Absolute Configuration of a Diterpene with an Acyclic 1,2-Diol Moiety and Cytotoxicity of Its Analogues from the Aerial Parts of *Aralia cordata*

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Aralia cordata Thunb. (Araliaceae) is a perennial herb which is distributed in Korea, China and Japan. Traditionally, the root of *A. cordata* has been widely used to treat rheumatism, lumbago and lameness in Japan.[1] Previous phytochemical investigations on this plant have reported the isolation of several kinds of diterpenes having pimarane and kaurane skeletons.[2] In our current phytochemical investigation on the aerial parts of this plant has led to the isolation of a new *ent*-pimarane diterpene, *ent*-15*S*,16-dihyroxypimar-8(14)-en-19-oic acid (**1**), together with three known diterpenes, *ent*-pimar-8(14),15-dien-19-oic acid (**2**)[3], *ent*-16α-hydroxykauran-19-oic acid (**3**)[4] and *ent*-kaur-16-en-19-oic acid (**4**)[5] (Figure 1). Although the structure of compound **1** was reported previously,[6] there has been no report of the isolation from natural sources. Moreover, there has been no report on the determination of the absolute configuration of the 1,2-dihydroxyethyl moiety in **1**. Therefore, in order to deduce the absolute configuration of this moiety, a CD method employing dimolybdenum tetraacetate [$Mo_2(AcO)_4$] developed by Snatzke and Frelek[7] was applied to **1**. The present paper reports the isolation and structure elucidation of isolated compounds (**1-4**) from the aerial parts of *A. cordata* and their cytotoxicity, as well as the determination of the absolute configuration of an acyclic 1,2-diol moiety in **1** using Snatzke's method.

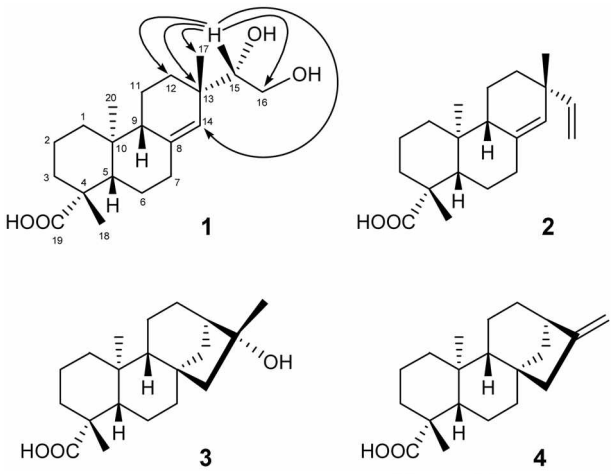

Figure 1. Structures of compounds **1-4** isolated from the aerial parts of *A. cordata* and key HMBC correlations (H → C) in **1**.

Compound **1** was obtained as a white amorphous powder with a negative optical rotation. $[\alpha]_D^{25}$ −39.5° (*c* 0.4, MeOH). The molecular formula of **1** was found to be $C_{20}H_{32}O_4$, on

Table 1. ^{1}H (300 MHz), ^{13}C (75 MHz) and HMBC data for compound **1**[a] (in CD_3OD)

Carbon	^{13}C	^{1}H (*J* in Hz)	HMBC (H → C)
1	40.6	1.02 ddd (3.0, 3.6, 13.5)	2, 3, 5, 10, 20
		1.62 m	
2	21.0	1.56 m	1, 3, 4, 10
		1.80 m	
3	39.5	0.82 m	1, 2, 4, 5, 19
		1.85 m	
4	45.1		
5	57.6	1.10 dd (2.4, 13.2)	4, 6, 7, 9, 10, 18, 19, 20
6	26.2	1.32 m	
		1.56 m	
7	38.3	2.15 m	5, 6, 8, 9, 14
		2.29 ddd (1.8, 4.2, 13.5)	
8	138.3		
9	50.9	1.61 m	5, 8, 10, 11, 12, 14, 20
10	40.8		
11	20.0	1.45 m	8, 9, 12, 13
		1.48 m	
12	32.1	0.84 m	9, 11, 13, 14, 17
		1.89 m	
13	38.5		
14	130.0	5.36 s	7, 9, 12, 13, 15, 17
15	80.5	3.50 dd (2.1, 8.7)	12, 13, 14, 16, 17
16	64.6	3.42 dd (8.7, 10.5)	13, 15
		3.68 dd (2.1, 10.5)	
17	23.9	0.90 s	12, 13, 15
18	29.7	1.20 s	3, 4, 5, 19
19	181.6		
20	15.0	0.76 s	1, 5, 9, 10

[a]Assignments made on the basis of DEPT, ^{1}H-^{1}H COSY, HMQC and HMBC experiments.

the basis of a quasimolecular ion peak at *m/z* 337.2380 $[M+H]^+$ in the HRFABMS. Its IR spectrum exhibited absorption bands for hydroxyl group (3450 cm^{-1}), carbonyl group (1695 cm^{-1}) and trisubstituted double bond (1640 and 842 cm^{-1}). The ^1H NMR spectrum of **1** displayed signals for an olefinic proton at δ 5.36 (s), carbinolic protons at δ 3.68 (dd, J = 2.1, 10.5 Hz), 3.50 (dd, J = 2.1, 8.7 Hz) and 3.42 (dd, J = 8.7, 10.5 Hz) and three tertiary methyl protons at δ 1.20, 0.90 and 0.76 (Table 1). The ^{13}C NMR and DEPT spectra revealed 20 carbon signals consisting of three methyls, eight methylenes, four methines and five quaternary carbons including a carboxyl carbon at δ 181.6 (Table 1). On the basis of the above observations, the presence of a pimarane diterpene skeleton could be inferred.[8-11] In addition, the olefinic carbon signals at δ 138.3 and 130.0 were indicative for a C-8/C-14 double bond in the *ent*-pimarane-type structure.[11,12] Furthermore, a major fragment ion peak at *m/z* 275 $[M\text{-}CH(OH)CH_2OH]^+$ in the EIMS spectrum indicated that **1** is an *ent*-pima-8(14)-ene derivative having a 1,2-dihydroxyethyl side-chain. The presence of a partial structure of 1,2-dihydroxyethyl moiety in **1** was further supported by the ^1H-^1H COSY experiment through the cross-peaks for the geminal coupling of the hydroxymethylene protons at δ 3.40 and 3.68 (H_2-16), and for both of them with the proton at δ 3.50 (H-15). The linkage position of a 1,2-dihydroxyethyl moiety was determined to be C-13 on the basis of the HMBC cross-peaks of H-15 (δ 3.49) with C-17 (δ 23.9), C-12 (δ 32.1), C-13 (δ 38.5), C-16 (δ 64.6) and C-14 (δ 130.0) (Figure 1).

The relative stereochemistry of the chiral groups on the rings in **1** can be determined through NMR spectra, owing to the structural rigidity. On the contrary, the sidearm, 1,2-dihydroxyethyl moiety, in **1** is flexible and attached to the rest of the molecule through a quaternary carbon, which prevents the use of NMR techniques. Furthermore, the UV-vis spectral region of acyclic 1,2-diols below about 190 nm prevents the use of chiroptical methods[13] for the direct analysis of their absolute configuration, unless a chemical derivatization is carried out on the chiral substrate by addition of a suitable chromophoric group. A possible way to solve this problem is application of a CD (circular dichroism) method employing dimolybdenum tetraacetate $[Mo_2(AcO)_4]$ as an auxiliary chromophore, which is one of the most useful method for rapid and effective determination of the absolute configuration of acyclic 1,2-diols.[7] In this method, the chiral substrate acts as a ligand of the metal center through ligation to the Mo^{2-} core.[7] As a consequence to the ligation, the conformational freedom of the flexible molecule is either very much reduced or totally restricted, which makes possible the absolute configurational assignment of the acyclic 1,2-diol moiety on the basis of the chiroptical data, independently of the rest of the molecule.

In order to deduce the absolute configuration of an acyclic 1,2-diol moiety in **1**, a CD method employing dimolybdenum tetraacetate $[Mo_2(AcO)_4]$ developed by Snatzke and Frelek[7] was applied to **1**, and obtained its CD spectrum in the region of 550-250 nm. According to the rule proposed by

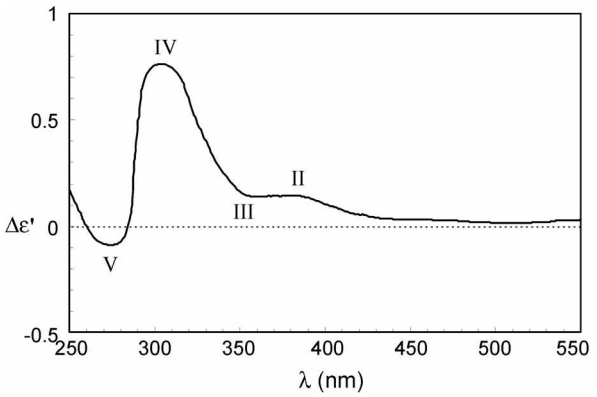

Figure 2. CD spectrum of compound **1** in DMSO solution of $Mo_2(AcO)_4$. The x-axis represents the wavelength and y-axis represents molar circular dichroism ($\Delta\varepsilon'$, $L \cdot mol^{-1} \cdot cm^{-1}$). A series of four bands above 250 nm (the absorption region of Dimolybdenum tetraacetate) is apparent and Roman numerals (II, III, IV, V) refer to Snatzke's band nomenclature.[7a,b]

Snatzke, the sign of the CD band around 305 nm, which has been assigned to a metal-to-ligand charge-transfer transition,[7a] correlates with the absolute configuration of the acyclic 1,2-diol moiety in the ligating structure.[7b] The rule states that a complex of a "*R*" or "*R,R*" 1,2-diol with dimolybdenum tetraacetate always gives rise to a negative CD band around 305 nm, whereas a complex having a "*S*" or "*S,S*" 1,2-diol always gives rise to a positive CD band around 305 nm.[7b] Thus, a positive CD band observed around 305 nm ("band IV") in the CD spectrum of **1** shown in Figure 2 leads to the assignment of the *S*-configuration for the chiral center (C-15) in the 1,2-dihydroxyethyl moiety. On the basis of the above evidences, the structure of compound **1** was determined to be *ent*-15*S*,16-dihyroxypimar-8(14)-en-19-oic acid.

Previous biological study on *A. cordata* has shown that some diterpenes isolated from *A. cordata* exhibited cytotoxic effects against human tumor cells.[14] Thus, all the isolates (**1**-**4**) were evaluated for *in vitro* cytotoxicity against SK-OV-3 (human ovarian cancer), HL-60 (human promyelocytic leukemia), B16F10 (murine melanoma) and L1210 (murine leukemia) using the MTT assay method,[15] and the results are presented in Table 2. Of the pimarane-type (**1** and **2**) and

Table 2. Cytotoxicity of compounds 1-4 from the aerial parts of *A. cordata*

Compound	IC_{50} (μg/mL)[a]			
	SK-OV-3	HL-60	B16F10	L1210
1	> 30	> 30	> 30	> 30
2	26.2 ± 1.2	29.4 ± 0.8	24.4 ± 1.4	20.1 ± 1.2
3	> 30	> 30	> 30	> 30
4	20.1 ± 1.3	22.6 ± 1.5	18.9 ± 0.9	15.8 ± 0.8
Adriamycin[b]	2.5 ± 0.2	2.8 ± 0.2	1.7 ± 0.1	1.4 ± 0.1

[a]The IC_{50} value is defined as the concentration of sample to reduce a 50% of absorbance relative to the vehicle-treated control and the values represent the mean = SD of three individual experiments. [b]Positive control.

kaurane-type diterpenes (**3** and **4**) tested, compounds **2** and **4** having an exomethylene group showed a moderate cytotoxicity against all the cell lines tested, with IC_{50} values ranging from 20.1 to 29.4 μg/mL and from 15.8 to 22.6 μg/mL, respectively, which was well accorded with the previous study.[14] Although compound **3** was known to exhibit a selective cytotoxicity against some cell lines such as 9PS (a chemically induced murine lymphocitic leukemia), A-549 (human lung carcinoma) and HT-29 (human colon adenocarcinoma),[16] it did not show any significant cytotoxicity against all the cell lines tested.

Experimental Section

General Experimental Procedures. Melting point was measured on an Electrothemal apparatus. Optical rotation was measured in MeOH on a JASCO DIP-370 digital polarimeter. IR spectrum was recorded on a JASCO 100 IR spectrometer. CD spectrum was recorded in DMSO on a JASCO J-715 spectrometer. HRFABMS and EIMS data were recorded on JEOL JMS-DX 300 and Hewlett-Packard 5989B spectrometers, respectively. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker DRX-300 spectrometer with tetramethylsilane (TMS) as internal standard. Two-dimensional (2D) NMR experiments (^{1}H-^{1}H COSY, HMQC and HMBC) were recorded on a Bruker Avance 600 spectrometer.

Plant Materials. The aerial parts of *A. cordata* were collected in November 2004, in Daejeon, Korea and identified by Prof. KiHwan Bae. A voucher specimen (CNU 1499) has been deposited in the herbarium at the College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and Isolation. The dried aerial parts of *A. cordata* (4 kg) were extracted three times with EtOH (50 L × 3) at room temperature for 3 days, filtered and concentrated to yield an EtOH extract (300 g). The EtOH extract was suspended in H_2O (2 L) and then partitioned successively with *n*-hexane (2 L × 3), EtOAc (2 L × 3) and *n*-BuOH (2 L × 3) to afford hexane- (85 g), EtOAc- (63 g) and BuOH-soluble fractions (82 g), respectively.

The hexane-soluble fraction (85 g) was subjected to silica gel column chromatography (80 × 10.0 cm) eluting with a stepwise gradient of *n*-hexane-acetone (100:1 → 1:2) to afford four fractions (A-D). Fraction A was rechromatographed on a silica gel column (50 × 5.0 cm) using *n*-hexane-acetone (50 : 1) to give compound **2** (1000 mg).

The EtOAc-soluble fraction (63 g) was subjected to silica gel column chromatography (80 × 10.0 cm) eluting with a stepwise gradient of $CHCl_3$-MeOH (100:1 → 1:2) to afford five fractions (E-I). Fraction F was rechromatographed on a silica gel column (50 × 3.5 cm) using *n*-hexane-acetone (20:1 → 15:1) to give compounds **3** (20 mg) and **4** (15 mg). Fraction G was further purified by silica gel column chromatography (50 × 2.5 cm) using *n*-hexane-acetone (10:1) to afford compound **1** (130 mg).

***ent*-15*S*,16-Dihyroxypimar-8(14)-en-19-oic acid (1):** White amorphous powder; mp: 211-213 °C; $[\alpha]_D^{25}$ −39.5° (*c* 0.4, MeOH); IR ν_{max} (KBr) cm^{-1}: 3450, 2935, 1695, 1640, 1460, 842; HRFABMS *m/z* 337.2380 $[M+H]^+$ (calc. for $C_{20}H_{32}O_4H^+$, 337.2379); EIMS *m/z* (rel. int.) 336 $[M]^-$ (5), 321 (27), 298 (15), 281 (17), 275 (88), 166 (29), 134 (37), 121 (100); ^{1}H and ^{13}C NMR data are listed in Table 1.

***ent*-Pimar-8(14),15-dien-19-oic acid (2):** Colorless needles; mp: 165-166 °C; $[\alpha]_D^{25}$ −120.2° (*c* 0.7, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}: 3400, 1690, 1460; FABMS *m/z* 303 $[M+H]^+$; ^{1}H and ^{13}C NMR data were in accordance with published data.[3]

***ent*-16α-Hydroxykauran-19-oic acid (3):** White amorphous powder; mp: 275-277 °C; $[\alpha]_D^{25}$ −104.4° (*c* 1.0, MeOH); IR ν_{max} (KBr) cm^{-1}: 3460, 1700; FABMS *m/z* 321 $[M+H]^+$; ^{1}H and ^{13}C NMR data were in accordance with published data.[4]

***ent*-Kaur-16-en-19-oic acid (4).** White amorphous powder; mp: 178-180 °C; $[\alpha]_D^{25}$ −110.5° (*c* 1.0, MeOH); IR ν_{max} (KBr) cm^{-1}: 3450, 1690, 1470; FABMS *m/z* 303 $[M+H]^+$; ^{1}H and ^{13}C NMR data were in accordance with published data.[5]

Determination of the absolute configuration of an acyclic 1,2-diol moiety in 1 using Snatzke's method. Dimolybdenum tetraacetate $[Mo_2(AcO)_4]$ was purchased from Fluka. DMSO, spectroscopy grade, was obtained from Fluka. According to the published procedure,[7] about 1:1 diol-to-molybdenum mixture was prepared using 0.7 mg/mL of a chiral substrate in DMSO. Soon after mixing, the CD spectrum was recorded and its evolution monitored until stationary (30-40 min).

Cytotoxicity assay. The cancer cell lines (SK-OV-3, HL-60, B16F10 and L1210) were maintained in RPMI 1640 which included L-glutamine (JBI) with 10% FBS (JBI) and 2% penicillin-streptomycin (GIBCO). Cells were cultured at 37 °C in a 5% CO_2 incubator. Cytotoxicity was measured by a modified Microculture Tetrazolium (MTT) assay.[15] Viable cells were seeded in the growth medium (180 μL) into 96 well microtiter plates (1×10^4 cells per each well) and incubated at 37 °C in 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 1.875 to 30 μg/mL by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to < 0.1%. After standing for 2 h, 20 μL of the test sample was added to each well. The same volume of DMSO was added to the control group well. Forty-eight hours after the test sample was added, 20 μL MTT was also added to the each well (final concentration, 5 μg/mL). Two hours later, the plate was centrifuged for 5 minutes at 1500 rpm, the medium was then removed and the resulting formazan crystals were dissolved with 150 μL DMSO. The optical density (O.D.) was measured at 570 nm using a Titertek microplate reader (Multiskan MCC/340, Flow). The IC_{50} value is defined as the concentration of sample to reduce a 50% of absorbance relative to the vehicle-treated control.

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