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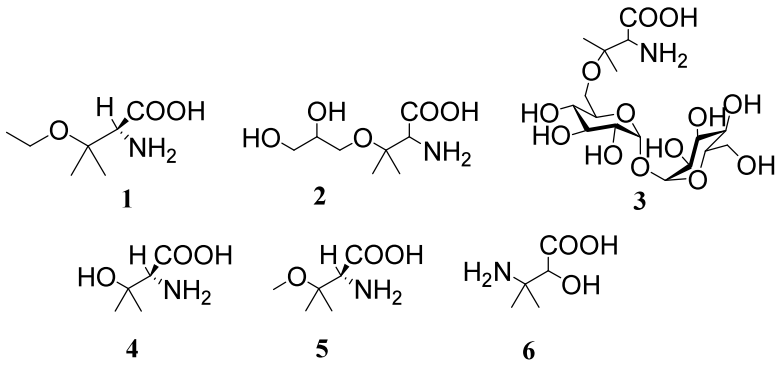

Unusual amino acid derivatives from the mushroom *Pleurocybella porrigens*

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Abstract —Three new amino acid derivatives (**1-3**) and three known ones (**4-6**) were isolated from the mushroom *Pleurocybella porrigens*. The structures of **1-6** were determined by the interpretation of spectroscopic data. Compounds **1, 3, 4,** and **5** were toxic to mouse cerebrum glial cells.

1. Introduction

The mushroom *Pleurocybella porrigens* (Angel's wings in English; Sugihiratake in Japanese) is widespread and common throughout temperature regions of the world. It has been eaten for a long time all over the world. However, in autumn 2004 in Japan, fifty-five people got poisoned by eating this mushroom, and seventeen people among them died of acute encephalopathy.

Epidemiological investigation found that the most patients had been on hemodialysis treatment for chronic renal failure and had digested the fruiting bodies of the fungus between one day and two weeks before the onset of neurological symptoms[1-3]. Therefore, highly possible cause of the incident was identified to be the fruiting bodies. The fruiting bodies cannot be cultivated artificially. Local people harvest and consume the fruiting bodies that grow in the forest during the late summer and autumn. However, there had been no report regarding toxicity of the fruiting bodies until the incident.

After the incident, several chemical investigations have been accomplished on the mushroom *P. porrigens*. Sasaki *et al*. performed the comparative chemical analyses on the fruiting bodies that were collected at the various areas including the region of acute encephalopathy incidents in Japan[4]. As a result, the high content of vitamin D analogues was found in the fruiting bodies collected in the regions where acute encephalopathy incidents happened. The involvement of aberrant calcium metabolism caused by the vitamin D-related compounds was claimed to cause the acute encephalopathy in the paper[4]. Ohta *et al.* isolated a cytotoxic ketonic fatty acid, 14-hydroxy-9-oxooctadeca-10, 12-dienoic acid, from the mushroom[5]. The ketonic acid exhibited toxicity to human myeloma cells and murine melanoma cells. Ohta *et al.* also suggested that *N*-

glycolylneuraminic acid in the mushroom might be related to the incidents[6]. In addition, we have reported the purification, characterization and cDNA cloning of a lectin from the mushroom[7]. However, relationship between the chemical constituents and the acute encephalopathy incidents has not been clarified yet.

Under these circumstances, we tried to isolate toxic compounds against a kind of brain cells, glial cells, from the mushroom. As a result, six compounds (**1**-**6**) including three novel ones (**1**-**3**) were isolated from the mushroom. Here we report the isolation, structural determination, and toxicity of the compounds.

2. Results and Discussion

Lyophilized fruiting bodies of *Pleurocybella porrigens* were successively extracted with hexane, EtOAc, EtOH, H_2O, and then boiled H_2O. The EtOH fraction which showed cytotoxicity to mouse glial cells was subjected to open silica gel column chromatography, eluting with stepwise by acetone and 90% MeOH. The 90% MeOH fraction was separated with repeated HPLC to afford compounds **1**-**6**.

Compound **1** was isolated as white crystals, and its HR-ESIMS analysis data indicated the molecular formula of $C_7H_{16}NO_3$. The analyses of ^{1}H-NMR, ^{13}C-NMR, DEPT and HMQC spectra indicated the presence of three methyls, a methylene, a methine, a quaternary carbon, and a carboxyl (Table 1). The moiety of 2-amino-3-methylbutanoic acid (2-hydroxyvaline) was constructed by the HMBC correlations (H2/C1, H2/C3, H2/C4, H2/3-CH_3, H4/C2, H4/C3, H4/3-CH_3, 3-CH_3/C2, 3-CH_3/C3, and 3-CH_3/C4) as shown in Fig. 1. The presence of an ethoxy group was suggested by COSY correlation between H1' and H2' and HMBC correlation from H2' to C1' and H1' to C2'. The linkage between the 2-hydroxyvaline moiety and the ethoxy group was determined by HMBC correlation from H1' to C3. Therefore, the planar structure of **1** was determined as 2-amino-3-ethoxy-3-methylbutanoic acid.

Compound **2** was isolated as white crystals and the HR-ESIMS analysis data indicated the molecular formula of $C_8H_{17}NO_5$. The analyses of ^{1}H-NMR, ^{13}C-NMR, DEPT and HMQC spectra indicated the presence of two methyls, two methylenes, two methines, a quaternary carbon, and a carboxyl (Table 1.). The HMBC data indicated that this compound also had a 2-hydroxyvaline moiety (Fig. 1). The HMBC correlations (H1'/C2', H1'/C3', H2'/C1', H2'/C3', H3'/C2', H3'/C1'), downfield-shifted chemical shift values (H2' δ 3.69; C2' δ 71.7; H3' δ 3.43, 3.46; C3' δ 63.3), and the molecular formula indicated the presence of a 2,3-dihydroxypropoxy residue. The linkage of this

residue to C3 through an oxygen atom was suggested by the HMBC correlation (H1'/C3). All the data suggested that **2** was 2-amino-3-(2,3-dihydroxypropoxy)- 3,3-dimethylpropanoic acid.

Compound **3** was isolated as white crystals, and the HR-ESIMS data analysis indicated the molecular formula of $C_{17}H_{31}NO_{13}$. The analyses of ^{1}H-NMR, ^{13}C-NMR, DEPT, HMQC and HMBC spectra indicated the presence of two methyls, two methylenes, eleven methines, a quaternary carbon, and a carboxyl (Table 1) and also suggested the involvement of a 3-hydroxyvaline residue as **1** and **2**. Judging from the results that the rest of the structure had the formula of $C_{12}H_{23}O_{12}$ and the characteristic signals of two anomeric protons (H1' δ 5.04; C1' δ 94.4; H1" δ 5.02; C1" δ 94.3) were observed in the NMR spectra, the presence of a disaccharide was indicated. Sugar composition analysis of the compound detected Glc only. The ^{1}H- and ^{13}C-NMR data of the saccharide part in **3** were very similar to those of trehalose (Table 1). The coupling constants of two anomeric protons (J = 3.7 Hz and 3.9 Hz) and the HMBC correlations (H1'/C1", H1"/C1') confirmed that the disaccharide was α,α-trehalose. Since the specific rotation of **3** ($[\alpha]^{30}_{D}$ +130 (H_2O, c 0.10)) was similar to that of α,α-trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) ($[\alpha]^{30}_{D}$ +178 (H_2O, c 7.0), the constituent sugar in **3** was deduced to be D-Glc. The ether bond between C6' and C3 was indicated by the HMBC correlation from H6' to C3 and the downfield-shifted chemical shifts (H6' δ 3.57, 3.73; C6' δ 60.9; C3 δ 75.8). Therefore, the structure of **3** was deduced to be as shown.

Compounds **4**, **5**, and **6** were identified as 2-amino-3-hydroxy-3-methylbutanoic acid, 2-amino-3-methoxy-3-methylbutanoic acid, and 3-amino-2-hydroxy-3-methylbutanoic acid, respectively, by the analyses of spectroscopic data. Compound **4** has been synthesized and isolated from this mushroom[8, 9]. Compounds **5** and **6** have

1 been already synthesized[10, 11]. However, this report is the first isolation of **5** and **6** from
2 a natural source.
3 The absolute configurations of **4** and **5** were determined to be *S* by comparison of
4 their specific rotation values with those reported previously[11, 12]: **4**, $[\alpha]^{30}_{D}$ +3.8 (H_2O, *c*
5 0.13), reported data, $[\alpha]^{30}_{D}$ +4.0 (H_2O, *c* 0.20); **5**, $[\alpha]^{30}_{D}$ +10 (H_2O, *c* 0.4), reported data,
6 $[\alpha]^{30}_{D}$ +11.0 (H_2O, *c* 3.0). The absolute configuration of **1** was deduced as *S*, because its
7 structure and specific rotation ($[\alpha]^{30}_{D}$ +9.4, H_2O, *c* 0.20) were very similar to those of **5**,
8 respectively. The stereochemistry of **2**, **3**, and **6** remains unknown.
9 Cytotoxicity of **1** and **3** - **6** against mouse cerebrum glial cells was evaluated.
10 Compounds **1**, **3**, **4**, and **5** showed weak toxicity to the cells at 10 μg/mL but **6** exhibited
11 no activity (Table 2). This result indicates that the 2-hydroxyvaline moiety is
12 indispensable to the cytotoxicity. However, the relationship between the cytotoxicity of
13 the compounds and the acute encephalopathy in human remains unsolved.
14

3. Experimental

3.1. General

^{1}H NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz. The HRESIMS spectra were measured on a JMS-T100LC mass spectrometer. A JASCO grating infrared spectrophotometer was used to record the IR spectra. HPLC separations were performed with a JASCO Gulliver system using a preparative column (Develosil C30-UG-5, Nomura chemical, Japan; Cosmosil HILIC Waters, Nakalai Tesque, Japan). Silica gel plate (Merck F_{254}) and silica gel 60 N (Merck 100-200 mesh) were used for analytical TLC and for flash column chromatography, respectively. Wakosil-II 5C18HG for sugar composition analysis was a product of Wako Pure Chemicals, Japan.

3.2. Fungus materials

Fruiting bodies of *P. porrigens* were collected in Yamanashi Prefecture, Japan, in October 2004.

3.3. Extraction and isolation

The fresh fruiting bodies of *P. porrigens* (4.0 kg) were lyophilized, and successively extracted with hexane, EtOAc, EtOH, H_2O, and boiled H_2O (3 L, four times, respectively). Each solution was concentrated under reduced pressure and the EtOH-soluble part (10.1 g) was fractionated by silica gel flash column chromatography, eluting with stepwise by acetone and 90% MeOH. The 90% MeOH fraction (6.0 g) was separated by reversed-phase HPLC to obtain 25 fractions (column, Develosil C30-UG-5

50×500 mm; solvent, 20% MeOH; flow rate, 25 ml/min; UV absorbance, 220 nm). The tenth fraction (*Rt* 75.0 min) was separated by HPLC (column, Cosmosil HILIC Waters 20×250 mm; solvent, acetonitrile/H_2O (85:15); flow rate, 5 ml/min; UV absorbance, 220 nm), to afford **5** (1.5 mg; *Rt* 14.5 min). The twenty-fourth fraction (*Rt* 192.5 min) was also separated by HPLC in the same manner, with acetonitrile/H_2O (90:10) to yield **1** (2.3 mg; *Rt* 12.1 min). Furthermore, the sixteenth fraction (*Rt* 122.2 min) was separated by HPLC in the same manner, with acetonitrile/H_2O (85:15) to yield **2** (0.8 mg; *Rt* 18.1 min). The fourth fraction (*Rt* 59.4 min) was separated by HPLC with acetonitrile/H_2O (80:20) to yield **3** (1.3 mg; *Rt* 25.8 min), **4** (4.0 mg; *Rt* 14.5 min), and **6** (0.7 mg; *Rt* 15.7 min).

3.3.1. Compound 1; $[\alpha]^{30}_{D}$ +9.4 (H_2O, *c* 0.20); HRESIMS *m/z* 184.0950 $[M+Na]^+$ (calcd for $C_7H_{15}NaNO_3$, 184.0980); mp 180-182°C; IR ν_{max} (KBr) cm^{-1}: 1734

3.3.2. Compound 2; $[\alpha]^{30}_{D}$ +12 (H_2O, *c* 0.08); HRESIMS *m/z* 230.1004 $[M+Na]^+$ (calcd for $C_8H_{17}NaNO_5$, 230.1017); mp 175-177°C; IR ν_{max} (KBr) cm^{-1}: 3628, 1732.

3.3.3. Compound 3; $[\alpha]^{30}_{D}$ +130 (H_2O, *c* 0.10); HRESIMS *m/z* 480.1693 $[M+Na]^+$ (calcd for $C_{17}H_{31}NaNO_{13}$, 480.1703); mp 140-142°C; IR ν_{max} (KBr) cm^{-1}: 3426, 1734.

3.3.4. Compound 4; $[\alpha]^{30}_{D}$ +3.8 (H_2O, *c* 0.13); ESIMS *m/z* 156.0637$[M+Na]^+$; ^{1}H-NMR (in D_2O): 1.08(3H, s, 3-CH_3) , 1.30(3H, s, 3-CH_3), 3.44(1H, s, H-2); ^{13}C-NMR (in D_2O): 24.0(3-CH_3), 28.1(3-CH_3), 64.2(C-2), 70.6(C-3), 172.9(C-1)

3.3.5. Compound 5; $[\alpha]^{30}_{D}$ +10 (H_2O, *c* 0.4) ESIMS *m/z* 170.0793 $[M+Na]^+$; ^{1}H-NMR (in D_2O): 1.05(3H, s, 3-CH_3), 1.27(3H, s, 3-CH_3), 3.09(3H, s, H-1'), 3.60(1H, s, H-2); ^{13}C-NMR (in D_2O): 20.9(3-CH_3), 22.9(3-CH_3), 49.9(C-1'), 61.6(C-2), 75.6(C-3), 172.7(C-1)

3.3.6. Compound 6; $[\alpha]^{30}_{D}$ +12 (H_2O, *c* 0.07); ESIMS *m/z* 156.0620 $[M+Na]^+$; ^{1}H-NMR (in D_2O): 1.18(3H, s, 3-CH_3) , 1.23(3H, s, 3-CH_3), 3.78(1H, s, H-2); ^{13}C-NMR (in D_2O): 22.1(3-CH_3), 22.2(3-CH_3), 56.7(C-3), 76.1(C-2), 177.5(C-1)

3.4. Sugar composition analysis

Sugar composition was determined as described previously[12]. Briefly, the sample (200 μg) was dissolved in 20 μl distilled water in a test tube to which 6 M TFA (20 μl) was added. The test tube was incubated at 100°C in a hot block bath. After 6 h, the tube was cooled to room temperature and the acid was removed by using a centrifugal concentrator at 35 °C. The dried sample was derivatized with *p*-aminobenzoic ethyl ether in the presence of borane-pyridine complex at 80 °C. After 1 h, the reaction mixture was cooled to room temperature. Distilled water (200 μl) and an equal volume of chloroform were added to the reaction mixture. After vigorous vortexing, the sample was centrifuged (6000 × g, 1 min). The upper aqueous layer was analyzed by reversed-phase HPLC under the following conditions: column, Wakosil-II 5C18HG (4.6 × 250 mm); solvent, A 0.02% TFA/CH_3CN (90/10), B 0.02% TFA/CH_3CN (50/50); program, 0-70 min (B conc. 0%), 70-80 min (B conc. 100%), 80-90 min (B conc. 0%); flow rate, 1.5 ml/min; temp., 45 °C; detection, absorbance at 360 nm. The monosaccharide and amino monosaccharide standards used were D-GlcNAc, D-GalNAc, D-Glc, D-Gal, D-Man, D-Xyl, and L-Fuc..

3.5. Bioassay

Primary cultured mouse glial cells were prepared from the cortex of embryonic day 18 C57BL/6 mice. All animal experiments were approved by the University of Yamanashi Animal Care and Use Committee. The cells were cultured in Dulbecco's modified Eagles medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37ºC in a humidified 5% CO_2 atmosphere. After 1 week of culture in this medium, the cells were passaged and used as a glial cell culture.

Samples at various concentrations were added to the glial cells cultured in D-MEM without serum. The cells were cultured for 48 hours. After incubation, the cell viabilities were analyzed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Dojindo, Kumamoto) colorimetric assay. Briefly, treated glial cells were incubated with MTT (250 μg/ml) in D-MEM without serum for 2 h. The reaction was terminated by adding 20% (w/v) sodium dodecylsulfate and 50% (v/v) dimethylformamide in water. The number of living cells was quantified by measuring absorbance at 570 nm.

Acknowledgement

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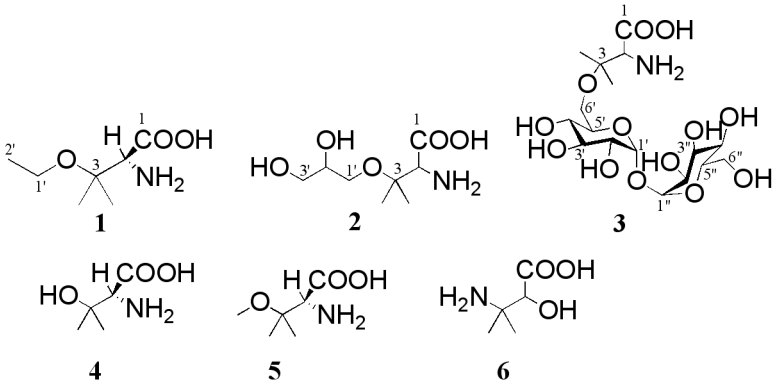
1 Figure legend

2

3 Fig. 1. COSY and HMBC correlations of **1** and **2**



1 **2** **3**

4 **5** **6**

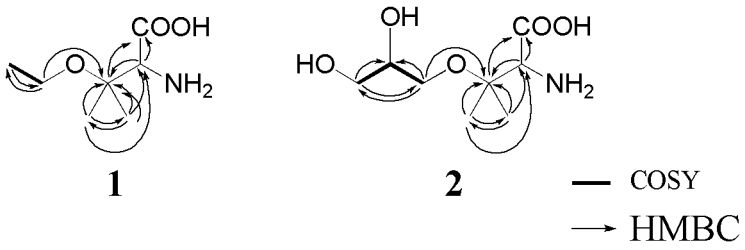


Figure 1. Kawaguchi *et al.*

Table 1. NMR data for **1**-**3** and treharose

1*			**2**		
Position	^{1}H (multiplicity, *J* Hz)	^{13}C	Position	^{1}H (multiplicity, *J* Hz)	^{13}C
1	-	172.7	1	-	172.7
2	3.65 (s)	62.0	2	3.61 (s)	62.4
3	-	75.5	3	-	75.6
3-CH_3	1.34 (s)	23.7	3-CH_3	1.34 (s)	23.6
	1.11 (s)	21.3		1.11 (s)	21.0
1’	3.42 (m)	58.4	1’	3.30 (dd, 10.1, 7.0) , 3.42 (dd, 10.1, 3.5)	63.7
2’	1.05 (t, 7.0)	15.7	2’	3.69 (m)	71.7
			3’	3.40 (dd, 11.1, 2.3) , 3.48(dd, 11.1, 4.7)	63.3
3			trehalose		
Position	^{1}H (multiplicity, *J* Hz)	^{13}C	Position	^{1}H (multiplicity, *J* Hz)	^{13}C
1	-	172.6	1	4.88 (d, 3.7)	94.3
2	3.64 (s)	62.1	2	3.24 (dd, 4.3, 3.7)	72.14
3	-	75.8	3	3.55 (dd, 9.0, 4.3)	73.65
3-CH_3	1.31 (s)	23.6	4	3.14 (dd, 9.8, 9.0)	70.81
	1.12 (s)	21.4	5	3.65 (ddd, 9.8, 4.6, 2.1)	73.19
1’	5.04 (d, 3.7)	94.4	6	3.47 (dd, 11.5, 4.6,), 3.56 (dd, 11.5, 2.1)	61.7
1”	5.02 (d, 3.9)	94.3			
2’	3.52 (m)**	71.8**			
2”	3.53 (m)**	71.9**			
3’	3.68 (m) **	73.4**			
3”	3.70 (m) **	73.5**			
4’	3.30 (dd, 9.7, 8.9)	70.1			
4”	3.41 (dd, 9.7, 8.9)	70.5			
5’	3.71 (m) **	72.9**			
5”	3.75 (m) **	72.9**			
6’	3.57 (m) , 3.73 (m)	60.9			
6”	3.72 (m)	61.1			

***** Concentration, **1** (6.4 mg/0.5 ml), **2** (2.2 mg/0.5 ml), **3** (3.6 mg/0.5 ml), treharose (12.0 mg/0.5 ml)

****** interchangeable between positions, n’ and n”

Table 2. Toxicity to glial cells at 10 μg/ml

Compound	Relative viability*
1	76 ± 2**
2	ND***
3	84 ± 3**
4	79 ± 1**
5	80 ±1**
6	101 ±1**

* The number indicates relative viability (%) of glial cells to that of control. Data are the mean ± SE.

** $p < 0.01$ vs control using Student's *t*-test..

*** ND; not determined