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(2*S*)-2-Amino-5-chloro-4-hydroxy-5-hexenoic acid, a new chloroamino acid, and related compounds from *Amanita gymnopus**

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Combining different chromatography systems, unusual nonprotein amino acids were isolated and unequivocally identified from a small amount (less than 100 g fresh weight) of *Amanita gymnopus* fruit body. Without obtaining crystals of these amino acids, on the basis of ^{1}H-NMR determination, high resolution mass spectrometry, chlorine analysis and oxidation with L-amino acid oxidase, one of them proved to be a new chloroamino acid, (2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid (G2). The other three were (2*S*)-2-amino-5-hexenoic acid (G1), (2*S*)-2-amino-4,5-hexadienoic acid (G3) and (2*S*)-2-amino-5-hexynoic acid (G4). Amino acid (G1) was also encountered for the first time in natural products. Amino acid (G3) has been reported from several kinds of fungi belonging to *Amanita*, subgenus *Lepidella*. The occurrence of amino acid (G4) was already reported from *Cortinarius claricolor*.

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

Introduction

Sporadic occurrence of unsaturated aliphatic amino acids of norleucine type is one of the characteristics of *Amanita* subgenus *Lepidella*, particularly section *Roanokenses* (*Lepidella* auct.) of the genus *Amanita* (Singer, 1986). Thus, (2*S*)-2-amino-4,5-hexadienoic acid (Chilton et al., 1968) and (2*S, Z*)-2-amino-5-chloro-4-hexenoic acid (Chilton and Tsou, 1972) are known from *A. smithiana*, and (2*S*)-2-amino-4-pentynoic acid, (2*S*)-2-amino-4,5-hexadienoic acid, and (2*S, Z*)-2-amino-5-chloro-6-hydroxy-4-hexenoic acid from *A.abrupta* Peck (Yamaura et al., 1986, Ohta et al., 1987). Fruit bodies of *A. virgineoides* Bas contain (2*S*)-2-amino-3-cyclopropanepropionic acid, a rare cyclopropane amino acid as a natural product (Ohta et al., 1986). (2*S*)-2-Amino-4,5-hexadienoic acid was also isolated from *A. neoovoidea* Hongo (section *Amidellae*) (Hatanaka and Kawakami, 1980), *A. pseudoporphyria* Hongo (section *Phalloideae*) (Hatanaka et al., 1985) and *A. miculifera* Bas & Hatanaka (section *Roanokenses*, Bas and Hatanaka, 1984; Hatanaka et al., unpublished). This amino acid appears to be widely distributed in subgenus *Lepidella*. Non-protein amino acid in higher fungi have recently been reviewed (Hatanaka, 1992).

This paper describes the non-protein amino acids of a still unexplored species of the subgenus *Lepidella*, *Amanita gymnopus* Corner & Bas (Corner and Bas, 1962; Hongo, 1974). Our study revealed a new chloroamino acid, (2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid. Moreover, (2*S*)-2-amino-5-hexenoic acid, (2*S*)-2-amino-4,5-hexadienoic acid and (2*S*)-2-amino-5-hexynoic acid were also identified. (2*S*)-2-Amino-5-hexynoic acid has been previously isolated from *Cortinarius claricolor* (Fr.) Fr. var. *tenuipes* Hongo (Aoyagi and Sugahara, 1985).

Materials and Methods

General Solvents were evaporated in a rotary evaporator below 40°C. Thin layer plates and cellulose powder used were "Avicel" of Funakoshi Pharmaceutical Co. Ltd. Solvent systems for paper and thin layer chromatography were as follows: n-BuOH-HOAc-H_2O

* Part 23 in the series "Biochemical studies of nitrogen compounds in fungi." Part 22, Hatanaka, S. I. et al. 1985. Trans. Mycol. Soc. Japan **26**: 61-68.

Table 1. Cellulose thin layer chromatography.

Amino acids	Rval-values Solvent systems*				Color reaction with ninhydrin
	A	B	C	D	
G1	1.32	1.07	1.60	1.18	Violet
2-Amino-5-hexenoic acid	1.33	1.07	1.60	1.17	〃
G3	1.04	1.04	1.40	1.10	Yellow→Violet
2-Amino-4,5-hexadienoic acid	1.04	1.03	1.40	1.10	〃
G4	0.88	0.90	1.08	0.94	Violet
2-Amino-5-hexynoic acid	0.88	0.91	1.03	0.95	〃
Alanine	0.50	0.60	0.38	0.65	〃

*Compositions of the solvent systems are indicated in Materials and Methods.

Table 2. Retention time of the amino acids on an automated analyzer.

Amino acids	Retention times
G1	94.52
2-Amino-5-hexenoic acid	94.72
G2	89.49
2-Amino-4,5-hexadienoic acid	89.52
G4	74.58
2-Amino-5-hexynoic acid	74.93

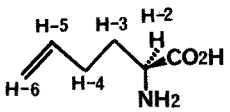
(63 : 10 : 27) (A), PhOH-H_2O (25 : 8, in NH_3 vapor) (B), t-AmOH-MeCOEt-NH_4OH (28%) (15 : 9 : 4 : 2) (C), and PrOH-NH_4OH (2 : 1) (D). An automated amino acid analyzer, Hitachi 835, programmed for biological fluid was used throughout the work. ^{1}H-NMR was determined with JEOL GSX-500, 500 MHz, in D_2O, with DSS as an internal standard ($\delta=0$), and ^{13}C-NMR, 125 MHz in D_2O, with dioxane as an internal standard ($\delta=67.4$). Mass spectra were measured with JEOL HX-110. Absolute configuration around C-2 was determined by a standardized method of our laboratory for a small amount of amino acid sample using L-amino acid oxidase (Murakami et al., 1985).

Authentic amino acids (2*S*)-2-Amino-4,5-hexadienoic acid was an isolate from *Amanita neoovoidea* Hongo (Hatanaka and Kawakami, 1980). Racemates of 2-amino-5-hexenoic acid and 2-amino-5-hexynoic acid were synthesized by alkylation of diethyl formylaminomalonate according to Black and Landor (1968).

Fungus Six fruit bodies (78.7 g) of *Amanita gymnopus* were collected in August 1987 in Tottori Prefecture and stored in ethanol in a cold room before extraction.

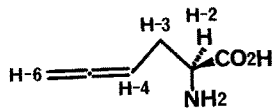
Extraction of amino acids and fractionation Fruit bodies were homogenized repeatedly in a mixer with 80% ethanol and filtered. The combined extract (2L) was passed through a column of Amberlite IR-120B (40 ml). After washing the column thoroughly with 80% ethanol and water successively, the amino acids were displaced

Table 3. ^{1}H-NMR-Spectrum of 2-amino-5-hexenoic acid.



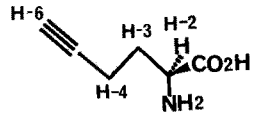
	G1	Authentic sample
H-2	δ 3.74 1H dd J 5.1, 7.0 Hz	3.74 1H dd 5.1, 7.0
H-3	1.87–2.03 2H m	1.87–2.03 2H m
H-4	2.16 2H ddd 6.5–7.7	2.16 2H ddd 6.7–7.7
H-5	5.88 1H ddt 10.4, 17.2, 6.5	5.88 1H ddt 10.3, 17.2, 6.5
H-6	5.07, 5.14 1H 1H d d 10.3 17.4	5.07, 5.14 1H 1H d d 10.3 17.2

Table 4. ^{1}H-NMR-Spectrum of 2-amino-4,5-hexadienoic acid.

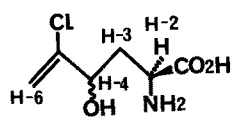


	G3	Authentic sample
H-2	δ 3.82 1H dd J 4.3, 7.2 Hz	3.82 1H dd 4.5, 7.2
H-3	2.50–2.65 2H m	2.50–2.66 2H m
H-4	5.11 1H ddt 7.7, 6.8	5.11 1H ddt 7.7, 6.8
H-6	4.84 2H d 6.8	4.84 2H d 6.8

Table 5. ^{1}H-NMR-Spectrum of 2-amino-5-hexynoic acid.



	G4	Authenic sample
H-2	δ 3.83 1H dd J 5.6, 7.4 Hz	3.85 1H dd 5.4, 7.3
H-3	1.98–2.17 2H m	1.99–2.18 2H m
H-4,6	2.33–2.43 3H m	2.33–2.46 3H m



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

with 2 N NH_4OH (400 ml). The ammonia eluate was then concentrated to ca. 40 ml and fractionated on a column of Dowex 1×4 (200–400 mesh, 23 mm × 900 mm), being eluted with 0.05, 0.5, and 2.0 N acetic acid, successively. Relevant fractions were combined and fractionated further with ion pair reverse phase chromatography using LiChroprep RP-8. The solvent used was 0.13% HFBA containing dilute acetonitrile (0→10% gradient). This procedure was repeated, and in some cases cellulose column chromatography was subsequently necessary. Purities of each fraction were checked by use of an amino acid analyzer.

Results and Disucssion

Two dimensional paper chromatography developed with the solvents (A) and (B) revealed the presence of unusual amino acid(s) near valine. Previous works (Hatanaka and Kawakami, 1980; Hatanaka et al. 1985; Ohta et al., 1986) have shown that a large number of unsaturated aliphatic amino acids located in this area. Coincidentally several unusual peaks appeared on amino acid analysis.

Lyophilized samples of four of these amino acids were designated G1, G2, G3, and G4 in the order of their Rf-values on thin layer chromatography using solvent (A). They were successfully purified by repeated chromatography with various systems described in Materials and Methods. Because of the the small amount of starting materials, none of these could be crystallized, but the purities of samples were high enough for the structural studies.

1. (2*S*)-2-Amino-5-hexenoic acid (G1), (2*S*)-2-amino-4,5-hexadienoic acid (G3) and (2*S*)-2-amino-5-hexynoic acid (G4)

Table 1 compares the Rval-values of G1, G3, and G4 on cellulose thin layer chromatograms developed with four different solvents, and Table 2 shows their retention times on an automated amino acid analyzer with those of authentic amino acids. To confirm these tentative identification ^{1}H-NMR spectra were compared with those of authentic samples, as shown in Table 3, 4, and 5. Oxidation with L-amino acid oxidase was not always complete: G1, 99%; G3, 88%; G4, 100% (L-Leu, 100%). Because we did not apply the amino acids in pure crystalline form, we cannot conclude at present, whether a small amount of D-enantiomer exists concomitantly in the G3 sample.

Except for optical purity, experimental results described above are consistent with each other and with identification of G1, G3, and G4 as (2*S*)-2-amino-5-hexenoic acid, (2*S*)-2-amino-4,5-hexadienoic acid, and (2*S*)-2-amino-5-hexynoic acid, respectively.

2. (2*S*)-2-Amino-5-chloro-4-hydroxy-5-hexenoic acid (G2)

Chromatographic behavior of G2 on thin layers corresponded to none of the natural or synthetic amino acids available in our laboratory.

The FAB-MS (glycerine, Xe) of G2 showed m/z=180, 182 ($[M+H]^+$) in the ratio 3 : 1. EI-MS exhibited also a pair of intense peaks at m/z=134, 136 ($[M-COOH]^+$) in the same ratio. These observations clearly indicated the presence of one chlorine atom. The elementary composition $C_6H_{10}NO_3Cl$ was obtained from an accurate mass measurement of the ion with a mass of 180 in the FAB-MS (Found: 180.0444, Calcd: 180.0427).

In NMR measurements, six carbons and six non-exchangeable protons were observed. The NMR data were thus well explained with the proposed structure, 2-amino-5-chloro-4-hydroxy-5-hexenoic acid. ^{13}C-NMR, δ: 174.56 (s, >COOH), 53.15 (d, $-\underline{C}H(NH_2)COOH$), 34.79 (t, $>\underline{C}H_2$), 72.12 (d, $>\underline{C}H(OH)$), 142.65 (s, $=\underline{C}(Cl)-$), 114.85 (t, $=\underline{C}H_2$). ^{1}H-NMR, δ: 3.89 (1H, t, J=5.5 Hz, $-\underline{C}H(NH_2)COOH$), 2.24 (2H, dd, J=5.5, 6.3 Hz, $>CH_2$), 4.47 (1H, t, J=6.3 Hz $-\underline{C}H(OH)$), 5.45 (1H, d, J=1.9 Hz, $=\underline{C}H_2$), 5.59 (1H, d, J=1.9 Hz, $=\underline{C}H_2$).

Chlorine content of the G2 was 17.5% (Calcd. for $C_6H_{10}NO_3Cl$: 19.9%). Absolute configuration around C-4 could not be determined. Over 70% percent of G2 were oxidized by L-amino acid oxidase, suggesting that most of G2 is present in the L-form.

3. Biological activities

Most of the non-protein amino acids of unsaturated norleucine-type have been found to function as antimetabolites. (2*S*)-2-Amino-5-hexynoic acid is also no exception (Aoyagi and Sugahara, 1985). 2-Amino-4,5-hexadienoic acid has been reported to exhibit a hypoglycemic activity (Chilton and Tsou, 1975). Another chloroamino acid, (2*S*)-2-amino-4-chloro-4-pentenoic acid from *A. pseudoporphyria*, has antibacterial activity (Moriguchi et al., 1987). It is, therefore, interesting to examine biological function and significance of the chloroamino acid reported here.

4. Chemotaxonomy

Fruit bodies of *A. gymnopus* have similar specific characters of the universal veil and gills to those of *A. ochrophylla* (Cooke) Clel. Bas (1969) separated these two species and proposed *Gymnopodae* Bas, subsect. nov. for them. In consequence, four subsections are now recognized in the section *Roanokenses* (Singer, 1986). The phylogenetic relationship among them is, however, uncertain.

Although non-protein amino acids of unsaturated norleucine-type are not confined to the section *Roanokenses*, they occur in many species in this taxon. Non-protein amino acids of this type in *A. gymnopus* all have a six carbon atoms and include a chlorine-containing amino acid. Their distribution pattern in subgenus *Lepidella* is species-specific, and those in *A. gymnopus* are not exceptional.

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