



A NEW CONJUGATED DIENE AMINO ACID, D,L-2-AMINO-3(*cis*), 5-HEXADIENOIC ACID, FROM *CLAVULINOPSIS HELVOLA*

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Key Word Index—*Clavulinopsis helvola*; Basidiomycetes; mushroom; non-protein amino acid; conjugated diene amino acid; L-azetidine-2-carboxylic acid; 3-methylanthionine; 2-amino-3,5-hexadienoic acid.

Abstract—A new amino acid, D,L-2-amino-3(*cis*),5-hexadienoic acid, was isolated from the fruiting bodies of *Clavulinopsis helvola*. © 1997 Elsevier Science Ltd

RESULTS AND DISCUSSION

Three unidentified ninhydrin-positive compounds (**1**, **2**, **3**) occurring in the fruiting bodies of *Clavulinopsis helvola* (Pers.; Fr.) Coner were isolated by ion-exchange chromatography. **1**, the most abundant compound, was identified to be L-azetidine-2-carboxylic acid which commonly occurs in some plant species and the mushrooms of *Clavulinopsis* sp. [1, 2].

The ninhydrin reaction of **2** was masked by the Cu^{2+} and positive iodo-platinate reaction showing that it was a sulphur-containing α -amino acid. From its elemental analysis, the molecular formula was estimated to be $\text{C}_7\text{H}_{14}\text{N}_2\text{O}_4\text{S}$. Reductive cleavage of **2** by Raney nickel generated L-Ala and L-2-aminobutyric acid in equimolar ratios. The presence of a methyl doublet in the ^1H -NMR spectrum showed that **2** was 3-methylanthionine. The presence of this amino acid in natural sources has been reported for yeast and *Phallus impudicus*, etc. [1].

The ninhydrin reaction of **3**, yellow coloured and completely masked by Cu^{2+} , suggested it to be an unsaturated α -amino acid. From its elementary analysis and EI mass spectrometry (m/z 127 $[\text{M}]^+$) the molecular formula was estimated to be $\text{C}_6\text{H}_9\text{NO}_2$. Catalytic hydrogenation of **3** absorbed 2 mol. of H_2 per mol. and produced D,L-norleucine. These results showed **3** is D,L-unsaturated norleucine having two double bonds or one triple bond. The ^{13}C -NMR analysis showed one carbonyl, four methine and one methylene carbon atoms and a UV_{max} at 227.3 nm which suggested the presence of a conjugated double

bond. Therefore, **3** was shown to be D,L-2-amino-3,5-hexadienoic acid. Since the coupling constant in the ^1H NMR spectrum was $J_{3,4} = 10.98$, a *cis* configuration was presumed [3]. Many unsaturated non-protein amino acids in Basidiomycetes and plants have been reported [1,2]. Particularly, for unsaturated norleucines, almost all possible structures have been determined. However this is the first report of an unsaturated norleucine containing a conjugated double bond. Also, it is interesting that this amino acid is a racemic mixture. Dardenne *et al.*, reported the isolation of a D,L-racemate of 2-amino-3-butenic acid from fruiting bodies of *Rhodophyllus nidorosus* [4]. Although they suggested that the D-form might be naturally synthesized, no experimental proof was provided. If the occurrence of a 3,4 double bond is responsible for racemization at C-2, it seems likely that the D,L-racemate of 2-amino-3,5-hexadienoic acid was formed from the L-isomer during isolation.

EXPERIMENTAL

Materials. The fruiting bodies of *Clavulinopsis helvola* (Pers.; Fr.) Coner were collected in 1992 in the forest of Nagano Pref.

General. TLC solvents were *n*-BuOH–HOAc– H_2O (4:1:2, solvent 1), *Pr*OH– NH_4OH (7:3, solvent 2). ^1H NMR and ^{13}C NMR were recorded using a Hitachi R-40 spectrometer and a JEOL JNM EX-270 spectrometer with DSS as the int. standard. EIMS were taken with a Shimadzu QP-2000. AA anal. were recorded using a Hitachi 835 automatic amino acid analyser.

Isolation of **1, **2** and **3**.** The fresh fruiting bodies (3 kg) were extracted $\times 3$ with 70% EtOH. The extract

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(30 l) was concd and then defatted by Et₂O extraction ($\times 3$). The defatted extract was passed through a column (5.5 $\times$ 80 cm) of Amberlite IR-120(H⁺). After the resin was washed with H₂O, the amino acids were eluted with 2 M NH₄OH (10 l). The eluate, concd to dryness and redissolved in H₂O, was applied on a column (4 $\times$ 50 cm) of Dowex 1 $\times$ 4(OAc). Elution was made using 4 l of 0.1 M HOAc and then the eluate was concd. The concentrate was divided and some portions were repeatedly chromatographed on a Dowex 50 $\times$ 4 (pyridinium) column (3 $\times$ 100 cm) with a linear gradient system made by 1.5 l of 0.2 M pyridine-HOAc buffer (pH 3.1) and 1.5 l of 2 M pyridine-HOAc buffer (pH 5). **1**, **2**, and **3** were isolated from the ninhydrin positive frs of the eluate and crystallized. **1**, **2** and **3** were repeatedly recrystallized $\times 3$ from H₂O-EtOH, and yielded 6.5 g, 340 mg and 615 mg of colourless crystals, respectively.

Physico-chemical properties and spectral data. **1**. Mp 202–204° [α]_D²⁰ –129.1° (H₂O; c 0.1895) and –118.1° (3N HCl; c 0.095). Found: C, 47.57; H, 7.19; N, 13.56. Calcd for C₄H₇NO₂: C, 47.51; H, 6.98; N, 13.86%. EIMS m/z : 101 [M]⁺, 73, 56 [M–45]⁺. IR $\nu_{\max}^{\text{KBr}}$ cm^{–1}: 3420, 2975, 2690, 2520, 1638, 1598, 1482, 1415, 1320, 1258, 1115, 1090, 925, 800, 770, 740, 562, 500. ¹H NMR (90 MHz, D₂O): δ 2.3–3.1 (2H, m , H-4), 3.8–4.4 (2H, m , H-3), 4.86 (1H, t , J = 9 Hz, H-2).

2. mp 228–230°. [α]_D²⁰ –16.9° (H₂O; c 1.32) and +45.9° (3N HCl; c 0.66). Found: C, 35.76; H, 6.68; N, 11.79. Calcd for C₇H₁₄N₂O₄S $\cdot$ H₂O: C, 34.99; H, 6.71; N, 11.66. IR $\nu_{\max}^{\text{KBr}}$ cm^{–1}: 3450, 3050, 2625, 2080, 1608, 1398, 1358, 1305, 1055, 983, 920, 840, 703. ¹³C NMR (270 MHz, D₂O): δ 22.68 (C-4), 35.81 (C-3') 45.34 (C-3), 57.92 (C-2'), 63.13 (C-2), 176.33 (C-1'), 176.46 (C-1). ¹H NMR (270 MHz, D₂O): δ 1.46 (3H, d , J = 7.25 Hz, H-4), 3.18 (2H, d , J = 5.61 Hz, H-2'), 3.55 (1H, m , H-3), 3.90 (1H, d , J = 3.96 Hz, H-2), 4.00 (1H, t , J = 5.61 Hz, H-2').

3. mp > 300°. Found: C, 56.96; H, 7.19; N, 10.86. C₆H₉NO₂ requires: C, 56.68; H, 7.14; N, 11.02%. IR $\nu_{\max}^{\text{KBr}}$ cm^{–1}: 2900, 2080, 1815, 1640, 1575, 1385, 1355, 1340, 1270, 1190, 1155, 1120, 1053, 990, 960, 900, 810, 710, 710, 655, 520. EIMS m/z : 127 [M]⁺, 110, 82 [M–45]⁺, 80, 67, 65, 55, 53. ¹³C NMR (270 MHz, D₂O): δ 53.22 (C-2), 122.69 (C-6), 124.38 (C-3), 131.59 (C-5), 136.15 (C-4), 175.55 (C-1). ¹H NMR (270 MHz, D₂O): δ 4.62 (1H, d , J = 10.25 Hz, H-2), 5.3–5.5 (3H, m , H-3 and H-6), 6.39 (1H, dd , J = 10.98 and 10.98 Hz, C-4), 6.76 (1H, ddd , J = 16.59, 10.61 and 10.61 Hz, H-5). UV $\lambda_{\max}^{\text{H}_2\text{O}}$ nm (log ϵ): 227.3 (4.30).

Reductive cleavage by raney nickel. A reaction mixt.

of **2** (5 mg) and freshly prepd Raney Ni (5 g) in 85% EtOH (10 ml) was refluxed for 1 hr and filtered. The filtrate was concd and analysed by TLC (solvents 1 and 2) and an AA analyser. Equimolar amounts of ala and 2-aminobutyric acid were detected.

Catalytic hydrogenation by Adam's PtO₂. **3**. (33.5 mg) was dissolved in 8 ml of HOAc and hydrogenated over Adam's PtO₂ at room temp. and pres. The absorption of H₂ was 2.02 mol mol^{–1}. Concn of the filtrate of the reaction mixt. gave colourless crystals. Recrystallization from H₂O, produced 18 mg of reaction product. TLC (solvent 1 and 2), AA anal., IR and EIMS (m/z : 132 [M + 1]⁺, 86, 74, 69) showed it to be norleucine.

Resolution of D,L-isomer. The absolute configuration was assigned by HPLC analysis of the enantiomeric derivatives with 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosyl-isothiocyanate (GITC) [5]. The filtrate of the desulphurization reaction of **2** by the Raney Ni previously used, was concd to dryness and dissolved in one ml of 50% aq. MeCN containing 0.4% Et₃N. An aliquot of the soln. was mixed with an equal vol. of 0.2% GITC MeCN soln., and reacted at room temp. for 30 min. A small aliquot of the reaction mixt. was analysed by HPLC. Conditions were as follows. Column: Millipore puresil C₁₈ (4 $\times$ 150 mm). Solvent: MeOH–10 mM pH 2.8 Na-Pi buffer (11:9). Flow rate: 0.5 ml min^{–1} at room temp. Detection: UV at 220 nm.

Two peaks of the derivatives of Ala and 2-aminobutyric acid appeared and their retention times coincided with that of the L-form. The same analysis made on **3** and the norleucine, hydrogenated product of **3**, showed that they were both the D,L-form.

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