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A novel dipeptide, *N*-γ-glutamyl boletine, and a cyclic iminium toxin from the mushroom *Tylopilus* sp. (Boletaceae)

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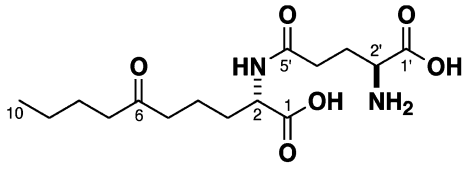
Abstract—*N*-γ-Glutamyl boletine and a toxin, 2-butyl-1-azacyclohexene iminium salt, were isolated from the mushroom *Tylopilus* sp. (Boltaceae). The absolute stereostructure of *N*-γ-glutamyl boletine was clarified based on spectroscopic analysis, acidic hydrolysis and total synthesis. *N*-γ-Glutamyl boletine exhibited moderate antibacterial activity. 2-Butyl-1-azacyclohexene iminium salt exhibited moderate acute toxicity against ddY mice. We proposed feasible biosynthetic pathway of piperidine alkaloids that the cyclic iminium compound might be biosynthesized from boletine, a new amino acid containing an δ-amino ketone moiety.

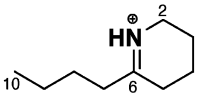
Various mushrooms have attracted a great deal of attention as health foods in Japan. The chemical constituents of mushrooms, especially the toxic varieties, have been well studied and numerous bioactive compounds have been reported. Nevertheless, food poisoning caused by the ingestion of mushrooms is still common, since the fungi have not been well identified compared to plants and animals. To prevent mushroom poisoning and to assure the safety of the food supply, we have searched for the bioactive constituents of a large variety of mushrooms.

Recently, a new variety of mushroom *Tylopilus* sp. (Boletaceae) was discovered at Mikawa, Aichi Prefecture, and its aqueous ethanol extract exhibited acute toxicity in mice. Since the Boletaceae are generally considered to be edible, this finding could be unique. Several bioactive compounds have been isolated from *Tylopilus* sp., but the toxic constituent has not been reported.[1] Therefore, we focused on the chemical constituents of this novel mushroom. Guided by acute toxicity against mice, a novel dipeptide, *N*-γ-glutamyl boletine (**1**), and an iminium compound **2**, a toxic principle, were obtained (Fig. 1). We report here the isolation and structure determination of these compounds.

The aqueous 80% EtOH extract of *Tylopilus* sp. was partitioned between water and EtOAc. Guided by acute toxicity against ddY mice, the water-soluble fraction was subjected to fractionation using column chromatography (TSK G-3000S polystyrene gel, Sephadex LH-20, ODS, and SiO_2) and reversed-phase HPLC. Final purification was achieved by reversed-phase HPLC (MeOH/aqueous NaCl) to give *N*-γ-glutamyl boletine (**1**, 2.8×10^{-3}% yield based on wet wt) and 2-butyl-1-azacyclohexene iminium salt (**2**, 7.1×10^{-4}% yield based on wet wt).[2] 2-Butyl-1-azacyclohexene iminium salt (**2**) exhibited moderate acute toxicity against ddY mice (LD_{99} 25 mg/kg). Although *N*-γ-glutamyl boletine (**1**) did not show acute toxicity against mice, it exhibited moderate antibacterial activity against the marine bacteria *Rhodospirillum salexigens* SCRC 113 strain (9 mm, 1 mg/disk).



N-γ-Glutamyl-boletine (**1**)



2-Butyl-1-azacyclohexene iminium salt (**2**)

Figure 1.

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The molecular formula of **1** was determined to be $C_{15}H_{26}N_2O_6$ by ESI MS (m/z 331.1870, calcd for $C_{15}H_{27}N_2O_6$ $[M+H]^+$ 331.1869). The NMR data for **1** are summarized in Table 1. The ^1H NMR, ^{13}C NMR, and HMQC spectra of **1** showed the presence of one methyl carbon (δ_c 12.8), eight methylene carbons, two methine carbons (δ_c 54.0, 54.5) connected to hetero atoms, and four carbonyl carbons (δ_c 173.3, 174.1, 176.9, 213.7). A detailed analysis of the COSY spectrum of **1** allowed three partial structures, C-2 to C-5, C-7 to C-10, and C-2′ to C-4′ (Fig. 2). The remaining connectivities of **1** were clarified by the HMBC correlations H-2/C-1 and C-5′, H-5/C-6, H-7/C-6, H-2′/C-1′, and H-4′/C-5′. Thus, the gross structure of *N*-γ-glutamyl boletine was determined to be a dipeptide consisting of glutamic acid and a new amino acid, which was named boletine, as shown in **1**.

The absolute stereostructure of **1** was elucidated as follows. Acidic hydrolysis of **1** followed by separation using reversed-phase HPLC gave glutamic acid (**3**) and cyclic amino acid **4** (Scheme 1). The absolute configuration of the glutamic acid (**3**) was determined to be L by chiral HPLC analysis.[3] The gross structure of cyclic amino acid **4** was determined by analysis of COSY, HMQC and HMBC spectra, but the stereochemistry of the carbon adjacent to the carboxyl group in **4** could not be determined.

Table 1. NMR data for *N*-γ-glutamyl boletine (**1**) in CD_3OD

Atom	^{13}C[a]	^1H[b]	HMBC[c]
1	176.9 s		H-2, 3
2	54.0 d	4.23 m 1H	H-3, 4
3	31.4 t	1.62 m 2H	H-2, 5
4	20.3 t	1.84 m 2H	H-2, 3, 5
5	41.0 t	2.51 m 2H	H-3
6	213.7 s		H-5, 7, 8
7	41.7 t	2.46 t (6.7) 2H	H-8, 9
8	26.8 t	1.51 tt (6.7, 6.9) 2H	H-7, 9, 10
9	22.4 t	1.31 tq (6.9, 6.9) 2H	H-7, 8, 10
10	12.8 q	0.92 t (6.9) 3H	H-8, 9
1′	173.3 s		H-2′, 3′
2′	54.5 d	3.64 m 1H	H-3′, 4′
3′a	27.0 t	2.10 m 1H	H-2′, 4′
3′b		2.23 m 1H	
4′	32.5 t	2.52 m 2H	H-2′, 3′
5′	174.1 s		H-2, 3′, 4′

[a] Recorded at 200 MHz. Multiplicity was based on the HMQC spectrum.
[b] Recorded at 800 MHz. Coupling constants (Hz) are in parentheses.
[c] Based on the correlation from each carbon atom.

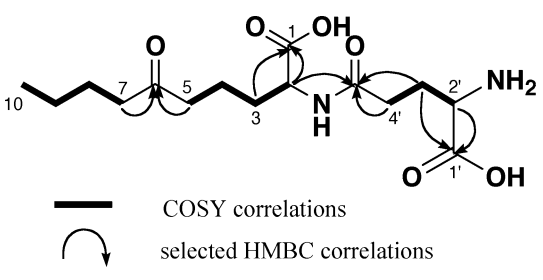
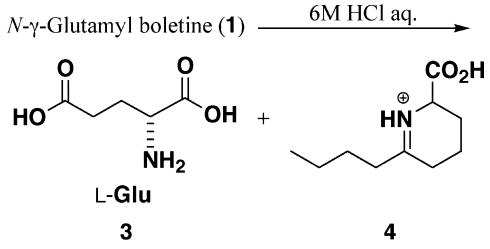


Figure 2. Gross structure of *N*-γ-glutamyl boletine (**1**).

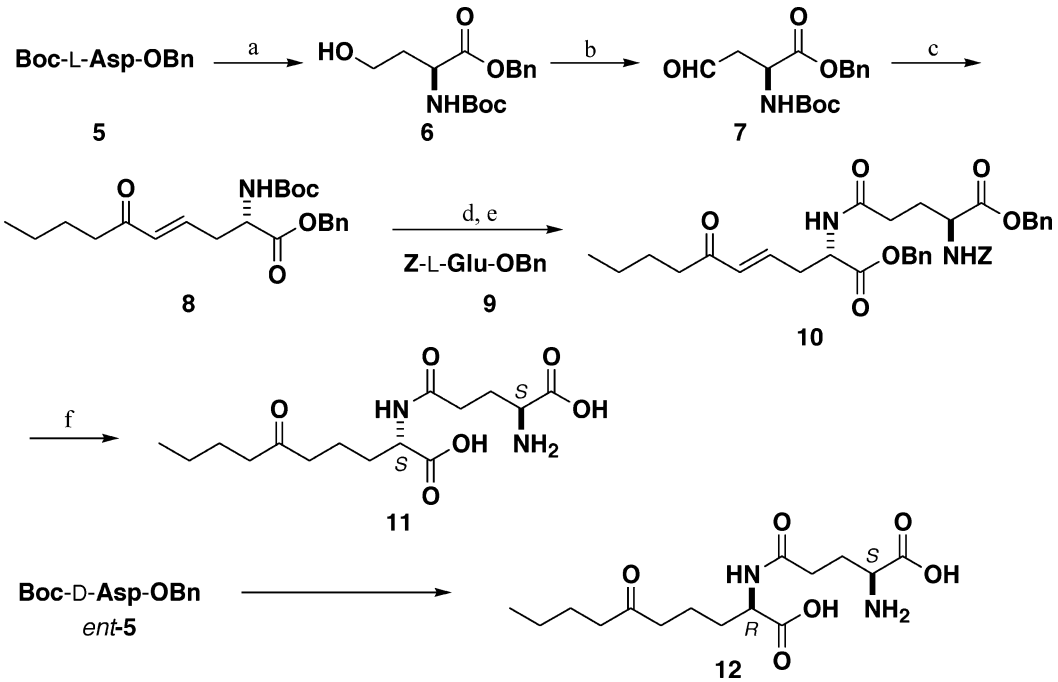


Scheme 1. Hydrolysis of *N*-γ-glutamyl boletine (**1**).

To confirm the absolute stereochemistry of *N*-γ-glutamyl boletine (**1**) and to obtain a greater supply for further biological studies, both diastereomers, (2*S*, 2′*S*)- and (2*R*, 2′*S*)-dipeptides **11** and **12**, were synthesized (Scheme 2). The synthesis of **1** started from commercially available Boc-L-Asp-OBn (**5**). Compound **5** was converted into a mixed anhydride and then subjected to reduction using sodium borohydride to give alcohol **6** in 69% yield.[4] Dess–Martin oxidation of alcohol **6** gave aldehyde **7** in 79% yield. The Horner–Wadsworth–Emmons reaction of aldehyde **7** with β-ketophosphonate gave α,β-unsaturated ketone **8** in 77% yield.[5,6] The geometry of the olefin in **8** was confirmed to be *E* based on the magnitude of the coupling constant between vinyl protons (16.0 Hz). Acidic deprotection of the Boc group in **8** followed by condensation with Z-L-Glu-OBn (**9**) gave dipeptide **10** in 53% yield. Finally, hydrogenation of **10** using $Pd(OH)_2$ led to deprotection of the Z and Bn groups and reduction of the olefin to give (2*S*, 2′*S*)-dipeptide **11** in quantitative yield. Similarly, (2*R*, 2′*S*)-dipeptide **12** was synthesized from Boc-D-Asp-OBn (*ent*-**5**) in 27% yield in six steps. Synthetic (2*S*, 2′*S*)-dipeptide **11** was identical to natural **1** with respect to TLC analysis and spectroscopic data (^1H and ^{13}C NMR, and optical rotation).[7] Thus, the absolute stereostructure in *N*-γ-glutamyl boletine (**1**) was shown to be 2*S* and 2′*S*. Synthetic *N*-γ-glutamyl boletine (**11**) also exhibited moderate antibacterial activity against *R. salexigens* (9 mm, 1 mg/disk) as with natural products. It is interesting to note that 2*R*-isomer **12** did not show any antibacterial activity.

2-Butyl-1-azacyclohexene iminium salt (**2**) showed an ion peak at m/z 140 $[M^+]$ in its FAB MS spectrum. The NMR data for **2** are summarized in Table 2. The ^1H NMR, ^{13}C NMR, and HMQC spectra of **2** showed the presence of one methyl carbon (δ_c 12.6), seven methylene carbons, and one carbonyl carbon (δ_c 192.3). Analysis of the COSY, HMQC, HMBC spectra of **2** allowed us to construct its entire framework (Fig. 3). Treatment of **2** with triethylamine gave an imine, the ^1H NMR data of which coincided with those of synthetic 2-butyl-1-azacyclohexene previously reported.[8] Thus, the structure was confirmed to be as shown in Fig. 3.

The biosynthesis of piperidine alkaloids has been well studied. Experiments using labeled precursors have established that a large group of piperidine alkaloids can be derived from lysine or acetic acid.[9] For example,



Scheme 2. (a) *N*-methylmorpholine, isobutyl chloroformate, THF, 0°C, 15 min; then $NaBH_4$, MeOH, 0°C, 1 h, 69%; (b) Dess–Martin periodinane, CH_2Cl_2, 0°C, 35 min, 79%; (c) $(MeO)_2P(O)CH_2C(O)nBu$, Cs_2CO_3, MeCN, rt, 35 min, 77%; (d) TFA, CH_2Cl_2, 0°C, 1.5 h, 79%; (e) **9**, WSCD·HCl, HOBt, $(i\text{-}Pr)_2NEt$, CH_2Cl_2, 0°C, 5 h, 53%; (f) H_2, $Pd(OH)_2$, EtOH, rt, 9 h, quant.

Table 2. NMR data for 2-butyl-1-azacyclohexene iminium salt (**2**) in CD_3OH

Atom	^{13}C[b]	^{1}H[c]	HMBC[d]
2	45.1 t	3.66 m 2H	H-2, 3
3	20.0 t	1.88 m 2H	H-3, 4
4a	18.0 t	1.84 m 1H	H-1, 2
4b		1.86 m 1H	
5[a]	28.0 t	2.70 m 2H	H-2, 3a
6	192.3 s		H-1, 3a, 7
7[a]	38.0 t	2.60 t (7.2) 2H	H-7
8	27.6 t	1.64 tt (7.2, 7.2) 2H	H-6, 8, 9
9	22.0 t	1.42 tq (7.2, 7.2) 2H	H-7
10	12.6 q	0.98 t (7.2) 3H	H-8

[a] Protons were almost not observed in CD_3OD due to deuterium exchange.
[b] Recorded at 200 MHz. Multiplicity was based on the HMQC spectrum.
[c] Recorded at 800 MHz. Coupling constants (Hz) are in parentheses.
[d] Based on the correlation from each carbon atom.

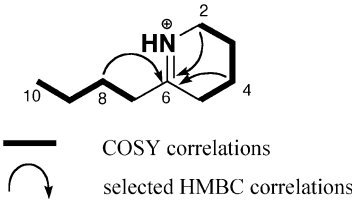
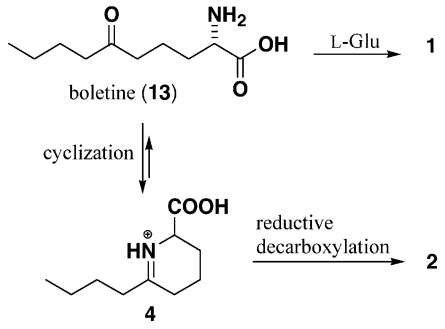


Figure 3. Gross structure of 2-butyl-1-azacyclohexene iminium salt (**2**).

anabasine, sedamine, and pelletierine, have been shown to arise from lysine. On the other hand, nigrifactin, coniine, and γ-conicerine can be derived from four to six acetate units in linear combination in their biosynthesis.[10] The structure of 2-butyl-1-azacyclohexene iminium salt (**2**) is quite similar to that of γ-conicerine. However, the difference between number of carbon atoms in these compounds suggests that the biosynthesis of **2** does not only involve acetate units.

Scheme 3 shows the possible biosynthetic pathways of compounds **1** and **2**. *N*-γ-Glutamyl boletine (**1**) might be biosynthesized by the condensation of boletine (**13**) and L-glutamic acid. Since boletine (**13**) has a δ-amino ketone moiety, however, intramolecular cyclization may easily occur to give a dehydrated form such as **4**. Decarboxylation of dehydrated boletine **4** may lead to substituted azacyclohexene **2**. However, reductive decarboxylation followed by cyclization may also be possible. Consequently, we assumed that both **1** and **2** were biosynthesized from the novel amino acid boletine (**13**).

In conclusion, *N*-γ-glutamyl boletine (**1**) and 2-butyl-1-azacyclohexene iminium salt (**2**) were isolated from the mushroom *Tylopilus* sp. Compound **1** was clarified to be a dipeptide consisting of L-glutamic acid and boletine, a novel amino acid, based on spectroscopic analysis and acidic hydrolysis. The total synthesis of **11** and its 2*R*-isomer **12** elucidated the absolute stereochemistry of **1**. Both natural and synthetic *N*-γ-glutamyl boletine **1** and **11** showed moderate antibacterial



Scheme 3. Possible biosynthetic pathways of compounds **1** and **2**.

activity. 2-Butyl-1-azacyclohexene iminium salt (**2**) exhibited moderate acute toxicity against ddY mice. Further biological studies of compounds **1** and **2** are in progress.

Acknowledgements

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2. Conditions for the isolation of *N*-γ-glutamyl boletine (**1**) and 2-butyl-1-azacyclohexene iminium salt (**2**): column, Develosil ODS-HG-5 (ϕ 20×250 mm); eluent, 30% MeOH/70% H_2O containing 0.1% NaCl; flow rate, 5.0 mL/min; detection at 215 nm. Desalting was achieved by the column chromatography (TSK G-3000S; eluent, 50% aqueous EtOH).
3. Conditions for the chiral HPLC analysis of glutamic acid (**3**): column, CROWNPAK CR(+) (ϕ 4.6×150 mm); eluent, aqueous $HClO_4$ (pH 2.0); flow rate, 0.4 mL/min; detection at 200 nm.
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7. Optical rotations: natural compound **1**, $[\alpha]_D^{30}$ −0.54° (*c* 0.28, MeOH); synthetic compound **11**, $[\alpha]_D^{30}$ −0.56° (*c* 0.33, MeOH).
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