Dyscusins A—C, Three New Steroids from the Leaves of *Dysoxylum cumingianum*

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Three new steroids dyscusins A—C (1—3), including a stigmastane-type sterol and two pregnanes, together with two known steroids were isolated from the leaves of *Dysoxylum cumingianum* (Meliaceae). Their structures were elucidated on the basis of extensive spectroscopic analyses. In a cytotoxicity assay, compound 1 showed ten-fold enhanced cytotoxicity against multi-drug resistant cancer cells (KB-C2) in the presence of 2.5 μM colchicine as compared with the absence of colchicine. This notable finding indicated that 1 possessed a multi-drug resistant reversal effect.

Key words *Dysoxylum cumingianum*; Meliaceae; steroid; multi-drug resistance

Our previous chemical study on the leaves of *Dysoxylum cumingianum* C. D.C. (Meliaceae) led to the isolation and characterization of new 14,18-cycloapotirucallanes, cumingianosides A—O,[1,2] and apotirucallanes, cumingianosides P and Q,[3] as well as trisnor- and tetranor-triterpene glucosides, cumindysosides A and B, respectively.[1,4] Among these compounds, cumingianosides A and C exhibited potent selective cytotoxicity against human leukemia cells (MOLT-4) with ED_{50} values of <0.00625 and <0.0045 μM, respectively.[1] We also recently isolated six new triterpenes from this plant, some of which exhibited a multidrug-reversing effect against multi-drug resistant (MDR) KB cells (KB-C2).[5] Our continuing investigation of this plant aimed at discovering potential new drug leads has resulted in the isolation and characterization of dyscusins A—C (**1**—**3**), a new stigmastane-type sterol and two new pregnanes, together with two known compounds. In this paper, we describe the structure elucidation of the new compounds and evaluation of their cytotoxicity against three human cancer cell lines, including a MDR cell line.

The 90% MeOH-soluble fraction (228.5 g) from the MeOH extract of the leaves of *D. cumingianum* was repeatedly subjected to column chromatography to give three new and two known steroids.

Compound **1** was obtained as a white amorphous powder. The molecular formula of **1** was elucidated as $C_{29}H_{50}O_4$ by high resolution-electrospray ionization (HR-ESI) MS. The ^{1}H-NMR spectrum showed the presence of two *tert*-methyl groups (δ_H 0.74, 1.45), two *sec*-methyl groups [δ_H 0.91 (d, J=6.8 Hz) and 1.02 (d, J=6.8 Hz)], an ethyl group [δ_H 0.97 (3H, t, J=7.4 Hz); 1.40 and 1.57 (each 1H, m)], an oxygenated methylene [δ_H 4.15 (d, J=10.2 Hz) and 4.41 (dd, J=10.2, 4.3 Hz), three oxygenated methines [δ_H 3.87 (dt, J=11.5, 3.7 Hz), 4.13 (br d, J=10.0 Hz) and 4.56 (d, J=3.7 Hz)], and a trisubstituted olefin [δ_H 5.70 (dd, J=5.0, 2.0 Hz)]. The ^{13}C-NMR spectrum displayed 29 carbon resonances due to two olefinic carbons [δ_C 126.5 (d), 144.7 (s)], three oxygen-bearing methine carbons (δ_C 72.3, 73.0, 78.1), an oxygen-bearing methylene carbon (δ_C 63.0), two sp^3 quaternary carbons (δ_C 36.7, 42.7), seven sp^3 methine carbons (δ_C 29.4, 32.4, 41.8, 49.1, 49.8, 50.8, 56.8), nine sp^3 methylene carbons (δ_C 21.0, 24.0, 24.5, 26.4, 28.0, 31.8, 32.4, 38.2, 39.5), and five methyl carbons (δ_C 12.1, 12.3, 18.1, 20.7, 21.3). The spectroscopic data suggested that **1** is a C_{29} steroidal compound. The locations of the hydroxy groups were determined to be at C-3, C-4, C-21, and C-22 based on the ^{1}H–^{1}H correlation spectroscopy (COSY) correlations of H_2-1–H_2-2–H-3–H-4 and of H-17–H-20–(H_2-21)–H-22–H_2-23–H-24–H_2-28–H_3-29, coupled with the heteronuclear multiple bond correlations (HMBC) of Me-19 with C-1 and C-5 and of Me-18 with C-17. The presence of a trisubstituted olefin between C-5 and C-6 was elucidated from the HMBC cross peak of H-6 with C-4. Furthermore, the stigmastane-type skeleton was indicated from the ^{1}H–^{1}H COSY correlations of H-6–H_2-7, H-9–H_2-11–H_2-12, and H_2-15–H_2-16, along with the HMBC correlations of Me-19 with C-9 and

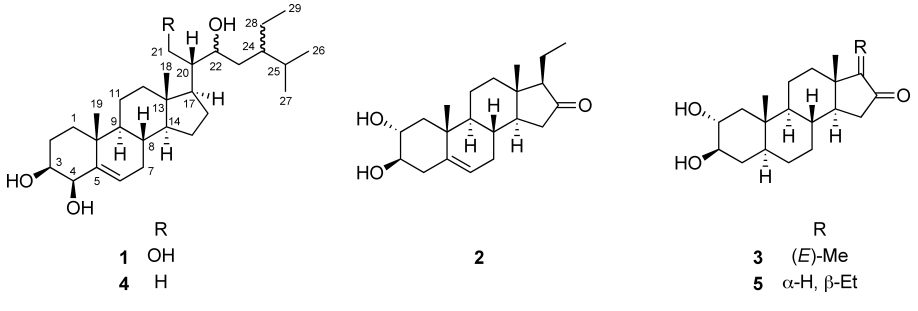

Fig. 1 Isolated Compounds from the MeOH Extract of the Leaves of *D. cumingianum*

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Table 1. ^{1}H- and ^{13}C-NMR Data for Compounds **2** and **3** in Pyridine-d_5+D_2O

Position	**2**		Position	**3**	
	^{1}H[a)]	^{13}C[b)]		^{1}H[a)]	^{13}C[b)]
1	2.39 (1H, dd, 12.5, 4.2)	46.3	1	2.25 (1H, dd, 12.5, 4.5)	46.1
	1.43 (1H, m)			1.26 (1H, m)	
2	4.16 (1H, ddd, 11.0, 9.0, 4.2)	72.5	2	4.05 (1H, ddd, 11.0, 9.0, 4.5)	73.0
3	3.85 (1H, ddd, 11.0, 9.0, 5.5)	76.3	3	3.85 (1H, ddd, 11.0, 9.0, 5.0)	76.6
4	2.72 (1H, m)	40.7	4	1.86 (1H, dd, 13.0, 5.0, 2.0)	37.1
	2.68 (1H, dd, 13.5, 5.5)			1.68 (1H, m)	
5	—	141.3	5	1.20 (1H, m)	45.1
6	5.41 (1H, d, 5.3)	120.9	6	1.25 (1H, m)	28.1
7	1.83 (1H, ddd, 17.0, 5.3, 2.5)	32.0		1.20 (1H, m)	
	1.57 (1H, m)		7	1.46 (1H, m)	32.1
8	1.49 (1H, m)	30.5		0.83 (1H, m)	
9	1.16 (1H, m)	50.3	8	1.37 (1H m)	33.6
10	—	38.5	9	0.75 (1H, m)	54.3
11	1.59 (1H, m)	20.8	10	—	37.6
	1.43 (1H, m)		11	1.62 (1H, qt, 13.0, 4.0, 3.0)	21.3
12	1.75 (1H, m)	37.8		1.30 (1H, m)	
	1.23 (1H, m)		12	2.10 (1H, dt, 12.5, 3.3)	36.3
13	—	41.7		1.43 (1H, m)	
14	1.31 (1H, m)	50.5	13	—	43.5
15	2.19 (1H, dd, 18.3, 7.8)	38.4	14	1.24 (1H, m)	50.0
	1.74 (1H, m)		15	2.19 (1H, dd, 17.1, 6.8)	38.0
16	—	218.2		1.97 (1H, dd, 17.1, 14.3)	
17	1.62 (1H, m)	64.8	16	—	205.2
18	0.58 (3H, s)	13.2	17	—	148.5
19	1.08 (3H, s)	20.6	18	0.87 (3H, s)	17.6
20	1.70 (1H, m)	17.9	19	0.86 (3H, s)	13.6
	1.24 (1H, m)		20	6.60 (1H, q, 7.8)	128.1
21	1.04 (1H, t, 7.5)	13.6	21	1.70 (3H, d, 7.8)	13.0

a) δ ppm (mult., *J* in Hz), 500 MHz. *b*) δ ppm, 125 MHz.

C-10, H-6 with C-8, H-15 with C-14, H-17 with C-16, Me-18 with C-12/C-13/C-14, and Me-26 and 27 with C-24 and C-25. The β-orientation of the C-17 side chain was also assigned from the nuclear Overhauser enhancement and exchange spectroscopy (NOESY) correlations of H-17 with H-14, H-14 with H-12α, H-12α with H-9, and H-9 with H-1α. The orientations of the hydroxy groups at C-3 and C-4 were determined to be β from the NOESY correlations of H-3 with H-1α/H-4 and of H-4 with H-6, as well as the small coupling constant of H-4 ($J_{3,4}$=3.7 Hz). The *R** configuration of C-20 was elucidated from the NOESY correlations of H-20 with Me-18 and H-16β and of H_2-21 with H-12β. The configurations of C-22 and C-24 still remain to be determined. On the basis of these findings, the structure of **1** was elucidated as shown in Fig. 1, and **1** has been named as dyscusin A.

A pseudo molecular ion peak at *m*/*z* 355.2260 ([M+Na]$^+$ Calcd for 355.2249) was observed in the positive-ion HR-ESI-MS of **2**, indicating the molecular formula $C_{21}H_{32}O_3$. The ^{1}H-NMR spectrum (Table 1) revealed the presence of two *tert*-methyl groups (δ_H 0.58, 1.08), an ethyl group [δ_H 1.04 (3H, t, *J*=7.5 Hz), 1.24, and 1.70 (each 1H, m)], two oxygenated methines [δ_H 3.85 (ddd, *J*=11.0, 9.0, 4.2 Hz) and 4.16 (ddd, *J*=11.0, 9.0, 5.5 Hz)], and a trisubstituted olefin [δ_H 5.41 (d, *J*=5.3 Hz)]. The ^{13}C-NMR spectrum (Table 1) and a distortion enhancement by polarization transfer (DEPT) experiment indicated the presence of 21 carbons, including a carbonyl carbon (δ_C 218.2), two sp^3 quaternary carbons (δ_C 38.5, 41.7), four sp^3 methines (δ_C 30.5, 50.3, 50.5, 64.8), and seven sp^3 methylenes (δ_C 17.9, 20.8, 32.0, 37.8, 38.4, 40.7, 46.3). The spectroscopic data suggested that **2** is a pregnane derivative. The assignments of the hydroxy groups at C-2 and C-3 were based on the ^{1}H–^{1}H COSY correlations of H_2-1–H-2–H-3–H_2-4, together with the HMBC correlations of Me-19 with C-1 and C-5. The location of the trisubstituted olefin was concluded to be at C-5 (C-6) from the HMBC cross peak of H-6 with C-4. Furthermore, the carbonyl group was determined to be at C-16 based on the ^{1}H–^{1}H COSY correlations of H-8–H-14–H_2-15, coupled with the HMBC correlations of H_2-15 with C-16, H-17 with C-16, and Me-18 with C-17. Furthermore, the ^{1}H–^{1}H COSY correlations of H-6–H_2-7–H-8–H-9–H_2-11–H_2-12, along with the HMBC correlations of Me-19 with C-9/C-10, Me-18 with C-12/C-13/C-14, and Me-21 with C-17, suggested that the planar structure of **2** is 2,3-dihydroxy-16-oxo-pregn-5(6)-ene. The orientations of the hydroxy groups were assigned as 2α and 3β, based on the coupling patterns of H-2 [δ_H 4.16 (ddd, *J*=11.0, 9.0, 5.5 Hz)] and H-3 [δ_H 3.85 (ddd, *J*=11.0, 9.0, 4.2 Hz)], as well as the NOESY cross peaks of H-2 with Me-19 and H-3 with H-1α. The β-configuration of the ethyl group was determined from the NOESY correlation of Me-18 with H_2-20. From these observations, the structure of **2** was fully determined as shown in Fig. 1, and **2** has been named as dyscusin B.

On the basis of HR-ESI-MS, compounds **2** and **3** have the same molecular formula ($C_{21}H_{32}O_3$). The ^{1}H- and ^{13}C-NMR spectra (Table 1) of **3** were similar to those of **2**, indicating that **3** is also a pregnane-type steroid. However, signals for a vinylic methyl [δ_H 1.70 (d, *J*=7.8 Hz), δ_C 13.0] were observed rather than signals for the terminal methyl of the ethyl

Table 2. Cytotoxicity (IC_{50}[a)] in μg/ml) of Compounds **1**—**3** and **5** against Human Cancer Cell Lines[b)]

	KB	KB-C2	KB-C2 (+2.5 μM colchicine)	MCF-7
1	11.6±0.47	19.6±0.83	1.64±0.04	NT[c)]
2	31.4±1.02	33.9±0.93	11.4±1.32	>100
3	40.9±0.19	37.9±1.83	18.2±0.56	58.1±3.01
5	15.4±0.63	30.6±1.42	30.4±2.64	26.1±0.23
Daunorubicin	0.44±0.05	7.87±0.24	11.6±1.4	0.22±0.02

a) Data are mean±S.E. from three or four experiments. *b*) Cell lines: KB, epidermoid carcinoma; KB-C2, colchicines-resistant KB; MCF7, breast carcinoma. *c*) Not tested.

group in **2**. The ^{1}H–^{1}H COSY and HMBC spectra of **3** were also similar to those found in **2**, except for the ^{1}H–^{1}H COSY correlation of H-20 [δ_H 6.60 (q, J=7.8 Hz)] with Me-21 [δ_H 1.70 (d, J=7.8 Hz)], as well as the HMBC correlations of Me-18 (δ_H 0.87) with C-17 (δ_C 148.5) and of H-20 with C-16 (δ_C 205.2) observed for **3**. These data indicated that a trisubstituted olefin was present at C-17(20). The orientations of the hydroxy groups at C-2 and C-3 in **3** were determined to be α and β, respectively, from the J-values of H-2 and H-3, as well as the analysis of the NOESY spectrum, and were the same as those found in **2**. The α-orientation of H-5 was elucidated from the NOESY cross peaks of H-3 with H-5 and of H-5 with H-9. The geometry of the double bond was assigned by comparison of the proton chemical shifts of H-20 and Me-21 with those of the structurally related *E*/*Z* isomers of 2β,3β-dihydroxy-5α-pregn-17(20)-(*E*/*Z*)-en-16-one,[6] which are the C-2 epimer of **3**. The signals due to H-20 and Me-21 in the *E*-isomer [H-20: δ 6.49; Me-21: δ 1.84 (in $CDCl_3$)] appeared at lower field as compared with those in the *Z*-isomer [H-20: δ 5.69; Me-21: δ 2.07 (in $CDCl_3$)]. The ^{1}H-NMR signals due to H-20 and Me-21 in **3** appeared at δ 6.50 and 1.85 (in $CDCl_3$), respectively, which were good agreement with those of *E*-isomer [H-20: δ 6.49; Me-21: δ 1.84 (in $CDCl_3$)],[6] indicating the geometry of C-17(20) double bond to be *E*. From the evidence described above, the structure of **3** was determined as shown in Fig. 1, and **3** has been named as dyscusin C.

Two known compounds were identified as 24-ethylcholest-5-en-3β,4β,22-triol (**4**)[7] and meliavosin (**5**)[8] by comparison of their spectroscopic data with that described in the literature.

Compounds **1**—**3** and **5** were evaluated for cytotoxicity against two drug-sensitive cancer cell lines (KB and MCF-7), as well as an MDR cancer cell line (KB-C2). The data are shown in Table 2. When tested alone, none of the four compounds showed significant cytotoxicity against KB or KB-C2, with IC_{50} values ranging from 11.6 to 40.9 μg/ml. However, in the presence of 2.5 μM colchicine, compound **1** did exhibit significant cytotoxicity against KB-C2 cells with an IC_{50} value of 1.64 μg/ml. In comparison, the IC_{50} value of **1** against the same cell line was ten-fold higher (19.6 μg/ml) in the absence of colchicine. This significant enhancement in cytotoxicity indicated that **1** might demonstrate an MDR-reversal effect. Because stigmasterol was not cytotoxic against KB or KB-C2 cells in the presence or absence of colchicine, the hydroxy groups on dyscusin A might play an important role in the cytotoxic activity. Smaller degrees of enhancement were also seen with **2** and **3**, but not **5**, in the presence of colchicine.

Experimental

General Experimental Procedures Optical rotations were measured with a JASCO DIP-370 digital polarimeter. MS were obtained on a Waters LCT PREMIER 2695. NMR spectra were measured on Bruker AVANCE-500 and 400 Fourier transform spectrometers (^{1}H-NMR: 500, 400 MHz, ^{13}C-NMR: 125, 100 MHz) using tetramethylsilane (TMS) as an internal standard. Column chromatography: silica gel 60N (63—210 μm, Kanto Kagaku, Japan), Toyo pearl HW-40 (TOSHO), MCI-gel CHP-20P (75—150 μm; Mitsubishi Chemical, Japan), YMC-pack ODS-A (YMC). Preparative HPLC: COSOMOSIL Cholester (250×20 mm; 5 μm; Nakalai Tesque, Japan), GPC (Gel-Permeation Chromatography) [Asahi pack GS-310 2G (MeOH, SHOWA DENKO, Japan)]. TLC: silica gel 60 F_{254} (Merck, Germany).

Extraction and Isolation The air dried leaves of *D. cumingianum* (8.7 kg) were extracted with MeOH at room temperature. After concentration, a portion (580 g) of the MeOH extract was partitioned with $CHCl_3$ and H_2O. After removal of the solvent by evaporation, the $CHCl_3$ layer was further partitioned with *n*-hexane and 90% aqueous MeOH to give *n*-hexane-soluble (50 g) and 90% MeOH-soluble (280 g) fractions. A part (230 g) of the 90% MeOH-soluble fraction was subjected to silica gel column eluting with solvent of increasing polarity ($CHCl_3$–MeOH–H_2O) to give 18 fractions. Fraction 7 was fractionated by flash SiO_2 CC [*n*-hexane–acetone (3 : 1→1 : 2)] to yield 15 fractions (7.1—7.15). Fraction 7.5 was subjected to a Toyo pearl HW-40 column [benzene–MeOH (1 : 1)] and then a YMC ODS-A column [MeOH–H_2O (3 : 2→1 : 0)] to afford 24-ethylcholest-5-en-3β,4β,22-triol (**4**) (25 mg) and fractions 7.5.1—7.5.14. Fraction 7.5.9 was purified by gel permeation chromatography (GPC) with MeOH to give compound **3** (2 mg). Fraction 7.10 was separated on MCI gel CHP-20P [MeOH–H_2O (2 : 1→1 : 0)] to yield 11 fractions (7.10.1—7.10.11). Fraction 7.10.8 was chromatographed on a YMC ODS-A column [MeOH–H_2O (2 : 1→1 : 0)] to yield fractions 7.10.8.1—7.10.8.4. Fraction 7.10.8.1 was further separated by SiO_2 CC [*n*-hexane–EtOAc (2 : 1→1 : 1)] to afford fractions 7.10.8.1.1—7.10.8.1.6. Fraction 7.10.8.1.3 was purified by GPC (MeOH) to give compound **2** (7 mg). Fraction 7.10.8.1.5 was also purified by GPC (MeOH) to yield meliavosin (**5**) (5 mg). Fraction 7.10.9 was separated by COSMOSIL Cholester to give compound **1** (10 mg).

Compound **1**: Amorphous powder; $[\alpha]_D$ −28.4 (c=0.85, pyridine); ^{1}H-NMR (pyridine-d_5+D_2O): δ_H 5.70 (1H, dd, J=5.0, 2.0 Hz, H-6), 4.56 (1H, d, J=3.7 Hz, H-4), 4.41 (1H, dd, J=10.2, 4.3 Hz, H-21a), 4.15 (1H, dd, J=10.2 Hz, H-21b), 4.13 (1H, br d, J=10.0 Hz, H-22), 3.87 (1H, dt, J=11.5, 3.7 Hz, H-3), 1.45 (3H, s, H-19), 1.36 (1H, m, H-17), 1.02 (3H, d, J=6.8 Hz, H-26), 0.97 (3H, t, J=7.4 Hz, H-29), 0.91 (3H, d J=6.8 Hz, H-27), 0.74 (3H, s, H-18); ^{13}C-NMR (pyridine-d_5+D_2O): δ_C 144.7 (C-5), 126.5 (C-6), 78.1 (C-4), 73.0 (C-3), 72.3 (C-22), 63.0 (C-21), 56.8 (C-14), 50.8 (C-9), 49.8 (C-17), 49.1 (C-20), 42.7 (C-13), 41.8 (C-24), 39.5 (C-12), 38.2 (C-1), 36.7 (C-10), 32.4 (C-7), 32.4 (C-8), 31.8 (C-23), 29.4 (C-25), 28.0 (C-16), 26.4 (C-2), 24.5 (C-15), 24.0 (C-28), 21.3 (C-19), 21.0 (C-11), 20.7 (C-26), 18.1 (C-27), 12.3 (C-29), 12.1 (C-18); HR-ESI-MS: m/z 485.3607 $[M+Na]^+$ (Calcd for $C_{29}H_{50}O_4Na$, 485.3601).

Compound **2**: White amorphous powder; $[\alpha]_D$ −157.4 (c=0.67, MeOH); ^{1}H- and ^{13}C-NMR data (pyridine-d_5+D_2O) see Table 1; HR-ESI-MS: m/z 355.2260 $[M+Na]^+$ (Calcd for $C_{21}H_{32}O_3Na$, 355.2249).

Compound **3**: White amorphous powder; $[\alpha]_D$ +13.6 (c=0.21, MeOH); ^{1}H- and ^{13}C-NMR data (pyridine-d_5+D_2O) see Table 1; HR-ESI-MS: m/z 333.2417 $[M+H]^+$ (Calcd for $C_{21}H_{33}O_3$, 333.2406).

Cell Lines and Cell Culture KB (human epidermoid carcinoma of the nasopharynx) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). KB-C2 (colchicine-resistant KB) cells were maintained in DMEM medium in the presence of 10% FBS and 5 μM colchicine. MCF7 (breast carcinoma) cells were cultured in RPMI1640 supplemented with 10% FBS. All cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2–95% air.

Cytotoxicity Assay Cells were seeded at each density (5×10^4 cells/well for KB and KB-C2, or 5×10^4 cells/well for MCF7) in 96-well plate and pre-incubated for 24 h. Test samples were dissolved in small amount of dimethyl sulfoxide (DMSO) and diluted in the appropriate culture medium (final concentration of DMSO <0.5%). After removal of pre-incubated culture medium, 100 μl of medium containing various concentrations (0.1, 0.5, 2, 2.5, 5, 10, 50, 100 μg/ml) of test compound were added and further incubated for 48 h. Cell viability was determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.[9] IC_{50} values (concentration in μg/ml required to inhibit cell viability by 50%) were calculated using the concentration–inhibition curve.

Cytotoxic activities are shown as mean±S.E. from three or four experiments.

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