Component Amino Acids of the Antibiotic Longicatenamycin. Isolation of 5-Chloro-D-tryptophan[1]

Tetsuo SHIBA, Yasuo MUKUNOKI, and Hitoshi AKIYAMA

Department of Chemistry, Faculty of Science, Osaka University, Toyonaka, Osaka 560

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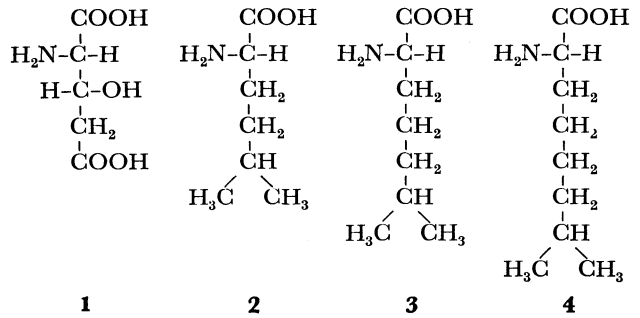
An amino acid composition of the peptide antibiotic longicatenamycin was investigated. Four new amino acids, *i.e.*, *threo*-β-hydroxy-L-glutamic acid, L-2-amino-5-methylhexanoic acid, L-2-amino-6-methylheptanoic acid, and L-2-amino-7-methyloctanoic acid, which were reported by Shoji *et al.*, were reconfirmed by comparison with the synthetic specimens. Racemic forms of the latter three amino acids were synthesized through acetaminomalonate method. Furthermore, 5-chloro-D-tryptophan was newly isolated from the hydrolyzate of the antibiotic. This structure was assigned by NMR, MS and ORD. A steric configuration of this amino acid was certified by degradation to D-aspartic acid through ozonization. 5-Chloro-DL-tryptophan was synthesized from 3-cyanopropionaldehyde and *p*-chlorophenylhydrazine by Fischer's indole synthesis.

Longicatenamycin is an antibiotic isolated from the strain S-520 of *Streptomyces diastaticus* by Shoji and his collaborators in 1970 and tentatively named S-520.[2] This antibiotic is active against Gram-positive bacteria including *Diplococcus pneumoniae*. The hydrochloride of the antibiotic has extremely poor solubilities in water and almost all organic solvents except lower alcohol or dimethyl sulfoxide. Longicatenamycin showed the positive Ehrlich reaction and the characteristic UV absorptions at 299 nm, 290, 283 and 227.[2] According to the preliminary investigation by Shoji *et al.*, longicatenamycin is a peptide containing several unusual amino acids. They also suggested that the antibiotic is a complex mixture composed of several closely related peptides which have three exchangeable positions for amino acid residues in their molecules.[3] This feature seems to be rather common in many peptide antibiotics.[4-7] Among the component amino acids they reported, two kinds of new amino acids are involved. The one is *threo*-β-hydroxy-L-glutamic acid (**1**)[8] and the other is a group of three homologous amino acids with long carbon chains, *i.e.*, L-2-amino-5-methylhexanoic acid (**2**), L-2-amino-6-methylheptanoic acid (**3**) and L-2-amino-7-methyloctanoic acid (**4**).[9] They assigned the structures of those four amino acids mainly by NMR studies.[8,9]

Since Dakin had pointed out a possibility of an existence of β-hydroxyglutamic acid in the hydrolyzate of casein in 1918,[10] much efforts have been made in vain for isolation of this amino acid. However, at the present time, an occurrence of the amino acid was denied after all negative results by many workers.[11,12] If Shoji's presumption is right,[8] this will be a first presentation for an occurrence of β-hydroxyglutamic acid in nature. In the present study, a sample of the acid **1** obtained from the hydrolyzate of longicatenamycin was compared with the authentic *threo* and *erythro*-β-hydroxy-DL-glutamic acids which had been early synthesized by Kaneko *et al.*[13] The natural L-amino acid **1** was completely identical with synthetic *threo* isomer in NMR, amino acid analysis and thin-layer chromatography.



1 **2** **3** **4**

Fig. 1.

Three other new amino acids **2**, **3** and **4** exceed, in their carbon chain lengths, leucine which is known as the longest naturally occurring amino acid of $(CH_3)_2CH(CH_2)_nCH(NH_2)COOH$ type. We synthesized these amino acids by means of acetaminomalonate method. Thus, diethyl acetaminomalonate was coupled with isoamyl bromide, isohexyl bromide and isoheptyl bromide respectively and each coupling product was hydrolyzed to yield the corresponding DL-α-amino acids. Three natural L-amino acids **2**, **3** and **4**, were identified with the synthetic 2-amino-5-methylhexanoic acid, 2-amino-6-methylheptanoic acid and 2-amino-7-methyloctanoic acid respectively in NMR, amino acid analysis and thin-layer chromatography.

The amino acid composition of the acid hydrolyzate of longicatenamycin was reinvestigated in our study by amino acid analysis. Our result as shown in Table 1 coincides with that of Shoji *et al.*[2] within the experimental errors.

The molar ratio of amino acid composition indicates that there are three groups each comprising the homologous amino acids and corresponding to one mole respectively, *i.e.*, i) Val, Ile ii) **2**, **3**, **4** iii) Orn, Lys. The low value of **1** may be ascribed to the possible degradation *via* retroaldol condensation during the acid hydrolysis. The loss due to such decomposition was confirmed in the reference experiment with the authentic β-hydroxyglutamic acid.

At the stage of this investigation, the characteristic UV absorption and positive Ehrlich reaction of longicatenamycin cannot be explained by the component amino acids isolated so far. In addition, a difference between an apparent molecular weight (566) calculated based on a cyclic pentapeptide consisting of each one mole of Gly, **1**, Val (Ile), **2** (**3**, **4**) and Orn (Lys)[14] and the measured values of molecular weight of 2,4-dinitrophenyl (969) and acetyl (*ca.* 900) derivatives of longicatenamycin from extinction coefficients of the absorption spectra at 350 and 289 nm respectively, sug-

Table 1. The molar ratio of component amino acids of longicatenamycin[a)]

Gly[b)]	1	Val	Ile	2	3	4	Orn	Lys	Amm
1.00	0.45	0.77	0.17	0.20	0.36	0.38	0.76	0.28	0.68

a) The ratio of 5-chloro-D-trypophan is not included. b) Gly: glycine; Val: D-valine; Ile: D-isoleucine; Orn: D-ornithine; Lys: D-lysine; Amm: ammonia; **1**: *threo*-β-hydroxy-L-glutamic acid; **2**: L-2-amino-5-methylhexanoic acid; **3**: L-2-amino-6-methylheptanoic acid; **4**: L-α-2-amino-7-methyloctanoic acid.

gests strongly a presence of a tryptophan-like substance which might escape from the amino acid analysis because of a possible decomposition in the acid hydrolysis.

An experiment to trace such a predicted amino acid was done first by an alkaline hydrolysis of the antibiotic.[15)] Thus, longicatenamycin was hydrolyzed with 10% barium hydroxide. From isobutyl alcohol extract of the hydrolyzate, a neutral amino acid with a molecular formula of $C_{11}H_{11}O_2N_2Cl$ was isolated in a crystalline state. This amino acid was positive to both ninhydrin and Ehrlich reactions, and showed UV absorptions at λ_{max}^{EtOH} 298 nm (ε 3800), 289 (5300), 282 (5200) and 226 (36700). From its NMR spectrum in C_5D_5N–D_2O as shown in Fig. 2 and mass spectrum, a structure of this new amino acid was assumed to be 5-chlorotryptophan. A position of chlorine atom on the indole nucleus was determined by the fact that a signal (Ha) in the lowest magnetic field can be assigned as H-4 in comparison with the spectrum of tryptophan itself and it shows only long range coupling of J=2 Hz with Hb.

A steric configuration of α-carbon atom cannot be clarified on this sample which was completely racemized during the alkaline hydrolysis. Therefore, the hydrolysis of longicatenamycin was carried out newly with 6 M hydrochloric acid in a sealed, evacuated tube. An optically active 5-chlorotryptophan thus obtained showed a positive ORD curve: $[\phi]_{320}$+3410° (peak), $[\alpha]_D^{25}$ +40° (methanol). Since L-tryptophan gave the negative ORD curve in methanolic solution, we assigned D-form for this amino acid. On the contrary, a configuration of α-carbon atom of *N*-carbethoxyacetyl-4-chlorotryptophan which was isolated as a hypocotyl swelling factor from immature seeds of *Pisum sativum* was assumed to be D-form by Marumo and Hattori only from the negative ORD curve.[16)] Both conclusions seem to be controversy though there are differences in substitution groups. Since any contribution of acyl or chlorine substituent on tryptophan molecule to the sign of Cotton effect is not known well, a suspection of the configuration of tryptophan derivative from the sign of ORD curve must be very prudent.

Therefore, we now oxidized (+)-5-chlorotryptophan with ozone to lead to an optically active aspartic acid[17)] which was purified as 2,4-dinitrophenyl (DNP) derivative. This DNP aspartic acid showed the negative value of the specific rotation: $[\alpha]_D^{20}$ −45° ($NaHCO_3$), indicating D-form of the amino acid unambiguously. Conclusively it is determined that (+)-5-chlorotryptophan in longicatenamycin has D-configuration at α-carbon atom.

For confirmation of the supposed structure of 5-chlorotryptophan, it was synthesized through the following novel method where the tryptophan synthesis by Komachiya *et al.*[18)] was applied (Scheme 1). 3-Cyanopropionaldehyde (**5**)[19)] was changed to 5-(2-cyanoethyl)-hydantoin (**6**) by Bücherer's method. A nitrile group in compound **6** was reduced to aldehyde using poisoned nickel catalyst according to the procedure by Komachiya *et al.*[18)] The hydantoin aldehyde (**7**)

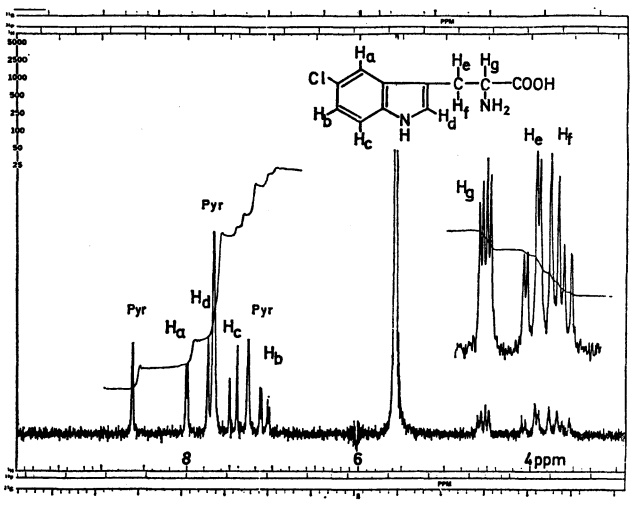
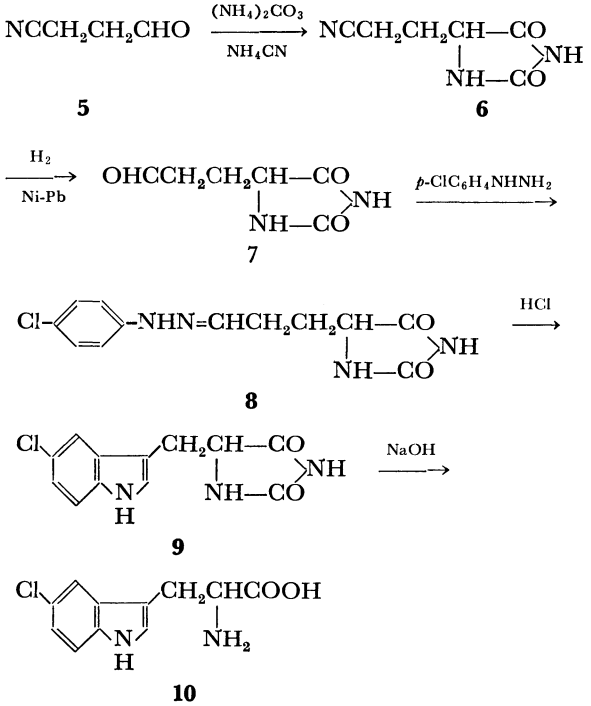


Fig. 2. NMR spectrum of 5-chlorotryptophan from longicatenamycin in C_5D_5N–D_2O (1 : 1) at 100 MHz.



Scheme 1.

thus obtained was converted to *p*-chlorophenylhydrazone (**8**) which was then cyclized to indole hydantoin (**9**) through Fischer's indole synthesis using dilute hydrochloric acid. Alkaline hydrolysis of the product **9** gave 5-chloro-DL-tryptophan. The synthetic amino acid was completely identical with the natural compound in IR, NMR, amino acid analysis and mass spectrum. This is the first occurrence of 5-chlorotryptophan in nature.

This amino acid had been already synthesized by Rydon and his collaborators but through tedious long path.[20,21] One of the advantage in our synthetic method is that a single product of 5-chloroindole compound is obtained in principle even if the indole ring is formed at either *ortho* position of the compound **8** in Fischer's indole synthesis. Moreover, the overall yield from 3-cyanopropionaldehyde dimethyl acetal in this synthesis was as good as about 40%.

Now, the experimental formula of longicatenamycin becomes $C_{36.6}H_{54.3}O_{9.0}N_{8.0}Cl_{1.0}$ (MW 786) on the basis of the molar ratio of the component amino acids mentioned before added with one mole of 5-chlorotryptophan.[22] Apparent molecular weights of mono-DNP (953) and diacetyl (871) derivatives calculated from this formula are consistent with the observed values mentioned before. This indicated that there is no more amino acid unidentified in the molecule of longicatenamycin.

Other than these amino acids, Shoji *et al.* reported one spot of a-II in the acidic area of two-dimensional paper chromatogram of the hydrolyzate of longicatenamycin, although they could neither isolate nor detect it in amino acid analysis.[3] In our experiment where the antibiotic was hydrolyzed with hydrochloric acid in the evacuated tube, no such spot appeared on paper-chromatogram. Therefore, this spot would be due to some degradation of 5-chlorotryptophan during the acid hydrolysis in the presence of air.

From all of the above results, it can be now concluded that longicatenamycin is composed of each one mole of glycine, *threo*-β-hydroxy-L-glutamic acid, 5-chloro-D-tryptophan, D-valine (D-isoleucine), L-2-amino-5-methylhexanoic acid (L-2-amino-6-methylheptanoic acid, L-2-amino-7-methyloctanoic acid) and D-ornithine (D-lysine).[14]

Experimental

All melting points are uncorrected. The following apparatuses were employed: Jasco Model IR-S Infrared Spectrophotometer, Hitachi 124 Spectrophotometer, Jasco Model ORD/UV-5-Optical Rotatory Dispersion Recorder, Varian T-60 Spectrometer, Varian XL-100 Spectrometer and Hitachi Amino Acid Analyzer KLA-3. For NMR measurement, tetramethylsilane was used as an internal reference substance. Paper electrophoresis was carried out in a pyridine-acetic acid-water (30 : 4 : 966) buffer solution using Toyo filter paper No. 51. For thin-layer chromatography on silica gel G, the solvent systems of *n*-butanol–acetic acid–water (4 : 1 : 2) and methyl ethyl ketone–pyridine–water–acetic acid (35 : 5 : 5 : 1) were employed mainly.

DL-2-Amino-5-methylhexanoic Acid (2). To a solution of diethyl acetaminomalonate (13.0 g, 0.06 mol) and sodium metal (1.38 g, 0.06 mol) in absolute ethanol (100 ml), there was added isoamyl bromide (8.72 g, 0.06 mol) and the mixture was heated under reflux for 36 hr. To an oily residue obtained after evaporation of the solvent, water (100 ml) was added and the product was extracted with ether (500 ml). Extract was washed with water and dried with magnesium sulfate. Oily substance obtained after evaporation of ether was crystallized on addition of *n*-hexane. The product was recrystallized from ether to give needles, yield, 5.5 g. These were hydrolyzed by refluxing with 47% hydrobromic acid (100 ml) for 18 hr. The amino acid obtained was purified by Dowex 50×8 (H^+ form) column. From the eluate with M ammonium hydroxide, crystals of DL-2-amino-5-methylhexanoic acid were obtained and recrystallized from hot water, yield, 2.2 g (25%); mp 273—275 °C (decomp.). R_f value on thin-layer chromatogram: 0.38 (methyl ethyl ketone–pyridine–water–acetic acid, 35 : 5 : 5 : 1).

Found: C, 57.86; H, 10.41; N, 9.67%. Calcd for $C_7H_{15}O_2N$: C, 57.90; H, 10.41; N, 9.65%.

DL-2-Amino-6-methylheptanoic Acid (3). Isohexyl bromide was prepared by bromination of isohexyl alcohol with mixed acid of 47% hydrobromic acid and concentrated hydrochloric acid.[23] Isohexyl alcohol was obtained by reduction of 4-methylpentanoic acid with lithium aluminum hydride.[24] The latter compound was synthesized from isoamylbromide by Grignard reaction with carbon dioxide.[25] A coupling reaction of isohexyl bromide (5.0 g, 30 mmol) with diethyl acetaminomalonate (6.5 g, 30 mmol) was carried out in a similar manner to that for **2**. Elution of the amino acid from Dowex-50 column was done using a mixed solvent of 1 M ammonium hydroxide and methanol (1 : 1), yield, 1.6 g (33%); mp 286—290 °C (decomp.). R_f value on thin-layer chromatogram: 0.50 (methyl ethyl ketone–pyridine–water–acetic acid, 35 : 5 : 5 : 1).

Found: C, 60.24; H, 10.70; N, 8.77%. Calcd for $C_8H_{17}O_2N$: C, 60.34; H, 10.76; N, 8.80%.

DL-2-Amino-7-methyloctanoic Acid (4). Isoheptyl bromide was prepared from isohexylbromide through Grignard reaction, lithium aluminum hydride reduction and bromination successively. Coupling of isoheptyl bromide (4.60 g, 26 mmol) and diethyl acetaminomalonate (5.56 g, 26 mmol) was carried out in a similar way to that mentioned above, yield, 1.57 g (35%); mp 271—273 °C (decomp.). R_f value on thin-layer chromatogram: 0.54 (methyl ethyl ketone–pyridine–water–acetic acid, 35 : 5 : 5 : 1).

Found: C, 62.16; H, 10.99; N, 8.07%. Calcd for $C_9H_{19}O_2N$: C, 62.39; H, 11.05; N, 8.09%.

Isolation of 5-Chloro-DL-tryptophan (Alkaline Hydrolysis). Longicatenamycin (1.0 g) was refluxed with 10% aqueous barium hydroxide (50 ml) for 48 hr. The reaction mixture was diluted with water (50 ml) and then neutralized with 2 M sulfuric acid. Precipitate of barium sulfate was filtered, and the filtrate was extracted with isobutyl alcohol (100 ml) three times. After evaporation of the solvent, the brown powder was obtained. This was purified by repeated crystallization with aqueous methanol and ether to obtain colorless crystals, yield, 50 mg; mp 250—255 °C (decomp.). UV: λ_{max}^{MeOH} 298 nm (ε 3800), 289 (5300), 282 (5200), 226 (36700). NMR (100 MHz, C_5D_5N–D_2O (1 : 1): H-4: δ 8.03 (1H, d, J=2.0 Hz), H-6: 7.11 (1H, dd, J=2.0, 9.0), H-7: 7.49 (1H, d, J=9.0), H-2: 7.78 (1H, s), H-β: 3.66 (1H, dd, J=4.0, 16.0), H-β′: 4.00 (1H, dd, J=10.0, 16.0), H-α: 4.55 (1H, dd, J=4.0, 10.0), MS: *m/e*, 238, 193, 164, 127.

Found: C, 55.04; H, 4.89; N, 11.74; Cl, 14.68%. Calcd for $C_{11}H_{11}O_2N_2Cl$: C, 55.35; H, 4.65; N, 11.74; Cl, 14.86%.

Isolation of 5-Chloro-D-tryptophan (Acid Hydrolysis). A suspension of longicatenamycin (250 mg) in 6 M hydrochloric acid (10 ml) was heated at 110 °C for 1 hr in a sealed, evacu-

ated tube. After dilution with water (20 ml), the hydrolyzate was neutralized with 2 M sodium hydroxide and extracted with isobutyl alcohol (300 ml). The residue obtained after evaporation of the solvent from the extract was dissolved in a mixture of methanol–acetic acid–water (1 : 1 : 1) and subjected to Dowex 50×2 column (1.5×30 cm) bufferized with 0.2 M pyridine–acetic acid buffer of pH 3.1. Elution was carried out gradiently with 0.2 M pyridine buffer of pH 3.1 (200 ml) and 2 M pyridine buffer of pH 5.0 (200 ml) successively. From fractions No. 32—40 of each 8 g, colorless crystals were obtained, yield, 10 mg; mp 238—245 °C (decomp.); ORD, $[\phi]_{320}+3410°$ (peak) (*c* 0.58, methanol), $[\alpha]_D^{25}$ +40° (*c* 0.58, methanol). This compound was identified with DL-isomer obtained by the alkaline hydrolysis in UV spectrum, thin-layer chromatography and amino acid analysis.

Ozonization of 5-Chloro-D-tryptophan. Optically active 5-chlorotryptophan (5 mg) obtained above was dissolved in 0.5 M hydrochloric acid (2 ml) and ozone was passed through the solution on ice-cooling for 2 hr. After addition of 30% aqueous hydroperoxide (0.3 ml), the reaction mixture was heated on boiling water bath for 10 min. Crystals obtained after evaporation were dissolved in a small amount of water and subjected to Dowex 50×2 column (0.5×10 cm) bufferized with 0.2 M pyridine–acetic acid of pH 3.1. Elution was carried out with the same buffer solution and fractions of each 4 g were collected fractionally. From fractions No. 11 and 12, colorless crystals of aspartic acid were obtained. This amino acid was 2,4-dinitrophenylated using sodium hydrogencarbonate in the usual manner. Crystals of DNP-aspartic acid were obtained from ether extract after acidification. These were purified by silica gel column (0.5×15 cm) using a mixed solvent of *n*-hexane–ether (9 : 1). $[\alpha]_D^{18}$ −8.0° (c 0.03, methanol), $[\alpha]_D^{20}$ −45° (*c* 0.11, 1% $NaHCO_3$).

5-[2-(4-Chlorophenylhydrazonoformyl)-ethyl]-hydantion (8). i) 5-(2-Cyanoethyl)-hydantoin (**6**) was prepared from 3-cyanopropionaldehyde (**5**)[26] through Bücherer's reaction.[18] According to the method by Komachiya *et al.*,[18] nitrile group in the hydantoin **6** was reduced to aldehyde using Raney nickel catalyst poisoned with lead acetate in acetic acid to yield 5-(2-formylethyl)-hydantoin (**7**). The reaction mixture starting from 15.3 g (0.10 mol) of **6** was filtered to remove the catalyst, and a solution of *p*-chlorophenylhydrazine (14.3 g, 0.10 M) in 100 ml of 1 M hydrochloric acid was added with stirring. Crystals deposited were collected by filtration and recrystallized from ethanol–water, to give the hydrazone **8**, yield, 15.4 g (55%); mp 147—149 °C (decomp.).

Found: C, 51.25; H, 4.70; N, 19.80; Cl, 12.56%. Calcd for $C_{12}H_{13}O_2N_4Cl$: C, 51.34; H, 4.67; N, 19.96; Cl, 12.63%.

ii) The same hydrazone **8** was obtained from semicarbazone of **7** (9.98 g, 47 mmol) and *p*-chlorophenylhydrazine 6.70 g (47 mM) in a similar way, yield, 12.3 g (94%), mp 147—148 °C (decomp.).

5-(5-Chloroindolyl-3-methyl)-hydantoin (9). The hydrazone **8** (1.40 g, 5.0 mmol) was dissolved in hot water (80 ml) containing 3 M hydrochloric acid (3.72 ml) on heating at 100 °C. After keeping at this temperature for 80 min, the reaction mixture was evaporated *in vacuo*. The residue was crystallized in addition of a small amount of water. The crystals were filtered off, washed with water and dried to give the crude product, yield, 1.15 g (85%). A pure sample was obtained by purification on silica gel column chromatography using chloroform–methanol (95 : 5) as an elution solvent, mp 127—132 °C (decomp.).

Found: C, 53.10; H, 4.18; N, 15.74%. Calcd for $C_{12}H_{10}O_2N_3Cl\cdot 1/2H_2O$: C, 52.85; H, 4.07; N, 15.41%.

5-Chloro-DL-tryptophan (10). The chloroindole derivative **9** (540 mg, 2.0 mM) was dissolved in M sodium hydroxide and heated at 150—155 °C for 1 hr in a sealed tube in which air was displaced with nitrogen. After neutralization to pH 5.9 with 3 M hydrochloric acid, the reaction mixture was extracted with isobutyl alcohol (80 ml). Extract was washed with water and evaporated *in vacuo*. A small amount of undissolved material in ethanol was removed, and the filtrate was again evaporated. The residue thus obtained was crystallized from methanol–ether, yield, 260 mg (55%). A pure sample was obtained by Dowex 50×2 column chromatography using a gradient buffer of 0.2 M pyridine–acetic acid of pH 3.1 and 2.0 M buffer of pH 5.0, mp 254—256 °C (decomp.).[27] R_f values on thin-layer chromatogram: 0.71 (*n*-butanol–acetic acid–water, 4 : 1 : 2); 0.10 (chloroform–methanol–water, 45 : 12 : 1).

Found: C, 55.14; H, 4.83; N, 11.79; Cl, 14.40%. Calcd for $C_{11}H_{11}O_2N_2Cl$: C, 55.35; H, 4.65; N, 11.74; Cl, 14.86%.

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26) This aldehyde was prepared from its dimethyl acetal which was supplied from Ajinomoto Co., Inc.

27) This melting point agreed with that of DL-isomer from the natural source. Rydon *et al.* reported mp 278—279 °C (decomp.) for the same compound.