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著者	Suzuki Tomohiro, Choi Jae-Hoon, Kawaguchi Takumi, Yamashita Kimiko, Morita Akio, Hirai Hirofumi, Nagai Kaoru, Hirose Tomoyasu, Omura Satoshi, Sunazuka Toshiaki, Kawagishi Hirokazu
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Tomohiro Suzuki, Jae-Hoon Choi,, Takumi Kawaguchi, Kimiko Yamashita, Akio Morita, Hirofumi Hirai, Kaoru Nagai, Tomoyasu Hirose, Satoshi Omura, Toshiaki Sunazuka*, Hirokazu Kawagishi*

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Tomohiro Suzuki^{a,†}, Jae-Hoon Choi^{a,†}, Takumi Kawaguchi^b, Kimiko Yamashita^a, Akio Morita^b, Hirofumi Hirai^b, Kaoru Nagai^c, Tomoyasu Hirose^d, Satoshi Omura^d Toshiaki Sunazuka^{d,*}, Hirokazu Kawagishi^{a,b,*}

^a*Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

^b*Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

^c*Department of Epigenetic Medicine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi 409-3898, Japan*

^d*Kitasato Institute for Life Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo, 108-8641, Japan*

*Corresponding authors. E-mail address: achkawa@ipc.shizuoka.ac.jp (H. Kawagishi), sunazuka@lisci.kitasato-u.ac.jp (T. Sunazuka)

[†] These authors contributed equally to this work.

Abstract

Makomotindoline (**1**) was isolated from Makomotake, *Zizania latifolia* infected with *Ustilago esculenta*. The structure was determined by the interpretation of spectroscopic data and synthesis. Makomotindoline (**1**), its L-Glc isomer (**2**) and its aglycon (**3**) were synthesized and their effects on rat glioma cells showed adverse effects on the cell growth.

Keywords: Makomotake, *Ustilago esculenta*, structure determination, glioma cell, synthesis

The fungus, *Ustilago esculenta*, penetrates into the aquatic perennial grass, *Zizania latifolia*. After penetration, the fungus incites the formation of an edible gall and inhibits inflorescence and seed production in the plant. The gall is called Makomotake in Japanese and Jiaobai in Chinese, and an edible product in Japan, China and other Asian countries. We have already reported osteoclast-forming suppressive nortriterpenes from it¹ and in the course of further search for new compounds from Makomotake, we succeeded in purifying a novel *N*-glucoside. Here we describe the isolation, structure determination, synthesis and biological activity of the compound and its analogues.

Fresh Makomotake was extracted with EtOH and then with acetone. After the solutions had been combined and concentrated under reduced pressure, the concentrate was partitioned between CH₂Cl₂ and H₂O, and then the H₂O soluble part was partitioned between EtOAc and H₂O. The EtOAc soluble part was fractionated by repeated chromatography, resulting in compound **1**.

Makomotindoline (**1**)² was purified as a colorless amorphous solid. Its molecular formula was determined as C₁₆H₂₁NO₇ by HRESIMS *m/z* 362.1191 [M+Na]⁺ (calcd for C₁₆H₂₁NO₇Na, 362.1216),³ indicating the presence of seven degrees of unsaturation in the molecule. The structure of **1** was elucidated by interpretation of NMR spectra³

including DEPT, COSY, HMQC, and HMBC (Fig. 1). The complete assignment of the protons and carbons was accomplished as shown in Table 1. The presence of the β -glucosyl group was suggested by the characteristic chemical shifts and coupling constants: δ_C 86.2 (C-1'), 71.3 (C-2'), 77.0 (C-3'), 69.8 (C-4'), 77.9 (C-5'), 61.0 (C-6'); δ_H 4.88 (d, $J = 9.2$ Hz, H-1'), 3.68 (dd, $J = 9.2, 9.5$ Hz, H-2'), 3.53 (dd, $J = 9.5, 8.9$ Hz, H-3'), 3.39 (dd, Hz, $J = 8.9, 9.5$ Hz, H-4'), 3.42 (m, H-5'), 3.61 (dd, $J = 12.2, 4.8$ Hz, H-6a'), 3.73 (dd, $J = 12.2, 1.6$ Hz, H-6b'). The presence of a 1,2-substituted phenyl group (C-3b to C-7a) in the aglycon was also suggested by the NMR data: δ_C 131.2 (C-3b), 124.9 (C-4), 121.6 (C-5), 130.9 (C-6), 109.6 (C-7), 148.5 (C-7a); δ_H 7.33 (d, $J = 7.3$ Hz, H-4), 6.92 (dd, $J = 7.3, 7.3$ Hz, H-5), 7.25 (dd $J = 7.3, 7.6$ Hz, H-6), 6.83 (d, $J = 7.6$ Hz, H-7). The structure of the other part, the condensed ring part, in the aglycon and the linkage position with the phenyl were elucidated by the correlations in the HMBC data (Fig. 1) and the molecular formula. The *N*-glucosidic bond and its position between the sugar and the aglycon were determined by the HMBC correlations (H-8a/H-1', H-1'/H-7a, H-1'/H-8a, Fig. 1) and the relatively high field chemical shift at C-1' (δ_C 86.2) compared with those of

Figure 1. HMBC correlations in **1**

O-glycosides.^{4, 5}

Table 1
¹H and ¹³C NMR data for **1** and **2** (in D₂O)

Position	1		2	
	¹ H δ (multiplicity, <i>J</i> in Hz)	¹³ C δ	¹ H δ (multiplicity, <i>J</i> in Hz)	¹³ C δ
Aglycone				
2	3.57 (m)	67.8	3.59 (m)	68.3
	4.02 (m)		4.05 (m)	
3	2.33 (m)	40.6	2.45 (m)	39.9
3a		87.8		87.8
3b		131.2		130.2
4	7.33 (d, 7.3)	124.9	7.39 (dd, 7.6, 0.9)	125.3
5	6.92 (dd, 7.3, 7.3)	121.6	6.93 (dd, 7.6, 7.3)	121.1
6	7.25 (dd 7.3, 7.6)	130.9	7.29 (ddd, 7.3, 7.9, 0.9)	131.3
7	6.83 (d, 7.6)	109.6	6.78 (d, 7.9)	108.2
7a		148.5		148.7
8a	5.59 (s)	99.7	5.51 (s)	98.4
Sugar				
1'	4.88 (d, 9.2)	86.2	4.89 (d, 9.2)	85.5
2'	3.68 (dd, 9.2, 9.5)	71.3	3.78 (dd, 9.2, 8.9)	70.1
3'	3.53 (dd, 9.5, 8.9)	77.0	3.59 (dd, 8.9, 8.8)	78.0
4'	3.39 (dd, 8.9, 9.5)	69.8	3.48 (dd, 8.8, 9.8)	69.9
5'	3.42 (m)	77.9	3.54 (ddd, 9.9, 4.9, 2.1)	78.0
6'	3.61 (dd, 12.2, 4.8)	61.0	3.73 (dd, 12.5, 4.9)	61.2
	3.73 (dd, 12.2, 1.6)		3.83 (dd, 12.5, 2.1)

To confirm the planar structure and determine the whole absolute configuration of

1, an optically active D-glucoside and L-glucoside, **1** and **2**,⁶were synthesized

chemically. Previously, we

completed total synthesis

of madindoline A,

involving an asymmetric

oxidative ring-closure

reaction of tryptophol to give **3** by

modified Sharpless epoxidation

[Ti(O-*i*-Pr)₄, (+)-DIPT, *t*-BuOOH,

CH₂Cl₂, -20 to -40°C (Scheme 1).^{7,8}

Interest in the synthetic applications of

3 led us to develop to the asymmetric

total synthesis of other optically active

indole alkaloid, (-)-physovenine,⁹ in a concise manner.¹⁰ Two diastereomers, **1** and **2**,

were prepared by the *N*-glucosidation of **3** with D- and L-glucoses^{11,12,13} in quantitative

yields, respectively (Scheme 2). The NMR data and the specific rotation ($[\alpha]_D^{21}$ -45,

H₂O, *c* 0.07)³ of synthetic **1** were completely identical with those of natural **1**,

indicating that the absolute configuration of **1** was as shown.

The effects of the compounds on C6 cell viability were analyzed.¹⁴ The natural

Scheme 1

Scheme 2

product (**1**) showed no effect. However, 100 μ M of the aglycone (**3**) significantly stimulated the cell growth at 24 h ($112.0 \pm 9.8\%$) and 48 h ($114.2 \pm 11.0\%$) treatment. On the other hand, L-glucoside (**2**) significantly reduced the cell number at 10 μ M ($92.4 \pm 9.1\%$) and 100 μ M ($83.9 \pm 8.0\%$) in 24 h treated cells, and at 100 μ M ($87.9 \pm 11.1\%$) in 48 h treated cells. Since dead cells were hardly observed by the treatment with **2** (data not shown), the reducing effect of the compound is thought to be growth retardation. Glycosylated natural products such as saponins or glycoalkaloids have been reported to show anti-proliferative activity or apoptotic effect in mammalian cells, especially cancer cells.¹⁵ Although they are expected to be utilized as anti-cancer agents, some of them show cytotoxicity even in normal cells. Tomato saponin called as tomatine, has been reported to be cytotoxic, and its deglycosylation abolished the cytotoxicity.¹⁶ It raises the possibility that each compound used in this study show distinct effect on mammalian cells.

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2. The material, Makomotake, was purchased from local market in Shizuoka, Japan.

Fresh Makomotake (16.7 kg) was extracted with EtOH (22 L, 3 times) and then with acetone (10 L, once). After the solutions had been combined and concentrated under reduced pressure, the concentrate was partitioned between CH₂Cl₂ and H₂O, and then the H₂O soluble part was partitioned between EtOAc and H₂O. The residue (20.0 g) obtained after removing EtOAc was fractionated by silica gel flash column chromatography (80% CH₂Cl₂/acetone, 50% CH₂Cl₂/MeOH, MeOH, each 2 L) to obtain seven fractions. Fraction 2 (eluted with 80% CH₂Cl₂/acetone, 1.28 g) was further separated by reversed-phase HPLC (Develosil C30-UG, 15% MeOH, 5 mL/tube), and nine fractions were obtained. Fraction 2-5 (65.7 mg) was further fractionated by reversed-phase HPLC (Cosmosil cholest, 10% MeOH) to afford compound **1** (1.8 mg; *R_t* 77.6 min). Compound **1**. mp 154-156°C; [α]_D²⁸ – 48 (*c* 0.30, H₂O); IR (neat); 1486, 1608, 3367 cm⁻¹; ¹H and ¹³C NMR, see Table 1; ESIMS *m/z* 362 [M+Na]⁺; HRESIMS *m/z* 362.1191 [M+Na]⁺ (calcd for C₁₆H₂₁NO₇Na, 362.1216).

3. ¹H NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500 spectrometer at 500 MHz, while ¹³C 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.

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6. Compound **2**. mp 131-132°C; $[\alpha]_D^{28} -12$ (c 0.30, H₂O); IR (neat); 1487, 1608, 3387 cm⁻¹; ¹H and ¹³C NMR, see Table 1; ESIMS *m/z* 362 [M+Na]⁺; HRESIMS *m/z* 362.1191 [M+Na]⁺ (calcd for C₁₆H₂₁NO₇Na, 362.1216).

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13. To solution of **3** (9.3 mg, 0.0525 mmol) in dry EtOH (0.53 mL), D-glucose (28.4 mg, 0.157 mmol) and acetic acid (9.0 mL, 0.157 mmol) at room temperature were added and the mixture was warmed up to 80°C with stirring for 16 h. Then, the reaction mixture was cooled to room temperature and concentrated. Flash chromatography (CHCl₃/MeOH = 5/1) gave the mixture of desired product and slight amount of D-glucose, which was further purified by Sep-Pak Plus C18 Cartridge (CH₃CN/H₂O = 1/9) to afford **1** (19.9 mg, 100%) as a colorless solid. According to the procedure of **1**, **2** (19.9 mg, 100%) was obtained as a colorless solid from **3** (10.3 mg, 0.0581 mmol) and L-glucose (31.4 mg, 0.174 mmol) with acetic acid (10 µL, 0.174 mmol) in EtOH (0.58 mL).
14. Rat glioma cell line C6 cells were maintained in the Dulbecco's modified Eagles medium (D-MEM, SIGMA, USA) supplemented with 10% fetal bovine serum (FBS), 10 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in humidified 5% CO₂ atmosphere. Cell number analysis was performed by 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H- tetrazolium bromide (MTT) assay. C6 cells were cultured in 96 well plates at 2000 cells /well cell density. After one-day culture, the cells were treated with

serial concentrations of the compounds in the medium without FBS. After 24 or 48 h incubation, the treated cells were subjected to the MTT assay. The treatment medium was replaced to the medium supplemented with 0.25 mg/mL of MTT without FBS, and the cells were incubated for 2 h. The incubation was terminated by addition of 20% (w/v) sodium dodecylsulfate (SDS) and 50% (v/v) dimethylformamide in water. The absorbance at 570 nm of the reaction mixture was measured by a microplate reader (Molecular Devices, USA). The data were expressed as means $\pm$ SD. The difference was analyzed by one-way ANOVA followed by Tukey-Kramer post-hoc test. P values less than 0.05 was considered significant.

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