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Synthesis of Tricholomic Acid. IV.[1] Synthesis of DL-*threo*-α-Amino-3-oxo-5-isoxazolidineacetic Acid[2]

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DL-*threo*-α-Amino-3-oxo-5-isoxazolidineacetic acid, an isomer of tricholomic acid, was synthesized in a 3-step sequence starting from dialkyl N-acyl-β-chloroglutamate. Several isolations have been attempted to separate the *threo*- and *erythro*-isomers from their mixture. The *threo*-isomer was devoid of good taste and flycidal activity which are characteristic of the *erythro*-isomer.

In the preceding paper[1] of this series, DL-*erythro*-α-amino-3-oxo-5-isoxazolidineacetic acid (DL-*erythro*-I) was synthesized and its optical resolution afforded L- and D-isomers, of which the former was proved to be identical with tricholomic acid.[4] The present paper deals with the synthesis of DL-*threo*-α-amino-3-oxo-5-isoxazolidineacetic acid (*threo*-I).

As reported in the preceding paper,[1] diethyl *threo*-β-chloroglutamate[5] hydrochloride (*threo*-II) was treated with hydroxylamine and alkali to give a hydroxamic acid, which, without isolation, was cyclized with alkali to *erythro*-I, but the yields were not satisfactory. A reason for the low yield is attributed to the side reactions such as the formation of pyrrolidone or diketopiperazine derivatives by the reaction between the free amino group and the ester group. If an amino-protecting group is removable under mild conditions without affecting the isoxazolidone ring formed, the protection of the amino group of II with such a group is expected to prevent the side reactions. The isoxazolidone ring is rather stable in alkali, while it easily opens in acid media even at room temperature. The ordinary N-acyl groups are eliminatable by acidic hydrolysis, but an acetyl group can be also removed to some extent by alkaline hydrolysis. In fact, the deacetylation by refluxing with alkali was applied to the synthesis of cycloserine.[6] N-Formyl groups in general can be eliminated with methanol-hydrogen chloride at room temperature. Similarly, trifluoroacetyl groups are eliminatable with aqueous ammonia at room temperature and hence the procedure was used also in the synthesis of cycloserine.[6]

Applicability of these amino-protecting techniques to the synthesis of tricholomic acid was investigated. *threo*-(or *erythro*-)II was acylated in the usual manner with trifluoroacetic anhydride, acetic anhydride or formic acid–acetic anhydride. N-Trifluoroacetyl derivative (III) was treated with a mole equivalent of hydroxylamine hydrochloride and two moles of sodium hydroxide solution at low temperature, and the mixture was stirred with further four moles equivalent of alkali to accomplish the complete saponification of the ester. Study of the ratio of *erythro*- to *threo*-I in the reaction product by means of paper electrophoresis revealed

1) Part III: H. Iwasaki, T. Kamiya, O. Oka, and J. Ueyanagi, *Chem. Pharm. Bull.* (Tokyo), **17**, 866 (1969).
2) Presented at Meeting of Kinki Branch, Pharmaceutical Society of Japan, May 1965.
3) Location: *Juso, Higashiyodogawa-ku, Osaka.*
4) T. Takemoto and T. Nakajima, *Yakugaku Zasshi*, **84**, 1183, 1230 (1964).
5) All amino acids and their derivatives have optical isomers (L, D and DL). Unless described particularly, individual compounds are DL form in this series of paper.
6) W.F. Runge, U.S. Patent 2794022 (1957); Pl. A. Plattner, A. Boller, H. Frick, A. Fürst, B. Hegedüs, H. Kirchensteiner, St. Majnoni, R. Schläpfer and H. Speigelberg, *Helv. Chim. Acta*, **40**, 1531 (1957).

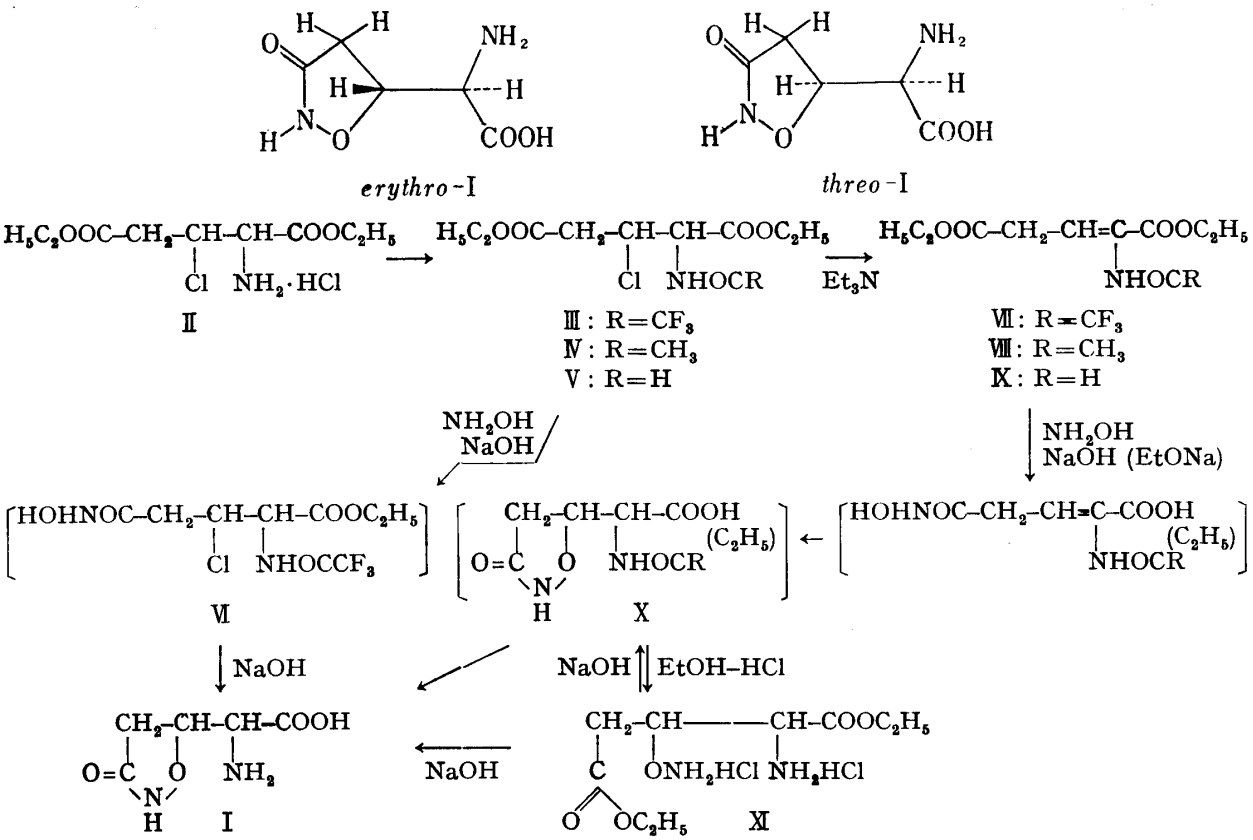

Chart 1

that *threo*-I was the major product from both of *threo*- and *erythro*-III as seen in Table I. Therefore, both *threo*- and *erythro*-III were supposed to be converted into *threo*-I *via* the same intermediate.

Table I

Synthetic method	Ratio of isomers *threo*-I/*erythro*-I	Total yield (%) *threo*-I	*erythro*-I
threo-III→VI→I	76·7/23·3	2·5	—
erythro-III→VI→I	82.7/17.3	—	—
VII→X→I	76.7/23.3	3.2	—
VIII→X→I	48.5/51.5	(1.6)	(1.7)
IX→X→I	60.5/39.5	(0.6)	(0.4)
VIII→X→XI→I	80.9/19.1	(2.44)	(0.66)

( ): yield calcd. from amino acid analysis

On the other hand, as already described in the preceding paper[1] N-benzoyl-β-chloroglutamate is easily converted into the unsaturated compound by elimination of hydrogen chloride with alkali. Taking account of this fact, there is ample possibility for assuming that the above reactions would have proceeded *via* an unsaturated compound (VII). N-Trifluoroacetyl (III), N-acetyl (IV) and N-formyl derivatives (V) were subjected to react with triethylamine in ethanol at room temperature, and found by thin-layer chromatography to undergo dehydrochlorination easily within 30 minutes. Of the reaction products, N-acetyl and N-formyl derivatives were isolated, and their structures were confirmed as diethyl 2-acetamido-2-pentenedioate (VIII) and diethyl 2-formamido-2-pentenedioate (IX), respectively, by elemental analysis and NMR spectra. Without isolation, VII was treated with hydroxylamine and sodium hydroxide in the same manner as in the case of III described above, and the

resulting reaction product was compared with that of the direct method from III. As seen in Table I, *threo*-I was the major compound and the ratio of *threo*-I to *erythro*-I was the same.

In our view the predominant formation of *threo*-I is attributed to the steric hindrance of the bulky trifluoroacetyl residue. N-Acetyl (VIII) and N-formyl (IX) derivatives were treated with hydroxylamine, and cyclized in a manner similar to the synthesis of cycloserine.[6] The resulting N-acyl-I, without isolation, was deacylated by hydrolysis: N-acetyl-I was boiled with sodium hydroxide solution and N-formyl-I was treated with methanolic hydrogen chloride at room temperature. In these cases, *threo*-I and *erythro*-I were formed almost in the equal ratio, indicating that the steric hindrance of acetyl and formyl residues is less than that of the trifluoroacetyl. However, the actual yield of *threo*-I by these methods was considerably lower than that in the case of the N-trifluoroacetyl derivative (VII), probably because of somewhat severe conditions applied to the deacylation.

Deacylation by boiling of the solution of N-acetyl derivative (X) in ethanolic hydrogen chloride, on the other hand, causes simultaneously the opening of the isoxazolidone ring to give an aminooxy derivative (XI). The crude XI was again cyclized with alkali to I. Amino acid analysis showed that the reaction product contained *threo*-I as the major component in the ratio of *threo*-I/*erythro*-I=81/19, and the result was quite different from the case of the deacetylation by boiling with alkali.

The experiments described above leads to the following conclusions: (1) since N-acyl-3-chloroglutamate undergoes easily dehydrochlorination in alkaline media, the reaction of N-acyl-β-chloroglutamate with hydroxylamine and alkali proceeds *via* the unsaturated compound to give the isoxazolidone derivative by the intramolecular addition of γ-hydroxamic acid onto the double bond, and (2) diethyl 2-trifluoroacetamido-2-pentenedioate (VII) afforded selectively *threo*-I in the cyclization due to the steric effect of the bulky trifluoroacetyl moiety.

The purification process was essentially the same as in the case of *erythro*-I described in the preceding paper[1] and the yield of crystalline *threo*-I was 3.2%, being calculated from *threo*-III. Paper chromatography could not distinguish *threo*-I from *erythro*-I, but paper electrophoresis, amino acid analysis and infrared spectrum showed a marked difference between the two isomers.

In order to confirm the stereochemical structure, *threo*-I was reduced catalytically to *threo*-β-hydroxyglutamine (XII), which was further hydrolyzed to *threo*-β-hydroxyglutamic acid (XIII), according to the method of Takemoto, *et al.*[4] Identification of XII and XIII was made by the direct comparison with authentic samples.

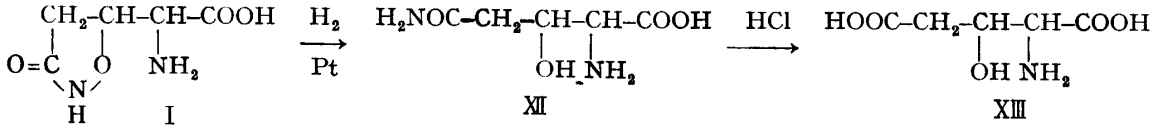

Chart 2

For the purpose of a large scale production, the method of separation of *threo*- and *erythro*-I was then studied. When either of the isomers is contained in excess in the mixture, the separation was carried out by subjecting the mixture to recrystallization from water. When the mixture contains nearly an equal amount of *erythro*- and *threo*-I, the separation was made as follows: (1) By means of Dowex 1×8 (acetate type) ion-exchange chromatography with acetic acid–methanol–water (1:2:7, v/v) as developer, *erythro*-I and *threo*-I were eluted in its order; (2) By means of Dowex 50 W×8 (pyridine salt form) ion-exchange chromatography with 0.2M pyridine acetate buffer (pH 5.25) as developer, *threo*-I was eluted ahead of *erythro*-I; (3) The separation was also carried out by taking advantage of difference in solubility in water of copper salts of *erythro* and *threo* isomers. That is, when copper ion is added to an aqueous solution of the mixture, the copper salt of *erythro*-I precipitated, while that of *threo*-I remained in the solution. The copper ion was removed by treatment with Amberlite IR-120.

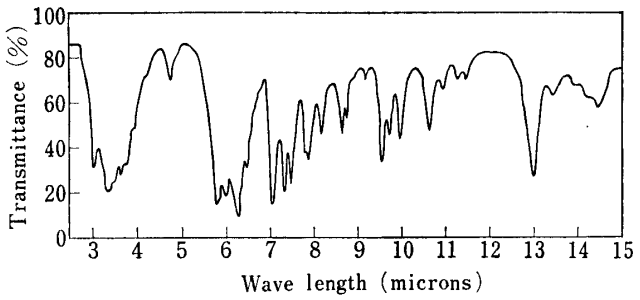

Fig. 1. IR Spectrum of *threo*-DL-α-Amino-3-oxo-5-isoxazolidineacetic Acid (KBr Disk)

The taste potency of *threo*-I was reported by Terasaki, *et al.*[7] to be almost negligible and its flycidal activity was hardly observed. That only L-*erythro*-derivative[1] of four stereoisomers has the biological activities is very interesting, indicating a close relationship between the biological activity and the steric configuration of the compound.

Experimental

Diethyl *threo*-N-Trifluoroacetyl-β-chloroglutamate (*threo*-III)——Ten grams (36.5 mmoles) of diethyl *threo*-β-chloroglutamate (*threo*-II) hydrochloride was added portionwise to 10 ml (71.5 mmoles) of trifluoroacetic anhydride with stirring under ice-cooling. After 10-min stirring under ice-cooling, the reaction was completed by warming at 60° for 10 min. The reaction mixture was evaporated *in vacuo* to yield an oil, which was dried overnight over KOH in a desiccator under reduced pressure. Vacuum distillation gave 10.7 g (90% yield) of *threo*-III as a colorless oil, bp 123—124° (0.04 mmHg). *Anal.* Calcd. for $C_{11}H_{15}O_5NF_3Cl$ (diethyl *threo*-N-trifluoroacetyl-β-chloroglutamate): C, 39.59; H, 4.53; N, 4.20; Cl, 10.63. Found: C, 39.62; H, 4.71; N, 4.07; Cl, 10.55.

***erythro*-III**——*erythro*-II (5.4 g) and trifluoroacetic anhydride (7 ml) were treated in the same manner as described in *threo*-isomer. Vacuum distillation gave 3.6 g (55% yield) of *erythro*-III as a colorless oil, bp 134—137° (0.07 mmHg). *Anal.* Calcd. for $C_{11}H_{15}O_5F_3Cl$ (diethyl *erythro*-N-trifluoroacetyl-β-chloroglutamate): C, 39.59; H, 4.53; N, 4.20. Found: C, 39.78; H, 4.83; N, 4.13.

Diethyl *threo*-N-Acetyl-β-chloroglutamate (IV)——Five grams of *threo*-II was mixed with 30 ml of acetic anhydride and the mixture was stirred at 80° for 1 hr. The reaction mixture was evaporated *in vacuo* to yield an oil. Vacuum distillation afforded 4.6 g (90% yield) of IV as an yellowish oil, bp 157° (0.04 mmHg). *Anal.* Calcd. for $C_{11}H_{18}O_5NCl$ (diethyl *threo*-N-acetyl-β-chloroglutamate): C, 47.23; H, 6.49; N, 5.01. Found: C, 47.07; H, 6.46; N, 5.18.

Diethyl *threo*-N-Formyl-β-chloroglutamate (V)——To a solution of 2.74 g of *threo*-II in 50 ml of 99% formic acid was added dropwise 15 ml of acetic anhydride, and the reaction mixture was kept at 80° for 2 hr, then left standing at room temperature overnight. The solution was added with 30 ml of formic acid and 15 ml of acetic anhydride, and the mixture warmed at 80°. Additional 30 ml of formic acid and 15 ml of acetic anhydride were added 2 hr later, and warming was continued for further 4 hr. The reaction mixture, from which had disappeared the starting material, was evaporated *in vacuo* to yield an oil. Vacuum distillation afforded 1.88 g (71% yield) of V as an yellowish oil, bp 165—168° (0.18 mmHg). *Anal.* Calcd. for $C_{10}H_{16}O_5NCl$ (diethyl *threo*-N-formyl-β-chloroglutamate): C, 45.20; H, 6.07; N, 5.27. Found: C, 45.72; H, 6.07; N, 5.16.

Diethyl 2-Acetamido-2-pentenedioate (VIII)——To a solution of 4.57 g of IV in 5 ml of EtOH was added 3 ml of triethylamine and the mixture was stirred at room temperature for 1 hr. The reaction mixture was evaporated *in vacuo* and the residue was extracted several times with EtOAc. The EtOAc solution was concentrated after filtration and left standing in a refrigerator to afford crystals, which were recrystallized from EtOAc to give 2.5 g (63% yield) of VIII as colorless needles, mp 75—76°. *Anal.* Calcd. for $C_{11}H_{17}O_5N$ (diethyl 2-acetamido-2-pentenedioate): C, 54,31; H, 7.04; N, 5,76. Found: C, 54.53; H, 7.07; N, 5.74. NMR (in $CDCl_3$) τ: 3.1 (1H, triplet, –CH=C–C), 6.7 (2H, doublet, $J=7$ cps, –OC–CH_2–C=C–).

Diethyl 2-Formamido-2-pentenedioate (IX)——To a solution of 2.25 g of V in 30 ml of EtOH was added 3 ml of triethylamine and the mixture was stirred at room temperature for 1.5 hr. The reaction mixture was evaporated *in vacuo* and the residue was extracted several times with EtOAc. An oil obtained by evaporation of the EtOAc solution was submitted to vacuum distillation to yield 440 mg (33% yield) of IX as an yellowish oil, bp 158—159° (1.1 mmHg). *Anal.* Calcd. for $C_{10}H_{15}O_5N$ (diethyl 2-formamido-2-pentenedioate): C, 52.39; H, 6.59; N, 6.11. Found: C, 52.19; H, 6.74; N, 6.08.

Reaction of *threo*-III with NH_2OH and Alkali——A solution of 1.4 g (4.2 mmoles) of *threo*-III in 5 ml of 80% EtOH was mixed with a solution of 314 mg (4.5 mmoles) of $NH_2OH \cdot HCl$ in 2 ml of H_2O. To this was added 2 ml (10 mmoles) of 5N NaOH under cooling at −5——3° with ice-NaCl, and the mixture was stirred at −5——3° for 2 hr. After dropwise addition of a mixture of 4 ml (20 mmoles) of 5N NaOH and

7) M. Terasaki, E. Fujita, S. Wada, T. Takemoto, T. Nakajima and T. Yokobe, *J. Japan. Soc. Food Nutr.*, **18**, 172, 222 (1965).

8 ml of H_2O below 0°, stirring was continued for 1 hr at 0°. The reaction mixture was allowed to stand at room temperature for 15 hr and, after being diluted with 150 ml of H_2O, was passed through a column of 50 ml of Amberlite IR-120 (H type). The resin was washed with 300 ml of water, then amino acids were eluted with 300 ml of 3% NH_4OH. The residue obtained by evaporation of the eluate was dissolved in 2 ml of 0.5N AcOH and submitted to Dowex 1×8 column chromatography (acetate type, 200—400 mesh, 3×90 cm) with 0.5N AcOH as developer. The fractions containing tricholomic acid on paper chromatography (MeOH–H_2O–pyridine, 160:40:8 v/v) were collected and evaporated *in vacuo* to dryness. A small portion of the residue was subjected to paper electrophoresis with 10% acetic acid as solvent for 4 hr at 28 V/cm. After the paper had been dried, the ratio of the amino acids was calculated according to the method of Kay, *et al.*[8] After a ninhydrin reagent had been sprayed on the paper, purple spots were cut out and eluted with 71% EtOH. The color of the eluates was compared at 575 mμ. The average value of three experiments was *threo*-I/*erythro*-I=76.7/23.3.

Reaction of *erythro*-III with NH_2OH and Alkali——*erythro*-III was treated in the same manner as in the case of *threo*-III and the ratio of *erythro*-I to *threo*-I was calculated (Table I).

Conversion of VIII into I——In 10 ml of EtOH were dissolved 2.19 g (9.0 mmoles) of VIII and 1.23 g (18 mmoles) of NaOEt. To the well-stirred solution was added dropwise below 5° a mixture of a suspension of 625 mg (9.0 mmoles) of powdered $NH_2OH \cdot HCl$ in 15 ml of EtOH and a solution of 615 mg (9.0 mmoles) of NaOEt in 5 ml of EtOH. The reaction mixture was stirred for 1 hr under ice-cooling, then for 2 hr at room temperature. After the pH of the solution had been adjusted to 4.0 with ethanolic HCl, the precipitating NaCl was filtered and the filtrate was evaporated *in vacuo*. The residue was boiled with 25 ml of 2N NaOH for 3 hr. The reaction mixture was diluted with H_2O and purified as described above with Amberlite IR-120 and Dowex 1×8, then subjected to the estimation of the ratio of *erythro*-I to *threo*-I (Table I). Yield of *erythro*-I was 1.7% and that of *threo*-I 1.6% from VIII.

Conversion of IX into I——A solution of 347 mg (5 mmoles) of $NH_2OH \cdot HCl$ in 3.2 ml (6.4 mmoles) of 2N NaOH was added dropwise at −5——3° with stirring into a solution of 573 mg (2.5 mmoles) of IX in 5 ml of EtOH and the mixture was stirred for 1 hr at this temperature. After addition of 3.1 ml (6.2 mmoles) of 2N NaOH, the mixture was stirred at −5——3° for 1 hr, then at room temperature for 2 hr. The reaction mixture was acidified by addition of 5.4 ml of 2.5% HCl–MeOH and allowed to stand at room temperature for 18 hr. Purification of the product was carried out by ion-exchange resin chromatography and the ratio of *erythro*-I to *threo*-I was estimated (Table I). Yield of *erythro*-I was 0.4% and that of *threo*-I 0.6%.

Conversion of VIII into I *via* Aminooxy Derivative (XI)——Under the same condition as in the case of VIII→I 2.43 g of VIII was treated with $NH_2OH \cdot HCl$ and NaOH, and the reaction mixture was evaporated *in vacuo*. The residue containing N-acetyl-I was dissolved in 50 ml of HCl-saturated MeOH. The mixture was boiled for 30 min, then filtered and allowed to stand at room temperature overnight. A crude XI obtained by evaporation of the solution was treated with 30 ml of 2N NaOH at room temperature for 3 hr. The reaction product was purified by ion-exchange resin chromatography as described above and the ratio and yield of *erythro*-I and *threo*-I was calculated by amino acid analysis (Table I). Yield: *erythro*-I, 0.66%; *threo*-I, 2.44%.

***threo*-α-Amino-3-oxo-5-isoxazolidineacetic Acid (*threo*-I)**——To a solution of 6.3 g (18.8 mmoles) of *threo*-III in 20 ml of 80% EtOH was added 3 ml of triethylamine and the mixture was stirred for 1 hr under ice-cooling to yield diethyl 2-trifluoroacetamido-2-pentenedioate (VII). Without isolation of VII, the reaction mixture was added dropwise with stirring below 0° with a solution of 1.4 g (20.2 mmoles) of $NH_2OH \cdot HCl$ and 4 ml (40 mmoles) of 10N NaOH in 16 ml of 50% EtOH. The mixture was stirred at 0° for 2 hr, then added an additional 8 ml of 10N NaOH below 5°, and the whole mixture was stirred at 0° for 30 min. After being left standing at room temperature for 16 hr, the reaction mixture was diluted with 400 ml of H_2O and passed through 200 ml column of Amberlite IR-120 (H type). The resin was washed with 1 liter of H_2O, then amino acids were eluted with 600 ml of 3% NH_4OH. The residue obtained by evaporation of the eluate *in vacuo* was dissolved in 5 ml of 0.5N AcOH and allowed to stand in a refrigerator to afford crystals (148 mg), which were recrystallized from H_2O to yield 80 mg of *threo*-I as colorless platelets, mp 213—214° (decomp.). The filtrate was concentrated to 5 ml and put on the column (3×100 cm) of Dowex 1×8 (acetate type, 200—400 mesh) and developed with 0.5N AcOH at a flow rate of 40 ml/hr. Fractions (5 ml each) containing *threo*-I were collected and concentrated to give a second crop (16 mg) of crystalline *threo*-I. A mixture of *threo*-I and *erythro*-I was 83 mg. The total yield of *threo*-I was 3.2% from *threo*-III. *Anal.* Calcd. for $C_5H_8O_4N_2$ (DL-*threo*-α-amino-3-oxo-5-isoxazolidineacetic acid): C, 37.50; H, 5.04; N, 17.50. Found: C, 37.43; H, 5.26; N, 17.27. Paper chromatography (Toyo No. 50): *n*-BuOH–AcOH–H_2O (120:30:50 v/v), *Rf* 0.1; MeOH–pyridine–H_2O (160:8:40, v/v), *Rf* 0.4. Paper electrophoresis (Toyo No. 50, 10% AcOH, 28 V/cm, 2.5 hr): −7 cm, (*erythro*-I: −8 cm). Amino acid autoanalysis (buffer pH 5.28, 30 ml/hr, column: 150 cm): *threo*-I, 125 min; (*erythro*-I, 135 min). IR: Fig. 1.

8) R.E. Kay, D.C. Harris and C. Entenman, *Arch. Biochem. Biophys.*, **63**, 14 (1956).

Separation of *threo*-I from *erythro*-I——(1) Dowex 1 Column Chromatography: A column (3×90 cm) of Dowex 1×8 (acetate type, 200—400 mesh) was washed thoroughly with a mixture of AcOH–MeOH–H_2O (1:2:7, v/v). A solution of 279 mg of a mixture of *erythro*-I and *threo*-I (1:1) in 2 ml of the AcOH·MeOH·H_2O was placed on top of the column, and run with the same solvent at a flow rate of 40 ml/hr. Fractions (5 ml each) containing *erythro*-I and *threo*-I were distinguished from each other by paper electrophoresis with 10% AcOH. The fractions 62—65 contained only *erythro*-I, while the fractions 68—77 *threo*-I, and the fractions 66—67 gave a mixture of the both. The fractions 62—65 were combined, concentrated by evaporation *in vacuo* and cooled to afford 80 mg of crystalline *erythro*-I, while the fractions 68—77 yielded 50 mg of crystalline *threo*-I.

(2) Dowex 50 W Column Chromatography: A column (3×97 cm) of Dowex 50 W×8 (pyridine salt type, 200—400 mesh) was washed thoroughly with 20% MeOH–0.2M pyridine acetate buffer (pH 5.25). A solution of 320 mg of a mixture of *erythro*-I and *threo*-I in 5 ml of the buffer was placed on top of the column, and developed with the same buffer at a flow rate of 30 ml/hr. When ninhydrin-positive fractions were checked by paper electrophoresis, the fractions 77—82 were found to contain only *threo*-I and the fractions 85—94 only *erythro*-I. The both of the fractions were evaporated *in vacuo* to dryness and crystallized from H_2O–EtOH to yield 70 mg of crystalline *threo*-I and 49 mg of crystalline *erythro*-I, respectively.

(3) Copper Salt Formation: A mixture (138 mg) of *erythro*-I and *threo*-I (1:1.35) dissolved in 2 ml of hot H_2O was added to a hot solution of 400 mg of cupric acetate in 6 ml of H_2O. The mixture was cooled to yield green-colored crystals, which were filtered and washed with H_2O. The crystal was dissolved in 100 ml of 1N HCl and passed through 100 ml of Amberlite IR-120 (H type). The resin was washed with 100 ml of H_2O. The amino acid was eluted with 200 ml of 3% NH_4OH and the eluate was evaporated *in vacuo* to give 44 mg of crystalline powder, which was a mixture of *erythro*-I and *threo*-I in a ratio of 9.4:1 by the amino acid analysis. The mother liquor obtained by removal of the crystalline copper salt was treated with Amberlite IR-120 in the same way as described above to yield 30 mg of a crystalline powder composed of *erythro*-I and *threo*-I in a ratio of 1:13.7.

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