Larrealignans A and B, Novel Lignan Glycosides from the Aerial Parts of *Larrea tridentata*

Akihito Yokosuka,* Yukiko Matsuo, Maki Jitsuno, Kohei Adachi, and Yoshihiro Mimaki*

Laboratory of Medicinal Pharmacognosy, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan.

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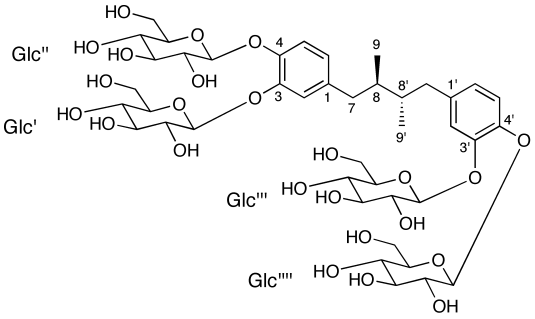
Two new lignan glycosides, named larrealignans A (1) and B (2), and a known lignan (3) were isolated from the aerial parts of *Larrea tridentata* (Zygophyllaceae). The structures of 1 and 2 were determined on the basis of spectroscopic analysis and the results of hydrolytic cleavage. The isolated compounds (1—3) and aglycones (1a, 2a) of 1 and 2 were evaluated for their cytotoxic activities against HL-60 human leukemia cells.

Key words lignan; lignan glycoside; *Larrea tridentata*; Zygophyllaceae; cytotoxic activity

Larrea tridentata (Sesse. and Moc. Ex DC.) Coville. (Zygophyllaceae) is an evergreen shrub that grows in the desert area of the American continents. The aerial parts (leaves and stems) of this plant are called Chaparral and are an alternative herbal medicine used for the treatment of various cancers, tuberculosis, menstrual pains, and diabetes in the United States.[1)] Previously, we have reported 25 triterpene glycosides from the aerial parts of *L. tridentata*.[2)] Further phytochemical analysis of the MeOH extract of this plant has resulted in the isolation of two new lignan glycosides, named larrealignans A (**1**) and B (**2**), and a known lignan (**3**). In this paper, we describe the structural elucidation of **1** and **2** on the basis of spectroscopic analysis, including various two-dimensional (2D)-NMR spectroscopic studies, and the results of hydrolytic cleavage. The cytotoxic activities of the isolated compounds (**1**—**3**) and the aglycones (**1a**, **2a**) of **1** and **2** against HL-60 human leukemia cells are also reported.

The aerial parts of *L. tridentata* (3.0 kg) were extracted with MeOH under reflux conditions. After removal of the solvent, the concentrated MeOH extract (940 g) was passed through a porous-polymer polystyrene resin (Diaion HP-20) column and successively eluted with MeOH in H_2O (3 : 7), MeOH–H_2O (1 : 1), MeOH, EtOH, and EtOAc. The MeOH eluate fraction (477 g) was subjected to column chromatography on silica gel and octadecylsilanized (ODS) silica gel, yielding compounds **1** (23.2 mg), **2** (14.5 mg), and **3** (11.2 mg). Compound **3** was identified by its physical and spectroscopic data as (*E*)-4,4′-dihydroxy-7,7′-dioxolign-8(8′)-ene.[3)]

Larrealignan A (**1**) was obtained as an amorphous solid and showed an accurate $[M+Na]^+$ ion at *m*/*z* 973.3557 in the high resolution (HR)-electrospray ionization (ESI)-time-of-flight (TOF)-MS, corresponding to the empirical molecular formula $C_{42}H_{62}O_{24}$. The IR spectrum of **1** suggested the presence of hydroxy groups (3376 cm^{-1}) and aromatic rings (1613, 1509 cm^{-1}). The UV spectrum showed an absorption maximum due to substituted aromatic rings (273 nm). The ^{1}H-NMR spectrum of **1** contained signals for two 1,3,4-trisubstituted aromatic rings at δ 7.60 (1H, d, J=8.2 Hz), 7.50 (1H, br s), and 6.81 (1H, br d, J=8.2 Hz); 7.60 (1H, d, J=8.2 Hz), 7.51 (1H, br s), and 6.81 (1H, br d, J=8.2 Hz), two methyl groups at δ 0.69 (3H, d, J=6.5 Hz) and 0.66 (3H, d, J=6.5 Hz), and four anomeric protons at δ 5.65 (1H, d, J=7.9 Hz), 5.64 (1H, d, J=7.3 Hz), 5.60 (1H, d, J=7.6 Hz), and 5.59 (1H, d, J=7.3 Hz). Enzymatic hydrolysis of **1** with β-D-glucosidase in an HOAc/NaOAc buffer (pH 5.0) at room temperature yielded *meso*-nordihydroguaiaretic acid (*meso*-NDGA) ($[\alpha]_D$ ±0)[4)] and D-glucose. The above data suggest that **1** is a *meso*-NDGA tetraglucoside.



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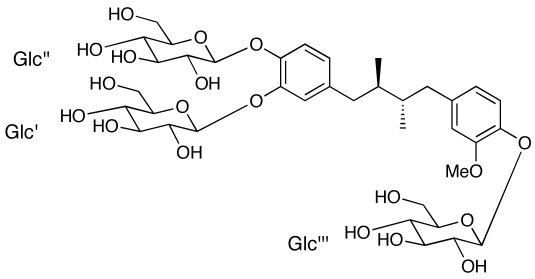

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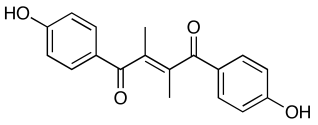

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Chart 1

The linkage positions of the glucosyl moieties to the aglycone were solved by detailed analysis of the one-dimensional (1D) totally correlated spectroscopy (TOCSY) and 2D-NMR spectra. The ^{1}H-NMR subspectra of the individual glucosyl

* To whom correspondence should be addressed. e-mail: yokosuka@toyaku.ac.jp; mimakiy@toyaku.ac.jp

units were obtained by using selective irradiation of non-overlapping proton signals in a series of 1D-TOCSY experiments.[5] Subsequent analysis of the ^{1}H–^{1}H shift correlation spectroscopy (COSY) spectrum resulted in the sequential assignments of all the proton resonances due to the four glucosyl units (Table 1). Using the ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC) and ^{1}H-detected heteronuclear single-quantum coherence (HSQC)-TOCSY spectra, the proton resonances were correlated with the corresponding one-bond coupled carbon signals, which allowed the carbon shifts to be completely assigned. The ^{1}H- and ^{13}C-NMR signals thus assigned were indicative of the presence of four terminal β-D-glucopyranosyl moieties (Glc′, Glc″, Glc‴, and Glc⁗) in **1**[6,7] (Table 1). The β-orientations of the anomeric centers of all of the glucosyl moieties were supported by the relatively large J values of their anomeric protons (7.3—7.9 Hz). In the ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) spectrum of **1**, long-range correlations were observed between the anomeric proton (H-1) of Glc′ at δ_H 5.65 and C-3 of the aglycone at δ_C 148.50, H-1 of Glc″ at δ_H 5.60 and C-4 of the aglycone at δ_C 146.93, H-1 of Glc‴ at δ_H 5.64 and C-3′ of the aglycone at δ_C 148.63, and between H-1 of Glc⁗ at δ_H 5.59 and C-4″ of the aglycone at δ_C 147.02. Accordingly, the structure of larrealignan A (**1**) was elucidated as shown in Chart 1.

Larrealignan B (**2**) was found to have a molecular formula

Table 1. ^{1}H- and ^{13}C-NMR Chemical Shift Assignments of **1** and **2** in C_5D_5N

	1				**2**			
	Position	^{1}H	J (Hz)	^{13}C	Position	^{1}H	J (Hz)	^{13}C
	1			137.36	1			137.72
	2	7.50 br s		120.42	2	7.58 d	1.5	121.11
	3			148.50	3			148.99
	4			146.93	4			147.46
	5	7.60 d	8.2	119.33[a]	5	7.62 d	8.5	119.81
	6	6.81 br d	8.2	123.97	6	6.89 dd	8.5, 1.5	124.46
	7a	2.15 m		38.98	7a	2.26 m		39.10
	b	2.66 m			b	2.72 m		
	8	1.68 m		39.79[b]	8	1.78 m		39.70
	9	0.69 d	6.5	15.80	9	0.77 d	7.0	16.17
	1′			137.42	1′			136.31
	2′	7.51 br s		120.53	2′	6.91 d	1.5	114.04
	3′			148.63	3′			149.65
	4′			147.02	4′			146.13
	5′	7.60 d	8.2	119.37[a]	5′	7.56 d	8.0	116.40
	6′	6.81 br d	8.2	124.00	6′	6.77 dd	8.0, 1.5	121.77
	7′a	2.12 m		38.72	7′a	2.22 m		39.02
	b	2.67 m			b	2.75 m		
	8′	1.68 m		39.47[b]	8′	1.75 m		39.70
	9′	0.66 d	6.5	15.87	9′	0.77 d	6.5	16.27
					3′-OMe	3.82 s		56.11
Glc′	1	5.65 d	7.9	103.90	Glc′ 1	5.66 d	7.6	104.51
	2	4.32 m		74.96	2	4.35 m		75.22
	3	4.34 m		78.02	3	4.34 m		78.19
	4	4.35 m		71.04	4	4.34 m		71.31
	5	4.02 m		78.59	5	4.02 m		78.80
	6a	4.50 br d	10.9	62.05	6a	4.50 br d	11.1	62.40
	b	4.36 m			b	4.37 dd	11.1, 4.1	
Glc″	1	5.60 d	7.6	104.09	Glc″ 1	5.58 d	7.6	104.58
	2	4.31 m		74.96	2	4.34 m		75.22
	3	4.33 m		77.96	3	4.33 m		78.29
	4	4.34 m		71.04	4	4.33 m		71.31
	5	4.04 m		78.59	5	4.02 m		78.84
	6a	4.52 br d	11.4	62.14	6a	4.53 br d	10.8	62.40
	b	4.37 br d	11.4		b	4.40 dd	10.8, 3.2	
Glc‴	1	5.64 d	7.3	104.02	Glc‴ 1	5.68 d	7.5	102.48
	2	4.32 m		74.96	2	4.38 dd	7.5, 9.5	74.97
	3	4.34 m		78.02	3	4.37 dd	9.5, 9.5	78.29
	4	4.35 m		71.04	4	4.36 dd	9.5, 9.5	71.27
	5	4.02 m		78.59	5	4.14 m		78.54
	6a	4.50 br d	11.2	62.05	6a	4.55 br d	11.0	62.40
	b	4.36 m			b	4.40 dd	11.0, 3.8	
Glc⁗	1	5.59 d	7.3	104.12				
	2	4.30 m		74.96				
	3	4.32 m		77.96				
	4	4.33 m		71.04				
	5	4.03 m		78.59				
	6a	4.52 br d	11.3	62.14				
	b	4.38 br d	11.3					

a, *b*) Assignments may be interchangeable.

of $C_{37}H_{54}O_{19}$ as determined by HR-ESI-TOF-MS analysis (*m/z* 825.3144 $[M+Na]^+$). The ^{1}H-NMR spectrum of **2** contained signals for three anomeric protons at δ 5.68 (d, J=7.5 Hz), 5.66 (d, J=7.6 Hz), and 5.58 (d, J=7.6 Hz), as well as signals for two 1,3,4-trisubstituted aromatic rings at δ 7.62 (1H, d, J=8.5 Hz), 7.58 (1H, d, J=1.5 Hz), and 6.89 (1H, dd, J=8.5, 1.5 Hz); 7.56 (1H, d, J=8.0 Hz), 6.91 (1H, d, J=1.5 Hz), and 6.77 (1H, dd, J=8.0, 1.5 Hz), two methyl groups at δ 0.77 (3H, d, J=7.0 Hz) and 0.77 (3H, d, J=6.5 Hz), and one methoxy group at δ 3.82 (3H, s). Enzymatic hydrolysis of **2** with β-D-glucosidase yielded D-glucose and an NDGA derivative (**2a**),[8] which gave *meso*-tetra-*O*-methyl-NDGA (**2b**, $[\alpha]_D$ $\pm$0)[9] by treatment of **2a** with trimethylsilyldiazomethane (TMS-CH_2N_2). The above data suggest that **2** is a triglucoside of **2a**. Using the same procedures as described for **1**, all the ^{13}C-NMR signals for the glucosyl moieties of **2** were assigned to the three terminal β-D-glucopyranosyl units (Glc′, Glc″, and Glc‴). In the HMBC spectrum of **2**, long-range correlations were observed between H-1 of Glc′ at δ_H 5.66 and C-3 of the aglycone at δ_C 148.99, H-1 of Glc″ at δ_H 5.58 and C-4 of the aglycone at δ_C 147.46, and between H-1 of Glc‴ at δ_H 5.68 and C-4′ of the aglycone at δ_C 146.13. Thus, the structure of larrealignan B (**2**) was characterized as shown in Chart 1 or its antipode in regard to the C-8 and C-8′ configurations.

A number of lignan glycosides have been isolated from the plant kingdom. Although lignan bisdesmosides have been reported from *Eucomia ulmoides*[10] (Eucommiaceae), *Sedum sarmentosum*[11] (Crassulaceae), and *Daphne pseudomezereum*[12] and *D. feddei*[13] (Thymelaeaceae), larrealignans A (**1**) and B (**2**) are believed to be the first representatives of lignan tetra- and trisdesmosides, respectively.

The isolated compounds (**1**—**3**) and the aglycones (**1a**, **2a**) of **1** and **2** were evaluated for their cytotoxic activities against HL-60 cells. Compounds **1**—**3** did not exhibit cytotoxicity even at a sample concentration of 20 μM. However, **1a** and **2a** showed moderate cytotoxicity with respective IC_{50} values of 7.0 and 4.9 μM, whereas etoposide used as a positive control gave an IC_{50} value of 0.33 μM.

Experimental

Optical rotations were measured using a JASCO P-1030 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO Fourier transform (FT)-IR 620 spectrophotometer, UV spectra on a JASCO V-630 spectrophotometer, and circular dichroism (CD) spectra on a JASCO J-720 instrument, respectively. NMR spectra were recorded on a Bruker DPX-500 spectrometer (500 MHz for ^{1}H-NMR, Karlsruhe, Germany) or a Bruker DRX-600 spectrometer (600 MHz for ^{1}H-NMR) using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as an internal standard. HR-ESI-TOF-MS data was recorded on a Waters-Micromass LCT mass spectrometer (Manchester, U.K.). Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan), silica gel (Fuji-Silysia Chemical, Aichi, Japan), and ODS silica gel (Nacalai Tesque, Kyoto, Japan) were used for column chromatography. TLC was carried out on Silica gel 60 F_{254} (0.25 mm thick or 0.5 mm thick, Merck, Darmstadt, Germany) and RP-18 F_{254S} (0.25 mm thick, Merck) plates, and spots were visualized by spraying with 10% H_2SO_4 followed by heating. HPLC was performed with a system composed of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), a RI-8010 (Tosoh) and a Shodex OR-2 (Showa-Denko, Tokyo, Japan) detector, and a Rheodyne injection port. The following materials and reagents were used for cell culture assay; microplate reader, Spectra Classic (Tecan, Salzburg, Austria); 96-well flat-bottom plate (Iwaki Glass, Chiba, Japan); JCRB 0085 HL-60 cells (Human Science Research Resources Bank, Osaka, Japan); fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD, U.S.A.); RPMI 1640 medium, etoposide, cisplatin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), (Sigma, St. Louis, MO, U.S.A.); penicillin G sodium salt, and streptomycin sulfate (Gibco, Grand Island, NY, U.S.A.). All other chemicals used were of biochemical reagent grade.

Plant Material The aerial parts of *L. tridentata* were obtained from Richters, Ontario, Canada. A small amount of the sample is preserved in our laboratory (07-003-LR).

Extraction and Isolation The aerial parts of *L. tridentata* (3.0 kg of dry weight) were extracted with hot MeOH (77 l). After removing the solvent, the MeOH extract (940 g), which showed cytotoxic activity against HL-60 cells (IC_{50} 6.8 μg/ml), was passed through a Diaion HP-20 column and successively eluted with 30% MeOH, 50% MeOH, MeOH, EtOH, and EtOAc. The MeOH, EtOH, and EtOAc eluate fractions exhibited cytotoxic activity against HL-60 cells (IC_{50} 2.7, 2.4, 11.0 μg/ml respectively). Column chromatography (CC) of the MeOH eluate portion (477 g) on silica gel and elution with a stepwise gradient mixture of $CHCl_3$–MeOH–H_2O (19 : 1 : 0; 9 : 1 : 0; 40 : 10 : 1), and finally with MeOH alone, gave six fractions (I—VI). Fraction VI was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (40 : 10 : 1; 20 : 10 : 1; 7 : 4 : 1) and ODS silica gel eluted with MeOH–H_2O (11 : 9; 2 : 1; 8 : 2) to afford **1** (23.2 mg) and **2** (14.5 mg). The EtOH eluate portion (40.7 g) on silica gel and elution with a stepwise gradient mixture of $CHCl_3$–MeOH (1 : 0; 19 : 1; 9 : 1), and finally with MeOH alone, gave 12 fractions (i—xii). Fraction x was chromatographed on ODS silica gel CC eluted with MeOH–H_2O (3 : 2; 7 : 3) to afford **3** (11.2 mg).

Larrealignan A (**1**): Amorphous solid. $[\alpha]_D^{25}$ −21.1 (c=0.10, MeOH). HR-ESI-TOF-MS *m/z*: 973.3557 $[M+Na]^+$ (Calcd for $C_{42}H_{62}O_{24}Na$: 973.3529). UV λ_{max} (MeOH) nm (log ε): 273 (3.48). IR ν_{max} (film) cm^{-1}: 3376 (OH), 2925 (CH), 1613 and 1509 (aromatic rings), 1454, 1267, 1070. ^{1}H-NMR (600 MHz, C_5D_5N) and ^{13}C-NMR (125 MHz, C_5D_5N) spectroscopic data, see Table 1. The CD spectrum showed no Cotton effect in the 600—200 nm region.

Enzymatic Hydrolysis of 1 Compound **1** (4.1 mg) was treated with β-D-glucosidase (EC 3.2.1.21, Sigma, 31.4 mg) in HOAc/NaOAc buffer (pH 5.0, 5 ml) at room temperature for 44 h. The reaction mixture was diluted with H_2O (5 ml) and extracted with EtOAc (10 ml×3). After concentration of the EtOAc-soluble phase, it was chromatographed on silica gel eluted with $CHCl_3$–MeOH (19 : 1) to yield *meso*-NDGA (**1a**; 0.9 mg). The H_2O-soluble phase was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (7 : 4 : 1) to yield a sugar fraction (2.0 mg). The sugar fraction was analyzed by HPLC under the following conditions: column, Capcell Pak NH2 SG80 Å (4.6 mm i.d.×250 mm, 5 μm, Shiseido, Tokyo, Japan); solvent, MeCN–H_2O (17 : 3); flow rate, 1.0 ml/min; detection, OR. Identification of D-glucose present in the sugar fraction was carried out by comparison of the retention time and optical rotation with that of an authentic sample. t_R (min): 16.45 (D-glucose, positive optical rotation).

meso-NDGA (**1a**): Amorphous solid. $[\alpha]_D^{26}$ ±0 (c=0.10, MeOH). ^{1}H-NMR (400 MHz, CD_3OD): δ 6.70 (2H, d, J=8.0 Hz, H-2, 2′), 6.63 (2H, d, J=2.0 Hz, H-5, 5′), 6.50 (2H, dd, J=8.0, 2.0 Hz, H-6, 6′), 2.68 (2H, dd, J=13.3, 5.0 Hz, H-7b, 7b′), 2.21 (2H, dd, J=13.3, 9.2 Hz, H-7a, 7a′), 1.74 (2H, m, H-8, 8′), 0.85 (6H, d, J=6.7 Hz, Me-9, 9′). The CD spectrum showed no Cotton effect in the 600—200 nm region.

Larrealignan B (**2**): Amorphous solid. $[\alpha]_D^{26}$ −26.8 (c=0.10, MeOH). HR-ESI-TOF-MS *m/z*: 825.3144 $[M+Na]^+$ (Calcd for $C_{37}H_{54}O_{19}Na$: 825.3157). UV λ_{max} (MeOH) nm (log ε): 275 (3.55). CD λ_{max} (MeOH) nm ($\Delta\varepsilon$): 272 (−1.0). IR ν_{max} (film) cm^{-1}: 3389 (OH), 2926 (CH), 1593 and 1512 (aronatic rings), 1454, 1265, 1072. ^{1}H-NMR (600 MHz, C_5D_5N) and ^{13}C-NMR (125 MHz, C_5D_5N) spectroscopic data, see Table 1.

Enzymatic Hydrolysis of 2 Compound **2** (10.0 mg) was subjected to enzymatic hydrolysis as described for **1** to give **2a** (2.5 mg) and a sugar fraction (4.3 mg). HPLC analysis of the sugar fractions under the same conditions as in the case of **1** showed the presence of D-glucose. t_R (min): 16.45 (D-glucose, positive optical rotation).

Compound **2a**: Amorphous solid. $[\alpha]_D^{25}$ +13.0 (c=0.07, MeOH). ^{1}H-NMR (400 MHz, CD_3OD): δ 6.72 (1H, d, J=8.0 Hz, H-5′), 6.71 (1H, d, J=8.0 Hz, H-5), 6.67 (1H, d, J=1.9 Hz, H-2′), 6.66 (1H, d, J=2.0 Hz, H-2), 6.60 (1H, dd, J=8.0, 1.9 Hz, H-6′), 6.53 (1H, dd, J=8.0, 2.0 Hz, H-6), 3.84 (1H, s, OMe), 2.76 (1H, dd, J=13.3, 5.0 Hz, H-7b′), 2.68 (1H, dd, J=13.5, 6.0 Hz, H-7b′), 2.31 (1H, dd, J=13.5, 8.7 Hz, H-7a), 2.23 (1H, dd, J=13.3, 9.5 Hz, H-7a′), 1.78 (1H, m, H-8), 1.75 (1H, m, H-8′), 0.88 (3H, d, J=6.8 Hz, Me-9), 0.88 (3H, d, J=6.8 Hz, Me-9′). The CD spectrum showed no Cotton effect in the 600—200 nm region.

Methylation of 2a TMS-CH_2N_2 in hexane (2.0 M, 0.5 ml) was added to a solution of **2a** (2.0 mg) in MeOH (4.0 ml), and then the mixture was stirred at room temperature for 15 h. The reaction mixture was subjected to preparative TLC using hexane–acetone (1 : 2) as the solvent to yield *meso*-tetra-*O*-

methyl-NDGA (**2b**, 2.0 mg).[9]

Cell Culture and Assay for Cytotoxic Activity against HL-60 Cells The cell growth was measured with an MTT reduction assay as described in a previous paper.[14] Briefly, HL-60 cells were maintained in RPMI 1640 medium containing heat-inactivated 10% (v/v) FBS supplemented with L-glutamine, 100 unit/ml penicillin G sodium salt, and 100 μg/ml streptomycin sulfate. The cells (4×10^4 cells/ml) were continuously treated with each compound for 72 h, and cell growth was measured with an MTT reduction assay procedure. A dose–response curve was plotted for **1a** and **2a**, and the concentration giving 50% growth inhibition (IC_{50}) was calculated.

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