Constituents of the Twigs of *Hernandia ovigera* that Inhibit the Transformation of JB6 Murine Epidermal Cells

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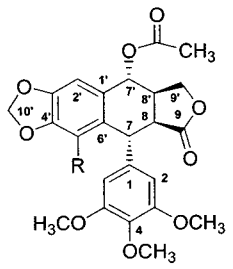
Activity-guided fractionation of an ethyl acetate extract of the twigs of *Hernandia ovigera*, using a soft agar assay with JB6 murine epidermal cells, led to the isolation of two new naturally occurring aryltetralin lignans, (7*R*,8*S*,7′*R*,8′*R*)-(+)-7′-acetyl-5′-methoxypicropodophyllin (**1**) and (7*R*,8*S*,7′*R*,8′*R*)-(+)-7′-acetylpicropodophyllin (**2**), of which **2** was previously identified as a synthetic product, along with eight known compounds, epiyangambin (**3**), caruilignan C, diasesartemin, (+)-epimagnolin A, *N*-methylcorydaldine, (−)-5′-methoxyyatein, (+)-syringaresinol dimethyl ether, and (−)-yatein. The structures and stereochemistry of **1** and **2** were determined using spectroscopic methods. Compounds **2** and **3** exhibited significant inhibition of the transformation of murine epidermal JB6 cells, with IC_{50} values of 0.15 and 0.4 μg/mL, respectively, while the other isolates were regarded as inactive (IC_{50} >4 μg/mL).

Hernandia ovigera L. (Hernandiaceae) is a coastal plant that grows throughout many subtropical and tropical regions of the world.[1] Previous phytochemical studies on *H. ovigera* have resulted in the isolation of several lignans,[2−6] as well as aporphine[7−11] and benzylisoquinoline alkaloids.[9−11] Recently, several lignans from this plant were found to inhibit Epstein−Barr virus early antigen activation induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in Raji cells.[12]

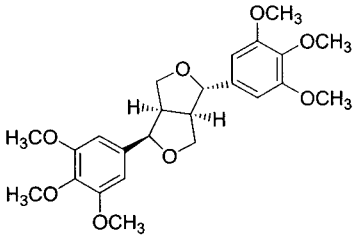
As part of an ongoing collaborative search for novel plant-derived cancer chemopreventive agents,[13,14] a methanolic extract of the twigs of *H. ovigera* was found to significantly inhibit the transformation of murine epidermal JB6 cells in a soft agar assay.[15] Bioassay-guided fractionation of an active ethyl acetate-soluble partition using the JB6 assay to monitor isolation led to the purification of two new naturally occurring aryltetralin lignans, **1** and **2**, one of which (**2**) was previously identified as the product of a synthetic scheme, along with eight known lignan and alkaloidal constituents. The structure elucidation of **1** and **2** and the biological evaluation of these isolates are described herein.

Eight compounds of previously known structure were isolated from the EtOAc extract of the twigs of *H. ovigera*, as described in the Experimental Section, and were identified, in turn, as epiyangambin (**3**),[16] caruilignan C,[17] diasesartemin,[18] (+)-epimagnolin A,[19] *N*-methylcorydaldine,[20] (−)-5′-methoxyyatein,[21] (+)-syringaresinol dimethyl ether,[22] and (−)-yatein,[23] by comparison of their physical and spectral data with reported values. All of these isolates were obtained from this species for the first time except for *N*-methylcorydaldine and (−)-yatein.[4,20] Diasesartemin was the major compound found in the present investigation, in a yield of greater than 0.012% w/w of the dried plant material.

Compound **1**, $[\alpha]^{20}_D$ +51.0° (*c* 0.069, MeCN), was obtained as a colorless gel. The molecular formula was determined as $C_{25}H_{26}O_{10}$ by HREIMS (obsd *m*/*z* 486.1547).



1 R = OCH_3
2 R = H



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Comparison of the ^1H and ^{13}C NMR spectral data (Table 1) obtained for **1** with those of a known aryltetralin lignan, 7′-acetyl-2′-methoxypicropodophyllin,[23] indicated that they are a pair of isomers, with the only differences being in the disposition of the methoxyl group. The methoxyl group in **1** was located at C-5′ as a result of the observed HMBC spectral cross-peak between H-2′ and the C-7′ (Table 1) and the spatial correlation between OCH_3-5′ and H-7 observed in a ROESY NMR spectrum (Figure 1). The *cis* configuration of the fused lactone ring in **1** was determined on the basis of the coupling constant value of 9.2 Hz for the pair H-8/H-8′, which is clearly different from the *trans* configuration (J = 14.8 Hz).[24] Furthermore, the chemical shift observed for C-9 (177.5 ppm) was in close agreement with expected values for a *cis* configuration of the fused lactone.[25]

The relative stereochemistry of **1** was determined to be H-7β, H-8α, H-7′β, and H-8′α, based on the spatial correlations between OCH_3-5′ and H-7β, H-8′α and H-8α and

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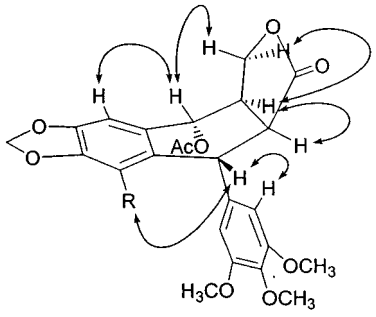
Table 1. NMR Spectral Data for **1** and **2**[a]

	1			**2**	
position	δ_H	δ_C	HMBC	δ_H	δ_C
1		141.2 s[b]			140.2 s
2/6	6.40 s	104.9 d	4, 3/5, 7	6.40 s	105.5 d
3/5		152.9 s			153.5 s
4		135.6 s			137.2 s
7	4.98 s-like	37.8 d	2/6, 8, 9, 6′	4.40 d (3.6)	44.3 d
8	3.34 dd (1.9, 9.2)	45.5 d	9, 1′, 7′	3.28 dd (3.6, 9.2)	45.5 d
9		177.5 s			177.5 s
1′		125.9 s			126.7 s
2′	6.57 s	104.9 d	1′, 3′, 7′	6.75 s	108.4 d
3′		148.8 s			148.4 s
4′		147.1 s			147.2 s
5′		141.2 s		6.55 s	109.8 d
6′		123.9 s			131.5 s
7′	5.67 d (2.4)	73.7 d	2′, 6′, acetate CO	5.73 d (4.8)	72.5 d
8′	3.04 m	39.1 d		2.98 m	39.7 d
9′a	4.24 dd (3.1, 9.7)	71.6 t	9	4.30 dd (2.9, 9.7)	70.9 t
9′b	4.43 dd (7.3, 9.7)		7′	4.42 dd (6.9, 9.7)	
10′a	5.99 d (1.3)	101.4 t	3′	5.97 d (1.4)	101.4 t
10′b	5.97 d (1.3)		4′	5.95 d (1.4)	
acetate CO		170.4 s			170.9 s
acetate CH_3	1.82 s	21.0 q		2.00 s	21.0 q
OCH_3-3/5	3.79 s	56.1 s	4	3.80 s	56.2 q
OCH_3-4	3.81 s	60.8 q	3/5	3.83 s	60.9 q
OCH_3-5′	3.89 s	59.9 q	5′		

[a] Spectra obtained at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR in $CDCl_3$. Figures in parentheses are coupling constants in Hz. [b] Carbon multiplicities were determined according to DEPT and HMQC spectra.



1 R = OCH_3
2 R = H

Figure 1. Selected ROESY correlations for **1** and **2**.

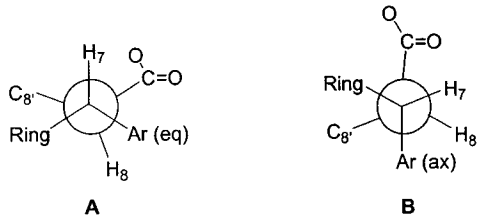


Figure 2. Newman diagrams showing dihedral relationships at C-7 and C-8 in **1**.

H-9′α, and H-7′β and H-2′ and H-9′β, as observed in the ROESY spectrum (Figure 1). Additionally, the proposed stereochemistry at H-7β and H-7′β was confirmed by the observed coupling constant values ($J_{7\beta,8\alpha}$ = 1.9 Hz, $J_{7'\beta,8'\alpha}$ = 2.4 Hz).

The absolute configuration of the stereogenic center at the C-7 position in **1** was assigned as *R* from circular dichroism (CD) spectroscopic evidence. It was previously reported that all 7α-aryltetralin lignans give a positive first Cotton effect at 290−280 nm in their CD spectrum, whereas 7β-aryl compounds give a negative effect.[26] A positive Cotton effect of **1** observed at 287 nm ($\Delta\epsilon$ +10.9) in the CD spectrum suggested that compound **1** contained a 7α-aryl group, which corresponds to a *R* configuration at the C-7 position. Accordingly, the stereochemistry at C-8, C-7′, and C-8′ could be deduced as *S*, *R*, and *R*, respectively.

At ambient temperature, the NMR spectra of **1** represent average boat conformations of conformer **A** with the C-7 aryl and C-7′ acetoxyl substituents in a flagpole orientation, and conformer **B** with these units in a quasi-equatorial orientation (Figure 2), due to its flexible *cis* lactone substituent, in contrast to the rigid *trans* lactone found in podophyllotoxin derivatives.[27] An estimate of the conformational equilibrium in **1** can be obtained from the relevant coupling constants and by the use of the modified Karplus equation,[28] while the coupling constant value for H-7/H-8 is most useful for its conformational analysis.[29] The calculated average dihedral angle of 66° for H-7/H-8 in **1** indicated that the molecule exists primarily in conformer **A** in deuterated chloroform at ambient temperature. Moreover, evidence for the consequent 7-aryl and 7′-acetoxy interaction in this boat form is the shielding of the acetate protons (δ 1.82 ppm) relative to their position in podophyllotoxin or its acetate derivatives (δ > 2.00).[29] The balance between these conformers for **1** was estimated taking $J_{eq,eq}$ = 1.5 Hz for **A** and $J_{ax,ax}$ = 9.5 Hz for **B**, and the weighted mean of these values corresponds to the observed $J_{7,8}$ of 1.9 Hz when the axial aryl contribution is about 95%. The tendency for **1** to assume a greater percentage of the flagpole conformation may be due to the spatial constraints exhibited between the OCH_3-5′ and the 7-aryl groups, which prevent it from existing in an unfavorable quasi-equatorial conformation.

Accordingly, compound **1** was assigned as the new aryltetralin lignan (7*R*,8*S*,7′*R*,8′*R*)-(+)-7′-acetyl-5′-methoxypicropodophyllin, a compound that exists primarily in a boat conformation with the C-7 aryl and C-7′ acetoxyl substituents occurring in a flagpole orientation at ambient temperature.

Compound **2**, $[\alpha]^{20}_D$ +44.9° (*c* 0.098, MeCN), was obtained as a white amorphous powder. The molecular formula was determined as $C_{24}H_{24}O_9$ by HREIMS (obsd *m*/*z* 456.1419). Comparison of the ^{1}H and ^{13}C NMR spectral data (Table 1) obtained for **2** with those of **1** indicated that

2 is an analogue of **1** with an additional aromatic proton signal (δ 6.55 ppm) but lacking a methoxy signal. Similar to the analysis of **1**, the relative stereochemistry of **2** was also determined as H-7β, H-7′β, H-8α, and H-8′α, based on the relevant coupling constant values and the spatial correlations, determined in a ROESY experiment (Figure 1). Moreover, the absolute configuration of the stereogenic center at the C-7 position in **2** was also assigned as *R* on the basis of the observed positive Cotton effect at 288 nm ($\Delta\epsilon$ +71.9) in a CD experiment, so the stereochemistry at C-8, C-7′, and C-8′ was confirmed as *S*, *R*, and *R*, respectively. Thus, compound **2** was assigned as the new naturally occurring aryltetralin lignan (7*R*,8*S*,7′*R*,8′*R*)-(+)-7′-acetylpicropodophyllin. Compound **2**, previously reported as a synthetic compound, exhibited ^{1}H NMR data similar to literature values.[29] The ^{13}C NMR spectral data were not reported previously for synthetic **2**.

All of these isolates from the twigs of *H. ovigera* were evaluated for their potential to inhibit the transformation of murine epidermal JB6 cells, with active principles then further tested for their cytotoxicity, according to an established protocol.[15] The results showed that compounds **2** and **3** exhibited significant inhibitory activity, with IC_{50} values of 0.15 and 0.4 μg/mL, respectively, while the other isolates were inactive (IC_{50} >4 μg/mL). When tested at higher concentrations, compounds **2** and **3** were also cytotoxic against JB6 cells, with IC_{50} values of 1.2 and 5.7 μg/mL, respectively. This 10-fold difference in activity provides some indication that compounds **2** and **3** may be suitable as potential cancer chemopreventive agents, but further testing will be required to establish efficacy.

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. UV spectra were measured on a Beckman DU-7 spectrometer. CD measurements were performed using a JASCO-710 CD spectropolarimeter. IR spectra were obtained with an ATI Mattson FT-IR spectrometer. NMR spectra were recorded on a Bruker DPX-300 NMR spectrometer or a Bruker DRX-500 NMR spectrometer with TMS as internal standard. HREIMS and LREIMS were recorded on a Finnigan MAT 95 mass spectrometer (70 eV). Reversed-phase HPLC was carried out using a 19 × 300 mm, 6-μm Prep Nova-Pak C_{18} column (Waters, Milford, MA) at 8 mL/min and monitored at 208 and 250 nm. For visualization of TLC plates, 10% (v/v) H_2SO_4 in EtOH was used.

Plant Material. The twigs of *H. ovigera* L. (Hernandiaceae) were collected at Momi, Ramsiki District, Kabupaten Manokwari, Irian Jaya, Indonesia, in October 2000, and identified by S.R. A voucher specimen (accession no. Ransiki 001) has been deposited at the Herbarium Bogoriense, Indonesian Institute of Science, Bogor, Indonesia.

Soft Agar Assay with JB6 Murine Epidermal Cells. This assay was carried out according to an established protocol.[15] In brief, 60 mL of medium was prepared by combining 40 mL of 2 × MEME medium, 10 mL of phosphate-buffered saline (PBS), 10 mL of fetal bovine serum (FCS), and gentamycin (50 μg/mL). An agar medium was prepared by adding this mixture to 40 mL of 1.25% Difco agar at 44 °C. This agar medium mixture was distributed into 6-well plates (35 mm well, 3 mL/well) and allowed to set (0.5% agar). JB6 cells, which had grown to about 70% confluency, were washed with Ca^{2+}- and Mg^{2+}-free PBS and harvested by treatment with 0.05% trypsin-EDTA. For each assay, a 1.5 mL aliquot of cell suspension (3 × 10^4 cells/mL) was added to 3 mL of the agar medium mixture followed by the addition of 4.5 μL of 16 μM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) plus a test compound. Aliquots of this mixture (1 mL) were layered onto the agar plates (in triplicate) and incubated at 37 °C in 5% CO_2/95% air for 14 days. Transformation frequency was determined as the average number of colonies (clusters containing ≥15 cells) counted in three randomly chosen 0.25 cm^2 areas on each well. The transformation responses of P^+ (TPA-sensitive) JB6 cells were expressed as colony-forming efficiency (number of colonies/10^4 cells plated). The relative transformation frequency was calculated by subtracting the mean number of control (0.1% DMSO treated) colonies from the mean number of TPA/test agent-induced colonies, dividing by the TPA-treated control group (minus the DMSO group), and multiplying by 100 to yield a percentage.

Cytotoxicity of compounds against JB6 cells was evaluated using trypan blue dye. Cells (1 × 10^4 cells/mL) were preincubated for 24 h and treated with various concentrations of test compounds. After 3 days, trypsinized cells were mixed with 0.4% trypan blue solution, and percent viability was determined by microscopic examination.[30]

Extraction and Isolation. The milled plant material (3.85 kg) was extracted by maceration with MeOH (20 L × 3). After filtration and evaporation of the solvent in vacuo, the resultant extract was diluted with H_2O to afford an aqueous MeOH solution (90%) and then partitioned with *n*-hexane and EtOAc, respectively, to afford dried *n*-hexane-soluble (11 g) and EtOAc-soluble (19 g) residues. The EtOAc extract exhibited significant inhibitory activity in the soft agar transformation assay with JB6 mouse epidermal cells (63% inhibition at 7.9 μg/mL) and was subjected to Si gel column chromatography by elution with increasing concentrations of MeOH in $CHCl_3$ to give seven fractions. Fractions 3 and 4 were active in the soft agar transformation assay (94.6% and 48.3% inhibition at 4 μg/mL, respectively). Chromatography of combined fractions 3 and 4 over Si gel by elution with gradient mixtures of $CHCl_3$−acetone (20:1 to 5:1) gave six subfractions (i−vi). Subfraction iii (1.5 g, 100% inhibition at 4 μg/mL) was further fractionated (SiO_2, stepwise, *n*-hexane−acetone, 10:1 to 7:3) to afford 11 additional subfractions (vii−xvii). (+)-Epimagnolin A[19] (35 mg), epiyangambin[16] (110 mg), and (−)-5′-methoxyyatein[21] (13 mg) were obtained from subfraction xiv (250 mg) by reversed-phase HPLC (CH_3CN−H_2O, 4.0:4.0; t_R 26.0, 28.8, and 34.0 min, respectively). Similarly, *N*-methylcorydaldine[20] (3.1 mg), caruilignan C[17] (1.4 mg), **1** (5.8 mg), and **2** (1.3 mg) were purified from subfraction xvii (52 mg) by reversed-phase HPLC (CH_3CN−H_2O, 3.5:4.5; t_R 9.5, 13.9, 37.6, and 39.4 min, respectively). In turn, subfraction v (600 mg, 100% inhibition at 4 μg/mL) was fractionated by reversed-phase HPLC (CH_3CN−H_2O, 3.2:4.8) to afford five further subfractions (xviii−xxii), followed by passage over Sephadex LH-20 with MeOH as the solvent system. (+)-Syringaresinol dimethyl ether[22] (2.5 mg) and (+)-epimagnolin A[19] (23 mg) were purified from subfraction xviii (50 mg) by reversed-phase HPLC (MeOH−H_2O, 4.8:3.2; t_R 16.9 min, and 20.0 min, respectively). Similarly, epiyangambin[16] (11 mg), (−)-5′-methoxyyatein[21] (5 mg), (−)-yatein[23] (23 mg), and diasesartemin[18] (450 mg) were subsequently obtained from subfractions xiv−xxii, by reversed-phase HPLC (MeOH−H_2O, 4.8:3.2; t_R 20.3, 19.4, 22.5, and 24.3 min, respectively).

(7*R*,8*S*,7′*R*,8′*R*)-(+)-7′-Acetyl-5′-methoxypicropodophyllin (1): colorless gel; $[\alpha]^{20}_D$ +51.0° (*c* 0.069, MeCN); UV (MeCN) λ_{max} (log ϵ) 213 (4.0) nm; CD (MeCN) nm $\Delta\epsilon_{287}$ +10.9, $\Delta\epsilon_{240}$ +541, $\Delta\epsilon_{233}$ +682; IR (dried film) ν_{max} 2923, 2851, 1772, 1729, 1591, 1507, 1459, 1236, 1128, 1020 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m*/*z* $[M]^+$ 486 (100), 426 (43), 368 (10), 353 (16), 295 (15), 168 (19), 57 (23); HREIMS *m*/*z* 486.1547 $[M]^+$ (calcd for $C_{25}H_{26}O_{10}$, 486.1526).

(7*R*,8*S*,7′*R*,8′*R*)-(+)-7′-Acetylpicropodophyllin (2): white amorphous powder; mp 210−213 °C (lit.[31] 212−214 °C); $[\alpha]^{20}_D$ +44.9° (*c* 0.098, MeCN); UV (MeCN) λ_{max} (log ϵ) 213 (4.1) nm; CD (MeCN) nm $\Delta\epsilon_{288}$ +71.9, $\Delta\epsilon_{270}$ −23.4, $\Delta\epsilon_{232}$ +268; IR (dried film) ν_{max} 2937, 2910, 2847, 1773, 1740, 1590, 1506, 1485, 1239, 1127, 1037 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m*/*z* $[M]^+$ 456 (79), 396 (55), 368 (16), 351 (14), 321 (13), 178 (36), 149 (38), 57 (100); HREIMS *m*/*z* 456.1419 $[M]^+$ (calcd for $C_{24}H_{24}O_9$, 456.1420).

Epiyangambin (3): white needles ($CHCl_3$−MeOH); mp 112−114 °C (lit.[16] 119−120 °C); $[\alpha]^{20}_D$ +81.3° (*c* 0.062, $CHCl_3$)

{lit.[16] $[\alpha]^{25}_D$ +122° ($CHCl_3$)}; UV, ^1H NMR, ^{13}C NMR, and EIMS data, consistent with literature values.[16]

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