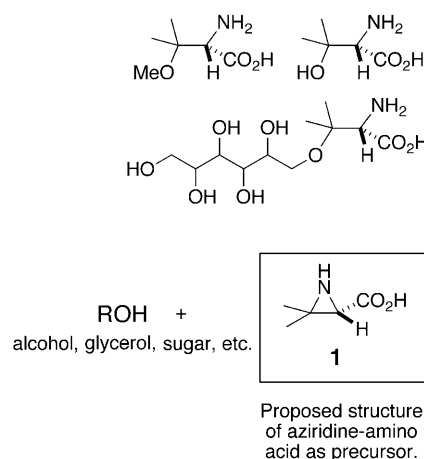


Proof of the Existence of an Unstable Amino Acid: Pleurocybellaziridine in *Pleurocybella porrigens***

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Angel's wing mushroom, *Pleurocybella porrigens* (Pers.) Singer, is distributed worldwide in temperate areas and has been eaten throughout the world, especially in Japan. Nonetheless, seventeen people who ate angel's wing mushroom died of acute encephalopathy in 2004 in Japan.^[1–3] Epidemiological investigation of the incident indicated that most patients were undergoing hemodialysis treatment for chronic renal failure and had digested the wild fruiting bodies before the onset of neurological symptoms. Although the mechanism of the acute encephalopathy has not yet been elucidated, several chemical investigations have reported that vitamin D analogues,^[4] fatty acids,^[5] and saccharides^[6] are potential causative agents of poisoning. Recently our group reported a lectin^[7] and cytotoxic amino acids,^[8] six of which were novel. The structural novelty and analogy of the amino acids is such that each acid has the β -hydroxyvaline unit attached to endogenous molecules (Scheme 1), which inspired us to conclude the occurrence of an aziridine amino acid (**1**) as the common precursor; one carbon atom of the aziridine ring is substituted with a carboxy group and the other with geminal

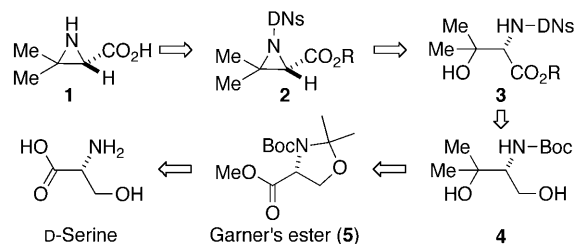


Scheme 1. Structure of the cytotoxic compounds^[8a] (top) and their proposed precursor **1**.

methyl groups (Scheme 1). Thus the instability of this molecule could be demonstrated by nucleophilic attack by endogenous molecules and successive ring opening. Therefore, we hypothesized that the labile feature of the precursor should be the reason why it could not be isolated from the mushroom in the previous study.

To confirm the existence of the aziridine as a component within the mushroom, we launched this unique project; synthesis of the proposed molecule and its esters, and isolation of the corresponding esters from the crude extract of the mushroom after esterification.

Our synthesis plan is illustrated in Scheme 2. As a result of the labile nature of **1**, utilizing appropriate protection of the nitrogen atom as well as the carboxylic group of **2** would play a crucial step in the total synthesis. Although racemic **1** has already been synthesized,^[9] employing a Mitsunobu reaction with **3** would be suitable for the preparation of an optically active compound. Recently we have developed a highly



Scheme 2. Retrosynthetic analysis of the aziridine amino acid **1**.

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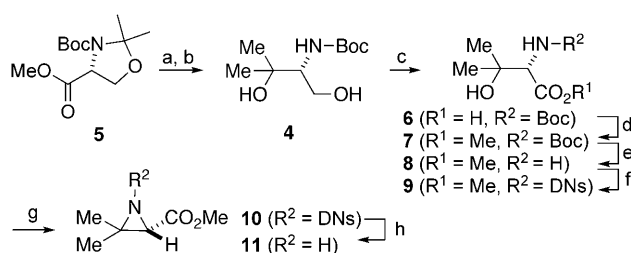
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efficient methodology for construction of secondary amines using nitrobenzenesulfonamides (Ns strategy).^[10] Since our Mitsunobu reaction, mediated by the 2,4-dinitrobenzenesulfonyl (DNs) group, proceeded under extra mild reaction conditions, the incorporation of DNs into **3** would enable the total synthesis of labile compound **1**.^[11] The change in the oxidation state of **4** into that of **3** could proceed by conversion of the geminal dimethyl alcohol unit in ester **5** and subsequent oxidation of **4**. Garner's ester (**5**)^[12] is readily obtained from both enantiomeric forms of serine, which would serve as an appropriate starting material.

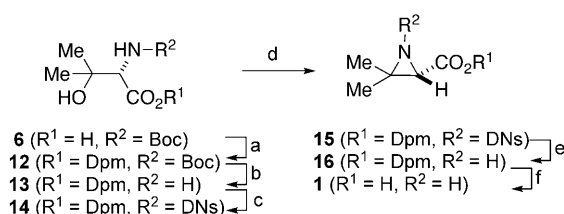
As shown in Scheme 3, we first synthesized methyl ester derivative **10** to confirm the existence of **1** in the mushroom's extracts. After treatment of **5** with excess MeMgBr and acidic



Scheme 3. Reagent and conditions: a) MeMgBr, THF, -20°C ; b) PPTS, MeOH, 96% (2 steps); c) TEMPO, PhI(OAc)₂, NaClO₂, MeCN, Buffer pH 6.4, 96%; d) CH₂N₂, Et₂O 87%; e) HCl gas, MeOH; f) DNsCl, 2,6-lutidine, CH₂Cl₂, 72% (2 steps); g) DEAD, Ph₃P, toluene, 83%; h) *n*-PrNH₂, CH₂Cl₂, 0°C –R.T., 52%. Boc = *tert*-Butoxycarbonyl, DEAD = diethyl azodicarboxylate, DNsCl = 2,4-dinitrobenzenesulfonyl, PPTS = pyridinium *p*-toluenesulfonate, TEMPO = 2,2,6,6-tetramethylpiperidine-1-oxyl.

hydrolysis, the resulting diol **4** was subjected to TEMPO-catalyzed oxidation^[13] to give the carboxylic acid **6**^[14] without loss of any functional group. After conversion of **6** into **7** by treatment with CH₂N₂, the protecting group was exchanged with the DNs group. Upon treatment of **9** with DEAD and PPh₃^[15] in toluene at room temperature, the desired aziridination reaction proceeded smoothly to produce **10** in 83% yield. Finally, removal of the DNs group in **10** was accomplished by treatment with *n*-propylamine to provide **11**.^[10]

Since hydrolysis of the methyl ester of **11** caused decomposition of its aziridine ring, a diphenylmethyl ester was considered to be a more suitable protecting group for the carboxy group in **1**. This ester counterpart has the advantage of not only facile incorporation and deprotection, but also provided a quantitative analysis of **16** because of its strong UV absorption. Furthermore, **16** seemed to be more stable than methyl ester **11** because of the steric hindrance around the aziridine ring.^[16] After treatment of the *N*-Boc β -hydroxy valine **6** with diphenyldiazomethane,^[17] the Boc protecting group in **12** was exchanged for a DNs group to provide the cyclization precursor **14** (Scheme 4). Upon treatment of **14** with DIAD and PPh₃, the desired cyclization reaction proceeded smoothly to give **15** in 70% yield. During this reaction, employment of DIAD gave a superior result compared to that of DEAD. After removal of the DNs group in **15**, hydrogenolysis of the diphenylmethyl group of **16**



Scheme 4. Reagent and conditions: a) Ph₂CN₂, CH₂Cl₂, 77%; b) HCl gas, MeOH;^[19] c) DNsCl, Na₂CO₃, THF/H₂O (2:1), 59% (2 steps); d) DIAD, Ph₃P, toluene, 70%; e) *n*-PrNH₂, CH₂Cl₂, 0°C –R.T., 90%; f) H₂, 5% Pd/C, MeOH, 67%. DIAD = diisopropyl azodicarboxylate, Dpm = diphenylmethyl.

proceeded smoothly to provide pleurocybellaziridine (**1**). During the course of the isolation, pleurocybellaziridine (**1**) fortunately crystallized in a mixture of CHCl₃ and MeOH (20:1) as colorless prisms.^[18]

With the desired compounds **11** and **16** in hand, we turned our attention to confirmation of the existence of **1** in *P. porrigens*. Considering the instability of **1**, we tried to isolate **11** or **16** from the extract of the mushroom after esterification. The lyophilized fruiting bodies were suspended in MeOH. The resulting extract was treated with CH₂N₂ or Ph₂CN₂, and then the resulting mixture was purified by repeated silica gel flash chromatography; the *R_f* values of the corresponding synthetic materials (**11** or **16**) were used to identify the components using TLC analysis. Although the methyl ester **11** showed partial decomposition during the separation steps and when concentrated to dryness, we finally succeeded in isolation of the esters. Their spectral data were identical with those of the synthetic compounds, and were the first confirmation of the existence of this labile amino acid, pleurocybellaziridine (**1**), in a natural source.

Interestingly, the content of **1** in the mushroom was extraordinarily high; 23 mg of **16** was obtained from a MeOH extract of the lyophilized fruiting bodies (4.0 g). Furthermore, the absolute configuration of **16** that isolated from the mushroom was determined by comparison of its specific rotation with that of the synthetic one: $[\alpha]_D^{20}$ (natural) = 25 ($c = 0.50$, CHCl₃); $[\alpha]_D^{20}$ (synthetic) = 24 ($c = 1.0$, CHCl₃). In contrast, only trace amounts of **1** could be detected in the H₂O fraction prepared according to the previous report,^[8a] and the synthetic **1** gradually decomposed in D₂O in an NMR tube (see Figures S8 and S10 in the Supporting Information). These results suggest that most of **1** decomposed during the previous isolation procedure.^[20]

Histological findings of the brain tissues affected by the encephalopathy showed demyelinating symptoms.^[21] It indicates that toxic substance(s) in the mushroom damaged oligodendrocytes which constitutes the myelin sheath in the brain. Therefore, we examined the toxicity of both pleurocybellaziridine (**1**) and **11** against rat CG4-16 oligodendrocyte cells. Given the results of an MTT assay, **1** significantly reduced the cell viability at concentrations of up to $10\ \mu\text{g mL}^{-1}$ (87 μM), but the methyl ester **11** showed only weak toxicity at $30\ \mu\text{g mL}^{-1}$ (233 μM ; Figure 1a). The cell staining also revealed that most of the cells treated with $30\ \mu\text{g mL}^{-1}$ (260 μM) of **1** showed red fluorescence, thus

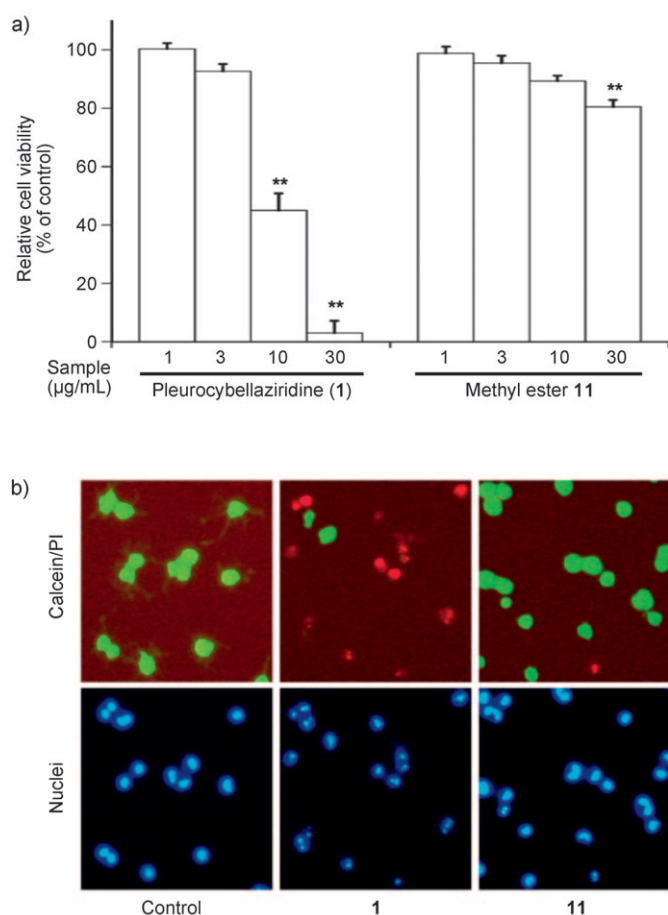


Figure 1. Toxicity of pleurocybellaziridine (**1**) and its methyl ester (**11**) for CG4-16 cells. CG4-16 cells treated with **1** or **11** were subjected to an MTT assay (a) and calcein-AM/PI staining (b). a) The values are represented as the mean of the relative percentage of surviving cells and the standard error of the mean is shown ($n=12$; n : the number of each experiment). Statistical analysis was performed by one-way ANOVA and then a Tukey-Kramer post hoc test. The differences between mean values were considered to be significant when p -values were less than 0.01 ($**p < 0.01$). CG4-16 cells were treated with 30 $\mu\text{g mL}^{-1}$ of **1** or **11**, and subjected to live/death staining. Upper panels represent a typical staining pattern of live (calcein, green) and dead (PI, red) cells. Lower panels represent nuclear staining with Hoechst 33342 for confirmation that the stained parts in upper panels were actual cells, not materials other than cells.

representing the dead ones, whereas only a few cells showed red fluorescence when treated with 30 $\mu\text{g mL}^{-1}$ of **11** (Figure 1b).^[22] In addition, the adducts previously isolated from the mushroom (Scheme 1) exhibited much less toxicity than **1** (see Figure S2 in the Supporting Information).^[8a] Furthermore, **1** could be detected in the assay media during and after the test (see Figures S3–S7 in the Supporting Information). These data suggest that **1** maybe the cause of the demyelinating symptom and the carboxylic residue and the aziridine skeleton are crucial for the cytotoxicity.

In summary, we synthesized a labile and toxic aziridine amino acid (**1**) and elucidated its presence in the angel's wing mushroom, *Pleurocybella porrigens*.

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