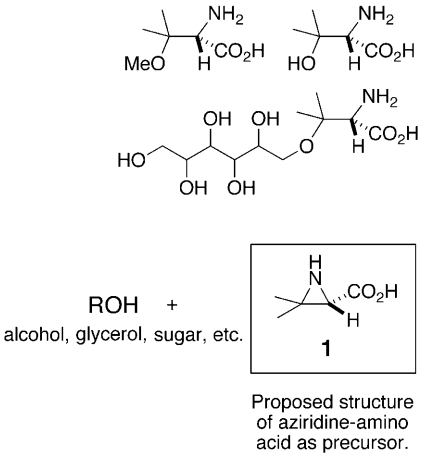
Natural Products

DOI: 10.1002/anie.201004646

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

Toshiyuki Wakimoto, Tomohiro Asakawa, Saeko Akahoshi, Tomohiro Suzuki, Kaoru Nagai, Hirokazu Kawagishi, and Toshiyuki Kan**

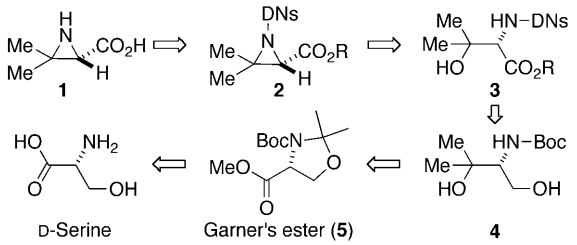
Angel's wing mushroom, *Pleurocybella porrigens* (Pers.) Singer, is distributed worldwide in temperate areas and has been eaten throughout the world, especially in Japan. Nonetheless, seventeen people who ate angel's wing mushroom died of acute encephalopathy in 2004 in Japan.[1–3] Epidemiological investigation 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 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.



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

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**.

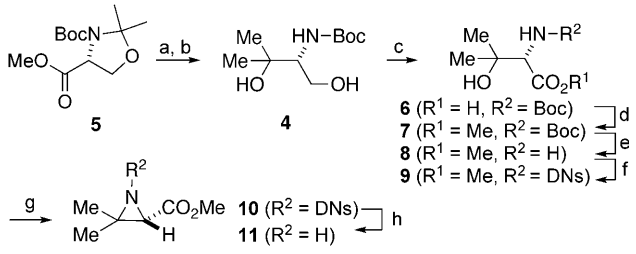
[*] Dr. T. Wakimoto,[+] Dr. T. Asakawa, S. Akahoshi, Prof. Dr. T. Kan
School of Pharmaceutical Sciences, University of Shizuoka and Global COE Program
52-1 Yada, Suruga-ku, Shizuoka 422-8526 (Japan)
Fax: (+81) 54-264-5745
E-mail: kant@u-shizuoka-ken.ac.jp
Dr. T. Suzuki, Prof. Dr. H. Kawagishi
Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University
836 Ohya, Suruga-ku, Shizuoka 422-8529 (Japan)
Fax: (+81) 54-238-4885
E-mail: achkawa@ipc.shizuoka.ac.jp
Dr. K. Nagai
Department of Epigenetics Medicine, Interdisciplinary Graduate School of Medicine and Engneering, University of Yamanashi
Yamanashi 409-3898 (Japan)
[+] Present address: Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033 (Japan)

[**] This work was partially supported by a Grant-in-Aid for Research and Development Projects for Application in Promoting New Policy of Agriculture Forestry and Fisheries from the Ministry of Agriculture, Forestry and Fisheries (MAFF) (Japan) and a Grant-in-Aid for Scientific Research on Priority from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (Japan). We also thank V. K. Deo (Shizuoka University) for valuable discussions, Dr. Toru Ogata (National Rehabilitation Center for Persons with Disabilities (Japan)), and Dr. Araceli Espinosa (UCLA) for providing CG4-16 cells.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201004646.

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.

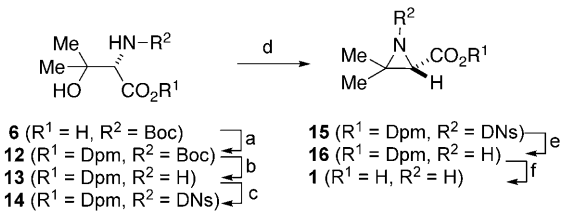
As shown in Scheme 3, we first synthesized methyl ester derivative **10** to confirm the existence of **1** in the mushroom's extracts. After treatment of **5** with excess MeMgBr and acidic



Scheme 3. Reagent and conditions: a) MeMgBr, THF, −20 °C; b) PPTS, MeOH, 96% (2 steps); c) TEMPO, $PhI(OAc)_2$, $NaClO_2$, MeCN, Buffer pH 6.4, 96%; d) CH_2N_2, Et_2O 87%; e) HCl gas, MeOH; f) DNsCl, 2,6-lutidine, CH_2Cl_2, 72% (2 steps); g) DEAD, Ph_3P, toluene, 83%; h) *n*$PrNH_2$, CH_2Cl_2, 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 CH_2N_2, the protecting group was exchanged with the DNs group. Upon treatment of **9** with DEAD and PPh_3[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 diphenymethyl 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 PPh_3, 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) Ph_2CN_2, CH_2Cl_2, 77%; b) HCl gas, MeOH;[19] c) DNsCl, Na_2CO_3, THF/H_2O (2:1), 59% (2 steps); d) DIAD, Ph_3P, toluene, 70%; e) *n*$PrNH_2$, CH_2Cl_2, 0 °C–R.T., 90%; f) H_2, 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 $CHCl_3$ 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 CH_2N_2 or Ph_2CN_2, 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 ($c = 0.50$, $CHCl_3$); $[\alpha]_D^{20}$ (synthetic) = 24 ($c = 1.0$, $CHCl_3$). In contrast, only trace amounts of **1** could be detected in the H_2O fraction prepared according to the previous report,[8a] and the synthetic **1** gradually decomposed in D_2O 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 $\mu g\,mL^{-1}$ (87 μM), but the methyl ester **11** showed only weak toxicity at 30 $\mu g\,mL^{-1}$ (233 μM; Figure 1a). The cell staining also revealed that most of the cells treated with 30 $\mu g\,mL^{-1}$ (260 μM) of **1** showed red fluorescence, thus

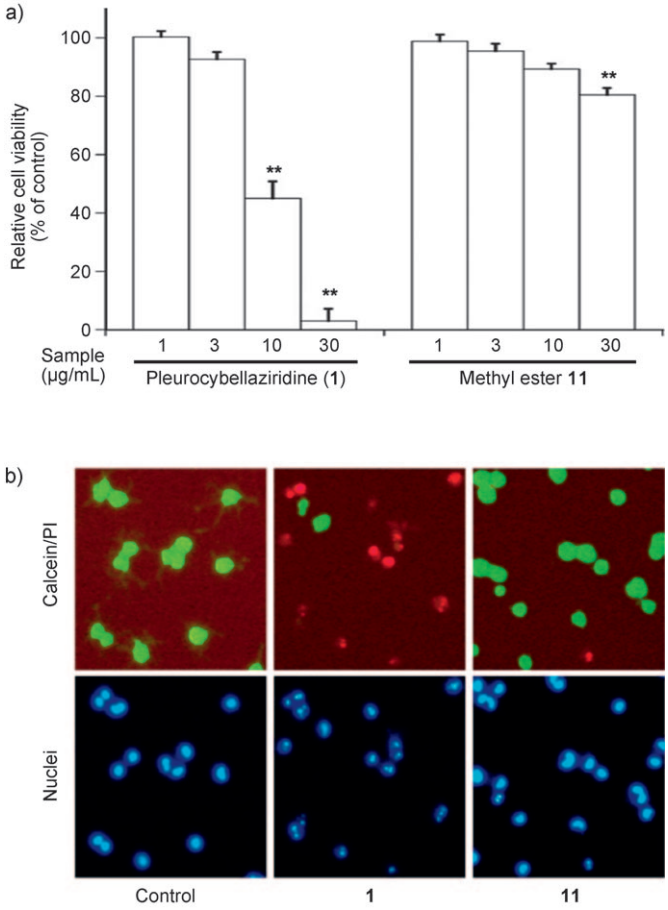


Figure 1. Toxicity of pleurocybellaziridine (**1**) and its methyl ester (**11**) for CG4-16 cells. CG4-16 cells treated with **1** or **11** were subjected to an MTT assay (a) and calcein-AM/PI staining (b). a) The values are represented as the mean of the relative percentage of surviving cells and 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 $\mu g mL^{-1}$ 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 Hoechst33342 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 $\mu g mL^{-1}$ 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*.

Received: July 28, 2010
Revised: November 12, 2010
Published online: December 23, 2010

Keywords: amino acids · aziridines · cytotoxicity · natural products · structure elucidation

[1] T. Kuwabara, T. Arai, N. Honma, M. Nishizawa, *Rinsho Shinkeigaku* **2005**, *45*, 239–245 (In Japanese with English abstract).
[2] K. Obara, S. Okawa, M. Kobayashi, S. Watanabe, I. Toyoshima, *Rinsho Shinkeigaku* **2005**, *45*, 253–256 (In Japanese with English abstract).
[3] K. Obara, C. Wada, T. Yoshioka, K. Enomoto, S. Yagishita, I. Toyoshima, *Neuropathology* **2008**, *28*, 151–156.
[4] H. Sasaki, H. Akiyama, Y. Yoshida, K. Kondo, Y. Amakura, Y. Kasahara, T. Maitani, *Biol. Pharm. Bull.* **2006**, *29*, 2514–2528.
[5] T. Hasegawa, M. Ishibashi, T. Takata, F. Takano, T. Ohta, *Chem. Pharm. Bull.* **2007**, *55*, 1748–1749.
[6] T. Tanaka, T. Hasegawa, T. Tatsuno, J. Date, Y. Ishigaki, Y. Nakamura, N. Tomosugi, F. Takano, T. Ohta, *J. Health Sci.* **2009**, *55*, 373–379.
[7] T. Suzuki, Y. Amano, M. Fujita, Y. Kobayashi, H. Dohra, H. Hirai, T. Murata, T. Usui, H. Kawagishi, *Biosci. Biotechnol. Biochem.* **2009**, *73*, 702–709.
[8] a) T. Kawaguchi, T. Suzuki, Y. Kobayashi, S. Kodani, H. Hirai, K. Nagai, H. Kawagishi, *Tetrahedron* **2010**, *66*, 504–507; b) Y. Aoyagi, T. Sugahara, *Phytochemistry* **1988**, *27*, 3306–3307.
[9] a) M. P. Sammes, A Rahman, *J. Chem. Soc. Perkin Trans. 1* **1972**, 344–346; b) T. Hiyama, H. Taguchi, H. Nozaki, *Bull. Chem. Soc. Jpn.* **1974**, *47*, 2909–2910.
[10] For reviews on synthetic strategies involving the Ns group, see: a) T. Kan, T. Fukuyama, *J. Syn. Org. Chem. Jpn.* **2001**, *59*, 779–789; b) T. Kan, T. Fukuyama, *Chem. Commun.* **2004**, 353–360.
[11] T. Fukuyama, M. Cheung, C.-K. Jow, Y. Hidai, T. Kan, *Tetrahedron Lett.* **1997**, *38*, 5831–5834.
[12] a) P. Garner, J. M. Park, *Org. Synth.*, *Coll. Vol. IX*, Wiley, New York, **1998**, pp. 300–306; b) A. Dondoni, D. Perrone, *Org. Synth.*, *Coll. Vol. X*, Wiley, New York, **2004**, pp. 320–326.
[13] M. Zhao, J. Li, E. Mano, Z. Song, D. M. Tschaen, E. J. J. Grabowski, P. J. Reider, *J. Org. Chem.* **1999**, *64*, 2564–2566.
[14] J. E. Dettwiler, W. D. Lubell, *J. Org. Chem.* **2003**, *68*, 177–179.
[15] For a review of Mitsunobu reactions, see: a) O. Mitsunobu, *Synthesis* **1981**, 1–28; b) D. L. Hughes, *Org. React.* **1992**, *42*, 335–656.
[16] Diphenylmethyl ester of **1** was stable enough to allow diode array detection (DAD) HPLC analysis: see Figure S3 in the Supporting Information.
[17] L. I. Smith, K. L. Howard, *Org. Synth.*, *Coll. Vol. III*, Wiley, New York, **1995**, pp. 351–355.
[18] Detailed experimental procedures and spectral data are described in the Supporting Information.
[19] Z. Tozuka, H. Takasugi, T. Takaya, *J. Antibiot.* **1983**, *36*, 142–146.
[20] **1** could be stabilized by salt formation with a counter ion in the natural environment.
[21] N. Arai, H. Kawagishi, unpublished data.
[22] **1** induced not only significant toxicity but also molphological changes in CG4-16 cells; see Figure S1 in the Supporting Information.

