Two Pimarane Diterpenoids from *Ephemerantha lonchophylla* and Their Evaluation as Modulators of the Multidrug Resistance Phenotype

Guo-Xiang Ma,* Tian-Shan Wang, Li Yin, and Yang Pan

Institute of Traditional Chinese Medicine and Pharmacology, Nanjing University of Traditional Chinese Medicine, Nanjing 210029, People's Republic of China

Yan-Long Guo

Division of Analytical Chemistry, China Pharmaceutical University, Nanjing 210009, People's Republic of China

Gerald A. LeBlanc

Department of Toxicology, Box 7633, North Carolina State University, Raleigh, North Carolina 27695-7633

Manfred G. Reinecke, Willam H. Watson, and Mariusz Krawiec

Department of Chemistry, Texas Christian University, Fort Worth, Texas 76129

Received January 29, 1997[⊗]

Two new pimarane diterpenoids, lonchophylloids A (**1**) and B (**2**), were isolated from the stems of *Ephemerantha lonchophylla*. The structures of **1** and **2** were established predominantly through the application of extensive ^{1}H-and ^{13}C-NMR, 1D- and 2D-homonuclear and heteronuclear correlation NMR experiments, and X-ray diffraction methods. Consistent with structure−activity predictions, both compounds were capable of sensitizing cells that expressed the multidrug resistance phenotype to the toxicity of the anticancer drug doxorubicin.

Introduction

The Chinese crude drug "Shi-Hu" is mainly prepared from the stems of *Dendrobium* species (Orchidaceae) and is used as a tonic and an antipyretic.[1] *Ephemerantha lonchophylla* (Hook. f.) P. F. Hunt et Summerh. is also used as source of "Shi-Hu" in some provinces of China.[2] Tezuka *et al*. reported the isolation and structure elucidation of four new phenolic compounds and one diterpene glycoside from *E. lonchophylla*.[3] Majumder *et al*. also isolated two new phenolic compounds from this plant.[4,5] In this paper, we report the isolation of two new pimarane-type diterpenoids (**1** and **2**) and the P-glycoprotein inhibition activity of these compounds.

Compound **1** was obtained as colorless needles upon recrystallization from petroleum ether−$CHCl_3$. The IR spectrum indicated the presence of hydroxyl groups (3442 and 3420 cm^{-1}), ketone carbonyl (1698 cm^{-1}), α,β-unsaturated ketone carbonyl (1666 cm^{-1}), and double bond (1650 cm^{-1}). The UV spectrum confirmed the presence of an α,β-unsaturated ketone carbonyl (277 nm, log ε 4.23). Its molecular formula was determined by high-resolution mass spectroscopy (M^+ *m/z* 332.1975, C_{20} $H_{28}O_4$ requires 332.1982).

The ^{13}C NMR spectrum together with the DEPT spectrum revealed 20 carbon signals including characteristic signals due to two trisubstituted double bonds [δ 126.9 (d), 140.6 (s), 124.4 (d), 145.6 (s)], two ketone carbonyl groups (δ 200.7, 214.3), an alcoholic carbon (δ 66.4), four methyl (δ 17.3, 22.8, 26.4, 27.3), four methylene, two methine, and three quaternary carbons. The ^{1}H NMR spectrum showed the presence of two oxygenated methylene protons at δ 4.45 and 4.34 (each d, J = 19.2 Hz), two olefinic protons at δ 5.60 (1H, brs) and 6.12 (1H, brs), one deuterium exchangeable singlet at δ 7.01, and four three-proton singlets at δ 0.93, 1.07, 1.13, and 1.19 due to four tertiary methyl groups. On the basis of the above observations, the presence of a pimarane diterpenoid skeleton could be inferred.[6] In addition, ^{13}C NMR signals at δ140.6 (s) and 126.9 (d) are characteristic for a C-8/C-14 double bond in the pimarane-type structure, and ^{1}H NMR absorptions at δ 4.45 and 4.34 are characteristic for a ketone group at C-15 and a hydroxyl group at C-16.[7] The presence of the pimarane skeleton was further verified using 2D NMR spectroscopy especially including HMBC measurements. The ^{1}H−^{13}C long-range COSY spectrum of this compound gave very good information for establishing the assignment of the partial structure of C-1−C-3 carbons. Long-range correlations between the H-20 proton at δ 0.93 and the methine carbons at δ 48.9 (C-9), 52.7 (C-5), a quaternary carbon at δ 39.9 (C-10), and the double bond carbon at δ 124.4 (C-1) in the HMBC spectrum indicated that one of the double bonds must be at position C-1/C-2 and C-1 is not substituted. The HMBC correlation between the H-1 proton at δ 6.12 and the double bond carbon at δ 145.6 (s, C-2) and the ketone carbonyl group carbon at δ 200.7 (C-3) showed that C-2 was substituted by OH and C-3 substituted by a ketone carbonyl group. Furthermore, the long-range correlation between the ketone carbonyl carbon and the two methyl protons at δ 1.19 (C-19), 1.07 (C-18) confirmed

* To whom correspondence should be addressed. Present address: Department of Chemistry, Texas Christian University, Box 298860, Forth Worth, TX 76129. Fax: (817) 921-7110. E-mail: gma@gamma.is.tcu.edu.

[⊗] Abstract published in *Advance ACS Abstracts*, December 1, 1997.

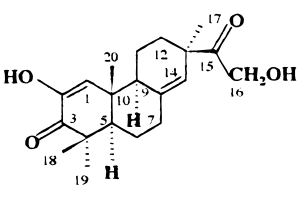
S0163-3864(97)00065-7 CCC: $15.00 © 1998 American Chemical Society and American Society of Pharmacognosy
Published on Web 01/23/1998

Table 1. NMR Data for **1** (in Acetone-d_6)

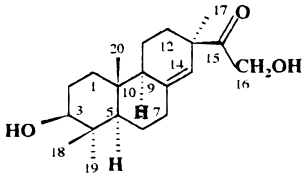
position	δ ^{13}C	δ ^{1}H (J = Hz)	H−H COSY	HMBC (H→C)
1	124.4	6.12 (s)		2, 3, 10
2	145.6	7.01 (s)		
3	200.7			
4	44.7			
5	52.7	1.94 (dd, 12.5, 3.0)	6α, 6β	
6	22.4	α1.68 (dt, 13.1, 3.0)	5, 7α, 7β	
		β1.62 (ddd, 13.1, 12.5, 4.8)	5, 7α, 7β	
7	35.9	α2.23 (td, 12.8, 5.6)	6α, 6β, 14	
		β2.49 (ddd, 12.8, 4.8, 1.9)	6β	5, 6, 8, 9, 14
8	140.6			
9	48.9	2.10 (t, 8.2)	14, 11α, 11β	
10	39.9			
11	21.0	α1.86 (ddd, 13.2, 3.7, 3.6)	12α, 12β, 9	
		β1.36 (ddt, 13.2, 10.9, 2.9)	12α, 12β, 9	
12	33.1	α2.34 (dd, 12.8, 2.9)	11α, 11β	9, 11, 13, 14
		β1.18 (ddd, 12.8, 10.9, 2.9)	11α, 11β	
13	47.6			
14	126.9	5.60 (dd, 5.6, 2.9)	7α, 9	7, 9, 12, 13, 17
15	214.3			
16	66.4	a4.45 (d, 19.2)		
		b4.34 (d, 19.2)		15
17	27.3	1.13 (s)		12, 13, 14,15
18	22.8	1.07 (s)		3, 4, 5, 18
19	26.4	1.19 (s)		4, 5, 19
20	17.3	0.93 (s)		1, 5, 9, 10

that the ketone must be at the C-3 position. On the other hand, in HMBC the long-range correlation between the H-17 protons at δ 1.13 and the double bond carbons at δ 126.9 (C-14) and 214.3 (C-15) verified that the other double bond was at C-8/C-14 and the other ketone carbonyl group at C-15. The latter was also confirmed by the long-range correlation between protons at δ 4.45 and 4.34 and the ketone carbonyl group carbon at δ 214.3. In addition, the ketone side chain was supported by the mass spectrum ion peaks at m/z 273 derived from M^+(332) − $[COCH_2OH]^+$(59). On the basis of the above information, the structure of **1** was determined as 2,16-dihydroxypimara-1(2),8(14)-diene-3,15-dione, exclusive of stereochemistry.

NOE difference spectroscopy allowed the assignment of the stereochemistry. NOEs were observed between H-17, H-14 (4%), and H-16a (3%), between H-19, H-18 (2%), and H-5 (7%), between H-18, H-20 (5%), and H-19 (6%), and between H-20, H-18 (3%), and H-16b (4%). From the NOE results and the coupling constants of 5-H (dd, 12.6, 3.0 Hz) and 9-H (t, 8.2 Hz),[6] the stereochemistry of **1** was determined as shown below. The chemical shifts of all protons and carbons were determined by DEPT, ^{1}H−^{1}H COSY, ^{1}H−^{13}C COSY, and ^{1}H−^{13}C long-range COSY (Table 1).



1



2

Compound **2** was obtained as colorless needles from petroleum ether−$CHCl_3$. The IR spectrum indicated the presence of a hydroxyl group (3440 cm^{-1}), ketone carbonyl (1718 cm^{-1}), and a double bond (1658 cm^{-1}). Its molecular formula was determined by high-resolution mass spectrometry (M^+ m/z 320.2336; $C_{20}H_{32}O_3$ requires 320.2360).

The ^{13}C NMR spectrum together with the DEPT spectrum revealed 20 carbon signals including characteristic signals due to one trisubstituted double bond [δ 123.2 (d), 142.3 (s)], a ketone carbonyl group (δ 214.7), two alcoholic carbons (δ 65.8, 78.9), four methyl (δ 27.4, 28.4, 15.7, 14.5), six methylene, two methine, and three quaternary carbons. The ^{1}H NMR spectrum showed the presence of a two-proton singlet at δ 4.35, one olefinic proton at δ 5.53 (1H, brs), and four three-proton singlets at δ 0.64, 0.80, 1.01, and 1.12. On the basis of the above observations, the presence of a pimarane diterpenoid skeleton could be inferred.[6] In addition, ^{13}C NMR signals at δ 142.3 (s) and δ 123.2 (d) are characteristic for a C-8/C-14 double bond in the pimarane-type structure, and the two-proton singlet in ^{1}H NMR at δ 4.35 is also characteristic for a ketone group at C-15 and a hydroxyl group at C-16.[7] The presence of the pimarane skeleton was further verified using 2D NMR spectroscopy, including HMBC measurements. The ^{1}H−^{13}C long-range COSY correlations between the H-3 proton at δ 3.25 and two methyl carbons at δ 28.4 and 15.7 indicate that the hydroxy group must be at position 3. The long-range HMBC correlation between protons at δ 1.12 (H-17) and carbons at δ 123.2 (C-14) and at 214.7 (C-15) confirmed that the double bond was at C-8/C-14 and the ketone carbonyl group at C-15. The latter was also confirmed by the long-range correlation between the protons at δ 4.35 and the ketone carbonyl group. On the other hand, the ketone side chain was supported by the mass spectrum ion peak at m/z 261 derived from M^+ (320) − $[COCH_2OH]^+$ (59). On the basis of the above information, the structure of **2** was determined as 3,16-dihydroxypimar-8(14)-en-15-one, exclusive of stereochemistry.

NOE difference spectroscopy allowed the assignment of the stereochemistry. NOEs were observed between H-17, H-14 (3%), and H-16 (2%), between H-19, H-18 (3%), H-3 (5%), and H-5 (5%), between H-18, H-20 (2%), H-19 (5%), and H-3 (4%), and between H-20, H-18 (5%), and H-16 (2%). From NOE results and the coupling constants of 3-H (dd, 11.5, 4.1 Hz), 5-H (dd, 12.4, 2.7 Hz), and 9-H (t, 8.4 Hz), the relative stereochemistry of **2** was determined as shown. The chemical shifts of all protons and carbons were determined with the help of DEPT, ^{1}H−^{1}H COSY, ^{1}H−^{13}C COSY, and ^{1}H−^{13}C-long-range COSY (Table 2).

The structure of **2** was confirmed by X-ray diffraction. Crystals were obtained by very slow evaporation of a petroleum ether (60−90 °C): chloroform (50:50) solution of compound **2**. Accurate lattice constants were a = 10.789(3) Å, b = 23.452(2) Å, c = 7.359(2) Å, V = 1861.9(7) Å^3. One molecule of composition $C_{20}H_{32}O_3$ formed the asymmetric unit. All unique diffraction maxima with 2θ = 157.4° were collected using $\omega - 2\theta$ scan and graphite-monochromated Cu Kα radiation (1.541 78 Å). A total of 2243 unique reflections were collected, and 1443 (64%) were judged observed and used in subsequent calculations. The structure was solved by direct methods[8] and expanded using Fourier techniques.[9] The

Table 2. NMR Data for **2** (in $CDCl_3$)

position	δ ^{13}C	δ ^{1}H (J = Hz)	H−H COSY	HMBC (H→C)
1	36.8	α 1.61 (m)		
		β 1.14 (m)		
2	27.4	α 1.54 (m)	3	
		β 1.18 (m)		
3	78.9	3.25 (dd, 11.5, 4.1)	2α	18, 19
4	39.0			
5	54.0	1.03 (dd, 12.6, 2.7)	6α, 6*β*	1, 9, 10, 20
6	22.0	α1.64 (m)	5	
		*β*1.37 (ddd, 14.1, 12.6, 5.7)	5, 7α, 7*β*	7
7	35.6	α2.03 (td, 13.8, 5.4)	6*β*, 14	8, 14, 6
		*β*2.38 (dt, 13.8, 5.4)	6*β*	8, 14, 5, 9, 6
8	142.3			
9	50.7	1.68 (t, 8.4)	7*β*, 14	11
10	38.3			
11	20.1	α1.19 (m)		
		*β*1.48 (ddd, 14.1, 12.6, 3.3)		
12	32.6	α2.33 (dt, 12.6, 5.3)		11, 13, 15
		*β*1.07 (m)	8	
13	46.8			
14	123.2	5.53 (d, 1.6)	7*β*, 9	7, 9, 12, 13, 17
15	214.7			
16	65.8	4.35 (s)		15
17	28.4	1.12 (s)		11, 12, 13, 14, 15
18	15.7	0.80 (s)		3, 5, 10, 18
19	27.4	1.01 (s)		4, 5, 19
20	14.5	0.64 (s)		1, 5, 9, 10

non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 1443 observed reflections [$I > 3.00\sigma(I)$] and 209 variable parameters. A computer-generated drawing of the final X-ray model of **2** is given in Figure 1. No absolute configuration is implied.

Structure−activity analyses, as described previously, suggested that both compounds **1** and **2** may inhibit P-glycoprotein.[10] Experiments were therefore performed to determine whether these compounds would inhibit P-glycoprotein as indicated by the increase in accumulation of doxorubicin by the B16/hMDR-1 cells. Both compounds increased the accumulation of doxorubcin in B 16/h MDR-1 cells but not in B16/F10 cells. This differential effect in the two cell lines is indicative of specific inhibition of P-glycoprotein by the compounds. Both compounds were weak inhibitors of P-glycoprotein with ED_{50} values of 193 and 195 μM for compounds **1** and **2**, respectively. In contrast, the ED_{50} values for the potent P-glycoprotein inhibitor verapamil was approximately 3 μM. These results indicate that while compounds **1** and **2** may be insufficiently active to serve as adjuvants to conventional cancer chemotherapy, they represent a new class of natural compounds that may contain medicinally-important P-glycoprotein inhibitors.

Experimental Section

General Experimental Procedures. The melting points were determined on a WU-1 mp apparatus and are uncorrected. NMR spectra (^{1}H, ^{13}C,COSY, NOE, HMQC, and HMBC) experiments were measured on a Bruker ACF-300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) in acetone-d_6 for **1** and $CDCl_3$ for **2**, the chemical shifts are reported in ppm with TMS as an internal standard, and coupling constants (J) are given in Hz. Optical rotations were obtained on a Perkin-Elmer Model 1412 polarimeter. UV and IR spectra were recorded on a Perkin-Elmer Lambda 2 spectrometer in MeOH and a Nicolet 5DX FT-IR spectrometer in KBr disks. EIMS was recorded on a Nicolet TTMS-2000 instrument. Column chromatography was carried out on silica gel. Fractions were monitored by TLC using phosphomolybdic acid in EtOH.

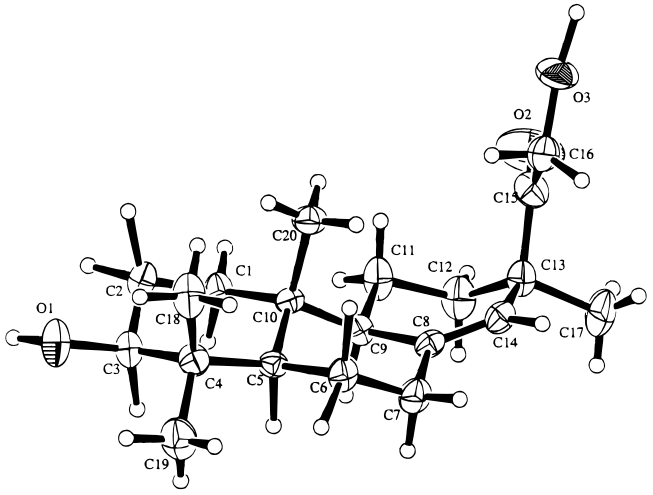


Figure 1. Computer-generated perspective drawing of the final X-ray model of compound **2**.

Plant Material. The stems of *E. lonchophylla* were collected from Yun-Nan, China, in May 1995 and authenticated by Dr. Guoxiang Ma, Institute of Traditional Chinese Medicine (TCM) and Pharmacology, Nanjing University of TCM, Nanjing, China. A voucher specimen was deposited in the Herbarium of Nanjing University of TCM, China (Nanjing 210029).

Extraction and Isolation. The fresh stems (50 kg) were chopped into small pieces and extracted three times with EtOH (300 L) at room temperature for 1 week, and the extract was evaporated *in vacuo* to yield crude extract (300 g). The EtOH extract was suspended in distilled water (1 L) and extracted with $CHCl_3$ (2 L × 4). The $CHCl_3$ layer was separated and evaporated under reduced pressure to give a brown residue (60 g). The $CHCl_3$ extract was subjected to silica gel column chromatography using a linear gradient petroleum ether−$CHCl_3$ system. The fractions 40−43 (petroleum ether−$CHCl_3$, 85:15) were further purified by repeated silica gel column chromatography to give lonchophylloid A (**1**, 35 mg). Fraction 50−55 (petroleum ether−$CHCl_3$, 8:2) gave lonchophylloid B (**2**, 1300 mg).

Lonchophylloid A (1): colorless needles ($CHCl_3$/petroleum ether); mp 147.5−148.5 °C; $[\alpha]^{25}_D$ −97.34° (*c* 1.0, EtOH); UV (MeOH) λ_{max} (log ϵ) 214 (4.62), 277 (4.23) nm; IR (KBr) ν_{max} 3442 (OH), 3420 (OH), 1698 (C═O, ketone), 1666 (C═O, α,*β*-unsaturated ketone), 1650 (C═C), 1450, 1401, 1221, 1025, 1013 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HREIMS 332.1975 (calcd for $C_{20}H_{28}O_4$, requires 332.1982); EIMS m/z 332 (M^+, 24), 273 (22), 255 (15), 165 (42), 152 (22), 137 (100), 121 (54), 109 (40), 93 (44), 79 (40).

Lonchophylloid B (2): colorless needles ($CHCl_3$/petroleum ether); mp 132−133 °C; $[\alpha]^{25}_D$ −9.93° (c 1.0,

EtOH); UV (MeOH) λ_{max} (log ϵ) 212 (3.42); IR (KBr) ν_{max} 3440 (OH), 1718 (C=O, ketone), 1658 (C=C) cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HREIMS 320.2336 (calcd for $C_{20}H_{32}O_3$, requires 320.2360); EIMS *m/z* 320 (M^+, 10), 275 (50), 261 (75), 187 (10), 175 (10), 161 (15), 147 (15), 119 (58), 95 (65), 91 (100).

Crystal data for **2**: $C_{20}H_{32}O_3$, MW = 320.47; orthorhombic; $P2_12_12_1$; a = 10.789(3) Å, b = 23.452(2)Å, c = 7.359(2) Å, V = 1861.9(7) Å^3, Z = 4, D_x = 1.143/cm^3, CuKα (λ = 1.541 78 Å), $F(000)$ = 704, R = 0.064, and Rw = 0.048 for 1443 unique observed reflections with $I > 3.00\sigma(I)$ (total 2243). The data were collected at a temperature of 23 ± 1 °C using a crystal of dimensions of 0.20 × 0.20 × 0.50 mm to maximum 2θ value of 157.4°, collected on a Rigaku AFC6S diffractometer with graphite-monochromated Cu Kα radiation. The structure was solved by direct methods and expanded using Fourier techniques and refined by full-matrix least-squares calculations. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined.

Cell Cultures. B16/F10 mouse melanoma cells and B16/F10 cells transfected with the human MDR-1 gene, designated B16/hMDR-1, were used in these experiments. Cells were transfected with a replication defective amphotropic retrovirus, LMDR1L6, containing the human MDR1 gene and transcribed from the MoMLV-LTR promoter as described by Choi et al.[11] The increased expression of P-glycoprotein and characterization of the multidrug resistance phenotype of these cells have been described previously.[10,12,13] Cells were cultured in RPMI medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum in a humidified atmosphere consisting of 5% CO_2 at 37 °C. The MDR phenotype of the B16/hMDR1 cells was maintained by intermittent culturing of the cells in 6 μM vinblastine.

P-Glycoprotein Inhibition. The inhibition of P-glycoprotein by compounds **1** and **2** was determined by measuring the ability of the compounds to increase the accumulation of the P-glycoprotein substrate doxorubicin in the B16/hMDR-1 cells but not the B16/F10 cells. B16/F10 and B16/hMDR-1 cells (2×10^5−1×10^6) were incubated in microfuge tubes with several concentrations of compounds **1** and **2** along with 50 μM doxorubicin (Pharmacia, Columbus, OH) for 4 h at 37 °C. Verapamil (Sigma, St. Louis, MO), a known P-gp inhibitor, was used as a positive control.[14] After incubation, the cells were pelleted by centrifugation, washed twice with 1 mL of ice-cold PBS, and placed in an extraction solution consisting of 0.6 N HCl in 50% ethanol/50% deionized water overnight.[13] Cells were then pelleted, and the extracted doxorubicin was measured fluorometrically at wavelengths of 470 nm excitation and 585 nm emission.[15] The inhibition of P-gp was quantified on a percentage basis, with 100% inhibition defined by the level of doxorubicin accumulation in the presence of the potent inhibitor verapamil. The potency of inhibition was calculated as the concentration of compound that increased the accumulation of doxorubicin to 50% of maximum (ED_{50}). ED_{50} values were calculated by Spearman−Karber regression analysis.[16]

Acknowledgment. We are grateful to the National Natural Science Foundation of China (Grant No. 39600186), Youth Foundation of Jiangsu Province (Grant No. BQ 96029), and the administration of National Traditional Chinese Medicine and Drug (Grant No. 95C014) for support of this research. We thank Mr. Peng Guoping at Institute of Traditional Chinese Medicine and Pharmacology, Nanjing University of Traditional Chinese Medicine, for assistance with NMR determination.

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NP970065O