KDR Kinase Inhibitor Isolated from the Mushroom *Boletopsis leucomelas*

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Three novel *p*-terphenyl compounds, named boletopsins A (**1**), B (**2**), and C (**3**), and four known analogues (**4**−**7**) were isolated from fruiting bodies of the mushroom *Boletopsis leucomelas*. Compounds **1**−**7** were tested for KDR kinase inhibitory activity, and boletopsin C (**3**) was found to have an IC_{50} value of 70.7 μM. Compound **3** also showed inhibition of proliferation of human umbilical vein endothelial cells, with an IC_{50} value of 9.04 μM.

Boletopsis leucomelas (Pers.: Fr.) Fayod (Thelephoraceae) is an edible mushroom with a black fruiting body. A series of *p*-terphenyl compounds, tentatively named Bl-I, Bl-II, Bl-III, Bl-IV, and Bl-V, have been isolated from the mushroom, and Bl-III showed 5-lipoxygenase inhibitory activity.[1] Moreover, *B. leucomelas* lectin (BLL) has been shown to induce apoptosis in human leukemic U937 cells.[2]

Tumor angiogenesis is an essential process in the growth of tumor cells, and tumor angiogenesis inhibitors represent a new strategy for the treatment of cancer. Two angiogenesis inhibitors have been approved in markets: sunitinib, for use in advanced kidney cancer and gastrointestinal stromal tumors, and sorafenib, for use in kidney and liver cancer. Both are classed as multitarget tyrosine kinase inhibitors and particularly inhibit the vascular endothelial growth factor receptor (VEGFR), which plays a critical role in regulating angiogenesis.[3,4] In particular, VEGFR2 (KDR) is the most necessary factor for proliferation and migration of vascular endothelial cells, which promote tumor angiogenesis.[5]

In this paper, we report the isolation and structure determination of novel *p*-terphenyl compounds, boletopsins A (**1**), B (**2**), and C (**3**), and four known analogues, Bl-IV (**4**), Bl-V (**5**), cycloleucomelone (**6**), and cycloleucomelone-2-acetate (**7**), from a MeOH extract of *B. leucomelas*. Structures for the new compounds **1**−**3** were determined by spectroscopic analysis. Antiangiogenic activity of the compounds was evaluated by measuring the inhibitory effects of KDR kinase and inhibitory effects on proliferation of human umbilical vein endothelial cells (HUVECs), as described herein.

Initially, the dry fruiting body of *B. leucomelas* was extracted with $CHCl_3$ and then MeOH. The MeOH extract was then partitioned between EtOAc and water. The EtOAc-soluble materials were subjected to silica gel column chromatography (CC), HPLC, and Sephadex LH-20 CC. Three novel *p*-terphenyl compounds (**1**−**3**) and four known analogues, Bl-IV (**4**), Bl-V (**5**), cycloleucomelone (**6**), and cycloleucomelone-2-acetate (**7**), were obtained.

The molecular formula of compound **1** was determined to be $C_{20}H_{14}O_8$ on the basis of HREIMS *m*/*z* 382.0688 $[M]^+$. The IR spectrum exhibited absorption peaks at 3399 cm^{-1} (OH) and 1730 cm^{-1} (carbonyl). The ^{13}C NMR and ^1H NMR spectra revealed an acetoxy carbonyl signal at δ_C 168.6 and an acetoxy methyl signal at δ_C 20.2 and δ_H 2.07 (s). Furthermore, the aromatic region [δ_H 6.95 (s), 6.79 (2H, d, J = 8.5 Hz), 7.06 (2H, d, J = 8.5 Hz), and 7.43 (s)] in the ^1H NMR spectrum suggested the presence of 1,4-disubstituted and 1,2,4,5-tetrasubstituted benzene rings. According to the NMR data, we proposed that **1** was an analogue of cycloleucomelone-leucoacetate, recently reported from *B. leucomelas*.[1,6] The signals at δ_H 8.00 (br s), 9.02 (br s), 9.11 (br s), 9.32 (br s), and 9.45 (br s) were assigned to the OH protons attached to C-2, C-8, C-1, C-7, and C-4′, respectively, based on HMBC correlations. Therefore, the acetyl group was considered to be at C-4, identifying **1** as a novel monoacetate of cycloleucomelone-leucoacetate, and it was named boletopsin A (**1**).

The molecular formula of compound **2** was determined to be $C_{22}H_{16}O_{10}$ on the basis of HRFABMS *m*/*z* 441.0823 $[M + H]^+$. The IR spectrum exhibited absorption peaks at 3399 cm^{-1} (OH) and 1757 cm^{-1} (carbonyl). The ^{13}C NMR and ^1H NMR spectra (Table 1) indicated the presence of two acetyl groups in **2**. Furthermore, the aromatic region in the ^1H NMR spectrum (Table 1) suggested the presence of 1,2,4-trisubstituted and 1,2,4,5-tetrasubstituted benzene rings. The two acetyl groups of **2** had the same chemical shift values as those of the known compound Bl-V (**5**),[1] indicating that compound **2** was an analogue of **5**. HRFABMS and NMR data indicated that **2** had one more OH group than **5**. The HMBC correlations, δ_H 6.50 (dd, J = 8.1, 2.0 Hz), 6.67 (d, J = 2.0 Hz), and 6.75 (d, J = 8.1 Hz), were assigned to H-6′, H-2′, and H-5′, respectively, indicating that the additional OH in **2** was located at the 3′ position. Compound **2** was named boletopsin B.

Compound **3** ($C_{20}H_{14}O_9$) had IR absorption peaks at 3307 cm^{-1} (OH) and 1735 cm^{-1} (carbonyl). The ^{13}C NMR and ^1H NMR spectra indicated that **3** had one acetyl group. The aromatic region in the ^1H NMR spectrum (Table 1) suggested the presence of 1,2,4-trisubstituted and 1,2,4,5-tetrasubstituted benzene rings. Thus, compound **3** was similar in structure to compound **1**. The acetyl group of compound **3** had nearly the same chemical shift as that of **1**, indicating that **3** was a structural analogue, and **3** was shown (by NMR and MS) to possess one more OH group than **1**. HMBC correlations assigned the signals of δ_H 6.52 (dd, J = 8.1, 2.1 Hz), 6.68 (d, J = 2.1 Hz), and 6.76 (d, J = 8.1 Hz) to H-6′, H-2′, and H-5′, respectively, indicating that the additional OH group was located at the 3′ position. Therefore, the acetyl group was considered to be at C-4, confirming that compound **3** was a novel monoacetate of cycloleucomelone-leucoacetate, and it was named boletopsin C (**3**).

The known compounds **4** and **5** were determined to be the triacetate (Bl-IV) and diacetate (Bl-V), respectively, of the cycloleucomelone-leucoacetate series. The known compounds **6** and **7** were identified as the quinine-type compounds cycloleucomelone and cycloleucomelone-2-acetate, respectively. Compounds **4**−**7** were identified on the basis of their spectroscopic data as compared with published data.[1,6]

Compounds **1**−**7** were all tested using the tyrosine kinase inhibitory assay kit with KDR. Only boletopsin C (**3**) showed

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Chart 1

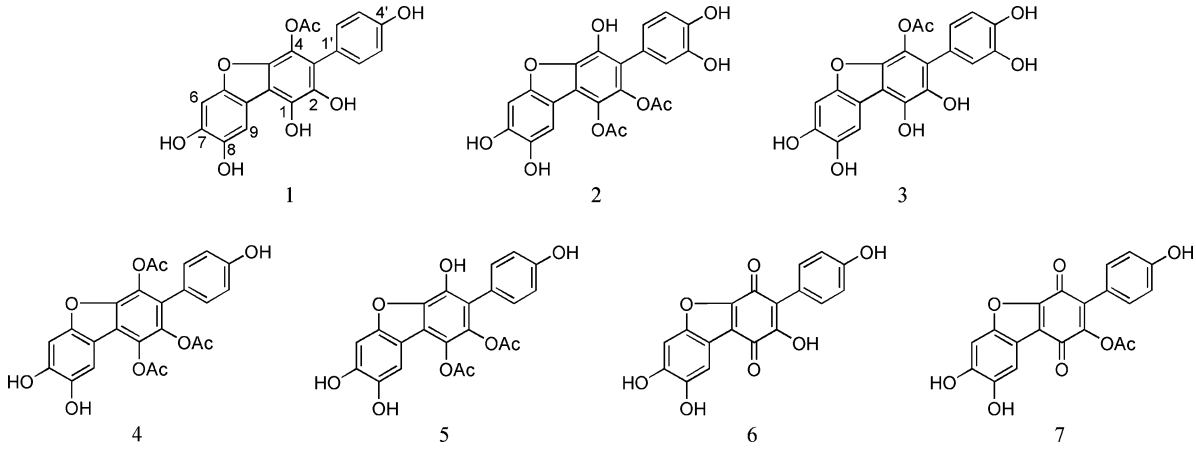

Table 1. NMR Spectroscopic Data (400 MHz, DMSO-d_6) for Compounds **1**−**3**

	boletopsin A (**1**)			boletopsin B (**2**)			boletopsin C (**3**)		
position	δ_C	δ_H (J in Hz)	HMBC	δ_C	δ_H (J in Hz)	HMBC	δ_C	δ_H (J in Hz)	HMBC
1	137.2[a], C			127.4[b], C			137.3[a], C		
2	137.8[a], C			135.5[b], C			137.6[a], C		
3	122.0, C			120.6, C			122.1, C		
4	124.5, C			137.2[b], C			124.5, C		
4a	141.5, C			142.2[b], C			141.5, C		
5a	149.3, C			149.7, C			149.4, C		
6	98.2, CH	6.95, s	5a, 7, 8, 9a	98.3, CH	7.06, s	5a, 7, 8, 9a	98.2, CH	6.95, s	5a, 7, 8, 9a
7	145.5, C			146.9, C			145.5, C		
8	141.9, C			142.5, C			141.9, C		
9	107.3, CH	7.43, s	5a, 7, 8, 9b	106.0, CH	7.06, s	5a, 7, 8, 9b	107.3, CH	7.43, s	5a, 7, 8, 9b
9a	114.1, C			112.4, C			114.2, C		
9b	113.5, C			117.2, C			113.4, C		
1′	123.7, C			123.2, C			124.1, C		
2′	131.1, CH	7.06, d (8.5)	3, 3′, 4′	117.6, CH	6.67, d (2.0)	3′, 4′, 6′	117.7, CH	6.68, d (2.1)	3, 3′, 4′, 6′
3′	114.6, CH	6.79, d (8.5)	1′, 2′, 4′	144.4[a], C			144.5[a], C		
4′	156.3, C			144.3[a], C			144.4[a], C		
5′	114.6, CH	6.79, d (8.5)	1′, 2′, 4′	114.9, CH	6.75, d (8.1)	1′, 3′, 4′	115.0, CH	6.76, d (8.1)	1′, 3′, 4′
6′	131.1, CH	7.06, d (8.5)	3, 3′, 4′	121.0, CH	6.50, d (8.1, 2.0)	3, 2′, 4′	121.2, CH	6.52, dd (8.1, 2.1)	3, 2′, 4′, 5′
$\underline{CH_3}CO$	20.2, CH_3	2.07, s		20.1, CH_3	2.03, s		20.3, CH_3	2.09, s	
				20.2, CH_3	2.44, s				
$CH_3\underline{C}O$	168.6, C			168.0, C			168.6, C		
				168.1, C					
1-OH		9.11, br s	1, 2, 9b					9.08, br s[b]	
2-OH		8.00, br s	1, 2, 3					7.97, br s[b]	
4-OH									
7-OH		9.32, br s	6, 7, 8					9.31, br s[b]	
8-OH		9.02, br s	7, 8, 9					9.02, br s[b]	
3′-OH								8.92, br s[b]	
4′-OH		9.45, br s	3′					8.92, br s[b]	

[a] Overlapping signals. [b] May be interchanged.

inhibitory activity toward KDR kinase, with an IC_{50} value of 70.7 μM. This indicated that the inhibitory activity arises from compounds with more than three catechol units in the *p*-terphenyl skeleton. KDR are expressed on vascular endotherial cells. KDR mainly plays a key role of proliferation of vascular endotherial cells during angiogenesis. We tested the inhibitory activity of boletopsins A, B, and C against proliferation of human umbilical vein endothelial cells in a MTT assay. Boletopsin C (**3**) showed antiproliferative activity with an IC_{50} value of 9.04 μM. As such, structures possessing more than three catechol units could be one possibility for the development of novel tumor angiogenesis inhibitors.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanaco MP apparatus. UV spectra were recorded with a Shimadzu UV-240 spectrophotometer. IR spectra were recorded with a Jasco IR Report-100 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were measured with Jeol JNM-AL400 MHz spectrometers using tetramethylsilane as the internal standard. Low- and high-resolution EIMS and FABMS spectra were measured with a Jeol JMS-700 spectrometer. Column chromatography was performed using silica gel 60N (63-210 μm) from Kanto Chemical and Sephadex LH-20 from GE Healthcare. HPLC was performed using a SSC-3461 pump and measured with a Jasco UV-970 detector.

Plant Material. *Boletopsis leucomelas* mushrooms were collected from Yamanashi Prefecture, Japan, in September 2005. A voucher specimen (KI-2005) was deposited at the Department of Pharmacognosy and Phytochemistry, Meiji Pharmaceutical University. Species identification was confirmed by one of the authors (K. Koyama).

Extraction and Isolation. Fruiting bodies of *B. leucomelas* (dry weight, 74.9 g) were extracted with $CHCl_3$ and then MeOH. The MeOH extract (13.3 g) was partitioned between EtOAc and water. The EtOAc fraction (2.92 g) was subjected to step-gradient silica gel CC with a solvent system consisting of $CHCl_3$−acetone (100:0, 1:1) followed by acetone−MeOH (1:1) to yield three fractions. The second fraction (1.93 g) was subjected to Sephadex LH-20 CC with a solvent system consisting of $CHCl_3$−acetone−MeOH (1:1:1) to yield five fractions. Fraction 2 (993 mg) of the second fractionation was subjected to three

chromatography steps: (1) step-gradient silica gel CC using *n*-hexane−$CHCl_3$ (1:1) and $CHCl_3$−acetone (10:1, 5:1, 2:1, 1:1, 0:100) followed by MeOH; (2) Sephadex LH-20 CC with $CHCl_3$−MeOH−H_2O (6:4:0.5); and (3) HPLC (using silica gel treated with 3% oxalic acid in MeOH) with $CHCl_3$−EtOAc (1:1) to yield **2** (9.5 mg), **4** (28.2 mg), **5** (47.6 mg), and cycloleucomelone-2-acetate (**7**) (4.5 mg). Fraction 3 (565 mg) of the second fractionation was subjected to two chromatography steps: (1) step-gradient silica gel CC with a solvent system consisting of $CHCl_3$−MeOH (100:1, 50:1, 10:1, 5:1, 2:1, 1:1, 0:100) and (2) Sephadex LH-20 CC using $CHCl_3$−acetone−MeOH (2:2:1) and $CHCl_3$−MeOH−H_2O (6:4:0.5) to yield **1** (165 mg) and cycloleucomelone (**6**) (42 mg). Fraction 4 (76.2 mg) of the second fractionation was subjected to three chromatography steps: (1) step-gradient silica gel CC (using silica gel treated with 3% oxalic acid in MeOH) with a solvent system consisting of $CHCl_3$−EtOAc (10:1, 5:1, 3:1, 2:1, 1:1) followed by EtOAc−MeOH (1:1); (2) Sephadex LH-20 CC with $CHCl_3$−MeOH−H_2O (6:4:0.5); and (3) HPLC (using silica gel treated with 3% oxalic acid in MeOH) with $CHCl_3$−EtOAc (1:1) to yield **3** (9.8 mg).

Boletopsin A (1): colorless needles; mp 206−208 °C; UV (MeOH) λ_{max} (log ε) 212 (4.59), 270 (4.31), 303 (4.44) nm; IR (KBr) ν_{max} 3399, 1730, 1614, 1523, 1471, 1434, 1322 cm^{-1}; ^1H NMR (DMSO-d_6) see Table 1; ^{13}C NMR (DMSO-d_6) see Table 1; EIMS *m*/*z* 382 $[M]^+$ (22), 340 (100); HREIMS *m*/*z* 382.0688 $[M]^+$ (calcd for $C_{20}H_{14}O_8$, 382.0689).

Boletopsin B (2): colorless powder; mp 163−165 °C; UV (MeOH) λ_{max} (log ε) 227 (4.79), 264 (4.46), 302 (4.53), 329 (4.59) nm; IR (KBr) ν_{max} 3399, 1757, 1614, 1527, 1473, 1428, 1372, 1312 cm^{-1}; ^1H NMR (DMSO-d_6) see Table 1; ^{13}C NMR (DMSO-d_6) see Table 1; FABMS *m*/*z* 441 $[M + H]^+$; HRFABMS *m*/*z* 441.0823 $[M + H]^+$ (calcd for $C_{22}H_{17}O_{10}$, 441.0822).

Boletopsin C (3): colorless powder; mp 180−182 °C (dec); UV (MeOH) λ_{max} (log ε) 239 (4.82), 271 (4.74), 315 (4.79) nm; IR (KBr) ν_{max} 3307, 1735, 1617, 1525, 1472, 1430, 1370, 1308 cm^{-1}; ^1H NMR (DMSO-d_6) see Table 1; ^{13}C NMR (DMSO-d_6) see Table 1; FABMS *m*/*z* 398 $[M]^+$; HRFABMS *m*/*z* 398.0640 $[M]^+$ (calcd for $C_{20}H_{14}O_9$, 398.0638).

KDR Kinase Assay. KDR (Millipore) was diluted with Protein Dilution Buffer B+ (Millipore). KDR kinase assays were performed on 96-well plates with KDR using a Universal tyrosine kinase assay kit (Takara Bio, Inc.). The assay was carried out according to the supplied manual with modifications. Samples were dissolved in DMSO. Mixtures containing 30 μL of phosphatase reacting solution, 5 μL of KDR diluted with buffer, 5 μL of test compound, and 10 μL of ATP-Na_2 (40 mM) were incubated for 2 h at 37 °C. The sample solution was removed, and the wells were washed four times with Tween-PBS. Blocking solution (100 μL) was added to each well, and the plates were incubated for 30 min at 37 °C. The blocking solution was then discarded, and 50 μL of antiphosphotyrosine (PY20)-HRP solution was added to each well, followed by a 30 min incubation at 37 °C. The antibody solution was then removed, and the wells were washed four times with Tween-PBS. The washing buffer was completely removed, 100 μL of HRP substrate solution (TMBZ) was added to each well, and the plates were incubated for 5 min at room temperature. Subsequently, 100 μL of stop solution (1 N H_2SO_4) was added to each well, and the absorbance at 450 nm was measured with a plate reader.

Cell Culture. HUVECs were purchased from Lonza Walkersville Inc. HUVECs were cultured using an EGM-2 Bulletkit (Lonza Walkersville, Inc.) at 37 °C in 5% CO_2.

Growth Inhibition Assay. HUVECs (3×10^3 cells/well) were seeded in 96-well plates with EGM-2 Bulletkit for 3 h at 37 °C in 5% CO_2. The medium was removed, replaced with 1% FBS-EBM-2, and incubated for 21 h at 37 °C in 5% CO_2. VEGF (1 nM) and the test compound were added to each well and incubated for 72 h at 37 °C in 5% CO_2. Cell proliferation was detected using the WST-8 reagent, and the inhibition of proliferation was measured at an absorbance wavelength of 450 nm using a plate reader.

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Supporting Information Available: Images of ^1H and ^{13}C NMR spectra for compounds **1**−**3** are available free of charge via the Internet at http://pubs.acs.org.

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