A new monoacylglycerol from the fruiting bodies of *Gymnopilus spectabilis*

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Bioassay-guided fractionation and chemical investigation of the MeOH extract of the wild mushroom *Gymnopilus spectabilis* resulted in the isolation of a new monoacylglycerol, together with five known compounds. The structure of the new compound (2'*S*)-1-*O*-(6,6-dimethoxyhexanol)-glycerol was elucidated on the basis of 1D and 2D NMR (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC and HMBC) spectroscopic data as well as chemical reactions. The known compounds were identified by comparison of their spectroscopic and physical data with the reported values. The metabolites were evaluated for their anti-proliferative activities against four human tumour cell lines (A549, SK-OV-3, SK-MEL-2 and HCT-15) and for their inhibitory effects on nitric oxide production in lipopolysaccharide (LPS)-activated murine microglial cells.

Keywords: *Gymnopilus spectabilis,* Cortinariaceae, monoacylglycerol, cytotoxicity, NO

During the course of our continuing search for structurally interesting and bioactive constituents of Korean wild mushrooms,[1–6] we have collected a plethora of endemic mushroom species in the mountainous regions during the hot humid summer, and prepared their MeOH extracts for use in anti-tumour activity screening tests. Among the collected uncultivated mushrooms, we found that the MeOH extract of *Gymnopilus spectabilis* showed significant cytotoxicity against several human tumour cell lines using a sulforhodamine B bioassay.[6] *G. spectabilis* (Cortinariaceae) is the hallucinogenic mushroom that is widely known as the 'big laughter mushroom' (Ohwaraitake in Japanese) as it can cause excessive laughing in those who consume it.[7] This hallucinogenic mushroom has been a rich source of diverse metabolites including fatty acids, phenolic compounds and alkaloids.[7–10] Some of these have shown antioxidant or cytotoxic properties in previous biological activity studies.[7–10] Interestingly, this mushroom has been reported to contain large amounts of unique gymnoprenols that possess the structure of a novel type of polyisoprenepolyol with 45 to 60 carbon atoms.[11–13] Recently, we also reported the isolation of gymnopilin derivatives with cytotoxic activities.[6] In continuing efforts to investigate the bioactive constituents of this source, we isolated one new monoacylglycerol (**1**), together with five known compounds (**2**–**6**) (Fig. 1). The structure of the new compound (**1**) was determined by spectroscopic methods, including 1D and 2D NMR (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC and HMBC) and HR-ESIMS analysis in addition to chemical reactions. The known compounds (**2**–**6**) were identified by comparison of their spectroscopic and physical data with the reported values. The isolated compounds (**1**–**6**) were tested for their cytotoxic activities against four human tumour cell lines (A549, SK-OV-3, SK-MEL-2 and HCT-15) and for their inhibitory effects on nitric oxide (NO) production in lipopolysaccharide-activated murine microglial cells. The isolation, structural elucidation and biological evaluation of these compounds are described in this paper.

The $CHCl_3$ soluble portion of the MeOH extract of *G. spectabilis* showed significant cytotoxicity against some human tumour cell lines, which led us to investigate the chemical components. Repeated column chromatography over silica gel and Sephadex LH-20, and semi-preparative HPLC resulted in the isolation and identification of one new monoacylglycerol (**1**), together with five known compounds (**2**–**6**) (Fig. 1). The known compounds were identified as 9,12,13-trihydroxyoctadeca-10(*E*),15(*Z*)-dienoic acid (**2**),[14] 4,6-decadiyne-1,3,8-triol (**3**),[7] 5α,8α-epidioxyergosta-6,22-dien-3β-ol (**4**),[15] 5α,8α-epidioxy-24(*R*)-methylcolesta-6-en-3β-ol (**5**)[16] and 2-allyl-1,4,5-trimethoxybenzene (**6**)[17] by comparing their spectroscopic and physical data with the reported values.

Compound **1** was isolated as a colourless gum with positive optical rotation $[\alpha]^{25}_{D}$ + 34.4 (in MeOH). The molecular formula was determined to be $C_{11}H_{22}O_6$ by the positive mode

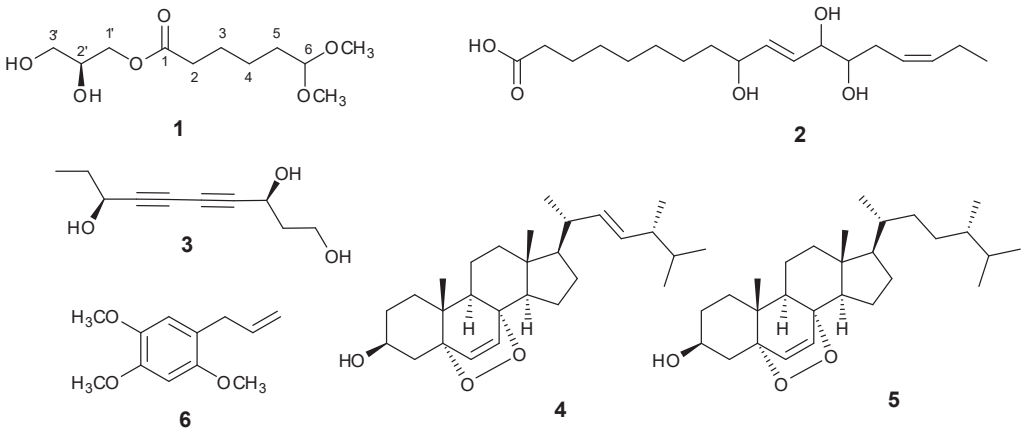

Fig. 1 The structures of compounds **1**–**6**.

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Table 1 ^{1}H and ^{13}C NMR data of compound **1** in CD_3OD[a]

Position	**1**	
	δ_H	δ_C
1		174.0
2	2.34 t (7.0)	33.7
3	1.61 m	24.8
4	1.40 m	24.7
5	1.61 m	33.5
6	4.32 t (6.0)	104.2
OCH_3	3.26 s	53.1
1′	4.17 dd (11.5, 4.5) 4.09 dd (11.5, 6.0)	65.3
2′	3.83 m	70.1
3′	3.59 dd (11.5, 5.5) 3.56 dd (11.5, 6.0)	62.4

[a] ^{1}H and ^{13}C NMR data were recorded at 500 and 125 MHz, respectively. Coupling constants (in Hz) are given in parentheses.

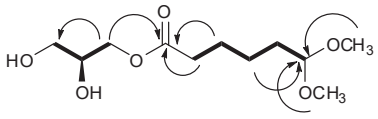

Fig. 2 Key ^{1}H-^{1}H COSY (bold) and HMBC (plain arrow) correlations of **1**.

HR-ESIMS ion at *m/z* 251.1491 $[M + H]^+$ (calcd. for $C_{11}H_{23}O_6$, 251.1495). The IR spectrum indicated that compound **1** possessed hydroxyl (3385 cm^{-1}) and carbonyl (1720 cm^{-1}) groups. The ^{1}H NMR spectrum of **1** (Table 1) displayed signals for the presence of an acetal methine proton at δ_H 4.32 (1H, t, J = 6.0 Hz, H-6), four aliphatic methylenes at [δ_H 2.34 (2H, t, J = 7.0 Hz, H-2), 1.61 (4H, m, H-3, 5), 1.40 (2H, m, H-4)], and two methoxyl groups integrating for 6H at δ_H 3.26 (s). The ^{13}C NMR spectrum exhibited 11 carbon signals, including a carbonyl carbon (δ_C 174.0) and an acetal carbon (δ_C 104.2). The NMR spectral data of **1** (Table 1) were similar to those of methyl 6,6-dimethoxyhexanoate.[18] However, in addition, presence of a glycerol moiety [δ_H 4.17 (1H, dd, J = 11.5, 4.5 Hz, H-1'a), 4.09 (1H, dd, J = 11.5, 6.0 Hz, H-1'b), 3.83 (1H, m, H-2'), 3.59 (1H, dd, J = 11.5, 5.5 Hz, H-3'a) and 3.56 (1H, dd, J = 11.5, 6.0 Hz, H-3'b); δ_C 70.1 (C-2'), 65.3 (C-1'), and 62.4 (C-3')] was observed in place of a methyl ester. The planar structure of **1** was determined by the ^{1}H-^{1}H COSY, HMQC and HMBC experiments (Fig. 2), in which HMBC cross peaks between H-1'/C-1 and OCH_3/C-6 were observed. Based on these data, the structure of **1** was established as 1-*O*-(6,6-dimethoxyhexanoyl)-glycerol. The sign of the specific optical rotation ($[\alpha]^{25}_D$ + 34.4) for compound **1** was similar to those of monoglycerides with an *S*-configuration,[19,20] suggesting that the absolute configuration at C-2' of **1** may be the same as 2'*S*. The absolute configuration at C-2' was determined using a dibenzoate chirality method.[21] The hydroxyl groups at C-2' and C-3' of **1** were esterified with benzoyl chloride in dry pyridine to obtain **1a**. The CD spectrum of **1a** exhibited a positive exciton couplet CD peak at 235 nm to reflect its 2'*S* configuration.[21] Thus, the structure of **1** was elucidated to be (2'*S*)-1-*O*-(6,6-dimethoxyhexanoyl)-glycerol.

It is possible that the dimethoxyacetal was an artefact arising from the addition of methanol to the aldehyde during the isolation. Consequently, a fresh sample of the fungus, *G. spectabilis* was extracted with ethanol and the extract was subjected to LC/MS. However it still showed a peak at the retention time of 0.814 minute with a molecular ion of 251.1479 $[M + H]^+$ corresponding to the dimethoxyacetal, which is therefore a genuine natural product.

Table 2 Cytotoxicity of compounds **1–6** against human tumour cell lines

Compounds	IC_{50}/μM[a]			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	> 30.0	> 30.0	26.43	> 30.0
2	> 30.0	> 30.0	16.30	> 30.0
3	> 30.0	> 30.0	22.26	> 30.0
4	10.02	20.41	6.45	11.46
5	> 30.0	27.20	23.49	> 30.0
6	> 30.0	> 30.0	> 30.0	> 30.0
Doxorubicin[b]	0.012	0.008	0.017	0.857

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

[b] Doxorubicin as a positive control.

Table 3 Inhibitory effects on NO production of compounds **1–6** in LPS-activated BV-2 cells

Compound	IC_{50}/μM[a]	Compound	IC_{50}/μM[a]
1	29.90	**5**	23.95
2	25.39	**6**	34.22
3	71.95	NMMA[b]	18.29
4	2.73		

[a] IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.

[b] NMMA as a positive control.

On the basis of the fact that the compounds **1–6** were isolated from the most active $CHCl_3$ soluble fraction, compounds **1–6** were evaluated for their anti-proliferative activities against four human cancer cell lines including A549, SK-OV-3, SK-MEL-2 and HCT-15 using the SRB bioassay.[22] The results (Table 2) show that compounds **1–5** had inhibitory activity against the proliferation of the SK-MEL-2 cell line with IC_{50} values ranging from 6.45 to 26.43 μM. In particular, compound **4** exhibited significant cytotoxic activities against all the tumour cell lines, A549, SK-OV-3, SK-MEL-2 and HCT-15 with IC_{50} values in the range of 6.45 to 20.41 μM. However, compound **6** was inactive (IC_{50} > 30 μM) against all the cell lines. Interestingly, among the isolates, the three aliphatic chain derivatives **1–3** showed a selective antiproliferative effect against SK-MEL-2.

Additionally, the anti-neuroinflammatory activities of the isolates **1–6** were evaluated by measuring the nitric oxide (NO) levels produced in LPS-activated microglia BV-2 cells. Microglia, the immune resident cells of the brain, have been implicated in the pathogenesis of a variety of neurodegenerative diseases including Parkinson's disease and Alzheimer's disease.[23,24] Under pathological conditions, microglia cells are over-activated and produce a variety of pro-inflammatory mediators including NO, which consequently leads to various neurodegenerative conditions of the central nervous system. In this test, the isolated compounds **1–6** inhibited NO levels in LPS-stimulated BV-2 cells with IC_{50} values in the range of 2.73 to 71.95 μM (Table 3). None of the compounds showed any significant cellular toxicity up to 20 μM (data not shown). In particular, compound **4** potently inhibited LPS-stimulated NO production with an IC_{50} value of 2.73 μM and it displayed greater activity than the positive control, N^G-nonomethyl-L-arginine (L-NMMA; a well-known NOS inhibitor, IC_{50} value of 18.29 μM).

In conclusion, a new monoacylglycerol (**1**) and five known compounds (**2–6**) have been isolated from the fruiting bodies of *G. spectabilis* during the investigation of the bioactive constituents. Compound **1** is chemically unusual with respect to the presence of a dimethoxy group at the terminal carbon of fatty acid analogues. In the bioassays, compounds **1–5**

had cytotoxicity against the SK-MEL-2 line, with IC_{50} values ranging from 6.45 to 26.43 μM. In addition, compounds **1**–**6** showed significant inhibitory effects on NO production in LPS-stimulated BV-2 with IC_{50} values in the range of 2.73 to 71.95 μM, without affecting viability.

Experimental

Optical rotations were measured on a Jasco P-1020 polarimeter. IR spectra were recorded on a Bruker IFS-66/S FTIR spectrometer. ESI and HR-ESI mass spectra were recorded on an SI-2/LCQ DecaXP Liquid chromatography (LC)-mass spectrometer. NMR spectra, including ^{1}H-^{1}H COSY, HMQC and HMBC experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Preparative HPLC was performed using a Gilson 306 pump with a Shodex refractive index detector. Column chromatography was performed with silica gel 60 (230–400 mesh). The packing material for molecular sieve column chromatography was Sephadex LH-20. Merck precoated silica-gel F_{254} plates and reversed-phase (RP)-18 F_{254s} plates were used for TLC. Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde-sulphuric acid.

Mushroom Material: The fresh fruiting bodies of *G. spectabilis* were collected at Donggureung, Guri of GyeongGi-do, Korea, in July 2006. A voucher specimen (SKKU-2006-7) of the mushroom was authenticated by one of the authors (K.R.L.) and was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Korea.

Extraction and isolation: The air-dried and powdered fruiting bodies of *G. spectabilis* (153 g) were extracted with 80% aqueous MeOH twice at room temperature and then filtered. The filtrate was evaporated under a vacuum to afford a MeOH extract (20 g), which was partitioned with *n*-hexane, $CHCl_3$, and *n*-BuOH sequentially using H_2O, yielding *n*-hexane (100 mg), $CHCl_3$ (2.3 g), and *n*-BuOH (1.9 g) soluble fractions. Each fraction was evaluated for cytotoxicity against A549, SK-OV-3, SK-MEL-2 and HCT-15 cell lines using a SRB bioassay. The most active fraction, the $CHCl_3$ soluble fraction (2.3 g) was separated on a silica-gel column [100 g, 1.8 × 35 cm, eluted $CHCl_3$-MeOH, 5:1, (2.1 L)] to give seven fractions [Fr. G1, 0.3 L; Fr. G2, 0.3 L; Fr. G3, 0.3 L; Fr. G4, 0.3 L; Fr. G5, 0.3 L; Fr. G6, 0.3 L; and Fr. G7, 0.3 L]. Fraction G2 (220 mg) was subjected to a Sephadex LH-20 column chromatography [50 g, 1 × 60 cm, eluted with CH_2Cl_2-MeOH, 1:1, (1.0 L)] to yield two fractions [Fr. G21, 0.5 L; and Fr. G22, 0.5 L]. Subfraction G22 (78 mg) was purified by semi-preparative reversed-phase HPLC using an Econosil RP-C18 column (10 μm column; 250 × 10 mm; 10 μm particle size; Shodex refractive index detector) with a solvent system of 100% MeOH to yield compounds **4** (5 mg) and **5** (3 mg). Fraction G5 (420 mg) was subjected to a Sephadex LH-20 column chromatography [50 g, 1 × 60 cm, eluted with CH_2Cl_2-MeOH, 1:1, (1.0 L)] to yield two fractions [Fr. G51, 0.5 L; and Fr. G52, 0.5 L]. Subfraction G51 (170 mg) was subjected to passage over a Waters Redi*Sep* C-18 reversed-phase column with 100% MeOH, and subsequently purified by semi-preparative reversed-phase HPLC with a solvent system of MeOH–H_2O (3:1) to give compound **3** (5 mg). Subfraction G52 (165 mg) was passed through a Waters Redi*Sep* C-18 reversed-phase column in a solvent system of MeOH–H_2O (7:3) and then purified by semi-preparative reversed-phase HPLC with solvent system of 100% MeOH to afford compounds **2** (3 mg) and **6** (4 mg). Fraction G6 (780 mg) was separated by Sephadex LH-20 column chromatography [100 g, 1.5 × 60 cm, and eluted with CH_2Cl_2-MeOH, 1:1, (1.5 L)] to give three fractions [Fr. G61, 0.5 L; Fr. G62, 0.5 L and Fr. G63, 0.5 L]. Subfraction G62 (450 mg) was chromatographed to a RP-C18 silica-gel column [100 g, 3 × 30 cm, eluted with MeOH–H_2O, 4:1, (3.0 L)] to give six subfractions [Fr. G621, 0.5 L; Fr. G622, 0.5 L; Fr. G623, 0.5 L; Fr. G624, 0.5 L; Fr. G625, 0.5 L; and Fr. G626, 0.5 L]. Subfraction G626 (38 mg) was purified by a Waters Silica gel Sep-Pak Vac 6cc with a solvent system of $CHCl_3$-MeOH (2:1) to yield compound **1** (5 mg).

*(2'S)-1-*O*-(6,6-dimethoxyhexanoyl)-glycerol* (**1**): Colourless gum. $[\alpha]^{25}_D$ + 34.4 (*c* 0.20, MeOH). IR (KBr) ν_{max} 3385 (OH), 2945, 1720 (C=O), 1615, 1450, 1032 cm^{-1}. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1. ESIMS (positive-ion mode) *m/z*: 251 $[M + H]^+$. HR-ESIMS (positive-ion mode) *m/z*: 251.1491 $[M + H]^+$ (calcd. for $C_{11}H_{23}O_6$, 251.1495).

Synthesis of **1a** *(benzoyl ester of* **1***)*: An ice-cooled solution of **1** (2.0 mg) in dry pyridine (0.75 mL) was mixed with benzoyl chloride (0.15 mL), and stirred at room temperature for 20 h. Two drops of MeOH were added to the reaction mixture which was stirred for 10 min, then diluted with EtOAc and aqueous Na_2CO_3, and the layers were separated. The organic layer was washed with brine, and the combined aqueous layer was extracted with EtOAc. The combined organic extract was dried over $MgSO_4$ and concentrated. The residual dark-brown oil was purified by a silica-gel Waters Sep-Pak Vac 6cc with *n*-hexane–EtOAc (4:1) to give **1a** (0.8 mg) as a colourless oil.

Benzoyl ester of **1** (**1a**): Colourless oil. CD (MeOH) λ_{max} (Δε) 235 (+27.3), 219 (–9.1) nm. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42 (2H, m, H-4), 1.63 (4H, m, H-3, 5), 2.37 (2H, t, *J* = 7.5 Hz, H-2), 3.28 (6H, s, OCH_3), 4.25 (1H, dd, *J* = 12.0, 6.0 Hz, H-1′a), 4.35 (1H, t, *J* = 6.5 Hz, H-6), 4.47 (1H, dd, *J* = 12.0, 4.0 Hz, H-1′b), 4.54–4.60 (2H, m, H-3′), 5.05 (1H, m, H-2′), 7.49–7.52 (4H, m, ArH), 7.60–7.63 (2H, m, ArH), 8.01–8.02 (4H, m, ArH). ESIMS (positive-ion mode) *m/z*: 459 $[M + H]^+$.

Cell cultures: All tumour cell cultures were maintained using RPMI1640 cell growth medium, supplemented with 5% foetal bovine serum (FBS), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Human tumour cell lines, A549 (non-small cell lung carcinoma), SK-OV-3 (ovarian malignant ascites), SK-MEL-2 (skin melanoma) and HCT-15 (colon adenocarcinoma), were provided by the National Cancer Institute (NCI). The murine microglial BV2 cell line was generously provided by Dr. E. Choi from Korea University (Seoul, Korea), and maintained in Dulbecco's modified Eagle (DMEM) medium supplemented with 5% FBS, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. All cells were incubated at 37 °C in a humidified incubator with 5% CO_2.

Cytotoxicity assessment: The cytotoxicity of the compounds against cultured human tumour cell lines was evaluated by the SRB method.[22] Each tumour cell line was inoculated in standard 96-well flat-bottomed microplates and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The attached cells were then incubated with the serially diluted compounds, with the control cultures receiving the carrier solvent (0.1% dimethyl sulphoxide). After continuous exposure to the compounds for 48 h, the culture medium was removed from each well and the cells were fixed with 10% cold trichloroacetic acid at 4 °C for 1 h. After washing with tap water, the cells were stained with 0.4% SRB dye and incubated for 30 min at room temperature. The cells were washed again and solubilised with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance was measured spectrophotometrically at 520 nm with a microtiter plate reader. Doxorubicin (purity ≥ 98%; Sigma) was used as a positive control. Tested compounds were demonstrated to be pure, as evidenced by NMR and HPLC analysis (purity ≥ 95%).

Measurement of NO production: BV-2 cells were plated into a 96-well plate (3 × 10^4 cells/well). After 24 h, cells were pre-treated with the compounds for 30 min, and stimulated with 100 ng mL^{-1} LPS for a further 24 h. Control cultures received the carrier solvent (0.1% dimethyl sulphoxide). Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 570 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate the NO_2^- concentration. N^G-monomethyl-L-arginine (NMMA, Sigma, St. Louis, MO, USA), a well-known NO synthase inhibitor, was tested as a positive control.[25]

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