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4-AMINOPYRIDINE-2,3-DICARBOXYLIC ACID FROM *CLITOCYBE ACROMELALGA*

FUKUSHI HIRAYAMA, KATSUHIRO KONNO, HARUHISA SHIRAHAMA* and TAKESHI MATSUMOTO†

Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060, Japan, †Department of Chemistry, Faculty of Science, Tokai University, Hiratsuka, Kanagawa 259-12, Japan

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Key Word Index—*Clitocybe acromelalga*, Tricholomataceae, Basidiomycetes, 4-aminopyridine-2,3-dicarboxylic acid

Abstract—A new pyridine derivative, 4-aminopyridine-2,3-dicarboxylic acid, was isolated from *Clitocybe acromelalga*, and its structure was determined by spectral and synthetic methods.

INTRODUCTION

The poisonous mushroom *Clitocybe acromelalga* (Japanese name; Dokusasako) is found only in Japan. Accidental ingestion of the fungus causes a violent pain and red coloration in the fingers and toes after a period of several days, and the pain continues over two to four weeks. The symptoms are similar to erythromelalgia or acromelalgia. These characteristic physiological properties prompted us to study the chemical constituents of this fungus. The mammalian toxins acromelic acid A(**2**) and B(**3**) [1-4] and clitidine (**4**) [5, 6], and the non-toxic amino acid betaine clithioneine (**5**) [7, 8] have already been isolated from this mushroom. Further investigation led to the isolation of a new pyridine derivative, 4-aminopyridine-2,3-dicarboxylic acid (**1**) whose structure was deduced from spectral analyses and confirmed by synthesis. Although compound **1** did not show lethal effects on mice, bioactivity is expected since pyridine-2,3-dicarboxylic acid is known to produce axon-sparing lesions similar to those observed in Huntington's disease [9].

RESULTS AND DISCUSSION

The water extracts of frozen fruit bodies were separated by acetone precipitation, dialysis, several chromatographic steps and paper electrophoresis. From a poisonous fraction containing acromelic acid A (**2**) and B (**3**) was isolated compound **1** which was finally purified by recrystallization from water. The yield was 0.000056% based on frozen fruit bodies

The acidic property of **1** was obvious from its behaviour on ion-exchange column chromatography and paper electrophoresis. Its EI and FD mass spectra showed base peaks at *m/z* 164 and 165 respectively and a peak at *m/z* 183 was observed by FAB mass spectrometry. The molecular formula was deduced as $C_7H_6O_4N_2$ from the $[M-H_2O]^+$ fragment in the HR-EI mass spectrum. The ^{1}H NMR spectrum in D_2O showed two doublet peaks (δ7.92, 1H and 6.95, 1H) and the ^{13}C NMR spectrum in DMSO-d_6 showed two doublet peaks (δ 111 and 139), and five singlet peaks (δ 113, 147, 160, 162 and 167). The MS and NMR data suggested that **1** was a pyridine substituted by $-CO_2H \times 2$, $-NH_2$, or $-CO_2H$, $-CONH_2$, $-OH$. Furthermore, the coupling constant (7.0 Hz) of the doublet peaks in the ^{1}H NMR spectrum and the chemical shifts of two doublet peaks in the ^{13}C NMR spectrum indicated that the three substituents were attached to C-2,-3 and -4 of the pyridine ring. Treatment of **1** with either hydrochloric acid–methanol or diazomethane–ether afforded the same product (**6**), whose HR-MS revealed the molecular formula as $C_9H_{10}O_4N_2$. The ^{1}H NMR spectrum of **6** in $CDCl_3$ showed two singlet peaks (δ 3.87, 3H and 3.95, 3H) due to two ester methyl groups, a broad singlet peak (δ6.08, 2H) accounted for NH_2, and two doublet peaks (δ 6.6, 1H and 8.18, 1H) for the aromatic protons These results suggested that **1** was an aminopyridinedicarboxylic acid. The substituted positions of the pyridine ring were deduced from a comparison with clitidine (**4**), which possesses a 4-aminopyridine-3-carboxylic acid moiety. Thus **1** was assigned the 4-aminopyridine-2,3-dicarboxylic acid structure.

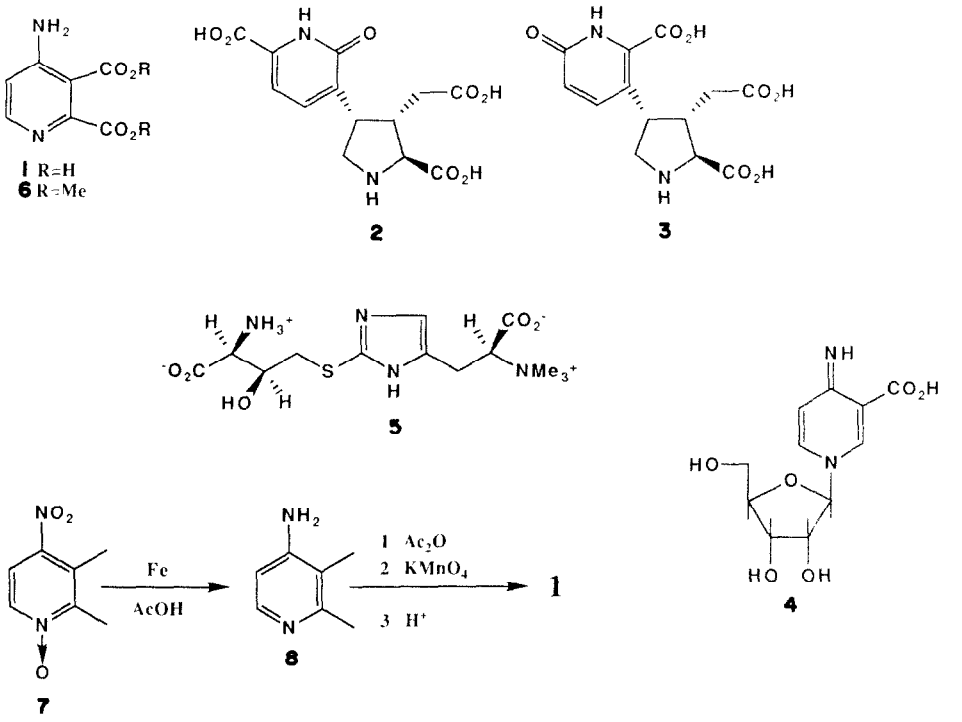
The structure of **1** was confirmed by the following synthesis Reduction of 2,3-dimethyl-4-nitropyridine 1-oxide (**7**) [10] with iron powder in acetic acid gave 4-amino-2,3-dimethylpyridine (**8**). Acetylation of **8** followed by oxidation with potassium permanganate and hydrolysis gave 4-aminopyridine-2,3-dicarboxylic acid (**1**) which was identical with natural product **1** (chromatographic behaviour and spectral data).

EXPERIMENTAL

^{1}H NMR (90 and 270 MHz) TMS in $CDCl_3$ and TSP in D_2O as int. standards ^{13}C NMR (25 MHz) DMSO in DMSO-d_6 as int. reference Paper electrophoresis pH 4.6 (pyridine–AcOH–H_2O, 3 3 494), 600 V, 1 5 hr Cellulose TLC two solvent systems, (A) *n*-BuOH–HCO_2H–H_2O, 6·1.2, (B) *n*-BuOH–HOAc–H_2O, 4·1·5. Mushrooms were collected in 1986 in Nagaoka city, Japan, and stored at −20°

Isolation of 4-aminopyridine-2,3-dicarboxylic acid (**1**) Frozen fruit bodies (3 6 kg) were extracted with H_2O (7 l) at 4° overnight.

*Author to whom correspondence should be addressed.



The extract was filtered off and the residue was extracted a further three times The combined extracts were concd under red pres to *ca* 300 ml. To the turbid soln was added Me_2CO (2 l) and the mixture was allowed to stand at 4° overnight The ppt formed in the brownish soup was then separated by decantation and dissolved in 200 ml H_2O Removal of the solvent (*in vacuo*) left a pasty soup, which was dialysed against H_2O (3 l) at 4° overnight Nondialysate was concd and dialysed a further three times. The combined dialysates were evapd *in vacuo*, and the residue (130 g) applied to a column of active charcoal (130 g, packed in H_2O) The column was eluted with a stepwise gradient of H_2O–EtOH (H_2O, 2 5, 5, 10 and 30% aq EtOH, each 4 l) The 2 5–10% aq EtOH fractions were collected and the solvent was removed *in vacuo* (7 5 g) The residue was chromatographed on a column of weakly basic ion-exchange resin (Amberlite IR-45, HCO_3^- form, 320 g) using H_2O–HCO_2H (2, 5, 10 and 20% aq HCO_2H each 2 l) as a solvent The eluate with 5–20% aq HCO_2H was concd *in vacuo* and the resultant paste (1 1 g) was subjected to paper electrophoresis (46 × 20 cm, 170 sheets) The blue fluorescent band at +9 cm was cut out and the strips extracted with H_2O The extracted mass (0 29 g) containing mainly **1**–**3** was placed on cellulose TLC (20 × 20 cm, 57 sheets) and developed with solvent system A The fluorescent band at R_f 0 41 was extracted with H_2O In this procedure **3** (R_f 0 28) was separated The extract (0 043 g) was chromatographed on cellulose TLC (20 × 20 cm, 10 sheets) developed twice with solvent system B Crude **1** was obtained from the band at R_f 0 44 which absorbed UV light In this procedure acromelic acid A (R_f 0 35) was separated Crude **1** (4 mg) was further purified by paper electrophoresis (46 × 20 cm, 5 sheets, UV absorbing band at +9 cm) and pure **1** (2 mg) was finally obtained by recrystallization from H_2O Found C, 45 99, H, 3 29, N, 15 39 $C_7H_6O_4N_2$ requires C, 46 16, H, 3 32; N, 15 38%, EIMS 70 eV *m/z* (rel int) 164 $[M-H_2O]^+$ (6), 138 $[M-CO_2]^+$ (35), 120 $[M-H_2O-CO_2]^+$ (39), 93 $[M-CO_2\times2-H]^+$ (26) and 44 (100), HR-EIMS *m/z* 164 0226 (Calc for $C_7H_4O_3N_2$ $[M-H_2O]^+$ 164 0222), 138 0435 (Calc for $C_6H_6O_2N_2$ $[M-CO_2]^+$ 138 0430) 120 0329 (Calc for $C_6H_4ON_2$ $[M-CO_2-H_2O]$ 120 0324) UV $\lambda_{max}^{H_2O}$ nm (log ε) pH 7 265 (3 96), pH 1 262 (3 98), pH 11 290 (3 32) and 245 (sh) (3 94), IR ν_{max}^{nujol} cm^{-1} 3330, 1650 and 1470, ^1H NMR (90 MHz) and ^{13}C NMR in the text, FAB and FDMS in the text

4-Amino-2,3-dimethylpyridine (**8**) To a soln of 2,3-dimethyl-4-nitropyridine *N*-oxide (**7**) (200 mg) in AcOH (6 ml) was added iron powder (550 mg) and the mixture was heated at 100 with stirring for 2 hr The cooled reaction mixture was diluted with water, adjusted to pH 10 -11 with 3 M aq NaOH, 4 ml of Et_2O was added, and the pptd Fe hydroxide removed by filtration The collected solid was washed with Et_2O the aq filtrate was extracted several times with Et_2O, and the combined ethereal soln was dried over Na_2SO_4 and evapd to a dry mobile powder The product was almost pure 4-amino-2,3-dimethylpyridine (**8**) (82 mg, 56%) EIMS 70 eV *m/z* (rel int) 122 $[M]^+$ (100), 107 $[M-Me]^+$ (7), 94 $[M-H-HCN]^+$ (18) and 80 $[M-Me-HCN]^+$ (45), HR-EIMS 122 0846 (Calc for $C_7H_{10}N_2$ 122.0845), UV λ_{max}^{MeOH} nm (log ε) 246 (3 95) and 266 (3 71); IR ν_{max}^{nujol} cm^{-1} 3446, 3346, 3206, 1640, 1590 and 1465, ^1H NMR (90 MHz, $CDCl_3$) δ 2 05 (3H, *s*), 2 45 (3H, *s*), 6 10 (2H, *br s*), 6 39 (1H, *d*, *J* = 5 5 Hz) and 7 97 (1H, *d*, *J* = 5 5 Hz)

4-Aminopyridine-2,3-dicarboxylic acid (**1**) A soln of **8** (400 mg) in Ac_2O (3 ml) was heated under reflux for 10 min, and cooled The excess Ac_2O and HOAc formed were evapd under red pres Purification of the residual oil by chromatography on a silica gel column ($CHCl_3$–7% MeOH–$CHCl_3$) afforded 4-acetamido-2,3-dimethylpyridine (446 mg, 83%) EIMS 70 eV *m/z* (rel int) 164 $[M]^+$ (50), 122 $[M-COCH_2]^+$ (100), 107 $[M-COCH_2-Me]^+$ (4), 94 $[M-COMe-HCN]^+$ (9) and 80 $[M-COCH_2-Me-HCN]^+$ (19), HR-EIMS 164 0947, (Calc for $C_9H_{12}ON_2$ 164 0948), UV λ_{max}^{MeOH} nm (log ε) 252 (4 00), IR ν_{max}^{nujol} cm^{-1} 3340, 1700, 1600 and 1465, ^1H NMR (90 MHz, $CDCl_3$) δ 2 09 (3H, *s*), 2 14 (3H, *s*), 2 45 (3H, *s*), 7 50 (1H, *br s*) 7 71 (1H, *d*, *J* = 5 5 Hz) and 8 18 (1H, *d*, *J* = 5 5 Hz)

To a soln of above product (135 mg) in H_2O (8 ml), was added $KMnO_4$ (494 mg) and the mixture kept at 90° with stirring until the violet colour disappeared (*ca* 2 hr) To the mixture was again added $KMnO_4$ (494 mg) and kept at 90° with stirring until the colour disappeared (*ca* 6 hr) The soln was filtered with suction and collected MnO_2 washed several times with hot H_2O. The combined filtrates were evapd to dryness and the residue was dissolved in H_2O (5 ml) The soln was acidified to pH 3 with conc HCl, and heated under reflux with stirring for 1 hr The reaction mixture was cooled and evaporated to dryness and the residue was subjected to CC on a strongly basic ion-exchange resin (Amberlite IRA-400, OH^- form, 50 g). The column was eluted with 500 ml each of H_2O, 0 2 M, 0 5 M and 1 M HCl. The eluates with 0.2–1 M HCl were combined and evapd to dryness. The residue was purified with paper electrophoresis (46 × 20 cm, 25 sheet, +9 cm) to afford crude **1**. Finally, pure **1** (11 mg, 7.3% from 4-acetamido-2,3-dimethylpyridine) was obtained by recrystallization from H_2O

Esterification of **1** (A) To a soln of **1** (*ca* 500 μg) in MeOH (3 drops) was added CH_2N_2 in Et_2O with stirring until the colour of the soln became yellow and generation of N_2 ceased The soln was further stirred for 10 min and then evapd *in vacuo*. Purification of the residue by silica-gel TLC (Me_2CO–$CHCl_3$ (2:3) R_f 0.53) afforded pure 4-amino-2,3-dimethoxycarbonylpyridine (**6**) FDMS *m/z*: 210 $[M]^+$; EIMS 70 eV *m/z* (rel. int.). 210 $[M]^+$ (9), 179 $[M-OMe]^+$ (7), 151 $[M-CO_2Me]^+$ (8) and 44 (100); HR-EIMS 210.0620. (Calc for $C_9H_{10}O_4N_2$ 210 0641); UV λ_{max}^{MeOH}: 250 and 313, ^1H NMR (270 MHz, $CDCl_3$): δ 3.87 (3H, *s*), 3 95 (3H, *s*), 6 10 (2H, *br s*), 6 60 (1H, *d*, $J = 5.8$ Hz) and 8 18 (1H, *d*, $J = 5$ 8 Hz)

(B) 5 drops of MeOH saturated with HCl gas was added to **1** (*ca* 500 μg) and the mixture was heated at 90° in a sealed tube for 4 hr. The soln was evapd *in vacuo* Dil $NaHCO_3$ soln was added to the residue and extracted several times with $CHCl_3$ Pure **6** was obtained from the $CHCl_3$ extract

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