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(2*R*)-2-AMINO-6-HYDROXY-4-HEXYNOIC ACID, AND RELATED AMINO ACIDS IN THE FRUITING BODIES OF *AMANITA MICULIFERA*[1]

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Abstract—(2*R*)-2-amino-6-hydroxy-4-hexynoic acid, (2*RS*)-2-amino-4,5-hexadienoic acid, (2*S*)-2-amino-4-hexynoic acid, and 2-amino-5-chloro-5-hexenoic acid were isolated and characterised from the fruit bodies of *Amanita miculifera*, with the first and the last mentioned amino acids being new natural products. Their structures were based on analysis of the results from elementary analysis, oxidation with $KMnO_4$, determination of optical rotations, ^1H NMR-spectra, and hydrogenation over Adams or Lindlar catalysts. (2*RS*)-2-Amino-6-hydroxy-4-hexynoic acid was synthesized and optically resolved by renal acylase to confirm its natural (2*R*)-configuration. The configuration at C-2 of 2-amino-4,5-hexadienoic acid was also assumed from its ORD spectrum, as well as that of its hydrogenation product. The optical rotation (2-amino-5-chloro-5-hexenoic acid) was not determined because it was isolated in trace amounts; it was also prepared from 2-amino-5-hexynoic acid, although in very low yield. A possible biosynthetic route leading to the non-protein amino acids in this fungus is presented.

INTRODUCTION

It has been known that the fruit bodies of the section *Roanokenses* (Singer [1] = *Lepidella*; [Bas, 1969] [2]), subgenus *Lepidella* of the genus *Amanita* contain various non-protein amino acids of specific chemical structure [3]. Interestingly several have unbranched unsaturated carbon-chains, i.e., unsaturated norvaline or norleucine, and chlorine-containing amino acids are also known. Most recently, (2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid was isolated and characterized from *Amanita gymnopus* Corner and Bas [4], and from an undescribed *Amanita* which also belongs to the section *Roanokenses* [5].

In 1984, Bas and one of us reported an undescribed *Amanita* sp. and named it *Amanita miculifera* Bas and Hatanaka [6]. This species also belongs to the section *Roanokenses*, and several specific non-protein amino acids were detected from its fruit bodies (Fig. 1, compounds **1**–**4**). Now we report details of their isolation and structural elucidation, giving special attention to their optical properties. Possible biosynthetic pathways and specific distribution pattern of non-protein amino acids of this species are also discussed.

RESULTS AND DISCUSSION

Although it is possible that the distribution pattern of non-protein amino acids might reflect phylogenetic relationships among fungal species, some knowledge of new biosynthetic relationships is first required to support this view. Figure 2 shows a presumed biosynthetic route leading to the formation of the specific non-protein amino acids found in *A. miculifera*. 2-Amino-4,5-hexadienoic acid **2** was first reported in 1968 from the fruiting bodies of *Amanita solitaria* (Fr.) Secr. sensu D. E. Stuntz [7] (now regarded as *A. smithiana* Bas in the section *Roanokenses*). Although the isolate by Chilton *et al.* was itself optically inactive, it was concluded that it belongs to the L-series of amino acids, because its hydrogenation product was optically similar to L-norleucine. Since then this amino

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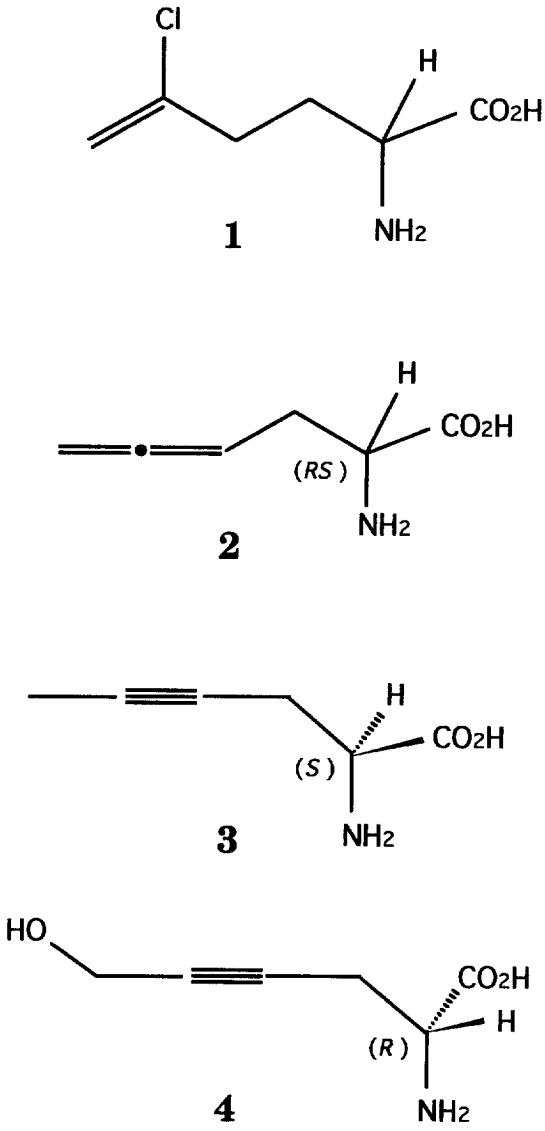

Fig. 1. Structures of the non-protein amino acids from *Amanita miculifera*.

acid has been reported to be distributed sporadically not only in the section *Roanokenses*, but in other sections such as *A. abrupta* Peck [8], and *A. gymnopus* [4] (both in the Roanokenses section), as well as *A. neoovoidea* Hongo [9] (section *Amidellae*), and in *A. pseudoporphyria* Hongo [10] (Phalloideae section). The possible chemical reactivity and the rather wide distribution of this allenic amino acid, suggests a role as a central intermediate in the biosynthesis of many specific, in particular, norleucine-type amino acids in fungi.

Interestingly **2** from this fungus gave no uniform optical rotation; and the $[\alpha]_D^{22}$-values in H_2O of this acid from *A. neoovoidea* and *A. pseudoporphyria* were reported $-24°$ (*c* 1.0) and $-52.1°$ (*c* 0.8), respectively. Furthermore, although no optical rotation was reported in the case of *A. abrupta*, it was completely oxidized by L-amino acid oxidase. Therefore it possibly occurs in many species in a partially racemized form. Regrettably its resolution form an isolate from *A. miculifera* by renal acylase, was unsuccessful.

L-2-Amino-4-hexynoic acid **3** was isolated first from the fruit bodies of *Tricholomopsis rutilans* (Fr.) Sing. [11], and then detected in other *Tricholomopsis* species [12]. Some years later it was also isolated from *A. pseudoporphyria*, amongst other amino acids of unsaturated norleucine-type [10]. The specific optical rotations in water of this amino acid isolated from *T. rutilans*, *A. pseudoporphyria*, and *A. miculifera* were determined to be $-54°$ (*c* 1.0), $-39°$ (*c* 1.0), and $-15°$ (*c* 0.8), at room temperature, respectively. Therefore, the optical purity of this amino acid again seems not to be uniform.

We then reported earlier that species of the pteridophyte, genus *Asplenium*, sometimes contain pure D- and sometimes partially racemized D-2-aminopimelic acid and *trans*-3,4-dehydro-D-2-aminopimelic acid. Furthermore the D: L ratio depends on the season, as well as on the plant species [13, 14].

It is worth emphasizing that **4** exists in pure D-form and its possible direct precursor, **3**, occurs predominantly in the L-form. Compound **2**, which is very likely metabolically related, is, however, a racemate. We cannot yet explain, what kind of chemical or enzymatic processes might account for these observations.

EXPERIMENTAL

Fungus

The fruiting bodies of *Amanita miculifera* (580 g, fresh weight) were collected in August 1975 in Nagano Prefecture and stored in a refrigerator for one day before extraction. We should like to propose to name this fungus "Haikaburi-Tengu-Take" (Ash-covered *Amanita*).

General

Evapn of solvents was carried out using a rotary evaporator on a water bath below 40°C. For PC, Whatman No. 50 and for cellulose chromatograpy, thin layer and column "Avicel" (Funakoshi Pharmaceutical Co., Ltd. Tokyo, Japan) were employed. The solvent systems used were as follows: *n*-BuOH-HOAc-H_2O (63:10:27) (A), Phenol-H_2O (25:8, in NH_3 vapor) (B), *t*-AmylOH-Pyridine-H_2O (35:35:30) (C), n-BuOH-methyl ethyl ketone-(conc.) NH_4OH-H_2O (15:9:4:2) (D) and *t*-AmylOH-methyl ethyl ketone-(conc.) NH_4OH-H_2O (72:45:2:1) (E).

Infrared spectra were measured using a Hitachi IR spectrometer model 260-30, whereas ^1H NMR-spectra were recorded on a Jeol JNM-PS-100 NMR spectrometer, 100 MHz, in D_2O with TPS as an int. standard ($\delta = 0$), and a Hitachi RMU6C mass spectrometer, respectively. Optical rotations were calculated from the results of the measurements of ORDs

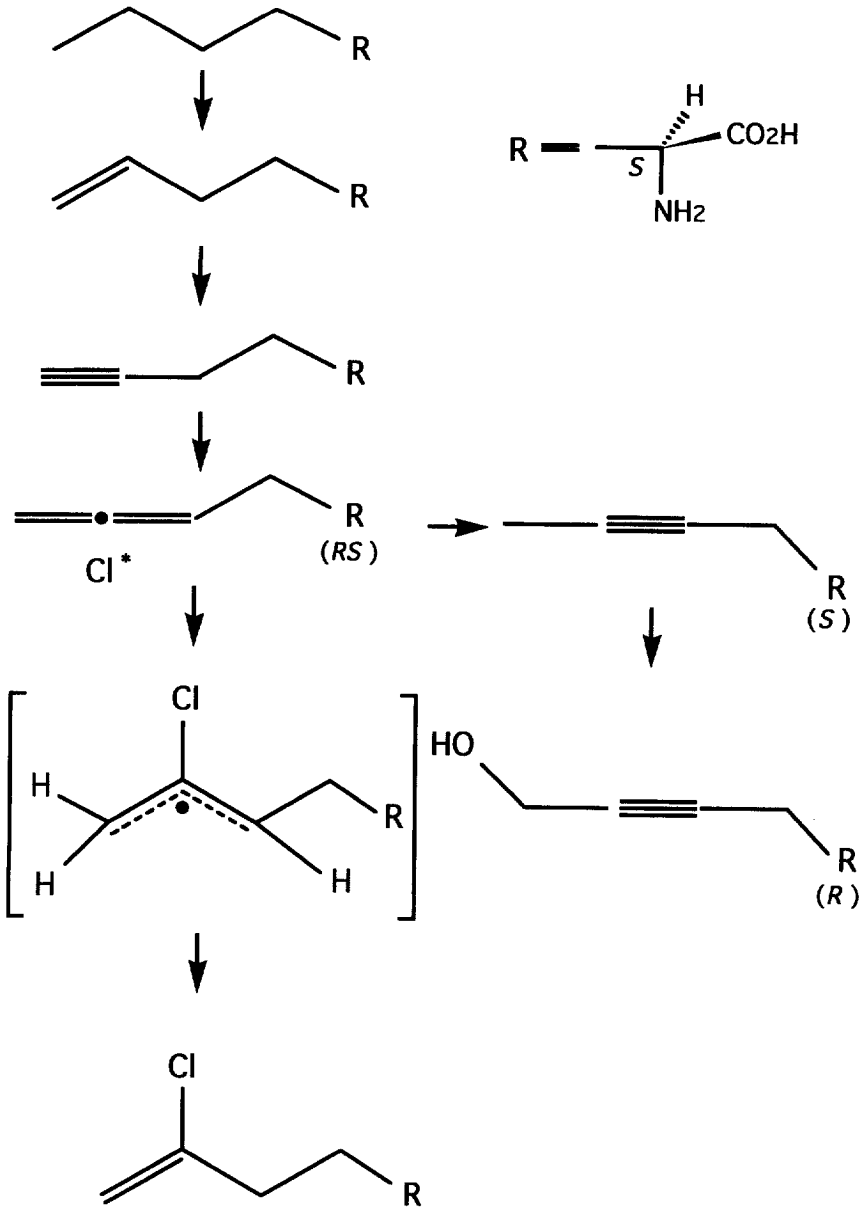

Fig. 2. Possible biosynthetic relationships among the non-protein amino acids in *Amanita miculifera*.

at 589 nm. Melting points were determined in capillaries and uncorrected.

Extraction, fractionation, and purification of the amino acids

On the day following collects on the fruiting bodies, these were then extracted (5 ×) *with* 80% *ethanol in a homogenizer. The combined extract* (4 *l*) *was next passed through a column of Amberlite IR* 120*B* (H^+) (100 ml), the resin was then washed successively thoroughly with 80% ethanol and water, and the retained amino acids were displaced with 2N NH_4OH (1 l). Ammonia was removed by evapn, giving the amino acid fraction. Although only one unusual spot near valine was detected on a two dimensional PC developed with the solvents A and B successively, careful fractionation on a cellulose column with the solvent A, D, or E revealed the existence of at least four unusual amino acids. We designated them **1**–**4** in the order of the Rf-values in the solvent E. To obtain each amino acid in crystalline form, the amino acid fraction was purified repeatedly mainly using cellulose chromotography with columns eluted with various solvent systems (see above). When an ion exchanger resin was used, Dowex-1 × 4 (200–400 mesh) in acetate form was the most successful. For the sepn of these amino acids, solvent system D with a cellulose column proved to be the most effective.

Isolation of the amino acids

As a result of the first fractionation of the total amino acids on a cellulose column (2.7 × 750 cm) eluted using solvent system A, several fractions were obtained containing **1–4** in different concn. Rechromatography under nearly the same conditions gave fractions I (**2**+**3**), II (**4**), and III (**1**), respectively.

Fraction I was further successively separated by cellulose column, (2.7 × 65 cm chromatography) using solvent system C, then by a cellulose column (2.7 × 75 cm) with solvent system D, and a cellulose column (1.5 × 65 cm) with solvent system E, respectively. As a result, amino acids **2** (576 mg) and **3** (50 mg) were obtained nearly homogeneous. From Fraction II, **4** (110 mg) was obtained following rechromatography with a cellulose column (2.7 × 75 cm) with solvent system D. Finally, Fraction III was placed on a cellulose column of 2.7 × 75 cm and developed with solvent system D, to yield **1** (18 mg).

Properties of the purified amino acids.

Compound **1** was recrystallized from aq. EtOH. Elemental analysis, Found: C, 44.19; H, 6.22; N, 8.54; Cl, 19.60%. $C_6H_{10}NO_2Cl$ requires: C, 44.05; H, 6.16; N, 8.56; Cl, 21.67%. mp. > 175°C (decomp.). ^{1}H NMR-spectra (δ): 2.1–2.4 (4H, m, -CH_2-CH_2-), 4.4 (1H, t, -CH-), 4.9, 5.1 (2H, CH_2=). MS: *m/z*, [M–Cl]$^+$; 128, [M–CO_2H]$^+$; 118, [-CH(NH_2)CO_2H]$^+$; 74. Hydrogenation over Adams catalyst; 2.10 equiv. mole H_2 absorbed with norleucine as product, with the latter identified by TLC comparison using solvent system A and E with visualization violet using ninhydrin. The yield of **1** was so small that its optical rotation could not be determined. Compound **2** was recrystallized from aq. EtOH. Elementary analysis, Found: C, 56,96; H, 7.27; N, 11.17%. Calcd for $C_6H_9NO_2$: C, 56,68; H, 7.13; N, 11.02%. mp. > 180°C (decomp.). ^{1}H NMR-spectra (δ): 2.54 (2H, m, -CH_2-), 3.76 (1H, q, –CH|), 4.80–5.02 (3H, m, $H_2C=C=CH$–). IR spectra (cm^{-1}): 1955, 845 (allene). Hydrogenation over Adams catalyst: 1.82 equiv. moles H_2, with the product being norleucine as before, as established by TLC comparisons using solvent systems A, B, C, and E. Oxidation with 1% $KMnO_4$ in 10% H_2SO_4 at room temp. gave Asp, which was identified by TLC comparison using solvent systems A, B and E with visualization with ninhydrin, which gave first a brown and then changed a violet color over time. For determination of its opticai activity, two solutions of 10 mg and 5 mg of this amino acid in H_2O and 3N HCl respectively were prepared and both gave no optical rotation when prescribed at 589 nm. The hydrogenation product, norleucine, was also inactive between 350 and 600 nm in the equivalent concn. in H_2O and HCl. Compound **3**. After the final chromatography prepn was further treated with a small amount of activated charcoal, the crystals obtained were recrystallized three times from aq. EtOH, yielding 50 mg of pure **3**. Elemental analysis, Found: C, 56.87; H, 7.18; N, 10.94%. Calcd for $C_6H_9NO_2$: C, 56.68; H, 7.13; N, 11.02%. mp. 199°C (decomp.). $[\alpha]_D^{22}$ −15° (H_2O; *c* 1.4,), −4° (3N HCl; *c* 0.8,). Hydrogenation over Adams and Lindlar catalysts revealed that the isolate absorbed 1.86 and 1.03 equiv. moles of H_2, respectively, with the only product identified as norleucine, by TLC comparison using solvents systems A, B, and E. Oxidation with $KMnO_4$ yielded Asp, as before, with its identification confirmed using TLC comparison with solvent systems A, B, and E; the colour of the reaction with ninhydrin was first yellow, then changed to brown with time, and finally to normal violet. Compound **4**. Following treatment with a small amount of activated charcoal, crude sample was recrystallized from aq. EtOH three times. Elemental analysis, Found: C, 50.11; H, 6.39; N, 9.77%. $C_6H_9NO_3$ requires C, 50.35; H, 6.34; N, 9.79%. mp. > 155°C. $[\alpha]_D^{22}$ +36° (H_2O; *c* 1), +10° (3N HCl; *c* 0.5,). ^{1}H NMR- (δ): 2.86 (2H, m, -CH_2-), 3.83 (1H, t, *J* = 5.5 Hz, −CH|−), 4.16 (2H, t, *J* = 2.0 Hz, $HOCH_2$-). MS: *m/z*, [M-H_2O]$^+$, 125; [M-CO_2H]$^+$, 98; [-CH(NH_2)CO_2H]$^+$, 74. IR-Spectra (cm^{-1}): 2260, 2230 (acetylene). On hydrogenation, the pure sample absorbed 2.08 and 1.08 equiv. moles H_2 over Adams and Lindlar catalysts, respectively. Hydrogenation product obtained with the former catalyst was purified and a few mg were isolated. When heated in a sealed tube, at 120–130°C with 55% HI (ca. 1 ml) and a small amount of red phosphorus for 16 h, norleucine was the only ninhydrin-positive substance formed. Reaction of **4** with ninhydrin gave a brown product.

Compound **4** seemed to be a D-amino acid, when the Lutz and Jirgensons rule was applied. It was, therefore, synthesized and resolved optically to confirm the natural occurrence of the D-isomer.

Synthesis of amino acids **4** *and* **1**

3.6.1. *Compound* **4**. Sodium (2.3 g) was dissolved in abs. EtOH (38 ml) under reflux. Diethyl formamidomalonate (20.3 g) in abs. EtOH (50 ml) was then added and refluxed further for 30 min. 1-Chloro-2-butyne-4-ol (10.5 g), which had been prepd according to Colonge and Poilane [15] (10.5 g) was added dropwise and reflux was a continued a further 5 h. The ppt. formed was removed by filtration and the filtrate concd under reduced pressure. The syrup was then dissolved in EtOH (140 ml), with NaOH solution (20 g in 100 ml H_2O) and the mixture refluxed for 1 h. The reaction mixt. was then concd to about half volume, and its pH was adjusted to ca. 2 by addition of conc. HCl under stirring in ice-water. The mixture was then heated until reflux began, which was continued for 1 h. The resulting mixture was then passed through 1 l Amberlite 120B (H^+), with the resin washed with EtOH and H_2O, successively, and the amino acid eluted with 10 l NH_4OH. Because the prod-

uct contained a small amount of Gly, it was subjected to further chromotagraphy using Dowex 1 × 4 ($^-$OAc, 200–400 mesh) eluted with 0.005 N acetic acid. Relevant fractions were combined and concentrated, yielding 6.06 g crude crystals (42% yield). Recrystallized from aq. EtOH gave colourless sample. Elemental analysis, Found: C, 50.25; H, 6,33; N, 9.99%. $C_6H_9NO_3$ requires: C, 50.34; H,6.34; N, 9.79%. mp. > 168°C (decomp.). ^{1}H NMR- (δ)2.86 (2H, m, -CH_2-), 3.83 (1H, t, J = 5.5 Hz, –CH|–), 4.16 (2H, t, J = 2.0 Hz, $HOCH_2$-). MS: m/z, $[M]^+$, 143; $[M\text{-}H_2O]^+$, 125; $[M\text{-}CO_2H]^+$, 98; $[\text{-}CH(NH_2)CO_2H]^+$, 74. The racemate (1.65 g) was next dissolved in ice-cold 2N NaOH (5.8 ml) and chloroacetyl chloride (1.1 ml) and 2N NaOH (7 ml) were added to it alternatively dropwise over 2 h while stirring. Ten ml H_2O was added and the mixt. was passed through a column of Dowex-50 (H^+, 50 ml). The eluate was concd to give a partially crystallised syrup (2.6 g). Which was dissolved in water (100 ml). $Co(CH_3CO_2)_2$ (36.2 mg) was then added, with the pH adjusted to 7.5 with 3N NaOH. Commercial renal acylase (20 mg) was added and incubated at 37°C. During the incubation a small amount of acylase was additionally provided several times, and at each calculation, the pH-value of the reaction mixt. was adjusted to 7.5 with 2N NaOH. Following incubation for 24 h, the mixt. was treated with a small amount of activated charcoal, filtered with a glass filter, and the filtrate was passed through Amberlite 120B (H^+, 80 ml). The water eluate and washings were combined and the thick syrup was hydrolyzed for 1 h in 50% ethanolic soln of NaOH (120 ml). The hydrolysate was treated as usual with Amberlite 120B (H^+), then subsequently purified with a column of Dowex 1 × 4 ($^-$OAc, 9 × 210 mm) and 0.05 N acetic acid. Relevant fractions were combined and concd, to give crystals (96.3 mg) (D-Form). Recrystallized from EtOH-H_2O. Elemental analysis, Found: C, 50,14; H, 6.33; N, 9.58%. $C_6H_9NO_3$ requires: C, 50.14; H, 6.33; N, 9.58%. mp. > 155°C gradually decomp. $[\alpha]_D^{22} = +38°$ (H_2O; *c* 1), 7.0° (3N HCl; *c* 1). From the remaining soln as well as washings from the former Amberlite-120B column, the free L-amino acid was also obtained (63.5 mg). Elemental analysis, Found: C, 50.08; H, 6.41; N, 9.65%. Calcd for $C_6H_9NO_3$:C, 50.35; H, 6.34; N, 9.79%. mp. > 150°C gradually decomp. $[\alpha]_D^{22} = -35°$ (H_2O; *c* 1), −10°(3N HCl; *c* 0.5).

3.6.2. *Compound* **1**. Sodium (1.23 g) was dissolved in abs. EtOH (20 ml), with diethyl formamidomalonate (10.7 g) in abs. EtOH (27 ml) subsequently added to soln and the mixture heated until reflux begun, this being maintained for 30 min. 1-Bromo-3-butyne (7.1 g), prepared according to Schulte and Reiss [16] was added dropwise under stirring and reflux was continued for a further 17 hr. After cooling, the salt was removed by filtration with the filtrate concd., refiltered. To the final filtrate, EtOH (67 ml) and NaOH (10.7 g in 54 ml H_2O) were added and the whole was heated until reflux began, this being maintained for 1.5 h. After cooling, the bulk of EtOH was then removed by evaporation under reduced pressure and the pH of the mixt. was adjusted to **2** with conc HCl (ca. 25 ml). It was hydrolyzed by reflux for 1.5 h. The temperature was again raised until reflux began, which was held for an additional 1.5 h. The solution was cooled, and the crude product was subjected to Amberlite 120B (H^+, 500 ml) chromatography as before. The ammonia eluate (5 l) was evaporated with *ca.* half of it fractionated using cellulose column (3 × 76 cm, solvent system A). Crude crystals were obtained (55 mg) and recrystallized from aq. EtOH. Elemental analyses, Found: C, 56.46; H, 7.05; N, 10.90%. $C_6H_{11}NO_2$ requires: C, 56,68; H, 7.14; N, 11, 02%. The MS, ^{1}H NMR- and IR-spectra agreed with those of the authentic DL-2-amino-5-hexynoic acid. This product (55 mg) was dissolved in 3N HCl (10 ml) and refluxed at 100–105°C, following which after cooling, the solution was evapd under reduced pressure, with the resulting residue purified on a cellulose column (2.5 × 50 cm) using water-saturated t-AmylOH. Under these conditions, first 2-amino-5-chloro-4-hexenoic acid was eluted, then **1**, and lastly unchanged amino acid. Yield of crude crystals: Compound **1**, 4 mg (6%). The MS and IR of the natural and synthetic product were superimposable, respectively, and TLC comparison using with various solvent systems were identical.

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