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# Isolation and identification of (2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid from an *Amanita* of the section *Roanokenses* (Amanitaceae)*

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**A chlorine-containing non-protein amino acid which was recently discovered from the fruit bodies of *Amanita gymnopus* (2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid, was isolated and crystallized for the first time from the fruit bodies of an unknown member of *Amanita* belonging to the section *Roanokenses*, subsection *Solitariae*. The results of elementary analyses, determination of optical rotations, $^1$H- and $^{13}$C-NMR-spectra, and some chemical reactions supported an earlier proposed structure.**

Key Words——(2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid; chloroamino acid; non-protein amino acid; *Amanita* sp.

Fruit bodies of several species belonging to the genus *Amanita*, subgenus *Lepidella*, in particular, section *Roanokenses*, are known to contain various kinds of non-protein amino acids of the unsaturated norleucine-type (Bas, 1969; Hatanaka, 1992; Singer, 1986).

Quite recently we reported on the non-protein amino acids from the fruit bodies of *Amanita gymnopus* Corner & Bas. Besides three known amino acids of the above type, evidence for the occurrence of a new chloroamino acid, (2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid, was presented. The amount of starting material was scanty, so that none of these could be obtained as pure crystals (Hatanaka et al., 1994).

We now report isolation and crystallization of the same chloroamino acid from the fruit bodies of an *Amanita* (Fig. 1) of section *Roanokenses* subsection *Solitaliae*. Some physical and chemical properties will also be described.

## Materials and Methods

**Fungus** The fruit bodies (344 g) were collected in August 1994 in Yamaguchi Prefecture and stored in EtOH in a refrigerator before use. The vouchers were deposited in the Tottori Mycological Institute.

This *Amanita* fungus apparently belongs to the section *Roanokenses* (=section ss. auct.) subsection *Solitariae* (Singer, 1986) due to its amyloid spores.

The appendiculate non-sulcate margin of the pileus and volval remnants that were present on pileus as persistent, medium-sized to large pyramidal warts, are almost absent or if present, inconspicuous on stipe and contain abundant subglobose elements cells in addition to rather frequent branching hyphae. However, it does not key out with any species of that subsection treated by Bas (1969) in his world monograph of *Amanita* section *Lepidella*, suggesting that our fungus may represent a new species. The taxonomy will be discussed intensively elsewhere in the near future.

**General** Cellulose thin layer chromatography (TLC), ninhydrin reaction, and automated amino acid analyses were the same as reported previously (Hatanaka et al., 1994). Solvent systems used for cellulose TLC were n-BuOH-HOAc-$H_2O$ (63 : 10 : 27) (A) and phenol-$H_2O$ (25 : 9, w/w) (B). Melting point was determined by using an IA9100 digital melting point apparatus and was not corrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. $^1$H- and $^{13}$C-NMR spectra were obtained with a Varian XL-300, in $D_2O$, with sodium 3-(trimethylsilyl)propanesulfonate ($\delta=0$) and with dioxane ($\delta=67.4$) as internal standards, respectively. Finally FAB-MS was determined with a JEOL JMS-AX505W, and EI-MS with JEOL-JMS-D-300.

**Hydrogenation and reduction** A few mg of the pure sample were hydrogenated by gently bubbling $H_2$ over Adams' catalyst for 30 min under atmospheric pressure at room temperature. The product was analyzed by an amino acid analyzer. The hydrogenated sample was filtered and concentrated to dryness. Hydriodic acid (57%, 1 ml) and a few mg of red phosphorus were added, and the mixture was then heated at 150°C for 5 h in a sealed glass tube. After cooling, it was treated with a

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Fig. 1. *Amanita* sp. used in the present study. Bar indicates ca. 5 cm.

small column of Amberlite IR-120B ($H^+$-form, 1 ml). The resin was washed with $H_2O$, and the amino acids were displaced with 2 M $NH_4OH$ (10 ml). The products were analyzed by an amino acid analyzer.

**Isolation of the amino acid** Fruit bodies (344 g) were homogenized repeatedly in a mixer with 80% EtOH and filtered. The combined filtrate (5.5 L) was passed through a column of Amberlite IR-120B ($H^+$-form, 100 ml). The resin was then washed thoroughly with 80% EtOH and $H_2O$, successively, and the amino acids retained were eluted with 2 M $NH_4OH$ (1 L). The ammonia eluate was concentrated, giving a thick syrup. It was then applied to a Dowex 1×4 column (200–400 mesh, $OAc^-$-Form, 23×900 mm), which had been equilibrated with 0.05 M HOAc. Fractionation was carried out also with the same solvent (3.5 ml/fraction).

## Results and Discussion

**Isolation of the amino acid** Even the two dimensional TLC revealed existence of unusual amino acid(s). Fractions, number 52–62, from the Dowex-column, were combined and concentrated, yielding the crude crystals of the chloroamino acid (388.5 mg). They showed a single peak and spot in an amino acid analyzer and in TLC, respectively. The yield was ca. 0.1% of the fresh fruit bodies and it was extraordinarily high compared to a single non-protein amino acid in plants and fungi.

A part of the crude crystals were recrystallized twice from 70% EtOH, giving fine needles.

**Structural studies** The FAB-MS showed a pair of ion peaks at m/z=180, 182 ($[M+H]^-$) with the ratio 3 : 1. EI-MS also exhibited the peaks at m/z=134, 136 ($[M-COOH]^+$) in the same ratio. These observations clearly indicated the presence of one chlorine atom. The result of the elementary analyses of the purified sample was in good agreement with the formula $C_6H_{10}NO_3Cl$. mp. ca. 170°C (Decomp.), $[\alpha]_D^{22}=-15.4°$ (c 1, $H_2O$), $+4.9°$ (c 0.5, 2 M HCl)

Found: C, 40.22; H, 5.62; N, 7.70; Cl, 19.84%
Calcd: C, 40.13; H, 5.61; N, 7.80; Cl, 19.74%

Table 1 shows $^1H$- and $^{13}C$-NMR-spectra, and they were compared with those of (2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid from *Amanita gymnopus*. In the $^1H$-NMR, six non-exchangeable protons were found. The chemical shift $\delta=3.89$ shows hydrogen of $\alpha$-carbon and double doublet at $\delta=2.24$ indicated two hydrogens of $\beta$-carbon. The signal $\delta=4.47$ can be assigned to the hydrogen of $\gamma$-carbon, and those of 5.45 and 5.59 indicated two hydrogens of terminal ethylene. In the $^{13}C$-NMR measurements, six carbon atoms were observed. The chemical shift 174.66 is characteristic to that of carboxyl group, 53.21 can be assigned to the $\alpha$-carbon, 34.82 to the $\beta$-carbon, and signal 72.16 to the hydroxylated carbon. The last two signals can also be reasonably explained.

Thus, the NMR data explained without any difficulties the structure, 2-amino-5-chloro-4-hydroxy-5-hexenoic acid discovered recently from *A. gymnopus*.

Comparison of its chromatographic behavior on TLC

Table 1. $^1$H- and $^{13}$C-NMR spectra of 2-amino-5-chloro-4-hydroxy-5-hexenoic acid.

| $^1$H-NMR | Amino acid from this fungus | 2-Amino-5-chloro-4-hydroxy-5-hexenoic acid (*A. gymnopus*) |
|---|---|---|
| H-2 | $\delta$ 3.89<br>1H<br>t<br>J (Hz) 5.6 | 3.89<br>1H<br>t<br>5.5 |
| H-3 | 2.24<br>2H<br>dd<br>5.6, 6.3 | 2.24<br>2H<br>dd<br>5.5,6.3 |
| H-4 | 4.47<br>1H<br>t<br>6.3 | 4.47<br>1H<br>t<br>6.3 |
| H-6 | 5.45, 5.59<br>1H 1H<br>d d<br>1.8 1.8 | 5.45, 5.59<br>1H 1H<br>d d<br>1.9 1.9 |
| $^{13}$C-NMR | | |
| C-1 | $\delta$ 174.66 | 174.56 |
| C-2 | 53.21 | 53.15 |
| C-3 | 34.82 | 34.79 |
| C-4 | 72.16 | 72.12 |
| C-5 | 142.69 | 142.65 |
| C-6 | 114.88 | 114.85 |

and retention time on an automated amino acid analyzer were also satisfactory, as shown in Table 2. The ninhydrin coloration was slightly brownish violet, although a large number of unsaturated amino acids react by turning light yellow to brown and in some cases the color turns violet with time. A brownish violet coloration of ninhydrin was also observed in some hydrogenated amino acids.

Among the final products of catalytic hydrogenation over Adams' catalyst, followed by treatment with hydriodic acid and red phosphorus, a small amount of norleucine was detected by TLC and amino acid analysis, supporting the proposed structure.

A great positive shift of the $[\alpha]_D^{22}$-values in water solution by acidifying suggested that most of this amino acid exists in the (2*S*)- or L-series of $\alpha$-amino acids. Determination of the absolute configuration of C-4 is now in progress (Fig. 2).

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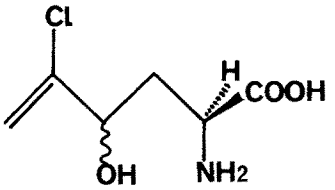


Fig. 2. (2*S*)-2-Amino-5-chloro-4-hydroxy-5-hexenoic acid.

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Table 2. Rval-values on thin layer chromatography, color reaction with ninhydrin, and retention times in an amino acid analyzer of 2-amino-5-chloro-4-hydroxy-5-hexenoic acid.

| Amino acid | Rval-values Solvent systems[a] (A) | (B) | Color reaction with ninhydrin | Retention times (min) |
|---|---|---|---|---|
| Amino acid from this fungus | 1.06 | 0.94 | brownish violet | 98.74 |
| 2-Amino-5-chloro-4-hydroxy-hexenoic acid (*A. gymnopus*) | 1.04 | 0.96 | brownish violet | 99.36 |
| Valine | 1.00 | 1.00 | violet | 108.24 |

a) Solvent systems for TLC: A, BuOH-HOAc-$H_2O$, 63 : 10 : 27: B, PhOH-$H_2O$, 25 : 9.