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Isoflavonoids from *Dalbergia olivari*☆

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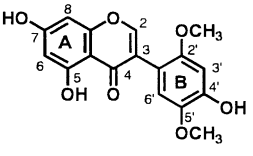
Abstract

Two isoflavonoids, named olibergin A (**1**) and B (**2**) were isolated from the stem bark of *Dalbergia oliveri* (Leguminosae). Along with three previously known compounds, they are inhibitors of Epstein-Barr virus early antigen activation induced by 12-*O*-tetradecanoylphorbol-13-acetate in Raji cells. Their structures were elucidated on the basis of spectroscopic analyses.

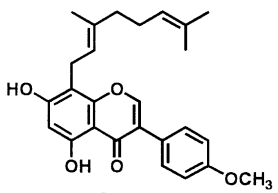
Keywords: *Dalbergia oliveri*; Leguminosae; Isoflavonoids; Anti-tumor-promoting effect; Epstein-Barr virus activation test

1. Introduction

Many isoflavonoids and neoflavonoids have been reported from *Dalbergia* species (family Leguminosae) (Dewick, 1988) and various isoflavonoids have been found to possess biological activities against several kinds of tumor cells (Li et al., 1993). Previously, we reported that some isoflavonoids isolated from plants of the Leguminosae show inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein–Barr virus early antigen (EBV-EA) activation and two-stage mouse skin carcinogenesis as potential cancer chemopreventive agents (anti-tumor promoters) (Konoshima et al., 1988, 1992, 1997; Zaizen et al., 1997, 2000; Ito et al., 2000). In the course of our continuing screening of various plant materials for anti-tumor promoting agents, the EtOH extract of the stems of *Dalbergia* species exhibited a significant anti-tumor promoting activity on TPA-induced EBV-EA activation. This paper describes the isolation and structure elucidation of two new isoflavonoids named olibergin A (**1**) and B (**2**) from the EtOH extract of *D. oliveri* Grmb. collected in Thailand, and the results of assays examining the inhibitory effects on TPA-induced EBV-EA activation of these new isoflavonoids.



1



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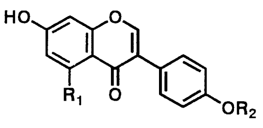

3 $R_1 = H, R_2 = CH_3$
4 $R_1 = OH, R_2 = CH_3$
5 $R_1 = OH, R_2 = H$

2. Results and discussion

2.1. Isolation and structure elucidation

The dried stem barks of *D. oliveri* was extracted with EtOH at room temperature. The acetone-soluble portions

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Table 1
NMR spectral data of isoflavonoids from *Dalbergia olivari*

	Olibergin A (**1**)[a]			Olibergin B (**2**)		
	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC
2	156.0 (*d*)	8.03		152.5 (*d*)	7.93	
3	121.5 (*s*)		H-2, H-6′	123.3 (*s*)		H-2, H-2′, H-6′
4	181.5 (*s*)		H-2	181.2 (*s*)		H-2
4a	106.0 (*s*)		H-6, H-8, 5-OH	106.3 (*s*)		H-6, 5-OH
5	163.8 (*s*)		H-6, 5-OH	160.9 (*s*)		H-6, 5-OH
5-OH		13.03			12.85	
6	99.8 (*d*)	6.26 (*d*, 2.2)	H-8, 5-OH	99.8 (*d*)	6.34	5-OH
7	165.2 (*s*)		H-6, H-8	161.0 (*s*)		H-6, H-1″
8	94.5 (*d*)	6.40 (*d*, 2.2)	H-6	104.9 (*s*)		H-6, H-1″
8a	159.0 (*s*)		H-2, H-8	155.0 (*s*)		H-2, H-1″
1′	110.9 (*s*)		H-2, H-3′	123.0 (*s*)		H-3′, H-5′
2′	153.5 (*s*)		H-3′, H-6′, 2′-OMe	130.1 (*d*)	7.47 (*d*, 8.8)	H-6′
3′	101.3 (*d*)	6.62		114.1 (*d*)	6.99 (*d*, 8.8)	H-5′
4′	148.7 (*s*)		H-3′, H-6′	159.8 (*s*)		H-2′, H-6′, 4′-OMe
5′	141.9 (*s*)		H-3′, H-6′, 5′-OMe	114.1 (*d*)	6.99 (*d*, 8.8)	H-3′
6′	116.9 (*d*)	6.96		130.1 (*d*)	7.47 (*d*, 8.8)	H-2′
1″				21.6 (*t*)	3.51 (*d*, 7.0)	
2″				120.8 (*d*)	5.26 (*m*)	H-1″, H-4″, H-5″
3″				139.0 (*s*)		H-1″, H-4″, H-5″
4″				16.3 (*q*)	1.84 (3H)	H-2″, H-5″
5″				39.7 (*t*)	2.08 (2H, *m*)	H-2″, H-4″, H-6″
6″				26.3 (*t*)	2.11 (2H, *m*)	H-5″
7″				123.6 (*d*)	5.05 (*m*)	H-6″, H-9″, H-10″
8″				132.2 (*s*)		H-6″, H-9″, H-10″
9″				17.7 (*q*)	1.60 (3H)	H-10″
10″				25.7 (*q*)	1.68 (3H)	H-9″
others	56.5 (*q*, 2′-OMe) 57.2 (*q*, 5′-OMe)	3.70 (3H, 2′-OMe) 3.79 (3H, 5′-OMe)		55.4 (*q*, 4′-0Me)	3.85 (3H, 4′-OMe) 6.05 (*br*, 7-OH)	

Values in (δ_H and δ_C) ppm. All signals correspond to 1H, and were observed as a singlet, unless otherwise stated. Figures in parentheses are coupling constants (*J*) in Hz.

[a] A spectrum was taken in acetone-d_6.

of the EtOH extract was subjected successively to silica gel column chromatography and preparative TLC to give **1** and **2** along with known formononetin (**3**), biochanin A (**4**), and genistein (**5**).

Olibergin A (**1**) was obtained as a colorless oil, and its molecular formula was determined as $C_{17}H_{14}O_7$ by HREIMS. The IR spectrum exhibited bands at ν_{max} 3469, 3259 *br*, 1622 cm^{-1} due to hydroxyl and conjugated carbonyl groups, respectively. The ^{1}H NMR spectrum (acetone-d_6) of **1** (Table 1) showed a singlet at δ 8.03 assigned as H-2, the isoflavone nucleus. The ^{1}H NMR spectrum further revealed the presence of two methoxyls (δ 3.70 and 3.79) and a chelated hydroxyl group (δ 13.03). In the aromatic proton region, signals due to *meta*-coupled protons at δ 6.26 and 6.40 (each 1 H, *d*, $J = 2.2$ Hz) and *para*-located protons at δ 6.62 and 6.96 as two singlets. Observation of MS fragment ion peaks at *m*/*z* 177 and 153 derived from a *retro*-Diels–Alder type cleavage followed by a hydrogen transfer, suggested the location of two methoxyls and a hydroxyl group on the B ring. In the HMBC spectrum, the hydrogen-bonded proton at δ_H 13.03 showed C–H three-bond correlations with the carbon signals at δ_C 106.0 (C-4a) and 99.8 (C-6), which were further correlated with the doublet at δ_H 6.40 (H-8). Correlation from C-1′ to H-2 and H-3′, from C-2′ to H-6′, and 2′-OCH_3, from C-5′ to H-3′ and 5′-OCH_3, and NOE between 2′-OCH_3 and H-3′ and H-2, as well as 5′-OCH_3 and H-6′, and H-2 and H-6′, indicated the locations of two methoxyl groups at C-2′, C-5′, and a hydroxyl group at C-4′ on the B ring. From these spectral data and further HMBC results (Table 1), structure **1** for olibergin A, is proposed.

Olibergin B (**2**) was obtained as a colorless oil having a molecular formula, $C_{26}H_{28}O_5$. The IR spectrum exhibited bands at ν_{max} 3383br and 1653 cm^{-1} due to hydroxyl and carbonyl groups, respectively. The signals at δ_H 7.93 assignable to H-2 and δ_C 152.5 (C-2) in the ^{1}H and ^{13}C NMR spectra (Table 1), respectively, were suggestive of an isoflavone type skeleton. The ^{1}H NMR spectrum further revealed the presence of a methoxyl group at δ 3.85, an isolated proton at δ 6.34, *ortho*-coupled A_2B_2-type protons at δ 7.47 and 6.99 (each 2 H, *d*, $J = 8.8$ Hz) assignable to H-2′, 6′ and H-3′, 5′, respectively, in addition to a chelated hydroxyl group proton at δ 12.85. The appearance of remaining signals at δ 5.26 (1 H, *m*), 5.05 (1 H, *m*), 3.51 (2 H, *d*, $J=7.0$ Hz), 2.11 (2 H, *m*), 2.08 (2 H, *m*), 1.84, 1.68, 1.60 (each 3 H, *s*) in the ^{1}H NMR spectrum, two characteristic MS fragment ions at *m*/*z* 351 and 297 arising from loss of [$\cdot C_5H_9$] and [$\cdot C_9H_{15}$] from the molecular ion, respectively, and NOE enhancement between the H-1″ (δ 3.51) and H-4″ (δ 1.84) suggested the presence of a geranyl moiety [$-CH_2CH=C(CH_3)\text{-}CH_2CH_2CH=C(CH_3)_2$] in the molecule. The location of the geranyl moiety at C-8 was confirmed by analysis of the HMBC spectrum. A hydrogen bonded proton at δ_H 12.85 showed C–H long-range correlation with a carbon resonance at δ_C 99.8 (C-6) bearing a singlet proton at δ_H 6.34 (H-6). Furthermore, on performing a NOE experiment, each 10% enhancement of H-3′ and 5′ signals were observed on irradiation of OCH_3 signal, indicating the location of OCH_3 at C-4′ on the B ring and a hydroxyl group at C-7 on the A ring. On the basis of these results, together with other HMBC correlations shown in Table 1, the structure of oliberatin B is proposed as **2**.

Three known isoflavones, formononetin (**3**) (Ingham, 1976), biochanin A (**4**) (Cook et al., 1978), and genistein (**5**) (Ingham, 1976) were isolated and identified by comparison with spectral data previously published in the literature.

2.2. *Inhibitory effects on EBV-EA induction*

The anti-tumor promoting activities of olibergin A (**1**) and B (**2**) were tested in a short-term in vitro assay of TPA-induced EBV-EA activation in Raji cells. Their inhibitory effects on the activation of the virus-genome, the viabilities of Raji cells and the 50% inhibitory concentration (IC_{50}) values are shown in Table 2. Although the inhibitory effect of olibergin A (**1**) at 1×10 mol ratio/TPA was not fully estimated, olibergin B (**2**) showed an inhibitory effect on EBV activation even at 1×10 mol ratio/TPA (8.7%), and only weak cytotoxicity against Raji cells even at 1×10^3 mol ratio was observed. These values of olibergin A (**1**) and B (**2**) corresponded to an IC_{50} value of 462 and 281 mol ratio/TPA, respectively. The IC_{50} value of olibergin B (**2**) was lower than that of β-carotene (IC_{50} 400), a vitamin A precursor commonly used in cancer preventive studies (Murakami et al., 1996). In view of the present findings, the lipophilic group (a geranyl side-chain) in olibergin B (**2**) may enhance the anti-tumor promoting effects, in a manner similar to our previous studies indicating that the presence of a prenyl and/or geranyl moiety in isoflavonoids plays an important role in the inhibition of EBV-EA induction (Ito et al., 2000).

3. Experimental

3.1. *General*

^{1}H NMR (400, 500, and 600 MHz), ^{13}C NMR (150 MHz), NOE, HMQC, and HMBC ($J=8$ Hz) spectra: in $CDCl_3$ (unless otherwise stated) with TMS as an int. standard. UV spectra: in MeOH and IR spectra: in $CHCl_3$. TLC: Kieselgel 60 F_{254} (Merck). Optical rotations: in $CHCl_3$ at 25 °C.

3.2. *Plant material*

The plant material used in this study, *Dalbergia olivari* Grmb.(Leguminosae) was collected at the Sakaerat Environment Research Station, Nakorn Ratsima Province, Thailand, in April 1996. A voucher specimen (NSR-092513) is preserved in the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

3.3. *Extraction and separation*

The dried stem bark (6.0 kg) of *D. oliveri* was extracted with EtOH at room temperature and the solvent was evaporated under reduced pressure to give the EtOH extract (1.3 kg). The acetone-soluble portion (6.4 g) of

Table 2
Inhibitory effects of new isoflavonoids on TPA-induced EBV-EA activation[a]

Compound	EBV-EA-positive cells (% viability)				IC_{50}[b]
	Compound concentration (mol ratio/32 pmol TPA)				(mol ratio/32 pmol TPA)
	1000	500	100	10	
olibergin A (**1**)	17.2±0.3 (60)	44.7±1.1 (>80)	78.5±1.4 (>80)	100.0±0.3 (>80)	462
olibergin B (**2**)	2.5±0.5 (60)	24.0±1.4 (>80)	71.9±1.3 (>80)	91.3±1.0 (>80)	281
β-carotene[c]	9.1±0.5 (60)	34.3±1.1 (>80)	82.7±1.8 (>80)	100.0±0.2 (>80)	400

[a] Mole ratio/TPA (32 pmol = 20 ng/ml), 1000 mol ratio = 32 nmol, 500 mol ratio = 16 nmol, 100 mol ratio = 3.2 nmol, and 10 mol ratio = 0.32 nmol. Values are EBV-EA activation (%)±SD in the presence of the test compound relative to the positive control (100%). Values in parentheses represent the surviving Raji cells measured by Trypan Blue staining. A minimum 60% survival rate of Raji cells 2 days after treatment with the compounds is required for an accurate result.

[b] IC_{50} represents mol ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol of TPA.

[c] Positive control substance.

the EtOH extract was subjected to Si gel cc eluted with $CHCl_3$–MeOH (96:4), acetone, CH_2Cl_2–MeOH (3:1) and MeOH, successively to obtain 7 fractions. Fraction 2 was subjected to preparative silica gel TLC (PTLC) developed with diisopropyl ether, hexane–EtOAc (4:1) to give **2** (2.4 mg). Fraction 3 was subjected to PTLC developed with diisopropyl ether to give **4** (12.8 mg), **3** (4.0 mg). Fraction 4 was subjected to PTLC with benzene–acetone (4:1) as developing solvents to obtain **1** (35.8 mg). Fraction 5 was further subjected to PTLC with CH_2Cl_2–MeOH (95:5), benzene–acetone (9:1) as developing solvents to obtain **1** (4.1 mg) and **5** (1.8 mg).

3.4. Olibergin A (1)

Colorless oil, UV λ_{max} nm: 228*sh*, 260, 295; IR ν_{max} cm^{-1}: 3469, 3259*br*, 1622; ^1H and ^{13}C NMR see Table 1. Differential NOE experiment data: enhancement of H-3′ (13% increase) and H-2 (1%) on irradiation of 2′-OCH_3; enhancement of H-6′ (13% increase) on irradiation of 5′-OCH_3; enhancement of H-6′ (2% increase) on irradiation of H-2; EIMS m/z (rel. int.): 330 $[M^+]$(100), 315 (24), 299 (30), 284 (20), 255 (6), 227 (5), 203 (5), 177 (6), 153 (19); HR-EIMS m/z 330.0720 ($[M^+]$, calcd for $C_{17}H_{14}O_7$: 330.0740).

3.5. Olibergin B (2)

Colorless oil; UV λ_{max} nm: 204, 266, 340; IR ν_{max} cm^{-1}: 3383*br*, 1653, 1610; ^1H and ^{13}C NMR see Table 1. Differential NOE experiment data: enhancements of H-3′, 5′ (10% increase) on irradiation of 4′-OCH_3; enhancement of H-4″ (6% increase) on irradiation of H-1″; EIMS m/z (rel. int.): 420 $[M^+]$(67), 368 (44), 351 (67), 337 (56), 322 (56), 297 (100), 292 (44), 278 (44), 254 (44), 235 (44), 225 (67), 217 (44), 189 (44), 165 (44); HR-EIMS m/z 420.1935 ($[M^+]$, calcd for $C_{26}H_{28}O_5$: 420.1937).

3.6. In vitro EBV-EA activating experiments

Raji cells in the exponential growth phase were maintained in RPMI-1640 medium (Sigma Chemical Co. MO, USA) supplemented with 10% fetal bovine serum, harvested by centrifugation and resuspended. To test the effect of the candidate compounds, each concentration of the candidate compounds was added to the Raji cells in medium supplemented with 32 pmol TPA and 4 mM *n*-butyric acid, and incubated for 48 h at 37 °C. After incubation, each sample was centrifuged at 1000 *g* for 10 min and the precipitates were resuspended in PBS and placed on 76×26 mm micro slide glass (MATSUNAMI GLASS IND., LTD, Tokyo, Japan). The smear samples were stained with NPC serum and human IgG serum to examine their specific activation. The activated early antigens were detected by immunohistochemical studies using fluorescence microscopy. The treated cells were observed with Trypan Blue staining for cell cytotoxicity.

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