The Structure of a New Dipeptide from the Mushroom *Clitocybe acromelalga*

K. Yamano and H. Shirahama
Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060, Japan

Z. Naturforsch. **49c,** 157–162 (1994); received December 13, 1993

Clitocybe acromelalga, Toadstool, GABA, Dipeptide, *N*-(γ-aminobutyryl)-L-glutamic Acid

From the poisonous mushroom *Clitocybe acromelalga,* a new glutamate-containing dipeptide was isolated. Its structure was deduced to be *N*-(γ-aminobutyryl)-L-glutamic acid (**10**) based on spectral data and was confirmed by synthesis.

Introduction

Clitocybe acromelalga (Japanese name: Dokusasako), 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.

γ-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*-(γ-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,** $[\alpha]_D^{26} + 18.0°$ (c 0.16, H_2O), showed a violet coloration with ninhydrin. The ^1H NMR spectrum (250 MHz) of **10** in D_2O indicated the presence of glutamic acid and γ-aminobutyric acid (GABA) moieties (Table). The FAB MS spectrum exhibited a protonated ion peak at m/z 233 $[M + H]^+$, corresponding to the molecular formula $C_9H_{17}O_5N_2$. These spectral data implied structures **10**-α, -γ and -*N* as possible structures (Fig. 2).

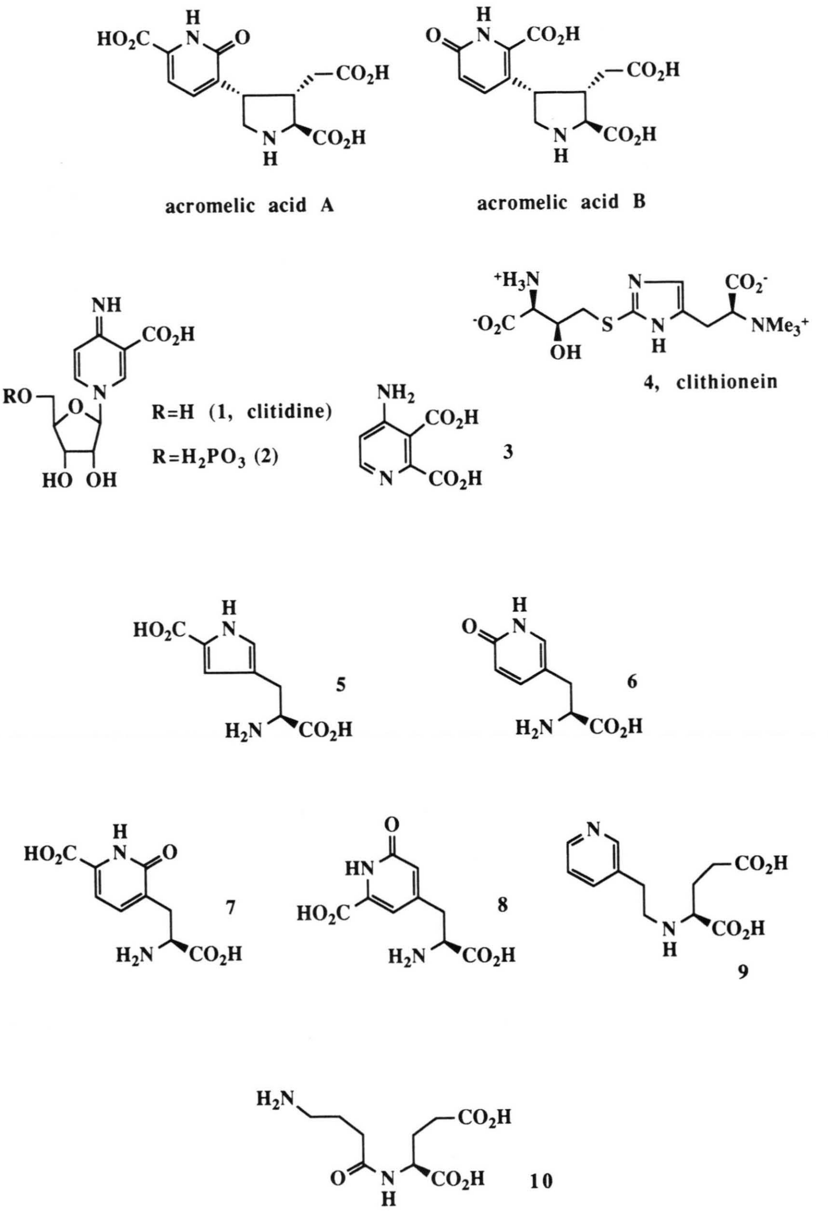
Confirmation of the structure for **10** was achieved by synthesis (Scheme 2). Selective peptide bond formation is necessary for the synthesis of **10**-α, and -γ. To achieve this coupling reaction, a commercially available γ-benzyl-L-glutamate was used for the starting material, whose *N*-protected form **11** was coupled with methyl γ-aminobutyrate TFA salt (GABA-Me-TFA) assisted by diphenyl-

---

Reprint requests to Prof. Dr. H. Shirahama.

Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0939–5075/94/0300–0157 $ 03.00/0

Fig 1.



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*-*t*-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, $[\alpha]_D^{26}$ (+20.8°, *c* 0.16, H_2O) and R*f* value on cellulose TLC (R*f* 0.46, ^{n}BuOH−

HCO_2H-H_2O, 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.

Scheme 1.

fruit bodies (6 kg)
│ **H_2O**
extracts
│ **acetone**
precipitates
│ **dialysis**
dialyzate (338 g)
│ **charcoal**
2.5~5% aq. EtOH fr. (41 g)
│ **IR-45 (HCO_2^- form)**
10~20% aq. HCO_2H fr. (3.7 g)
│ **PEP (pH 4.6, 600 V, 1.5 h)**
0~+3 cm fr.
│ **HPLC (Shodex Sugar SC-1821)**
R*t* 10~16 min
│ **cellulose TLC (^{n}BuOH-HCO_2H-H_2O, 6:1:2)**
R*f* 0.45
(10, 3.2 mg)

Table. ^{1}H NMR data of **10**.

10	*cf.* GABA (250 MHz, D_2O)
δ 1.86 (3 H, m)	δ 1.80 (2 H, m)
2.11 (1 H, m)	2.19 (2 H, t, *J* = 7.5)
2.32 (2 H, t, *J* = 7.0)	2.90 (2 H, t, *J* = 7.3)
2.39 (2 H, t, *J* = 7.6)	
2.91 (2 H, t, *J* = 7.6)	
4.31 (1 H, dd, *J* = 5.5, 9.2)	

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_2O 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_2O (7 l × 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_2O (3 l × 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_2O). The column was eluted stepwise with several concentrations of aq. EtOH (H_2O, 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_2O-HCO_2H (H_2O, 5, 10 and 20% aq. HCO_2H, each 6 l) as a solvent. The eluate with 10−20% aq. HCO_2H was concentrated *in vacuo* and a portion

10-α **10-γ** **10-*N***

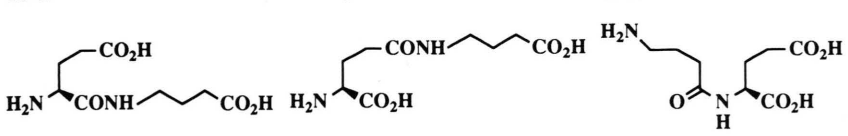
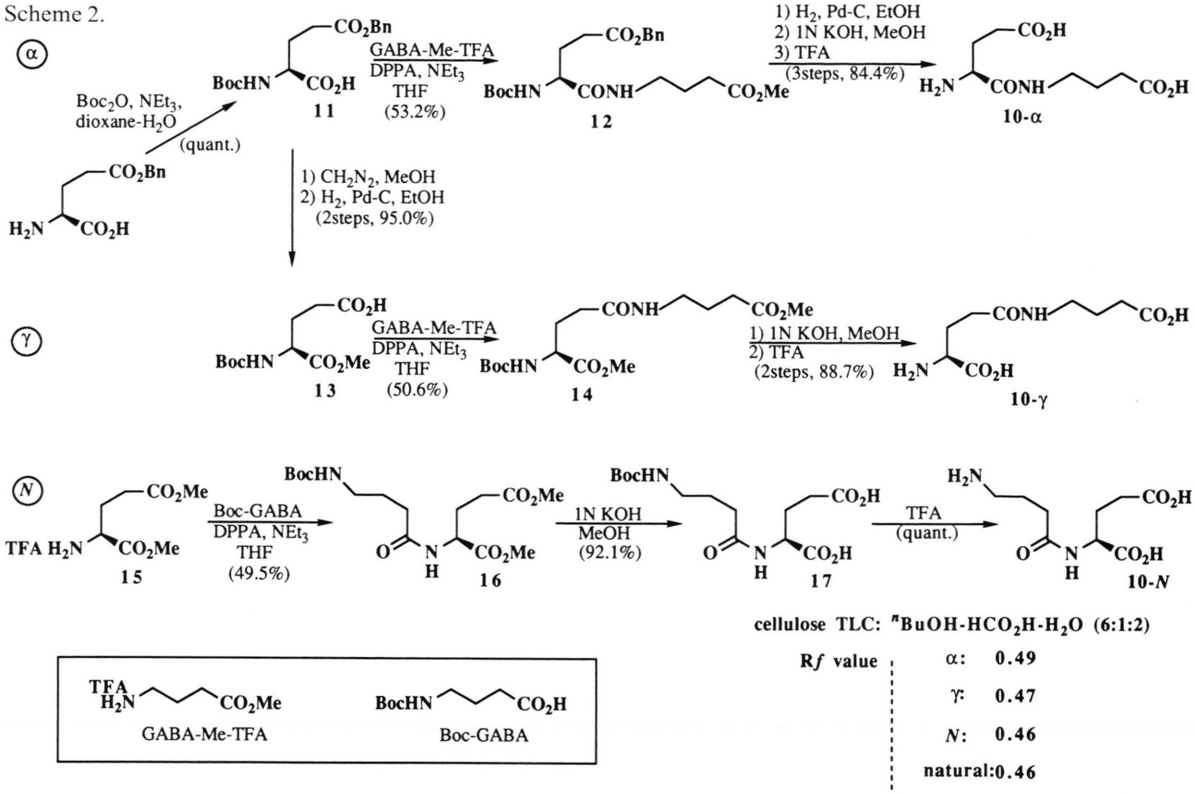


Fig. 2. Possible structures.

Scheme 2.



cellulose TLC: ^{n}BuOH-HCO$_2$H-H$_2$O (6:1:2)

Rf value
α: 0.49
γ: 0.47
N: 0.46
natural: 0.46

of the resultant paste (3.7 g) was subjected to paper electrophoresis (46 × 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_2O. The solvent was then removed. The residue was separated by HPLC (Shodex Sugar SC-1821: H_2O, 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 (^{n}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:** $[\alpha]_D^{26}+18.0°$ (*c* 0.16, H_2O); IR ν_{max} (nujol) cm^{-1}: 3680$-$2400 and 1770$-$1590; ^{1}H NMR (250 MHz, D_2O) δ ppm: 1.86 (3 H, m), 2.11 (1 H, m), 2.32 (2 H, t, $J = 7.0$ Hz), 2.39 (2 H, t, $J = 7.6$ Hz), 2.91 (2 H, t, $J = 7.6$ Hz) and 4.31 (1 H, dd, $J = 5.5$, 9.2 Hz); HR-FAB MS found: m/z 233.1131 $[M+H]^+$, calcd for $C_9H_{17}O_5N_2$: 233.1138.

Synthesis of **12**

To a stirred solution of γ-benzyl-L-glutamate (300 mg, 1.26 mmol) in H_2O-dioxane (2 ml $-$ 1 ml) was added triethylamine (154 mg, 1.52 mmol, 1.2 equiv.) and subsequently a solution of di-*t*-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 × 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 × 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 ν_{max} (neat) cm^{-1}: 3680$-$3120, 1758$-$1620, 1580$-$1478, 1450, 1393, 1370, 1245, 1168, 870 and 757; ^{1}H NMR (250 MHz,

$CDCl_3$) δ ppm: 1.42 (9 H, s), 1.76−1.98 (3 H, m), 2.13 (1 H, m), 2.31−2.61 (4 H, m), 3.28 (2 H, q, $J = 6.5$ Hz), 3.64 (3 H, s), 4.10 (1 H, m), 5.15 (2 H, s), and 4.31 (1 H, dd, $J = 5.5$, 9.2 Hz); EIMS (probe) 70 eV *m/z* (rel. int.): 436 $[M]^+$ ($C_{22}H_{32}O_7N_2$) (1), 380 (2), 363 (2), 305 (3), 292 (17), 236 (15), 192 (61), 129 (6), 112 (10), 108 (8), 102 (5), 91 (100), 84 (30), 79 (7), 69 (5), 65 (5), 57 (84) and 41 (23); HR-MS found: *m/z* 436.2221 $[M]^+$, calcd for $C_{22}H_{32}O_7N_2$: 436.2210.

10-α

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 H_2O, 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**: $[\alpha]_D^{25}$ + 1.3° (*c* 0.83, H_2O); IR ν_{max} (nujol) cm^{-1}: 3740−2400 and 1775−1590; ^1H NMR (250 MHz, D_2O) δ ppm: 1.81 (2 H, m), 2.14 (2 H, dt, $J = 7.0$, 7.5 Hz), 2.41 (2 H, t, $J = 7.5$ Hz), 2.50 (2 H, t, $J = 7.0$ Hz), 3.26 (2 H, t, $J = 6.5$ Hz) and 3.98 (1 H, t, $J = 7.0$ Hz); HR-FAB MS found: *m/z* 233.1134 $[M+H]^+$, calcd for $C_9H_{17}O_5N_2$: 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 ν_{max} (neat) cm^{-1}: 3680−3120, 1780−1610, 1580−1490, 1450, 1373, 1235 and 1170; ^1H NMR (250 MHz, $CDCl_3$) δ ppm: 1.42 (9 H, s), 1.78−2.00 (3 H, m), 2.17 (1 H, m), 2.25 (2 H, m), 2.38 (2 H, t, $J = 7.0$ Hz), 3.30 (2 H, dt, $J = 6.5$ Hz), 3.68 (3 H, s), 3.74 (3 H, s) and 4.27 (1 H, m); EIMS (probe) 70 eV *m/z* (rel. int.): 360 $[M]^+$ ($C_{16}H_{28}O_7N_2$) (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 $C_{16}H_{28}O_7N_2$: 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 H_2O, 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**: $[\alpha]_D^{23}$ + 2.8° (*c* 0.08, H_2O); IR ν_{max} (nujol) cm^{-1}: 3660−2400 and 1730−1590; ^1H NMR (250 MHz, D_2O) δ ppm: 1.78 (2 H, m), 2.18 (2 H, m), 2.30−2.60 (4 H, m), 3.19 (2 H, t, $J = 6.5$ Hz) and 4.05 (1 H, t, $J = 6.5$ Hz); HR-FAB MS found: *m/z* 233.1131 $[M+H]^+$, calcd for $C_9H_{17}O_5N_2$: 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 × 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, $CDCl_3$), 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 $[M]^+$ ($C_{16}H_{28}O_7N_2$) (1), 304 (5), 303 (5), 287 (17), 273 (6), 259 (2), 242 (25), 227 (4), 217 (85), 201 (14), 185 (10), 176 (18), 158 (20), 144 (48), 131 (26), 116 (100), 98 (9), 86 (41), 84 (57), 69 (17), 57 (96) and 41 (53); HR-MS found: *m/z* 360.1874 $[M]^+$, calcd for $C_{16}H_{28}O_7N_2$: 360.1896.

10-*N*

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 × 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 H_2O, and it was evaporated. Purification of the product was performed by paper electrophoresis to yield **10-*N*** (28.6 mg, quant.). **10-*N*:** $[\alpha]_D^{26} + 20.8°$ (*c* 0.16, H_2O); IR ν_{max} (nujol) cm^{-1}: 3680–2400 and 1770–1590; ^1H NMR (250 MHz, D_2O) δ 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* 233.1121 $[M + H]^+$, calcd for $C_9H_{17}O_5N_2$: 233.1137.

Elliott K. A. C. (1965), γ-Aminobutyric acid and other inhibitory substances. Brit. Med. Bull. **21,** 70–75.

Garyaev A. P., Dumpis M. A., Poznjakova L. N., Zaitzev Y. V. and Piotrivsky L. B. (1991), Excitatory action of some aspartate- and glutamate-containing dipeptides after intracerebroventricular injection in mice. European J. Pharmacol. **197,** 157–160.

Hirayama F., Konno K., Shirahama H. and Matsumoto T. (1989), 4-Aminopyridine-2, 3-dicarboxylic acid from *Clitocybe acromelalga.* Phytochemistry **28,** 1133–1135.

Ishida M. and Shinozaki H. (1991), Novel kainate derivatives: potent depolarizing actions on spinal motoneurons and dorsal root fibres in newborn rats. Brit. J. Pharmacol. **104,** 873–878.

Konno K., Hashimoto K., Ohfune Y., Shirahama H. and Matsumoto T. (1988), Acromelic acids A and B. Potent neuroexcitatory amino acids isolated from *Clitocybe acromelalga.* J. Am. Chem. Soc. **110,** 4807–4815.

Konno K., Hayano K., Shirahama H., Saito H. and Matsumoto T. (1982), Clitidine, a new toxic pyridine nucleoside from *Clitocybe acromelalga.* Tetrahedron **38,** 3281–3284.

Konno K., Shirahama H. and Matsumoto T. (1984), Clithioneine, an amino acid betaine from *Clitocybe acromelalga.* Phytochemistry **23,** 1003–1006.

Miura O. (1936), Studies on the pharmacological effect of *Clitocybe acromelalga,* Ichimura and Biological effect of *Clitocybe acromelalga,* Ichimura on a rooster. Tohoku J. Exp. Med. **30,** 150–169 and 196–202.

Shinozaki H. and Ishida M. (1991), Recent advance in the study of glutamate receptor agonists. Asia Pacific J. Pharmacol., 293–316.

Yamano K. and Shirahama H. (1994), Clitidine 5′-mononucleotide, a toxic pyridine nucleotide from *Clitocybe acromelalga.* Phytochemistry, in press.

Yamano K., Konno K. and Shirahama H. (1991), A new amino acid, L-3-(2-carboxy-4-pyrrolyl)alanine from the poisonous mushroom *Clitocybe acromelalga.* Chem. Lett., 1541–1542.

Yamano K. and Shirahama H. (1992), New amino acids from the poisonous mushroom *Clitocybe acromelalga.* Tetrahedron **48,** 1457–1464.

Yamano K., Hashimoto K. and Shirahama H. (1992), Novel neuroexcitatory amino acid from *Clitocybe acromelalga.* Heterocycles **34,** 445–448.

Yamano K. and Shirahama H. (1993), Isolation of L-*N*-[2-(3-pyridyl)ethyl]-glutamic acid from the poisonous mushroom *Clitocybe acromelalga.* A possible intermediate in the biogenesis of acromelic acids. Chem. Lett., 21–24.

Yamano K., Konno K. and Shirahama H. (1993), A novel neuroexcitatory amino acid from *Clitocybe acromelalga.* A possible intermediate in the biogenesis of acromelic acid A. Heterocycles **35,** 125–128.

Yamano K. and Shirahama H. (1993), New amino acids from *Clitocybe acromelalga.* Possible intermediates in the biogenesis of mushroom toxins, acromelic acids. Tetrahedron **49,** 2427–2436.