Bioactive Constituents of *Thuja occidentalis*

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An ethyl acetate-soluble extract of the combined leaves and twigs of *Thuja occidentalis* was found to inhibit 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ornithine decarboxylase (ODC) in cultured mouse epidermal ME 308 cells. Bioassay-guided fractionation of this extract led to the isolation of six active constituents (**1**−**6**), namely, (+)-7-oxo-13-*epi*-pimara-14,15-dien-18-oic acid (**1**), (+)-7-oxo-13-*epi*-pimara-8,15-dien-18-oic acid (**2**), (+)-isopimaric acid (**3**), (1*S*,2*S*,3*R*)-(+)-isopicrodeoxypodophyllotoxin (**4**), (−)-deoxypodophyllotoxin (**5**), and (−)-deoxypodorhizone (**6**). Compounds **1** and **4** are new natural products, and their structures and stereochemistry were determined using spectroscopic methods. Compounds **1**−**6** were evaluated for inhibition of the transformation of murine epidermal JB6 cells, inhibition of ornithine decarboxylase induction with murine epidermal ME 308 cells, and cytotoxic activity against KB cells.

Thuja occidentalis L. (Cupressaceae), commonly known as eastern arborvitae or white cedar, is endemic to eastern North America[1] and is grown in northern Europe as an ornamental tree in parks and churchyards.[2] Extracts of this plant have shown antiviral[3] and benzo(*a*)pyrene−protein binding inhibitory[4] activities. Previous phytochemical studies on *T. occidentalis* have resulted in the isolation of several diterpenes (dehydroabietane, neothujic acids III and IV),[5,6] lignans [(−)-matairesinol, (−)-thujaplicatin methyl ether, (−)-wikstromol, *epi*-pinoresinol],[7] monoterpenes (α-thujone, *β*-thujone, fenchone),[8] and a sesquiterpene alcohol [(+)-occidentalol].[9]

Cancer chemoprevention is a strategy for reducing cancer mortality and involves the use of natural, dietary, or pharmaceutical agents to delay, inhibit, or reverse the development of cancer before malignancy occurs.[10−12] In our current work in this field, a battery of mechanism-based in vitro assays is employed to facilitate the search for potential cancer chemoprevention agents.[13] One such assay involves the inhibition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ornithine decarboxylase (ODC) in cultured mouse epidermal ME 308 cells.[14] This enzyme catalyzes the decarboxylation of ornithine to form putrescine; it is highly inducible by growth-promoting stimuli such as growth factor, hormones, and tumor promoters.[15,16] ODC activity and polyamine levels are related to the process of tumorigenesis.[15,16] In addition, the JB6 epidermal cell assay is utilized in our project to evaluate the ability of test compounds to inhibit anchorage-independent growth induced by the tumor promoter TPA.[17]

In the present investigation, bioassay-monitored fractionation of *T. occidentalis* using the ODC-inhibitory test system as a monitor has resulted the isolation of two new (**1** and **4**) and four known compounds (**2**, **3**, **5**, and **6**) as active principles. The known constituents were identified as (+)-7-oxo-13-*epi*-pimara-8,15-dien-18-oic acid (**2**),[18] (+)-isopimaric acid (**3**),[19] (−)-deoxypodophyllotoxin (**5**),[20] and (−)-deoxypodorhizone (**6**),[20] respectively. The structure elucidation and the determination of the absolute configurations of **1** and **4** and the biological evaluation of **1**−**6** are the subjects of this article.

Results and Discussion

The molecular formula of compound **1** was determined as $C_{20}H_{28}O_3$ by HREIMS (obsd *m*/*z* 316.2035). Comparison of its ^{1}H and ^{13}C NMR data with 7-oxo-13-*epi*-pimara-8,-15-dien-18-oic acid[18] and several of its analogues[19,21] indicated that **1** is a diterpene acid. The functional groups present in the molecule of **1** could be assigned as an α,*β*-unsaturated carbonyl (ν_{max} 1660 and 1608 cm^{-1}; δ_C 199.9), a vinyl group (ν_{max} 3075, 985, and 905 cm^{-1}; δ_C 112.1 and 146.4), and a carboxylic acid (ν_{max} 1725 cm^{-1}; δ_C 181.0). In the ^{1}H NMR spectrum, three tertiary methyl signals (δ_H 0.88, 1.10 s, 1.25), a typical ABX system of a vinyl group [δ_H 4.98 dd (J = 1.9, 10.5 Hz); δ_H 5.00 dd (J = 1.9, 17.6 Hz); and δ_H 5.80 dd (J = 10.5, 17.6 Hz)], and an olefinic proton signal (δ_H 6.70 s) were also observed.[19,21] Thus, analysis of its ^{1}H and ^{13}C NMR data suggested that **1** is based on a pimarane skeleton.[18,19] The relative location of the vinyl group in **1** was established from HMBC NMR spectral observations, in which cross-peaks were observed between δ_H 5.80 (H-15) and δ_C 38.7 (C-13), 25.9 (C-17), and 145.2 (C-14). Additional correlations were observed between δ_H 5.00 (H-16) and δ_C 38.7 (C-13) and 146.4 (C-15). The position of the olefinic proton at C-14 was determined from correlations between δ_H 6.70 (H-14) and δ_C 51.7 (C-9), 34.2 (C-12), and 25.9 (C-17). Moreover, the H_3C-19 signal at δ_H 1.25 showed correlations with the signals of δ_C 37.0 (C-3), 46.1 (C-4), and 181.0 (C-18). The location of the carbonyl group at the C-7 position was confirmed by HMBC NMR spectral observations, with cross-peaks observed between δ_H 2.36 (H-5) and δ_C 199.9 (C-7), 14.4 (C-20), 35.7 (C-10), 46.1 (C-4), and 38.0 (C-6). Of the possible ways of placing a conjugated −C═CH− unit, only a structure with a C-8(C-14) double bond is compatible with the lack of any proton vicinal to the ring vinyl proton. The proposed location of a double bond between C-8 and C-14 in **1** is in agreement with the formation of prominent fragment ions at *m*/*z* 167 and 122 in the EIMS.[21,22]

It has been established that in the pimaric acid series the equatorial C-13 methyl group gives rise to a ^{13}C NMR signal near 30 ppm, while in the 13-epimeric sandaracopi-

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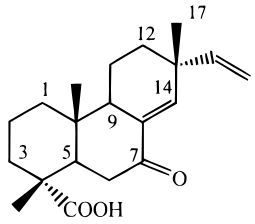
Chart 1



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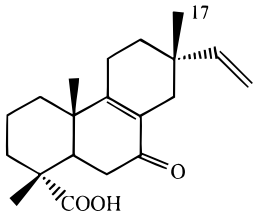

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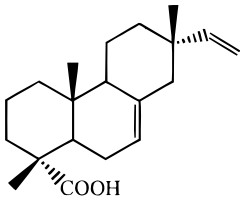

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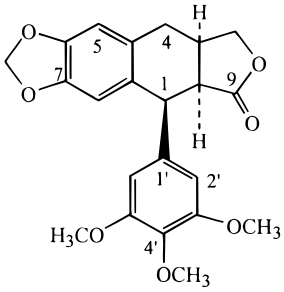

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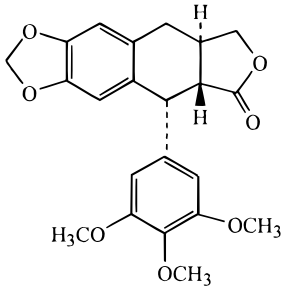
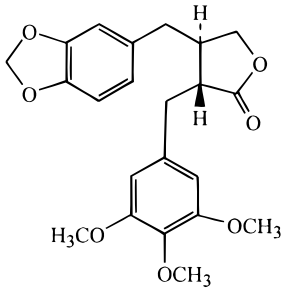


5



6

maric acid series, the axial 13-methyl signal resonates near 26 ppm.[19,23,24] The increased shielding of the axial methyl is due to a γ-gauche interaction with C-11.[21] Thus, compound **1**, with a 13-methyl group resonating at 25.9 ppm, was assigned an axial orientation. The CD spectrum of compound **1** showed maxima at 332 nm ($\Delta\epsilon$ +4.47), 247 nm ($\Delta\epsilon$ −4.35), and 215 nm ($\Delta\epsilon$ +15.2), similar to data published for (+)-7-oxo-13-*epi*-pimara-8,15-dien-18-oate.[18] The CD data obtained for **1** and the established rules for the CD spectra of enones[25,26] indicate that this compound belongs to the normal 5α-pimarane series, and thus, the absolute configuration at C-13 is *R*. Moreover, in a NOESY experiment, the Me-17 signal at δ_H 1.10 showed a correlation with δ_H 6.70 (H-14), strongly supporting the axial assignment of the C-13 methyl group. Accordingly, the structure of **1** was determined as (+)-7-oxo-13-*epi*-pimara-14,15-dien-18-oic acid.

The HREIMS of compound **4** showed a molecular ion peak at *m*/*z* 398.1366, indicating a molecular formula of $C_{22}H_{22}O_7$. The ^{1}H and ^{13}C NMR spectra of this isolate were similar to those of the known lignan, (−)-deoxypodophyllotoxin (**5**).

Comparison of spectral data obtained for **4** with those of **5**, a compound also obtained in this investigation, indicated that they are a pair of isomers, with the only differences being the relative stereochemistry at H-1 and H-2 for these lignans, thereby affecting the chemical shifts and coupling constants observed. ^{1}H NMR signals corresponding to H-1 and H-2 for compound **4** were observed at δ_H 4.36 (1H, d, J = 3.0 Hz, H-1) and 3.33 (1H, dd, J = 3.0, 9.5 Hz, H-2), in contrast to δ_H 4.60 (1H, d, J = 2.3 Hz, H-1) and 2.78 (1H, m, H-2) for compound **5**.[20]

The relative stereochemistry at the C-1, C-2, and C-3 positions in **4** was assigned based on ^{1}H NMR coupling constant values and a NOESY experiment. A *cis* arrangement was evident for the pair H-1/H-2 (J = 3.0 Hz). In addition, the NOESY experiment indicated correlations between H-1 and H-2, and H-2 and H-3, supporting the conclusion that H-1/H-2 and H-2/H-3 have the same orientation. The absolute configuration of the stereogenic center at the C-1 position in **4** was assigned on the basis of circular dichroism (CD) spectroscopic evidence. In exciton coupled circular dichroism (ECCD),[27,28] the interaction between chirally disposed strong electronic transition dipoles leads to "split" Cotton effects (CE), the signs of which are directly related to the chiral twist between the corresponding chromophores. We assumed that the π-electron systems of the two benzene ring chromophores make the most significant contributions to the exciton coupled CEs.[27] The negative helicity observed for **4** is in agreement with observed negative exciton coupled CD experimental data showing a negative Cotton effect at 210 nm ($\Delta\epsilon$ −14.0) and positive effect at 204 nm ($\Delta\epsilon$ +9.46). In contrast, for compound **5**, with a known absolute configuration (*R*) at the C-1 position, the CD curve showed opposite signs at 209 nm ($\Delta\epsilon$ +18.2) and 203 nm ($\Delta\epsilon$ −26.0), respectively. Thus, compound **4** has *S* configuration at the C-1 position. In turn, the stereochemistry at C-2 and C-3 was deter-

Table 1. Transformation of Murine Epidermal JB6 Cells and Inhibition of 12-*O*-Tetradecanoylphorbol 13-Acetate (TPA)-Induced Ornithine Decarboxylase Activity in Cultured ME 308 Cells by Compounds **1**−**6**[a]

compound	JB6 cell line	ME 308 cell line
1	11.7	0.50
2	12.6	0.98
3	13.2	0.86
4	0.18	0.55
5	0.004	<0.08
6	3.75	6.5
positive control[b]	0.021	0.6

[a] Data are expressed in μM. [b] 13-*cis*-Retinoic acid.

mined as *S* and *R*, respectively. Accordingly, compound **4** was assigned as the novel natural product lignan (1*S*,2*S*,3*R*)-(+)-isopicrodeoxypodophyllotoxin. Compound **4** has been reported in the literature as a synthetic product and exhibited ^{1}H NMR data similar to literature values.[29] The ^{13}C NMR spectrum was not reported for synthetic **4**.

Compounds **1**−**6** were active as inhibitors of JB6 cell transformation, with the greatest activity being demonstrated by **4** and **5** (Table 1). There was a general correlation with inhibitory activity observed in the mouse 308 cell culture system, suggesting that inhibition of cell transformation may result from an early response in the phorbol ester−protein kinase C pathway. Considering the known cytotoxic potential of lignans such as **4**−**6**, it was anticipated that this could be a contributory factor in mediating these responses. To examine such a possibility, compounds **4**−**6** were evaluated with JB6 cells and found to be active as general inhibitors of growth (data not shown). Therefore, the inhibition of compounds on JB6 cell transformation might be due to cytotoxicity.

On the other hand, the diterpenes **1**−**3** were not effective inhibitors of cell growth with cultured JB6 cells or cultured KB cells (IC_{50} > 60 μM). Nevertheless, as summarized in Table 1, they were effective inhibitors of JB6 cell transformation and TPA-induced ornithine decarboxylase activity. In previous studies, pimarane diterpenoids have shown a variety of biological activity, as exemplified by antituberculous activity,[30] inhibitory effects on mycelial growth of fungi,[31] and inhibition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced inflammation on the mouse ear.[32] Accordingly, these diterpenoid acids are worthy of evaluation in additional biological test systems in relation to cancer chemoprevention.

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer model 241 polarimeter. UV spectra were obtained with a Beckman DU-7 spectrometer. CD measurements were performed using a JASCO-600 CD spectrometer. IR spectra were obtained with a Midac Collegian FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured with TMS as internal standard, using Bruker Avance DPX-300 and DRX-500 MHz spectrometers. 1D NOE, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HMBC, and HMQC NMR experiments were also performed on these instruments, using standard pulse sequences. Low- and high-resolution mass spectra were obtained on a Finnigan MAT-90 instrument. Column chromatography was carried out on Si gel (70−230 mesh). Reversed-phase HPLC was carried out using a Waters 600E Multisolvent Delivery System pump, a Waters 996 photodiode array detector, and a YMC-Pack ODS-AQ column (250 × 20 mm), at a flow rate of 8 mL/min, with the UV detector set at 210 nm. TLC was performed on Whatman aluminum-backed plates coated with 0.25-mm layers of Si gel 60 F_{254}.

Soft Agar Transformation Assay with JB6 Mouse Epidermal Cells. This assay was employed to determine the inhibition of transformation of the murine epidermal cell line JB6 by treatment with the test materials. The assay for promotion of anchorage independence was performed as described previously.[33−35] Briefly, 60 mL (2×) of medium were prepared by mixing 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 mixture was prepared by adding 60 mL of the medium to 40 mL of 1.25% Difco agar at 44 °C. The agar−medium mixture was poured into a 6-well plate (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 removed from plates with 0.05% trypsin−EDTA. For each assay, a 2.5-mL aliquot of cell suspension (3 × 10^4 cells/mL) was added to 5 mL of the agar−medium mixture followed by the addition of 7.5 μL of 16 μM TPA or TPA plus a test compound. This mixture was layered 1 mL of 0.33% agar/well onto agar plates and incubated at 37 °C in 5% CO_2/95% air for 14 days. Colony-forming efficiency was determined after 14 days of incubation at 37 °C. Transformation frequency was determined as the average number of colonies (clusters containing 16 or more cells) counted in three randomly chosen 0.25 cm^2 areas on each well in triplicate for each treatment group. The transformation responses of both P^+ (sensitive) and P^- (resistant) JB6 cell variants were expressed as colony-forming efficiency (number of colonies/10^4 cells plated). The relative transformation frequency for P^+ and P^- cells in each test group was calculated by subtracting the mean number of control (0.1% DMSO)-induced colonies from the mean number of TPA- and TPA/test agent-induced colonies, expressed as a percentage by multiplying by 100.

Cytotoxicity of compounds against JB6 cells (P^+ and P^-) was evaluated using trypan blue dye. Cells (1 × 10^4 cells/mL) were preincubated for 24 h and treated with various concentrations of compounds. After 4 days, trypsinized cells were mixed with 0.4% trypan blue solution and counted under a microscope.[36]

Inhibition of TPA-Induced Ornithine Decarboxylase Activity with Cultured Mouse Epidermal 308 Cells. Plant extracts, fractions, and pure isolates were evaluated as inhibitors of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ornithine decarboxylase (ODC), using cultured mouse epidermal ME 308 cells, as described previously.[14]

Cytotoxicity to KB Cells. Cytotoxicity was evaluated against KB (human epidermal carcinoma of the nasopharynx) cells, using an established protocol. [37]

Plant Material. The leaves and twigs of *Thuja occidentalis* were collected in December, 1979, at the Morton Arboretum, Lisle, IL. These were stored at ambient temperature at the University of Illinois Pharmacognosy Field Station (Downers Grove, IL) before being milled when the present investigation began. A voucher specimen (accession number XT-227-LP-01) has been deposited at the University of Illinois Pharmacognosy Field Station.

Extraction and Isolation. The plant material (5 kg) was extracted exhaustively by maceration with MeOH−H_2O (9:1; 3 × 10 L). After filtration and evaporation of the solvent, the resultant extract was diluted with H_2O to afford an aqueous MeOH solution (80%) and then partitioned with petroleum ether and EtOAc, respectively, to afford dried petroleum ether-soluble (12.6 g) and EtOAc-soluble (85.8 g) residues. The EtOAc extract exhibited significant activity in the ODC assay (IC_{50} 0.2 μg/mL) and was subjected to Si gel column chromatography by elution with increasing concentrations of MeOH in $CHCl_3$ to give 17 fractions. Fractions 4, 9, and 10 were active in the ODC assay with IC_{50} values of 1.4, <0.5, and <0.1 μg/mL, respectively. Chromatographic separation of bioactive fraction 4 over Si gel with mixtures of EtOAc in petroleum ether of increasing polarity yielded compound **3** (50.0 mg, 0.001% w/w; elution with petroleum ether−EtOAc, 3:1). Further chromatography of combined fractions 9 and 10 over Si gel with gradient mixtures of petroleum ether−EtOAc yielded a pure lignan **5** [140.0 mg (0.0014% w/w), elution with petroleum ether−EtOAc, 2:1] as a major compound. Purifica-

tion of subfraction 25 from fractions 9 and 10, eluted with petroleum ether−EtOAc, 1:1, by passage over Sephadex LH-20 with MeOH as the solvent system, afforded **6** [15.0 mg (0.0003% w/w)].

Further purification was also carried out by reversed-phase semipreparative HPLC. From subfraction 20 (obtained by elution with petroleum ether−EtOAc, 2:1, of fractions 9 and 10), compound **4** (t_R 22.6 min) was obtained by HPLC, using acetonitrile−H_2O (60:40) for elution. In addition, from subfraction 15, eluted with petroleum ether−EtOAc, 2:1 from fraction 4, compounds **1** and **2** (t_R 31.6 and 38.9), respectively, were also purified by HPLC, using acetonitrile−H_2O (65:35) as solvent system.

(+)-7-Oxo-13-*epi*-pimara-14,15-dien-18-oic acid (1): colorless oil; $[\alpha]^{20}_D$ +63.8° (*c* 0.03, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 248 (3.2) nm; CD (MeCN) nm $\Delta\epsilon_{332}$ +4.47, $\Delta\epsilon_{247}$ −4.35, $\Delta\epsilon_{215}$ +15.2; IR ν_{max} (film) 3075, 2946, 1725, 1660, 1608, 985, 905 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (3H, s, H-20), 1.10 (3H, s, H-17), 1.25 (3H, s, H-19), 2.36 (1H, m, H-5), 4.98 (1H, dd, J = 1.9, 10.5 Hz, H-16), 5.00 (1H, dd, J = 1.9, 17.6 Hz, H-16), 5.80 (1H, dd, J = 10.5, 17.6 Hz, H-15), 6.70 (1H, s, H-14); ^{13}C NMR ($CDCl_3$) δ 14.4 (C-20), 16.4 (C-19), 18.0 (C-2), 19.0 (C-11), 25.9 (C-17), 34.2 (C-12), 35.7 (C-10), 37.0 (C-3), 38.0 (C-6), 38.7 (C-13), 38.9 (C-1), 44.5 (C-5), 46.1 (C-4), 51.7 (C-9), 112.1 (C-16), 135.2 (C-8), 145.2 (C-14), 146.4 (C-15), 181.0 (C-18), 199.9 (C-7); EIMS *m/z* $[M]^+$ 316 (75), 167 (49), 162 (100), 149 (74), 133 (64), 122 (80), 105 (63); HREIMS *m/z* 316.2035 $[M]^+$ (calcd for $C_{20}H_{28}O_3$, 316.2031).

(+)-7-Oxo-13-*epi*-pimara-8,15-dien-18-oic acid (2): colorless oil; $[\alpha]^{20}_D$ +72.0° (*c* 0.03, $CHCl_3$) {lit.[18] $[\alpha]_D$ +77.6° (*c* 0.76, $CHCl_3$)}; IR, ^1H NMR, ^{13}C NMR, and EIMS data, consistent with literature values.[18]

(+)-Isopimaric acid (3): colorless needles; mp 175 °C; $[\alpha]^{20}_D$ +60.0° (*c* 0.05, $CHCl_3$) {lit.[21] $[\alpha]_D$ +76°(EtOH)}; ^1H NMR, ^{13}C NMR, and EIMS data, consistent with literature values.[19]

(1*S*,2*S*,3*R*)-(+)-Isopicrodeoxypodophyllotoxin (4): colorless oil; $[\alpha]^{20}_D$ +12.5° (*c* 0.06, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 217 (4.0), 241 (3.5), 291.5 (3.2) nm; CD (MeCN) nm $\Delta\epsilon_{287}$ +15.2, $\Delta\epsilon_{249}$ −20.8, $\Delta\epsilon_{210}$ −14.0, $\Delta\epsilon_{204}$ +9.46; IR ν_{max} (film) 2920, 1770, 1738, 1615, 1591, 1517, 1495, 945 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.48 (1H, dd, J = 5.5, 15.3 Hz, H-4), 2.89 (1H, J = 6.2, 15.3 Hz, H-4), 3.03 (1H, m, H-3), 3.33 (1H, dd, J = 3.0, 9.5 Hz, H-2), 3.78 (6H, s, OCH_3-5′ and 6′), 3.82 (3H, s, OCH_3-4′), 3.97 (1H, dd, J = 3.2, 9.2 Hz, H-10), 4.36 (1H, d, J = 3.0 Hz, H-1), 4.44 (1H, dd, J = 7.5, 9.2 Hz, H-10), 5.92 (2H, d, J = 1.4 Hz, $-OCH_2O-$), 6.32 (2H, s, H-2′ and 6′), 6.58 (1H, s, H-8), 6.67 (1H, s, H-5); ^{13}C NMR ($CDCl_3$) δ 32.2 (C-4), 33.2 (C-3), 45.5 (C-1), 46.5 (C-2), 56.4 (OCH_3-3′ and 5′), 61.0 (OCH_3-4′), 72.9 (C-10), 101.2 ($-OCH_2O$-), 105.1 (C-6′ and 2′), 109.0 (C-5), 110.0 (C-8), 128.4 (C-4a), 130.7 (C-1a), 136.4 (C-4′), 138.4 (C-1′), 146.9 (C-6), 147.0 (C-7), 153.5 (C-3′ and 5′), 178.6 (C-9); EIMS *m/z* $[M]^+$ 398 (100), 367 (19), 283 (17), 230 (14), 105 (15); HREIMS *m/z* 398.1366 $[M]^+$ (calcd for $C_{22}H_{22}O_7$, 398.1359).

(−)-Deoxypodophyllotoxin (5): colorless needles; mp 167 °C [lit.[20]168 °C]; $[\alpha]^{20}_D$−103.0° (*c* 0.11, $CHCl_3$) {lit.[20] $[\alpha]^{25}_D$ −129.7° (*c* 0.99, MeOH)}; ^1H NMR, ^{13}C NMR, and EIMS data, consistent with literature values.[20]

(−)-Deoxypodorhizone (6): colorless oil; $[\alpha]^{20}_D$ −18.3° (*c* 0.1, $CHCl_3$); {lit.[20] $[\alpha]^{25}_D$ −19.3° (*c* 1.0, MeOH)}; ^1H NMR, ^{13}C NMR, and EIMS data, consistent with literature values.[20]

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