The Structure of a New Dipeptide from the Mushroom Clitocybe acromelalga

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Introduction

Clitocybe acromelalga (Japanese name: Dokusa-sako), found only in Japan, is a famous toadstool that has a unique biological activity. The accidental ingestion of this fungus causes violent pain, a marked reddish edema on the hands and feet after several days, and this pain continues for about a month (Miura, 1936). These physiologically characteristic properties prompted us to study the toxic constituent of this mushroom. Although it was difficult to reproduce the symptoms in experimental animals, on the basis of a lethal effect in mice acromelic acids A and B were isolated as the toxic principles (Konno et al., 1988). Acromelic acids are the extraordinarily potent neuroexcitatory amino acids. Because of their remarkable physiological activity, they attract significant interest both pharmacologically and physiologically. Not only the complete study of these acids (Ishida and Shinozaki, 1991; Shinozaki and Ishida, 1991) but also investigation of other toxins is expected. Further separation of the water extracts of this mushroom led to isolation of nine new compounds with structures 1–9 (Fig. 1) (Konno et al., 1982; Yamano and Shirahama, 1994; Hirayama et al., 1989; Konno et al., 1984; Yamano et al., 1991; Yamano and Shirahama, 1992; Yamano et al., 1992; Yamano and Shirahama, 1993; Yamano et al., 1993; Yamano and Shirahama, 1993). Amino acids 6–8 exhibited a weakly depolarizing activity in the preparation of the spinal cord of a newborn rat. New pyridine nucleoside 1 and nucleotide 2 also showed a lethal effect in mice on intraperitoneal injection.

\(\gamma\)-Aminobutyric acid (GABA), which plays an important role in the central inhibitory process in mammals (Elliott, 1965), was also found from this fungus. Its function is just opposite to that of glutamic acid (Glu) within the central nervous system. The title mushroom biosynthesizes such compounds with opposite activities as instrumental in animals. \(N-\gamma\)-aminobutyryl-L-glutamic acid (10) was also isolated in the course of further investigation. Isolation of 10 is the first example of a natural product. We wish to describe the determination of structure and synthesis of 10.

Results and Discussion

The water extracts were diluted with acetone to give a copious residue which was dialyzed against water. The dialyzate was further fractionated by chromatography and paper electrophoresis (Scheme 1). Each step of the fractionation was carried out by monitoring a lethal effect in mice. A new compound 10 was contained in the poisonous fraction.

A new dipeptide 10, \([\alpha]_D^{20} + 18.0^\circ\) (c 0.16, H2O), showed a violet coloration with ninhydrin. The \(^1\)H NMR spectrum (250 MHz) of 10 in D2O indicated the presence of glutamic acid and \(\gamma\)-aminobutyric acid (GABA) moieties (Table). The FAB MS spectrum exhibited a protonated ion peak at \(m/z \text{ 233} [\text{M} + \text{H}]^+\), corresponding to the molecular formula \(\text{C}_9\text{H}_{17}\text{O}_3\text{N}_2\). These spectral data implied structures 10-\(\alpha\), -\(\gamma\) and -\(N\) as possible structures (Fig. 2).

Confirmation of the structure for 10 was achieved by synthesis (Scheme 2). Selective peptide bond formation is necessary for the synthesis of 10-\(\alpha\), and -\(\gamma\). To achieve this coupling reaction, a commercially available \(\gamma\)-benzyl-L-glutamate was used for the starting material, whose \(N\)-protected form 11 was coupled with methyl \(\gamma\)-aminobutyrate TFA salt (GABA-Me-TFA) assisted by diphenyl-
phosphoryl azide (DPPA) to yield an adduct 12 in 53% yield. It was deprotected to give 10-α in 84% yield from 12. Further, a glutamate derivative 11 was converted into a γ-free glutamate 13 in 94% yield which produced 10-γ (45% yield from 14) in a way similar to that employed in the synthesis of 10-α. On the other hand, the synthesis of 10-N was achieved starting from dimethylglutamate 15 which was coupled with γ-(N-tert-butyloxycarbonyl)aminobutyric acid (Boc-GABA) to provide an adduct 16 in 50% yield. Removal of protective groups yielded 10-N in 91% yield from 16.

The NMR spectrum, $[\alpha]_D^{20} +20.8^\circ$ (c 0.16, H$_2$O) and Rf value on cellulose TLC (Rf 0.46, *BuOH–
HCO_{2}H-H_{2}O, 6:1:2) of synthetic 10-N were completely coincident with those of the natural compound.

Garyaev et al. (1991) discussed the excitatory activity of some synthetic glutamate-containing dipeptides. They reported that the compound corresponding to 10 exhibited a seizure activity to convulsant potency of 11.4, relative to L-glutamate.

**Experimental**

**General procedure**

IR spectra were measured on a JASCO model IR-S spectrophotometer with a NaCl cell. Mass spectra were obtained on a JEOL model JMS-DX 300 spectrometer. Optical rotations were taken with a JASCO model DIP-360 automatic polarimeter. \( ^{1}H \) NMR spectra were recorded in D_{2}O on a Hitachi R-250 H (250 MHz) using 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (DSS) as an internal standard.

**Mushroom material**

Fruiting bodies of *Clitocybe acromelalga* were collected in autumn in Nagaokashi, Niigata-ken, Japan, and frozen upon collection, and stored at -20 °C.

**Isolation of 10**

Frozen fruit bodies (6 kg) were extracted with H_{2}O (71 × 3) at 4 °C overnight. The combined extracts were concentrated *in vacuo* to about 1 l. To this turbid solution acetone (2.5 l) was added and the mixture was allowed to stand at 4 °C overnight. The supernatant was decanted and the lower muddy layer was then evaporated. The copious residue was dialyzed against H_{2}O (31 × 4) at 4 °C overnight. The combined dialyzate was evaporated and the residue (338 g) was applied to a column of charcoal (300 g, packed in H_{2}O). The column was eluted stepwise with several concentrations of aq. EtOH (H_{2}O, 2.5, 5, 10 and 30% aq. EtOH, each 10 l). The 2.5–5% aq. EtOH fraction was collected and the solvent was removed *in vacuo* (42 g). The residue (6 g × 7) was chromatographed on a column of weakly basic ion-exchange resin (Amberlite IR-45, HCO_{2}~ form) using H_{2}O–HCO_{2}H (H_{2}O, 5, 10 and 20% aq. HCO_{2}H, each 61 l) as a solvent. The eluate with 10–20% aq. HCO_{2}H was concentrated *in vacuo* and a portion
of the resultant paste (3.7 g) was subjected to paper electrophoresis (46 x 20 cm, pH 4.6, 600 V, 1.5 h). An area of 0~3 cm was cut off and the strips were extracted with H$_2$O. The solvent was then removed. The residue was separated by HPLC (Shodex Sugar SC-1821: H$_2$O, 52 °C, 1 ml/min, R$_t$ 10~16 min). After removal of the solvent, the residue was placed on cellulose TLC and developed by a mixed solvent ("BuOH-HCO$_2$H-H$_2$O, 6:1:2). A new dipeptide 10 was isolated from the band at R$_f$ 0.45 (3.2 mg). 10: [a]$_D^2$ + 18.0° (c 0.16, H$_2$O); IR v$_{max}$ (nujol) cm$^{-1}$: 3680-2400 and 1770-1590; $^1$H NMR (250 MHz, D$_2$O) $\delta$ ppm: 1.86 (3H, m), 2.11 (1H, m), 2.32 (2H, t, J = 7.0 Hz), 2.39 (2H, t, J = 7.6 Hz), 2.91 (2H, t, J = 7.6 Hz) and 4.31 (1H, dd, J = 5.5, 9.2 Hz); HR-FAB MS found: m/z 233.1131 [M + H]$^+$, calcd for C$_9$H$_{17}$O$_5$N$_2$: 233.1138.

Synthesis of 12

To a stirred solution of γ-benzyl-L-glutamate (300 mg, 1.26 mmol) in H$_2$O-dioxane (2 ml~1 ml) was added triethylamine (154 mg, 1.52 mmol, 1.2 equiv.) and subsequently a solution of di-r-butyl dicarbonate (Boc$_2$O, 331 mg, 1.52 mmol, 1.2 equiv.) in dioxane (1 ml). The reaction mixture was allowed to stand at room temperature overnight and then neutralized with 1 N HCl. This solution was extracted with AcOEt (10 ml x 3). The combined extracts were dried over anhydrous sodium sulfate and evaporated to yield the N-protected form 11 (420 mg, quant.). To the mixture of 11 (100 mg, 0.30 mmol) and triethylamine (66.0 mg, 2.2 equiv.) in THF (1 ml) was added the solution of GABA-Me-TFA (69.3 mg, 0.30 mmol, 1.0 equiv.) in THF (0.5 ml) at 0 °C. To this mixture was subsequently added diphenylphosphoryl azide (DPPA, 82.0 mg, 0.30 mmol, 1.0 equiv.) in THF (1 ml) and allowed to stand at room temperature overnight. Water was added to the reaction mixture which was extracted with AcOEt (100 ml x 3). The combined extracts were dried over anhydrous sodium sulfate and evaporated. The residue was chromatographed on silica gel column (100 g, hexane-ether) to give an adduct 12 (69.0 mg, 53.2%). 12: IR v$_{max}$ (neat) cm$^{-1}$: 3680-3120, 1758-1620, 1580-1478, 1450, 1393, 1370, 1245, 1168, 870 and 757; $^1$H NMR (250 MHz,
The coupling product 12 (69.0 mg, 0.16 mmol) was stirred with palladium carbon powder (70.0 mg) in EtOH (3 ml) at room temperature under hydrogen gas for 1 h. This solution was filtered through Celite and evaporated. The residue was dissolved in MeOH (3 ml). To this stirred solution was added 1 N KOH (0.8 ml, 0.8 mmol, 5.0 equiv.), and the mixture was allowed to stand at room temperature overnight. After neutralization of the reaction mixture with 1 N HCl, it was extracted with AcOEt (10 ml × 3). The combined extracts were evaporated. The residue was stirred in TFA (1 ml) at room temperature. After 45 min, to the mixture was added H2O, and it was evaporated. Purification of the product was performed by paper electrophoresis to yield 10-α (31.0 mg, 3 steps 84.4%).

10-α: [α]D25 + 1.3° (c 0.83, H2O); IR νmax (nujol) cm−1: 3740−2400 and 1775−1590; 1H NMR (250 MHz, CDCl3) δ ppm: 1.81 (2H, m), 2.14 (2H, dt, J = 7.0, 7.5 Hz), 2.41 (2H, t, J = 7.5 Hz), 2.50 (2H, t, J = 7.0 Hz), 3.26 (2H, t, J = 6.5 Hz) and 3.98 (1H, t, J = 7.0 Hz); HR-FAB MS found: m/z 233.1134 [M + H]+, calcd for C9H17O3N2: 233.1138.

Synthesis of 14

The compound 11 (200 mg, 0.60 mmol) was dissolved in MeOH (10 ml). To this solution was added an ethereal solution of diazomethane until the yellow color was developed, and the solvent was then removed. This resultant residue and palladium carbon (200 mg) was stirred in EtOH at room temperature under hydrogen gas for 1 h. This reaction mixture was filtered through Celite. The filtrate was evaporated to provide 13 (147 mg, 2 steps 95%). To the mixture of 13 (100 mg, 0.38 mmol) and triethylamine (86.0 mg, 0.85 mmol, 2.2 equiv.) in THF (1 ml) was added the solution of GABA-Me-TFA (90.0 mg, 0.39 mmol, 1.0 equiv.) in THF (1 ml) at 0 °C. To this mixture was subsequently added DPPA (106 mg, 0.39 mmol, 1.0 equiv.) in THF (1 ml) and allowed to stand at room temperature overnight. Water was added to the reaction mixture which was extracted with AcOEt (100 ml × 3). The combined extracts were dried over anhydrous sodium sulfate and evaporated. The residue was chromatographed on silica gel column (100 g, hexane-ether) to give an adduct 14 (70.0 mg, 50.6%).

14: IR νmax (neat) cm−1: 3680−3120, 1780−1610, 1580−1490, 1450, 1373, 1235 and 1170; 1H NMR (250 MHz, CDCl3) δ ppm: 1.42 (9H, s), 1.78–2.00 (3H, m), 2.17 (1H, m), 2.25 (2H, m), 2.38 (2H, t, J = 7.0 Hz), 3.30 (2H, dt, J = 6.5 Hz), 3.68 (3H, s), 3.62 (3H, s), 3.74 (2H, s) and 4.27 (1H, m); EIMS (probe) 70 eV m/z (rel. int.): 360 [M]+ (C16H28O7N5) (1), 304 (8), 301 (10), 287 (8), 273 (4), 260 (4), 245 (5), 213 (2), 211 (3), 201 (4), 187 (13), 184 (42), 172 (6), 159 (25), 152 (10), 144 (18), 142 (12), 127 (5), 124 (5), 118 (15), 116 (31), 101 (20), 86 (60), 84 (43), 73 (8), 57 (100) and 41 (55); HR-MS found: m/z 360.1906 [M]+, calcd for C16H28O7N5: 360.1896.

10-γ

To the stirred solution of 14 (70.0 mg, 0.19 mmol) in MeOH (3 ml) was added 1 N KOH (0.95 ml, 0.95 mmol, 5.0 equiv.), and the mixture was allowed to stand at room temperature overnight. After neutralization of the reaction mixture with 1 N HCl, it was extracted with AcOEt (10 ml × 3). The combined extracts were evaporated. The residue was stirred in TFA (1 ml) at room temperature. After 45 min, to the mixture was added H2O, and it was evaporated. Purification of the product was performed by paper electrophoresis to yield 10-γ (40.0 mg, 2 steps 88.7%).

10-γ: [α]D25 + 2.8° (c 0.08, H2O); IR νmax (nujol) cm−1: 3660−2400 and 1730−1590; 1H NMR (250 MHz, D2O) δ ppm: 1.78 (2H, m), 2.18 (2H, m), 2.30−2.60 (4H, m), 3.19 (2H, t, J = 6.5 Hz) and 4.05 (1H, t, J = 6.5 Hz); HR-FAB MS found: m/z 233.1131 [M + H]+, calcd for C9H17O3N2: 233.1138.

Synthesis of 16

To the mixture of Boc-GABA (1.14 g, 5.62 mmol) and triethylamine (1.70 g, 16.8 mmol,
3.0 equiv.) in THF (10 ml) was added the solution of dimethylglutamate TFA salt (1.63 g, 5.64 mmol, 1.0 equiv.) in THF (15 ml) at 0 °C. To this mixture was subsequently added DPPA (1.55 g, 5.63 mmol, 1.0 equiv.) in THF (10 ml) and allowed to stand at room temperature overnight. Water was added to the reaction mixture which was extracted with AcOEt (100 ml x 3). The combined extracts were dried over anhydrous sodium sulfate and evaporated. The residue was chromatographed on silica gel column (100 g, hexane-ether) to give an adduct 16 (1.0 g, 49.5%). 16: IR ν max (neat) cm⁻¹: 3620–3160, 1770–1630, 1565–1498, 1450, 1370, 1250 and 1172; ¹H NMR (250 MHz, CDCl₃), δ ppm: 1.42 (9H, s), 1.80 (2H, m), 2.03 (1H, m), 2.14–2.34 (3H, m), 2.42 (2H, m), 3.19 (2H, m), 3.67 (3H, s), 3.73 (3H, s) and 4.59 (1H, m); EIMS (probe) 70 eV m/z (rel. int.); 360 [M⁺]⁺ (C₁₆H₂₈O₇N₂) (1), 304 (5), 303 (5), 287 (17), 273 (6), 259 (2), 242 (25), 227 (4), 217 (85), 169 (78), 150 (84), 138 (78) 123 (91), 101 (100), 98 (9), 86 (41), 84 (57), 69 (17), 57 (96) and 41 (53); HR-MS found: m/z 360.1874 [M⁺], calcd for C₁₆H₂₈O₇N₂: 360.1896.

To the stirred solution of 16 (90 mg, 0.25 mmol) in MeOH (3 ml) was added 1 N KOH (1.25 ml, 1.25 mmol, 5.0 equiv.), and the mixture was allowed to stand at room temperature overnight. After neutralization of the reaction mixture with 1 N HCl, it was extracted with AcOEt (10 ml x 3). The combined extracts were evaporated to give 17 (76.4 mg, 92.1%). The compound 17 (32 mg, 0.01 mmol) was stirred in TFA (1 ml) at room temperature. After 45 min, to the mixture was added H₂O, and it was evaporated. Purification of the product was performed by paper electrophoresis to yield 10-N (28.6 mg, quant.). 10-N: [α]D +20.8° (c 0.16, H₂O); IR ν max (nujol) cm⁻¹: 3680–2400 and 1770–1590; ¹H NMR (250 MHz, D₂O) δ ppm: 1.86 (3 H, m), 2.11 (1 H, m), 2.32 (2H, t, J = 7.0 Hz), 2.39 (2H, t, J = 7.6 Hz), 2.91 (2H, t, J = 7.6 Hz) and 4.31 (1H, dd, J = 5.5, 9.2 Hz); HR-FAB MS found: m/z 233.1121 [M + H]⁺, calcd for C₉H₁₇O₅N₂: 233.1137.