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NOTE

Gymnopilin K: a new cytotoxic gymnopilin from *Gymnopilus spectabilis*

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In our continuing search for structurally interesting and bioactive metabolites from Korean wild mushrooms,[1–5] we have collected scores of endemic Korean mushroom species in the mountainous areas during the hot humid summer and prepared MeOH extracts from them for antitumor activity. Among the collected wild mushrooms, the extract of *Gymnopilus spectabilis* showed significant cytotoxicity against some human tumor cell lines using a sulforhodamine B bioassay. The hallucinogenic mushroom *G. spectabilis* (Cortinariaceae) is widely known as big laughter mushroom (Ohwaraitake in Japanese) as it can cause excessive laughing in those who consume it.[6] This hallucinogenic mushroom has been a rich source of unique metabolites. Chemical constituents of this mushroom have been reported to have gymnoprenols possessing the structure of a novel type of polyisoprenepolyol with 45–60 carbon atoms as major substances.[7–9] Some gymnopilins showing depolarizing activity as bitter principles, were also isolated.[10] In addition, a cytotoxic fatty acid (ostopanic acid),[11] antioxidant phenolics (bisnoryangonin and hispidin)[12] and a hallucinogenic alkaloid (psilocybin)[13] have been isolated from this source. Column chromatographic separation of its MeOH extract resulted in the isolation of a new gymnopilin named gymnopilin K (**1**), together with four known compounds, including gymnopilin A9 (**2**),[10] gymnopilin A10 (**3**),[10] gymnopilene (**4**)[9] and gymnoprenol A9 (**5**)[10] (Figure 1). The structure of **1** was elucidated on the basis of 1D and 2D NMR spectroscopic data analysis as well as chemical reactions, and the known compounds **2–5** were identified by comparison of physical and spectroscopic data with literature values. Here, we describe the isolation and structural elucidation of **1** as well as the cytotoxic activities of compounds **1–5**.

The air-dried and powdered fruiting bodies of *G. spectabilis* (153 g) were extracted with 80% aqueous MeOH two times at room temperature and then filtered. The filtrate was evaporated under vacuum to afford a MeOH extract (20 g), which was partitioned with *n*-hexane, $CHCl_3$ and *n*-BuOH subsequently using H_2O, yielding *n*-hexane (100 mg), $CHCl_3$ (2.3 g) and *n*-BuOH fractions (1.9 g). Each fraction was evaluated for cytotoxicity against A549, SK-OV-3, SK-MEL-2 and HCT-15 cell lines using a sulforhodamine B bioassay. We selected the $CHCl_3$ soluble fraction for current phytochemical investigation because the $CHCl_3$ soluble fraction had significant cytotoxicity against the tested tumor cell lines. The active $CHCl_3$ soluble fraction (2.3 g) was separated on a silica gel column with $CHCl_3$-MeOH (5:1) to yield seven fractions (G1–G7). Fraction G3 (250 mg) was separated on a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column with CH_2Cl_2-MeOH (1:1) and then purified by RP-C_{18} preparative HPLC (Econosil RP-18 10 μ column (Alltech, Nicholasville, KY, USA), 250×10 mm) using a solvent of MeCN-MeOH-H_2O (9:1:0.3) to yield compounds **4** (4 mg) and **5** (5 mg). Fraction G5 (420 mg) was separated on a Sephadex LH-20 column with CH_2Cl_2-MeOH (1:1) to afford two subfractions (G51–G52). Fraction G51 (170 mg) was subjected to passage over a Waters Sep-Pak Vac 6 cc (Waters, Milford, MA, USA) (100% MeOH) and then purified by RP-C_{18} preparative HPLC (80% MeOH) to yield compound **3** (5 mg). Fraction G6 (780 mg) was separated on a Sephadex LH-20 column with CH_2Cl_2-MeOH (1:1) to obtain two subfractions (G61–G62). Fraction G62 (450 mg) was separated on a RP-C_{18} silica gel column (75% MeOH) to yield six subfractions (G621–G626). Fraction G621 (27 mg) was purified by RP-C_{18} preparative HPLC (80% MeOH) to yield compound **2** (4 mg) and fraction G622 (35 mg) was purified by RP-C_{18} preparative HPLC (70% MeOH) to afford compound **1** (7 mg).

Compound **1** was obtained as a colorless gum with a negative specific rotation value $[\alpha]_D^{25}$−8.7 (*c* 0.35, MeOH). Its molecular formula was determined to be $C_{51}H_{98}O_{14}$ from the $[M+Na]^+$ peak at *m/z* 957.6859 (calcd for $C_{51}H_{98}NaO_{14}$, 957.6854) in the positive-ion high resolution (HR)-ESI-MS spectrum. The IR spectrum of **1** showed a broad hydroxyl band at 3440 cm^{-1} and a carbonyl absorption band at 1715 cm^{-1}. The physicochemical properties of **1** are summarized in Supplementary Information. The ^1H- and ^{13}C-NMR spectral data of **1** are shown in Table 1.

The ^1H- and ^{13}C-NMR spectra (Table 1) of **1** were very similar to those of **2**, with an apparent difference being the absence of signals for *cis*-vinyl methyls at δ_H 1.63 (3H, s, H-18) and 1.62 (3H, s, H-18); δ_C

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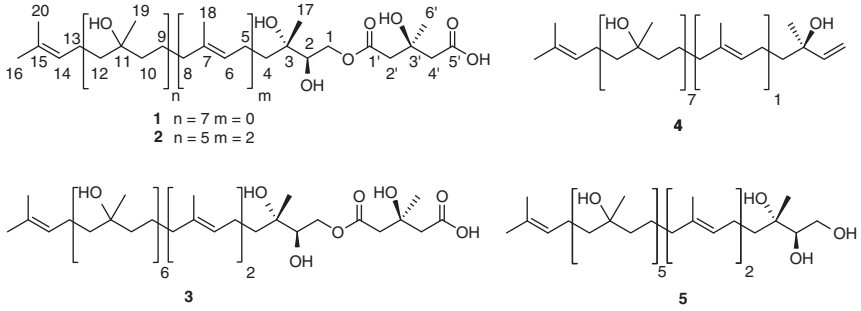

Figure 1 Structures of compounds **1**–**5**.

Table 1 ^{1}H- and ^{13}C-NMR data of compounds 1 and 2*

	1			*2*	
Position	δ_C	δ_H	*HMBC*	δ_C	δ_H
1	65.9 t	4.06 (dd, 11.0, 8.5)	C-2, 3, 1′	65.8 t	4.05 (dd, 11.0, 8.5)
		4.38 (dd, 11.0, 2.0)			4.40 (dd, 11.0, 2.0)
2	74.5 d	3.65 (dd, 8.5, 2.0)	C-1, 3, 4, 17	74.5 d	3.65 (dd, 8.5, 2.0)
3	73.2 s			73.2 s	
4	39.2 t	1.42 (br s)	C-2, 3, 9, 10, 17	39.1 t	1.42 (br s)
5				26.6 t, 22.5 t	2.15–1.95 (m)
6				124.6 d	5.12 (m)
7				134.9 s	
8				41.1 t, 40.1 t,	2.15–1.95 (m)
9	18.2 t	1.42 (br s)	C-3, 4, 10, 11	18.2 t	1.42 (br s)
10	42.2 t	1.42 (br s)	C-4, 9, 11, 12, 19	42.2 t	1.42 (br s)
11	72.2 s			72.2 s	
12	42.2 t	1.42 (br s)	C-10, 11, 13, 14, 19	42.2 t	1.42 (br s)
13	22.5 t	2.10 (m)	C-11, 12, 14, 15	22.1 t	2.15–1.95 (m)
14	124.6 d	5.12 (m)	C-12, 13, 15, 16, 20	124.6 d	5.12 (m)
15	130.8 s			130.8 s	
16	24.7 q	1.67 (s)	C-14, 15, 20	24.7 q	1.67 (s)
17	22.0 q	1.13(s)	C-2, 3, 4	21.5 q	1.13(s)
18				14.8 q	1.63 (s), 1.62 (s)
19	25.7 q	1.13 (s)	C-10, 11, 12	25.7 q	1.13 (s)
20	16.3 q	1.61 (s)	C-14, 15, 16	16.5 q	1.62 (s)
1′	171.6 s			171.8 s	
2′, 4′	45.5 t	2.68 (s), 2.67 (s)	C-1′, 3′, 5′, 6′	45.9 t	2.65 (s), 2.63 (s)
3′	69.7 s			69.9 s	
5′	177.9 s			177.8 s	
6′	26.6 q	1.13 (s)	C-2′, 3′, 4′	26.6 q	1.13 (s)

*NMR data were obtained in 500 MHz for ^{1}H and 125 MHz for ^{13}C in CD_3OD, and values in parentheses are coupling constants in Hz.

14.8 (C-18) and 14.8 (C-18), allylic methylenes at δ_H 2.15-1.95 (8H, m, H-5, H-8); δ_C 26.6 (C-5), 22.5 (C-5), 41.1 (C-8), and 40.1 (C-8), and double bonds at δ_H 5.12 (2H, m, H-6); δ_C 124.6 (C-6) and 134.9 (C-7) in **2**.[10] The intensity of the ^{1}H-NMR signal at δ_H 5.12 (1H, m, H-14) and two vinyl methyls at δ_H 1.67 (3H, s, H-16) and 1.61 (3H, s, H-20), which were correlated with H-14 in the HMBC spectrum, indicated that compound **1** has only one isoprene unit with a trisubstituted double bond at the terminal position of **1**. Compound **1** was assigned to be a successively hydrated isoprenoid structure, which had the same number of repeated isoprene units compared to those of **2** by the molecular formula $C_{51}H_{98}O_{14}$ obtained from the analysis of the HR-ESI-MS data. This suggested that compound **1** may be produced by the saturation of C-6/7 double bond in **2** by the addition of tertiary alcohols. The full NMR assignments of **1** were performed by the analysis of the ^{1}H-^{1}H COSY, DEPT, HMQC and HMBC spectroscopic data (Table 1).

The absolute configurations of C-2 and C-3 of **1** were established on the basis of the modified Mosher's method in combination with the NOESY experiment.[14] Treatment of **1** with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*S*)-MTPA-Cl] and 4-(dimethylamino)pyridine (DMAP) in pyridine gave the (*R*)-MTPA esters **1r**. Similar treatment of **1** with (*R*)-(-)-MTPA-Cl afforded the (*S*)-MTPA esters **1s**. Analysis of the ^{1}H-NMR chemical shift differences ($\Delta\delta_{S-R}$; see Supplementary Information) of the two MTPA esters allowed the assignment of the absolute configuration of C-2 as *R* (Figure 2). In the NOESY spectrum of **1**, H_2-1 and H-2 showed consistent correlations with H_3-17 and H_2-4, respectively. However, there was no apparent correlation between H-2 and H_3-17 in its 1D

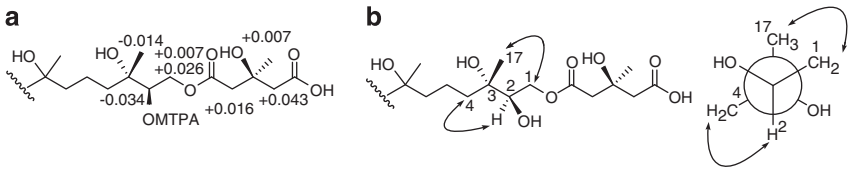

Figure 2 $\Delta\delta$ Values (δ_S–δ_R) in p.p.m. of the two MTPA esters derived from **1** (**a**) and Key NOE correlations in the NOESY spectrum of **1** (**b**).

Table 2 Cytotoxic activities of compounds 1–5 isolated from *Gymnopilus spectabilis*

	IC_{50}(μM)[a]			
Compound	*A549*	*SK-OV-3*	*SK-MEL-2*	*HCT-15*
1	10.80	9.59	6.74	12.02
2	15.28	11.14	3.26	9.96
3	7.15	10.54	4.20	16.41
4	18.45	22.14	14.40	24.10
5	26.30	14.32	12.80	25.14
Doxorubicin[b]	0.012	0.008	0.017	0.857

[a]IC_{50} (inhibitory concentration) value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.
[b]Doxorubicin as a positive control.

NOE spectrum (Figure 2), which indicated that H-2 and H_3-17 in **1** were in the *anti* configuration. These NOESY data, in combination with the result from the modified Mosher's method, allowed us to assign the absolute configuration of C-3 as *S*. Finally, the absolute configuration of C-3′ of **1** was determined by the alkaline methanolysis of **1**. Treatment of **1** with 3% sodium methoxide (NaOMe) in MeOH yielded methyl (*S*)-3-hydroxy-3-methylglutarate, which was identified by comparison of its ^{1}H-NMR, specific rotation $[\alpha]_D^{25}$+6.1 (*c* 0.04, $CHCl_3$), and MS data (see Supplementary Information).[15,16] Thus, the structure of **1** was established as shown in Figure 1 and the compound was named gymnopilin K.

Compounds **1**-**5** were evaluated for their antiproliferative activities against four human cancer cell lines including A549, SK-OV-3, SK-MEL-2, and HCT-15 using the sulforhodamine B bioassay.[17] All tested compounds showed inhibitory activity against proliferation of the tested cell lines with inhibitory concentration $(IC)_{50}$ values ranging between 3.26-26.30 μM (Table 2). In particular, all compounds exhibited significant cytotoxicity against the SK-MEL-2 cell line with IC_{50} values in the range of 3.26-14.40 μM (Table 2). In the structure-activity relationship (SAR), it appears that the methyl (*S*)-3-hydroxy-3-methylglutarate moiety in the molecule improves cytotoxicity, as compounds **1–3** (IC_{50} values ranging between 3.26 and 16.41 μM) with the above moiety were more active than compounds **4** and **5** (IC_{50} values ranging between 12.80 and 26.30 μM). This SAR study provides valuable data for future synthetic and pharmacological studies with the aim of obtaining more potent cytotoxic compounds.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)