissolved in 5 m*l* of water and subjected to Dowex-50 column (W×8, H^+ form, 200—400 mesh, 10 m*l*). Eluate with 20 m*l* of N ammonium hydroxide was evaporated *in vacuo* to yield alanine. Three mg each of the samples of alanine were mixed with 0.7 g of menthol. Hydrogen chloride gas was passed through the melted mixture at 110°C for one and a half hour.[11] Excess of menthol was removed by sublimation at 100°C under reduced pressure of 3 mmHg. To the residue thus obtained, a few m*l* of trifluoroacetic anhydride was added. After the mixture had been allowed to stand at room temperature overnight, excess of trifluoroacetic anhydride was removed by evaporation *in vacuo*. The residue was dissolved in 1 m*l* of acetone and subjected to gaschromatography. Gas chromatography was carried out on a column of 3m-length packed with 1.5% NPGS on 60—80 mesh Chromosorb W at column temperature of 150°C under excess pressure of 1.8 atm, and at detector temperature of 180°C and injection temperature of 250°C. All the three samples of menthyl trifluoroacetylalaninate were shown to be of complete DL-form.

Isolation of L-Cystine from Acid Hydrolyzate of III. Hydrolisys of 1.7 g each (0.05 mol) of III, which was synthesized by either the imino ether or dehydration method, was carried out by refluxing in 50 m*l* of 6N hydrochloric acid with 0.5 g of anisole for 3 hr. The reaction mixture was cooled and extracted with ether. The aqueous layer was concentrated *in vacuo* to dryness. A solution of the residue in 30 m*l* of water was adjusted to pH about 8.5 with 28% aqueous ammonia. Air was bubbled through the solution until the nitroprusside reaction became negative by a spot test. The reaction mixture was allowed to stand at 0°C for 2 days. The precipitate thus formed was filtered off and then dissolved in 15 m*l* of N hydrochloric acid. The solution was adjusted to pH 5.5—6.0 with an aqueous sodium acetate solution. After standing at 0°C for 2 days, the precipitate formed was filtered off and dried at 80°C *in vacuo* for 4 hr to give 0.33 g (55%) and 0.37 g (62%) of L-cystine respectively; $[\alpha]_D^{20}$ −218° (*c* 1.00, N HCl).

Found: N, 11.51 and 11.62; S, 26.09 and 26.38%. Calcd for $C_6H_{12}O_4N_2S_2$: N, 11.66; S, 26.69%.

Amino Acid Analysis of L-Isoleucine and D-Alloisoleucine in Acid Hydrolyzate of VII and VIII. *a)* A suspension of 0.05 g of VIII in 10 m*l* of 6N hydrochloric acid was refluxed for 2 hr. The hydrolyzate was extracted with ether. An aqueous layer was used for amino acid analysis. The ratio of D-alloisoleucine and L-isoleucine was found to be 51.4 : 48.6.

b) In a similar way, 0.1 g each of VII and VIII, which was treated with ammonia in ethanol at room temperature for 3 days, was hydrolyzed by refluxing in 10 m*l* of 6N hydrochloric acid for 3 hr, and the hydrolyzate was applied to amino acid analysis. Ratios of D-alloisoleucine and L-isoleucine for both compounds were found to be 54.7 : 45.3 and 54.0 : 46.0% respectively.