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Potent Cytotoxic Arylnaphthalene Lignan Lactones from *Phyllanthus poilanei*

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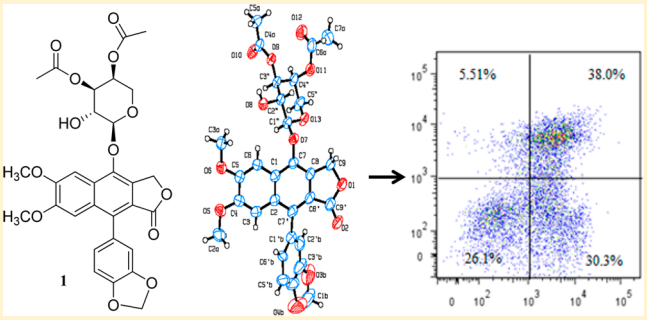
Supporting Information

ABSTRACT: Two new (**1** and **2**) and four known arylnaphthalene lignan lactones (**3**–**6**) were isolated from different plant parts of *Phyllanthus poilanei* collected in Vietnam, with two further known analogues (**7** and **8**) being prepared from phyllanthusmin C (**4**). The structures of the new compounds were determined by interpretation of their spectroscopic data and by chemical methods, and the structure of phyllanthusmin D (**1**) was confirmed by single-crystal X-ray diffraction analysis. Several of these arylnaphthalene lignan lactones were cytotoxic toward HT-29 human colon cancer cells, with compounds **1** and 7-*O*-[(2,3,4-tri-*O*-acetyl)-α-L-arabinopyranosyl)]diphyllin (**7**) found to be the most potent, exhibiting IC_{50} values of 170 and 110 nM, respectively. Compound **1** showed activity when tested in an in vivo hollow fiber assay using HT-29 cells implanted in immunodeficient NCr *nu/nu* mice. Mechanistic studies showed that this compound mediated its cytotoxic effects by inducing tumor cell apoptosis through activation of caspase-3, but it did not inhibit DNA topoisomerase IIα activity.



Cancer is a serious threat to human health, and the discovery and development of promising new agents to treat this condition is therefore an urgent need. Natural products and their semisynthetic derivatives are used widely in cancer chemotherapy.[1,2] As an example, etoposide (VP-16) is a semisynthetic aryltetralin lignan lactone glycoside modeled on the natural product podophyllotoxin. It targets DNA topoisomerase II (topo II) and has been utilized for decades to treat several types of cancer.[3] However, side effects have been reported for etoposide, including myelosuppression and the development of secondary leukemias linked to topo II inhibitory activity.[4] Thus, it is highly desirable to discover new agents to treat cancer showing diverse mechanisms of action.

Etoposide is a semisynthetic epipodophyllotoxin glycoside derived from podophyllotoxin that is a naturally occurring aryltetralin lignan lactone containing four stereogenic centers at its C-7, -8, -7′, and -8′ positions. Podophyllotoxin is well known to occur in *Podophyllum peltatum* and *P. emodi* var. *hexandrum* (syn. *Sinopodophyllum hexandrum*) (Berberidaceae).[3,5,6] The latter plant has been found to contain both aryltetralin and arylnaphthalene lignan lactones.[7] In a manner different from aryltetralin lignan lactones, arylnaphthalene lignan lactones are based on a naphthalene unit rather than a tetrahydronaph-

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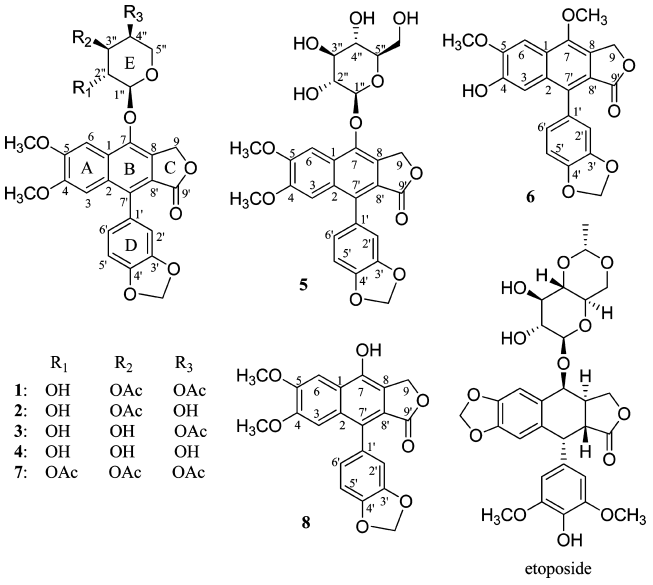
thalene unit and hence exclude the four stereogenic centers from their aglycones.

Thus far, arylnaphthalene lignan lactones are isolated mainly from plants belonging to six families, including the Acanthaceae, from the species *Acanthus mollis*,[8] *Justicia ciliata*,[9] *J. patentiflora* (syn. *Mananthes patentiflora*),[10,11] and *J. procumbens*;[12−14] the Lamiaceae, from *Hyptis verticillata*;[15] the Linaceae, from *Linum leonii*;[16] the Myristicaceae, from *Knema furfuracea*;[17] the Phyllanthaceae, from *Cleistanthus collinus*,[18] *Phyllanthus acutissima*,[19] *P. myrtifolius*,[20,21] *P. oligospermus*,[22] *P. piscatorum*,[23] and *P. taxodiifolius*;[24] and the Rutaceae, from *Haplophyllum cappadocicum*,[25] *H. buxbaumii*,[26,27] *H. dauricum*,[28] and *H. patavinum*.[29]

Many naturally occurring arylnaphthalene lignan lactones have been reported to possess potent cytotoxicity toward small panels of human cancer cell lines.[9,10,16,19,22,24,30,31] For example, justicidin A (a methylated diphyllin derivative) isolated from *Justicia ciliata* showed cytotoxicity toward several human cancer cell lines with IC_{50} values in the range 2−7 ng/mL.[9] Justicidin B, a dehydroxylated diphyllin, was cytotoxic toward SKW-3 chronic lymphoid leukemia cells.[16] Acutissimalignan A, a diphyllin methylated arabinoside derivative, showed cytotoxicity toward P-388 murine lymphocytic leukemia cells with an IC_{50} value of 20 ng/mL.[19] Patentiflorin A (diphyllin quinovoside) inhibited MCF-7S human breast carcinoma and KB human epidermoid carcinoma cell growth with IC_{50} values of 3 and 4 nM, respectively.[10] Cleistanthin A exhibited cytotoxicity toward KB cells with a GI_{50} value of 5.6 nM,[24] and cleistanthin B showed cytotoxicity toward human tumor cells selectively when it was tested for cytotoxicity against a broad spectrum of normal and tumor cell lines.[30]

In an in vivo experiment, five-week-old athymic male nude mice (BALB/c Slc-nu) bearing tumor xenografts established by subcutaneous injections of HCT116 cells were treated by intraperitoneal (ip) injection of daurinol (1, 5, 10, and 20 mg/kg, 3 times weekly for 2 weeks); this compound displayed antitumor effects at several doses used when compared with the vehicle control.[31] In an effort to develop these agents into new anticancer drugs, several analogues of diphyllin glycosides have been synthesized and evaluated.[32,33] Many of these substances showed potent cytotoxicity toward a number of human cancer cells, with some of them acting as topo II poisons but others showing a lack of this activity.[32,33]

Phyllanthus is a large plant genus with many species used in Asian traditional medicine systems.[19−24] As part of a search for novel anticancer agents from higher plants and other organisms,[2] an initial crude chloroform-soluble extract of *Phyllanthus poilanei* Beille (Phyllanthaceae), collected in Vietnam, was found to exhibit cytotoxicity toward the HT-29 human colon cancer cell line. No previous phytochemical investigation on this species has been reported thus far, so it was selected as a target plant for further investigation. Using column chromatography guided by inhibitory activity against the HT-29 cell line, two new (**1** and **2**) and four known (**3**−**6**) arylnaphthalene lignan lactones were isolated. The cytotoxicity of the compounds, including two semisynthetic derivatives (**7** and **8**), was evaluated against the HT-29 human colon cancer and the CCD-112CoN normal human colon cell lines. Compound **1** was evaluated using an in vivo hollow fiber assay against HT-29 cells, and preliminary mechanism of action studies on this compound have been conducted.



RESULTS AND DISCUSSION

Plant samples of *P. poilanei* collected in Vietnam at different times and locations were extracted with MeOH and then partitioned with *n*-hexane and $CHCl_3$. When the cytotoxic $CHCl_3$ partitions were subjected to chromatographic separation guided by inhibitory activity against the HT-29 cell line, two new [phyllanthusmins D (**1**) and E (**2**)] and four known [phyllanthusmins A (**6**),[22] B (**3**),[22] and C (**4**)[22] and cleistanthin B (**5**)[26]] arylnaphthalene lignan lactones were purified. The various plant collections and the compounds isolated from each collection are summarized in Table 1.

Table 1. Plant Collections and Their Isolates

plant code	collection information	isolate
A06024[a]	Dinh Khanh District,[f] November 2004	**1**−**4** and **6**
A06025[b]	Dinh Khanh District,[f] November 2004	**1**
A06473[c]	Cam Xuyen District,[g] December 2008	**1** and **4**
A06474[d]	Cam Xuyen District,[g] December 2008	**1**
AA06024[e]	Dinh Khanh District,[f] August 2011	**1**−**5**

[a]Initial collection of the combined leaves, twigs, flowers, and fruits of *P. poilanei*. [b]Initial collection of the stems of *P. poilanei*. [c]Second collection of the combined leaves, twigs, flowers, and fruits of *P. poilanei*. [d]Second collection of the stems of *P. poilanei*. [e]Large collection of the combined leaves, twigs, and stems of *P. poilanei*. [f]Located in Khanh Hoa Province, Vietnam. [g]Located in Hatinh Province, Vietnam.

Compound **1** was isolated as a new compound in the form of colorless fine needles, mp 210−211 °C. A sodiated molecular ion peak at *m*/*z* 619.1444 (calcd 619.1422) observed in the HRESIMS in conjunction with ^{13}C NMR spectroscopic data corresponded to a molecular formula of $C_{30}H_{28}O_{13}$. The UV (λ_{max} 260 nm) and IR [ν_{max} 3446 (hydroxy), 1747 (γ-lactone), 1619, 1507, and 1481 (aromatic) cm^{-1}] spectra showed the absorption characteristics of an arylnaphthalene lignan lactone.[8] The ^1H NMR data of **1** (Table 2)[34] exhibited resonances for two substituted aromatic rings at δ_H 6.81, 6.83, 6.97, 7.09, and 7.94, a lactone methylene group at δ_H 5.47 and 5.56, a methylenedioxy group at δ_H 6.05 and 6.10, two methoxy groups at δ_H 3.81 and 4.03, two acetyl groups at δ_H 2.14 and 2.23, and proton resonances for a sugar moiety in the range δ_H 3.60−4.99.[24] Analogous resonances consistent with the presence of

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Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data of 1 and 2[a]

	1		2	
position	δ_C,[b] type	δ_H,[c] (J in Hz)	δ_C,[d] type	δ_H,[e] (J in Hz)
1	127.1 C		127.2 C	
2	131.0 C		131.0 C	
3	106.4 CH	7.09 d (2.0[f])	106.4 CH	7.10 s
4	150.3 C		150.4 C	
5	152.2 C		152.2 C	
6	100.8 CH	7.94 s	100.9 CH	7.95 s
7	144.2 C		144.3 C	
8	131.4[g] C		131.4 C	
9	67.6 CH_2	5.47 d (15.2)	67.6 CH_2	5.46 ddd (9.6, 2.4, 1.2[f])
		5.56 d (15.2)		5.57 ddd (11.4, 3.6, 1.8[f])
1′	128.4 C		128.4 C	
2′	110.8 CH	6.83 overlapped	110.9 CH	6.84 d (0.6[f])
3′	147.7 C		147.7 C	
4′	147.7 C		147.7 C	
5′	108.4 CH	6.97 d (8.0)	108.4 CH	6.97 dd (5.4, 1.2[f])
6′	123.7 CH	6.81 overlapped	123.8 CH	6.82 dd (6.0, 1.2[f])
7′	136.9 C		136.9 C	
8′	119.4 C		119.4 C	
9′	170.0 C		169.9 C	
1″	105.4 CH	4.86 d (7.6)	105.7 CH	4.84 d (6.0)
2″	70.0 CH	4.31 t (8.8)	70.3 CH	4.33 t (6.6)
3″	73.3 CH	4.99 dd (10.0, 3.6)	75.9 CH	4.92 br d (7.8)
4″	68.1 CH	5.30 br s	67.2 CH	4.12 m
5″	64.9 CH_2	3.60 d (13.2)	66.7 CH_2	3.56 d (12.3)
		4.06 overlapped		4.09 d (11.4)
OMe-4	56.0 CH_3	3.81 s	56.0 CH_3	3.81 s
OMe-5	56.5 CH_3	4.03 s	56.5 CH_3	4.03 s
OCH_2O-3′,4′	101.4 CH_2	6.05 s	101.4 CH_2	6.05 s
		6.10 s		6.10 s
OAc-3″	170.8 C	2.14 s	171.2 C	2.25 s
	21.1 CH_3		21.3 CH_3	
OAc-4″	170.4 C	2.23 s		
	21.0 CH_3			

[a]Measured in $CDCl_3$ and assignments of chemical shifts are based on the analysis of 1D and 2D NMR spectra. The overlapped signals were assigned from ^{1}H–^{1}H COSY, HSQC, and HMBC spectra without designating multiplicity. CH_3, CH_2, CH, and C multiplicities were determined by DEPT 90, DEPT 135, and HSQC experiments. [b]Data (δ) measured at 100.6 MHz and referenced to the solvent residual peak at δ 77.16.[34] [c]Data (δ) measured at 400.1 MHz and referenced to the solvent residual peak at δ 7.26.[34] [d]Data (δ) measured at 150.9 MHz and referenced to the solvent residual peak at δ 77.16.[34] [e]Data (δ) measured at 600.2 MHz and referenced to the solvent residual peak at δ 7.26.[34] [f]The unusual value may result from the restricted rotation of the D ring. [g]Present in pairs at room temperature at δ_C 131.43 and 131.42.

these functionalities appeared in the ^{13}C NMR data of **1** (Table 2).[34,35] The lactone moiety was located at the C-8 and C-8′ positions, as supported by the HMBC correlations (Figure 1) between H-9/C-7, C-8, C-8′, and C-9′ (Figure 1). The methylenedioxy group could be located at the C-3′ and C-4′ positions, as indicated by the HMBC correlations between

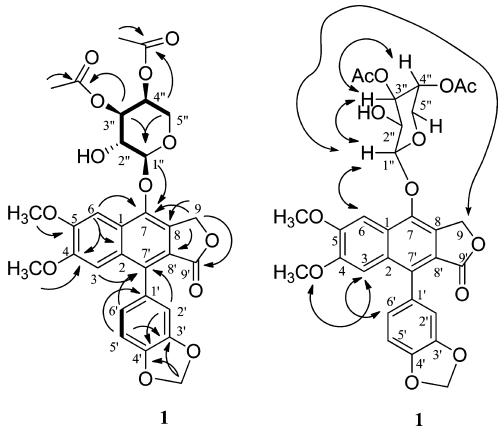

Figure 1. COSY (–, ^{1}H → ^{1}H), key HMBC (↷, ^{1}H → ^{13}C), and selected NOESY (↔, ^{1}H → ^{1}H) correlations of **1**.

these methylene protons and C-3′ and C-4′. Two methoxy groups were assigned at the C-4 and C-5 positions from the HMBC correlations between these methoxy groups and C-4 and C-5. The sugar unit was assigned to the C-7 position, as supported by the HMBC correlation between H-1″ and C-7. Two acetyl groups were placed at the C-3″ and C-4″ positions of the sugar residue, as indicated by the HMBC correlations between the H-3″ and H-4″ resonances and the acetyl carbonyl groups. The resonances at δ 3.52 for H-3″ and at δ 3.71 for H-4″ appearing in the ^{1}H NMR data of **4** (Table S1, Supporting Information) were deshielded to δ 4.99 (H-3″) and δ 5.30 (H-4″) in the ^{1}H NMR spectrum of **1**, due to the electron-withdrawing effects that resulted from the acetyl carbonyl groups linked at the C-3″ and C-4″ positions. This was also supported by the molecular weight of 596 Da of **1**, or 42 atomic mass units more than that of **3**, representing the presence of a diacetylglycosyl residue in **1** rather than a monoacetylglycosyl unit as in **3**. Thus, compound **1** was proposed as an *O*-acetyl analogue of the known compounds phyllanthusmins B (**3**) and C (**4**), with both being characterized from *Phyllanthus oligospermus* in a previous study.[22]

Comparison of the NMR data of compound **1** with those of phyllanthusmins B (**3**) and C (**4**) (Table 2 and Tables S1 and S2, Supporting Information) showed that these compounds displayed closely similar NMR resonances for the diphyllin aglycone unit but different resonances for their saccharide portions. An L-arabinopyranosyl residue of **1** could be proposed based on the several lines of evidence that follow. First, both the NOESY correlations and the specific rotation value of **1** were consistent with those of the known compound **3**, as reported[22] and isolated in the present study. Second, the NOESY correlations and specific rotation value of **1** were consistent with those determined for **4** (phyllanthusmin C) in this investigation. The latter compound, when isolated from *P. poilanei*, showed a specific rotation value {$[\alpha]^{20}_D$ −8 (*c* 0.1, $CHCl_3$)} that coincided with a synthetic version of compound **4** {$[\alpha]^{20}_D$ −8 (*c* 1, $CHCl_3$)}.[33] Finally, both **1** and **4** were acetylated to form the same compound, 7-*O*-[(2,3,4-tri-*O*-acetyl)-α-L-arabinopyranosyl)]diphyllin (**7**), which exhibited the same specific rotation value of $[\alpha]^{20}_D$ −12 (*c* 0.1, $CHCl_3$), and were closely comparable to the same compound synthesized from diphyllin and L-arabinose {$[\alpha]^{20}_D$ −13 (*c* 0.3, $CHCl_3$)}.[32]

The H-1″ doublet at δ_H 4.86 with a coupling constant of 7.6 Hz indicated the presence of an anomeric proton in an axial

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orientation in **1**.[22,36] The NOESY correlations between H-1″ and H-3″ and H-3″ and H-4″ suggested that H-1″, H-3″, and AcO-4″ are all axial (Figure 1). Thus, the structure of this new compound (phyllanthusmin D, **1**) was assigned as 7-*O*-[(3,4-di-*O*-acetyl)-α-L-arabinopyranosyl]-4,5-dimethoxy-3′,4′-methylenedioxy-2,7′-cycloligna-7,7′-dieno-9,9′-lactone, or 7-*O*-[(3,4-di-*O*-acetyl)-α-L-arabinopyranosyl]diphyllin. This was confirmed by analysis of its single-crystal X-ray diffraction data. As shown in Figure 2, this compound existed as an atropisomeric mixture

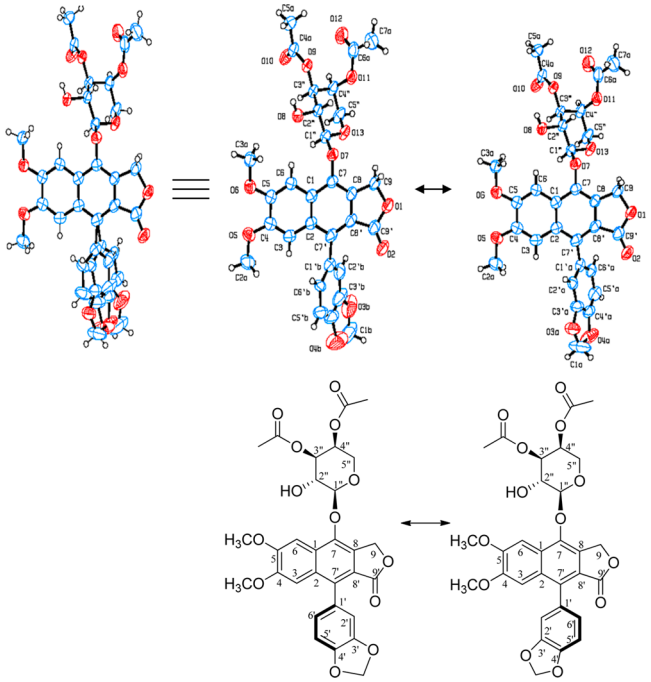

Figure 2. ORTEP plots for the molecular structure of **1** drawn with 50% probability displacement ellipsoids (oxygen atoms are red, carbon atoms are blue, and the small white circles represent hydrogen atoms, which are drawn with an artificial radius).

because of hindered rotation of ring D about the C-1′−C-7′ bond, which resulted in duplication of the C-8 resonance at δ_C 131.42 and 131.43 (Table 2) and an optical activity indicated by its ECD spectrum (Figure S17, Supporting Information).[8] The same hindered rotation would likely be observed in **3**−**5** and other arylnaphthalene lignan lactones, as indicated by the duplicated resonances in their NMR spectra (Tables S1, S2, and S4, Supporting Information)[19,24] and optical activity shown in their ECD spectra.[8] These atropisomers interconvert slowly, hence permitting the two diastereomers observed in the NMR spectra, but the rotation barrier is too small for the individual diastereomers to be isolated at room temperature.[37]

Compound **2** was isolated as an amorphous, colorless powder. The similar UV and IR spectra to those of **1** indicated that **2** is also an arylnaphthalene lignan lactone. The molecular formula of $C_{28}H_{26}O_{12}$ deduced from ^{13}C NMR data and a sodiated molecular ion peak at *m*/*z* 577.1319 (calcd 577.1316) observed in the HRESIMS and the similar NMR data to those of **1** demonstrated that this compound is a diphyllin monoacetylarabinoside, a regioisomer of phyllanthusmin B (**3**).[22] Comparison of the ^1H and ^{13}C NMR data of **2** with those of **3** showed that the resonances for H-3″ and C-3″ of **2** were deshielded, but those for H-4″ and C-4″ were shielded (Table 2 and Tables S1 and S2, Supporting Information). This indicated that the acetyl group is attached to C-3″ in **2** rather than to C-4″ in **3**, as supported by the HMBC correlation between the H-3″ and the acetyl carbonyl carbon resonance. A doublet at δ_H 4.84 showing a coupling constant of 6.0 Hz displayed in the ^1H NMR spectrum of **2** supported the presence of the anomeric proton in an axial orientation.[22,36] The NOESY correlations between H-1″ and H-5″$_{ax}$ and H-3″ and H-4″ and H-5″$_{ax}$ suggested that H-1″, H-3″, and OH-4″ are all axial (Figure S19, Supporting Information).

Although sample limitations precluded hydrolysis of **2** to yield the sugar unit or acetylation of **2** into 7-*O*-[(2,3,4-tri-*O*-acetyl)-α-L-arabinopyranosyl)]diphyllin (**7**) to determine its absolute configuration, it may be assumed that this compound has the same absolute configuration as that of **1**, **3**, and **4**, from the consistent NOESY correlations (Figure S19, Supporting Information) and specific rotation values {$[\alpha]^{20}_D$ −3 (*c* 0.1, $CHCl_3$) for **1**, $[\alpha]^{20}_D$ −4 (*c* 0.1, $CHCl_3$) for **2**, $[\alpha]^{20}_D$ −6 (*c* 0.1, $CHCl_3$) for **3**, and $[\alpha]^{20}_D$ −8 (*c* 0.1, $CHCl_3$) for **4** (Supporting Information)}. Therefore, the structure of **2** (phyllanthusmin E) was defined as 7-*O*-[(3-*O*-acetyl)-α-L-arabinopyranosyl]-4,5-dimethoxy-3′,4′-methylenedioxy-2,7′-cycloligna-7,7′-dieno-9,9′-lactone, or 7-*O*-[(3-*O*-acetyl)-α-L-arabinopyranosyl]-diphyllin.

Altogether, six arylnaphthalene lignan lactones were characterized from the different parts of *P. poilanei* collected in different locations in Vietnam. As summarized in Table 1, the most cytotoxic isolate, phyllanthusmin D (**1**), was isolated from all parts of *P. poilanei*, and this compound can be regarded as a major cytotoxic principle of *P. poilanei*. The known arylnaphthalene lignan lactones isolated from *P. poilanei* were identified by analysis of their spectroscopic data and comparison of these data with literature values,[22,26,35] and full assignments of their ^1H and ^{13}C NMR spectroscopic data are listed in Tables S1−S4 (Supporting Information). To partially discern the effects of both the arabinose unit and the acetyl group in the mediation of cytotoxicity of the diphyllin lignans, two previously reported analogues were prepared. 7-*O*-[(2,3,4-Tri-*O*-acetyl)-α-L-arabinopyranosyl]diphyllin (**7**) was produced by acetylation of **1** and **4** using a standard method[38] and was identified by its molecular formula of $C_{32}H_{30}O_{14}$, as determined by HRESIMS and comparison of its spectroscopic data (Supporting Information) with those reported for a synthetic sample.[32] The aglycone diphyllin (**8**) was generated by hydrolysis of phyllanthusmin C (**4**), and its structure was determined by comparison of its spectroscopic data with reference values.[12,35]

All arylnaphthalene lignans isolated from *P. poilanei* in the present study and their semisynthetic analogues were evaluated for their cytotoxicity against HT-29 human colon cancer cells, using paclitaxel and etoposide as the positive controls (Table 3).[38] Compounds **1**−**4**, **7**, and **8** were found to be cytotoxic, of which **1** and **7** were the most potently active, with IC_{50} values of 170 and 110 nM, respectively, but compounds **5**, **6**, and etoposide were inactive. Inspection of the lignan structures and their cytotoxicity showed that compounds containing more lipophilic acetyl groups exhibit higher potency, so the presence of one or more acetyl groups linked to the arabinose residue improved the resultant cytotoxicity. Compounds **2** and **3** exhibited the same activity, indicating that the acetyl group linked to C-3″ contributed to this activity equally when linked to C-4″. Phyllanthusmin C (**4**) showed a higher cytotoxicity than diphyllin (**8**) and cleistanthin B (**5**), implying that the α-L-arabinose unit at C-7 played an important role in generating

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Table 3. Cytotoxicity toward HT-29 and CCD-112CoN Cells of 1−8[a]

compound	HT-29[b]	CCD-112CoN[c]
1	0.17	>100
2	1.8	NT[d]
3	1.8	NT
4	3.2	>100
5	>10[e]	NT
6	>10	NT
7	0.11	NT
8	7.6	NT
paclitaxel[f]	0.001	23.0

[a]IC_{50} values were calculated using nonlinear regression analysis with measurements performed in triplicate and representative of two independent experiments in which the values generally agreed within 10%. [b]Represented as IC_{50} values (μM) toward the HT-29 cells. [c]Represented as IC_{50} values (μM) toward the CCD-112CoN cells. [d]NT = compound was not tested. [e]Showing borderline cytotoxicity with an IC_{50} value of 12.0 μM. [f]Positive control.

this effect and was more active than a β-D-glucose unit in mediating compound cytotoxicity toward HT-29 cells. Diphyllin (**8**) was active, but phyllanthusmin A (**6**) was inactive, showing that the C-4 and C-5 methoxy groups and the C-7 hydroxy group are all required for diphyllin to mediate its cytotoxicity. The new compound phyllanthusmin D (**1**) showed a much greater IC_{50} value than etoposide, indicating that this compound is more potently cytotoxic against HT-29 cells.

Investigation of the cytotoxic arylnaphthalene lignans reported in previous studies showed that most active compounds are analogues of diphyllin or diphyllin glycosides. A methylated diphyllin derivative, justicidin A, was highly cytotoxic, but its close analogue, chinensinaphthol methyl ether, did not show such activity,[9] indicating the importance of the substituents on the A and D rings of these compounds. Cleistanthoside A tetraacetate showed potent cytotoxicity with IG_{50} values in the nanomolar range, but its precursor, cleistanthoside A, was inactive,[24] suggesting the importance of acetylation of the glycose unit of cleistanthoside A. The structure−cytotoxicity relationships of arylnaphthalene lignan lactones observed from the present study are consistent with observations made from these previous studies.[9,24]

The two cytotoxic isolates **1** and **4** were tested for their cytotoxicity toward CCD-112CoN human normal colon cells using a previous protocol.[39] Both compounds were found to be noncytotoxic toward this cell line (Table 3), indicating some selectivity for HT-29 human colon cancer cells.

The new cytotoxic compound, phyllanthusmin D (**1**, IC_{50}, 170 nM), isolated from *P. poilanei*, was tested further in an in vivo hollow fiber assay for its possible antitumor efficacy.[40,41] Immunodeficient NCr *nu/nu* mice implanted with HT-29 human colon cancer cells placed in hollow fibers were treated once daily by **1** at doses of 5.0, 10.0, 15.0, or 20.0 mg/kg, or the vehicle control, or paclitaxel (5 mg/kg), by ip injection for 4 days. The relative HT-29 cell growth values from all mice were calculated. The results showed that the values from the treatment of **1** at 10.0, 15.0, or 20.0 mg/kg (ip) were all statistically significantly different from those at a dose of 5 mg/kg (ip), and they showed a dose-dependent tendency (Figure 3). No gross toxicity was observed in the mice treated at the doses employed.

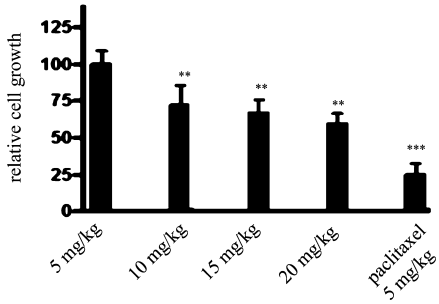

Figure 3. Effect of phyllanthusmin D (**1**) on the growth of human colon cancer HT-29 cells implanted in NCr *nu/nu* mice tested in an in vivo hollow fiber assay. Mice were treated with the indicated doses of **1** once daily by intraperitoneal injection from day 3 to day 6 after implantation of the HT-29 cells facilitated in hollow fibers. On day 7, the mice were sacrificed, and fibers were retrieved and analyzed (Experimental Section). The results are shown as the average percentage cell growth relative to control [columns, mean in each group (n = 6 for the control group and n = 3 for the treatment group); bars, SE; $^{**}p \leq 0.05$ and $^{***}p \leq 0.01$ for significant differences from the 5 mg/kg (**1**) treatment].

The enzyme DNA topoisomerase is an established molecular target of etoposide, on which this compound acts to form DNA double-strand breaks via stabilization of the intermediate topo II-DNA covalent complex to initiate the cell death pathway.[3] In the present study, several diphyllin arabinosides, including phyllanthusmins C (**4**) and D (**1**) and 7-*O*-[(2,3,4-tri-*O*-acetyl)-α-L-arabinopyranosyl)]diphyllin (**7**), together with etoposide, were tested for their ability to inhibit DNA topo IIα (Figure 4), using a method reported previously.[42,43] Consistent with a previous report that etoposide showed

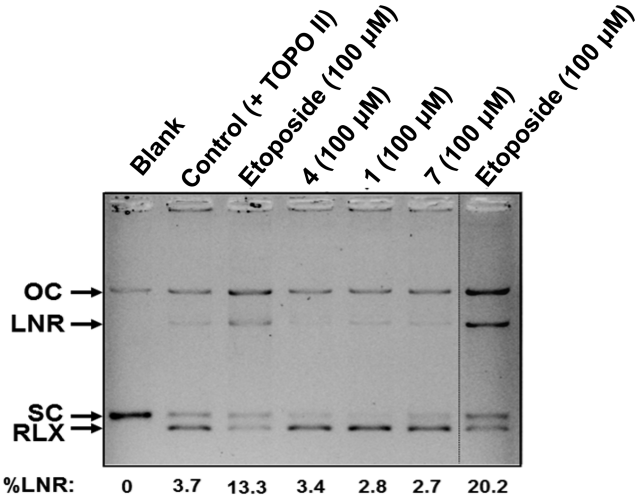

Figure 4. Evaluation of arylnaphthalene lignan lactones {phyllanthusmins C (**4**) and D (**1**) and 7-*O*-[(2,3,4-tri-*O*-acetyl)-α-L-arabinopyranosyl)]diphyllin (**7**)] from *P. poilanei* for activity as topoisomerase IIα (topo IIα) inhibitors. Topo II-DNA covalent complexes induced by test samples and etoposide were trapped by rapidly denaturing the complexed enzyme with sodium dodecyl sulfate (SDS), digesting the enzyme, and releasing the cleaved DNA as linear DNA. The formation of linear DNA was detected by separating the SDS-treated reaction products using ethidium bromide gel electrophoresis and quantified by accounting for the relationship between fluorescence and relative band intensity for open circular (OC), linear (LNR), supercoiled (SC), and relaxed (RLX) configurations of DNA (Experimental Section).

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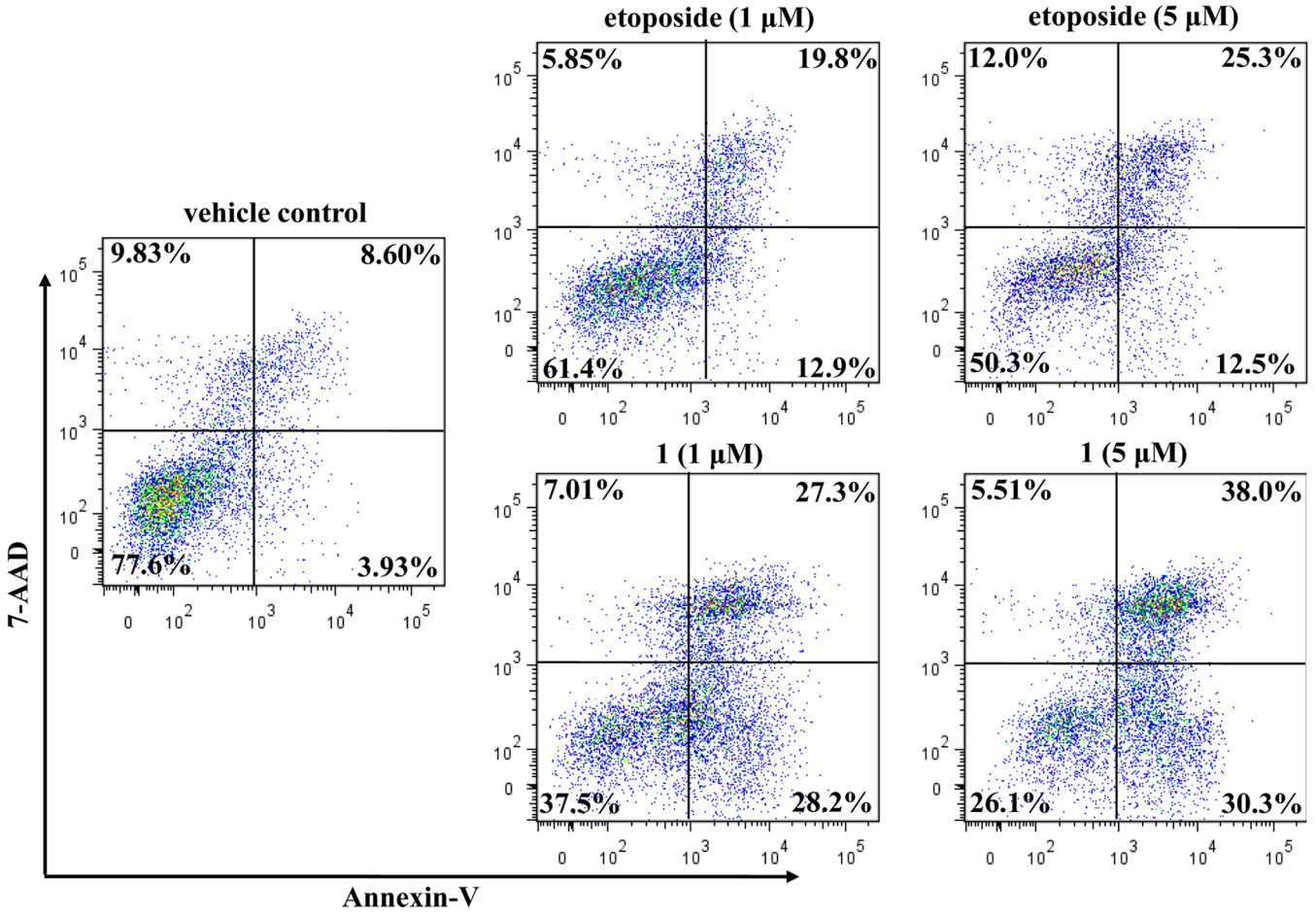

Figure 5. HT-29 cell apoptosis induction by phyllanthusmin D (**1**) and etoposide. HT-29 cells were treated with 1 or 5 μM phyllanthusmin D (**1**), 1 or 5 μM etoposide, or the vehicle control for 72 h, followed by an annexin V staining method (Experimental Section). Lower left quadrant: percentage of viable cells; lower right quadrant: percentage of apoptotic cells; upper left quadrant: percentage of necrotic cells; upper right quadrant: percentage of late-stage apoptotic cells or dead cells.

topo IIα inhibitory activity,[3] this agent exhibited similar activity in the present study (Figure 4). However, arylnaphthalene lignan lactones investigated herein neither inhibited topo IIα-mediated DNA strand passage/catalytic activity (conversion of supercoiled DNA to relaxed DNA) nor induced topo IIα-mediated DNA cleavage (linearized double-strand DNA) compared to the control, indicating that these substances are not topo IIα inhibitors. Previous reports have documented that some diphyllin glycosides inhibited topo II, but other close analogues of these compounds did not.[32,33] This indicates that the glycosidic moiety of these lignans plays a key role in topo II inhibition. Some specific diphyllin analogues might exert their cytotoxicity through a mechanism of action different from that of etoposide,[32,33] as supported by additional chemical and biological studies for an arylnaphthalene lignan lactone, daurinol, which did not induce the formation of open circular and linear DNA that originated from the topoisomerase-DNA cleavable intermediate.[31]

Apoptosis, or programmed cell death, occurs during normal cellular differentiation and the development of multicellular organisms.[44,45] To remain malignant, cancer cells must evade apoptosis to avoid elimination, and many anticancer agents induce cancer cell apoptosis.[45,46] A previous study showed that an 8-day treatment of etoposide induced HT-29 human colon cancer cell apoptosis, but shorter term treatment with this compound did not show this activity.[47] After HT-29 cells were treated with compound **1** or etoposide at different concentrations, annexin V flow cytometry was performed following a previous protocol.[48] Treatment of HT-29 cells with 1 or 5 μM phyllanthusmin D (**1**) for 72 h resulted in 28.2% or 30.3% of HT-29 cells undergoing early apoptosis, respectively, while the analogous values for the vehicle control or 1 or 5 μM etoposide treatments were 3.9%, 12.9%, and 12.5%, respectively (Figure 5). Also, **1** induced 27.3% (at 1 μM) and 38.0% (at 5 μM) HT-29 cell apoptosis at the late stage, while the vehicle control or 1 or 5 μM etoposide treatments induced 8.6%, 19.8%, or 25.3% HT-29 cell apoptosis at this stage, respectively (Figure 5). These results indicated that compound **1** showed a higher potency than etoposide in inducing HT-29 cell apoptosis.

Caspase-3, a key effector of programmed cell death and a well-known anticancer drug target, is activated only during cell apoptosis and contributes fundamentally to this process.[45,46] Following a previous procedure,[49] both **1** and etoposide were tested for their caspase-3 activation in HT-29 cells (Figure 6). After 24 h incubation, phyllanthusmin D (**1**) induced activation of caspase-3 at as low a dose as 1 μM, as well as at 5 and 10 μM. In contrast, under the same experimental conditions, etoposide did not induce caspase-3 activation, which is consistent with the known resistance of HT-29 cells to etoposide.[50] These results again indicate a fundamental difference in the mechanism(s) of action of these agents.

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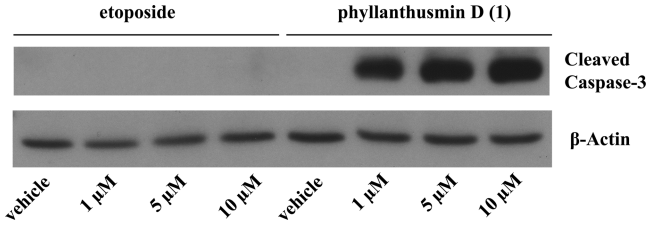

Figure 6. Caspase-3 activation by **1** in HT-29 cells. HT-29 cells were incubated with phyllanthusmin D (**1**) and etoposide with different concentrations for 24 h, and caspase-3-like activity was determined by Western blotting using rabbit monoclonal cleaved caspase-3 (Asp175) antibody. The data shown are a representative blot from three independent experiments with similar results (Experimental Section).

EXPERIMENTAL SECTION

General Experimental Procedures. The melting point was measured using a Fisher Scientific apparatus and is uncorrected. Optical rotations were measured on a PerkinElmer model 343 polarimeter. UV spectra were recorded on a Hitachi U2910 UV spectrophotometer. ECD measurements were performed using a JASCO J-810 spectropolarimeter. IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer. ^{1}H and ^{13}C, DEPT, HSQC, HMBC, NOESY, and COSY NMR spectra were recorded at room temperature on Bruker Avance DRX-400, DRX-600, or DRX-800 MHz NMR spectrometers. ESIMS and HRESIMS were measured on an LCT-TOF or a Q-TOF mass spectrometer in the positive-ion mode. Column chromatography was conducted using silica gel (65 × 250 or 230 × 400 mesh, Sorbent Technologies, Atlanta, GA, USA). Analytical thin-layer chromatography (TLC) was performed on precoated silica gel 60 F254 plates (Sorbent Technologies). Sephadex LH-20 was purchased from Amersham Biosciences, Uppsala, Sweden. For visualization of TLC plates, sulfuric acid reagent was used. Fluorescence was tested using a Spectroline (model ENF-260C) UV light source. All procedures were carried out using anhydrous solvents purchased from commercial sources and employed without further purification. Reagents for chemical synthesis were purchased from Sigma except where indicated, and reactions were monitored by TLC using precoated silica gel plates. Crystallographic data were collected through the Service Crystallography at Advanced Light Source (SCrALS) program at the Small-Crystal Crystallography Beamline 11.3.1 at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory, with a Bruker APEXII CCD detector (Bruker Analytical X-ray Instruments, Inc., Madison, WI, USA).

Plant Material. Initial collections of separate samples of the combined leaves, twigs, flowers, and fruits (acquisition number A06024) and the stems (acquisition number A06025) of *Phyllanthus poilanei* were collected from a shrub at the road transect from Suoi Cat village to Hon Ba peak (12°07.873′ N; 106°01.532′ E), Dinh Khanh District, Khanh Hoa Province, Vietnam, in November 2004, by D.D.S., T.N.N., and Pham Huy Hoang. A voucher herbarium specimen (*DDS 13619*) representing this collection was identified as *Phyllanthus poilanei* Beille by D.D.S. and deposited at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, IL, USA, under the accession number FM-2256257.

Second collections of separate samples of the combined leaves, twigs, flowers, and fruits (acquisition number A06473) and the stems (acquisition number A06474) of *P. poilanei* were obtained from a liana-like shrub in the forest occurring at the south end of Kego Lake, across from Mui Tru Ranger Station (18°06.530′ N; 106°00.891′ E), Kego Nature Reserve, Cam Xuyen District, Hatinh Province, Vietnam, in December 2008, by D.D.S., T.N.N., and Vuong Tan Tu. A voucher herbarium specimen (*DDS 14308*) representing this collection was identified as *Phyllanthus poilanei* Beille by D.D.S. and deposited at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, IL, under the accession number FM-2287526.

A larger sample of the combined leaves, twigs, and stems (acquisition number AA06024) of *P. poilanei* was collected from a liana in the Hon Ba mountain region, 25 km from Soi Cat on a peak along roadside forest (12°06.745′ N; 108°58.80′ E), Dinh Khanh District, Khan Hoa Province, Vietnam, in August 2011, by D.D.S., T.N.N., and Bui Van Thanh. A voucher herbarium specimen (*DDS 14886*) representing this collection was identified as *Phyllanthus poilanei* Beille by D.D.S. and deposited at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, IL, under the accession number FM-2300873.

Extraction and Isolation. The milled air-dried leaves, twigs, flowers, and fruits of *P. poilanei* (sample A06024, 2000 g) were extracted with MeOH (7 L × 6) at room temperature. The solvent was evaporated in vacuo, and the dried MeOH extract (170.0 g, 8.5%) was resuspended in 10% H_2O in MeOH (1000 mL) and partitioned with *n*-hexane (700 mL × 2 and 500 mL) to yield an *n*-hexane-soluble residue (D1, 22.4 g, 1.1%). The aqueous MeOH layer was then partitioned with $CHCl_3$ (800, 700, and 600 mL) to afford a $CHCl_3$-soluble extract (D2, 3.0 g, 0.15%), which was washed with a 1% aqueous solution of NaCl, to partially remove tannins. The $CHCl_3$-soluble extract exhibited cytotoxicity toward the HT-29 cell line (IC_{50} < 5.0 μg/mL). Both the *n*-hexane- and water-soluble extracts were inactive in the bioassay system used. The $CHCl_3$-soluble extract (2.8 g) was subjected to silica gel column chromatography (2.5 × 45 cm), eluted with a gradient of *n*-hexane−acetone. Eluates were pooled by TLC analysis to give 13 combined fractions (D2F1−D2F13). Of these, D2F4−D2F6 (IC_{50} < 2 μg/mL) were combined and further chromatographed over a silica gel column (2.5 × 20 cm), eluted with a gradient of *n*-hexane−acetone to yield seven pooled subfractions (D2F4F1−D2F4F7). Fractions D2F11 and D2F12 (IC_{50} < 5 μg/mL) were combined and further chromatographed over a silica gel column (2.5 × 20 cm), eluted with a gradient of *n*-hexane−acetone, to yield five combined subfractions (D2F11F1−D2F11F5). Subfraction D2F4F2 was chromatographed over silica gel, with a gradient of *n*-hexane−acetone, and purified by separation over a Sephadex LH-20 column, eluted with CH_2Cl_2−MeOH (1:1), affording phyllanthusmin D (**1**, 20 mg). The combined subfractions D2F4F3−D2F4F5 were separated by silica gel chromatography, eluted with *n*-hexane−acetone (3:1), and purified by passage over a Sephadex LH-20 column, eluted with a mixture of CH_2Cl_2−MeOH (1:1), to afford phyllanthusmin A (**6**, 2.0 mg), phyllanthusmin B (**3**, 1.0 mg), and phyllanthusmin E (**2**, 1.5 mg). Fractions D2F11F2−D2F11F4 were combined and chromatographed over silica gel, eluted by *n*-hexane−acetone (2:1), and purified by separation over a Sephadex LH-20 column, using CH_2Cl_2−MeOH (1:1) for elution, affording phyllanthusmin C (**4**, 7.0 mg).

The milled air-dried stems of *P. poilanei* (sample A06025, 580 g) were extracted with MeOH (3 L × 4 and then 2 L × 2) at room temperature. The solvent was evaporated in vacuo, and the dried MeOH extract (47.4 g, 8.2%) was resuspended in 10% H_2O in MeOH (500 mL) and partitioned with *n*-hexane (500, 300, 200 mL), to yield an *n*-hexane-soluble residue (D1, 1.4 g, 0.24%). The aqueous MeOH layer was partitioned with $CHCl_3$ (500, 300, and 300 mL) to afford a $CHCl_3$-soluble extract (D2, 2.0 g, 0.34%), which was followed by washing with a 1% aqueous solution of NaCl, to partially remove tannins. The $CHCl_3$-soluble extract exhibited cytotoxicity toward the HT-29 cell line (IC_{50} < 5.0 μg/mL). Both the *n*-hexane- and water-soluble extracts were inactive in the bioassay system used. The $CHCl_3$-soluble extract (1.8 g) was subjected to silica gel column chromatography (2.5 × 45 cm), eluted with a gradient of *n*-hexane−acetone. Fractions were pooled by TLC analysis to give 13 combined fractions (D2F1−D2F13). Of these, D2F4−D2F6 (IC_{50} < 2 μg/mL) were combined, further chromatographed over a silica gel column, eluted with a gradient of *n*-hexane−acetone, and purified by separation over a Sephadex LH-20 column, eluted with CH_2Cl_2−MeOH (1:1), affording phyllanthusmin D (**1**, 7.0 mg).

The milled air-dried combined leaves, twigs, flowers, and fruits of *P. poilanei* (sample A06473, 851 g) were extracted with MeOH (3 L × 4, 2 L × 2) at room temperature. The solvent was evaporated in vacuo, and the dried MeOH extract (96 g, 11.3%) was resuspended in 10% H_2O in MeOH (500 mL) and partitioned with *n*-hexane (500, 300, 200 mL), to yield an *n*-hexane-soluble residue (D1, 10.2 g, 1.2%). The

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aqueous MeOH layer was then partitioned with $CHCl_3$ (500, 300, and 300 mL) to afford a $CHCl_3$-soluble extract (D2, 3.0 g, 0.35%), which was followed by washing with a 1% aqueous solution of NaCl, to partially remove tannins. The $CHCl_3$-soluble extract exhibited cytotoxicity toward the HT-29 cell line (IC_{50} < 10.0 μg/mL). Both the *n*-hexane- and water-soluble extracts were inactive in the bioassay system used. The $CHCl_3$-soluble extract (2.8 g) was subjected to silica gel column chromatography (2.5 × 45 cm), eluted with a gradient of *n*-hexane−acetone. Fractions were pooled by TLC analysis to give 11 combined fractions (D2F1−D2F11). Of these, fractions D2F8 and D2F9 (IC_{50} < 5.0 μg/mL) were combined, further chromatographed over a silica gel column (2.5 × 20 cm), eluted with a gradient of *n*-hexane−acetone, and purified by separation over a Sephadex LH-20 column, eluted with CH_2Cl_2−MeOH (1:1), affording phyllanthusmins C (**4**, 2.0 mg) and D (**1**, 3.0 mg).

The milled air-dried stems of *P. poilanei* (sample A06474, 517 g) were extracted with MeOH (2 L × 6) at room temperature. The solvent was evaporated in vacuo, and the dried MeOH extract (75 g, 14.5%) was resuspended in 10% H_2O in MeOH (600 mL) and partitioned with *n*-hexane (500, 400, and then 300 mL), to yield an *n*-hexane-soluble residue (D1, 1.0 g, 0.2%). The aqueous MeOH layer was then partitioned with $CHCl_3$ (500, 300, and 300 mL) to afford a $CHCl_3$-soluble extract (D2, 2.0 g, 0.38%), which was followed by washing with a 1% aqueous solution of NaCl, to partially remove tannins. The $CHCl_3$-soluble extract exhibited cytotoxicity toward the HT-29 cell line (IC_{50} < 10.0 μg/mL). Both the *n*-hexane- and water-soluble extracts were inactive in the bioassay system used. The $CHCl_3$-soluble extract (1.8 g) was subjected to silica gel column chromatography (2.5 × 45 cm), eluted with a gradient of *n*-hexane−acetone. Fractions were pooled by TLC analysis to give 11 combined fractions (D2F1−D2F11). Of these, fraction D2F10 (IC_{50} <5.0 μg/mL) was chromatographed over a silica gel column, eluted with a gradient of *n*-hexane−acetone, and purified by separation over a Sephadex LH-20 column, eluted with CH_2Cl_2−MeOH (1:1), to afford phyllanthusmin D (**1**, 2.0 mg).

In an attempt to accumulate a greater quantity of phyllanthusmin D (**1**) for in vivo biological evaluation, a larger re-collection of the combined leaves, twigs, and stems of *P. poilanei* was made. The milled air-dried combined leaves, twigs, and stems of this sample (AA06024, 3200 g) were extracted with MeOH (7 L × 6) at room temperature. The solvent was evaporated in vacuo, and the dried MeOH extract (278.0 g, 8.7%) was resuspended in 10% H_2O in MeOH (1000 mL) and partitioned with *n*-hexane (800 mL × 3 and 500 mL × 3), to yield an *n*-hexane-soluble residue (D1, 27.0 g, 0.84%). The aqueous MeOH layer was partitioned with $CHCl_3$ (800 mL × 3 and 500 mL × 3) to afford a $CHCl_3$-soluble extract (D2, 8.5 g, 0.27%), which was followed by washing with a 1% aqueous solution of NaCl, to partially remove tannins. The aqueous MeOH layer was further partitioned with EtOAc (800 mL × 3 and 500 mL × 3) to afford an EtOAc-soluble extract (D3, 10.0 g, 0.31%), which was also washed with a 1% aqueous solution of NaCl. The $CHCl_3$-soluble extract exhibited cytotoxicity toward the HT-29 cell line (IC_{50} <5.0 μg/mL). However, all of the *n*-hexane-, EtOAc-, and water-soluble extracts were inactive in the bioassay system used. The $CHCl_3$-soluble extract (8.0 g) was subjected to silica gel column chromatography (4.5 × 45 cm), eluted with a gradient of *n*-hexane−acetone. Fractions were pooled by TLC analysis to give 11 combined fractions (D2F1−D2F11). Of these, D2F4−D2F6 (IC_{50} <5 μg/mL) were combined and further chromatographed over a silica gel column (2.5 × 20 cm), eluted with a gradient of *n*-hexane−acetone, to yield phyllanthusmins B (**3**, 1.0 mg), D (**1**, 10.5 mg), and E (**2**, 1.0 mg). Fraction D2F8 was separated by silica gel chromatography, eluted with *n*-hexane−acetone (2:1), and purified by passage over a Sephadex LH-20 column, eluted with a mixture of CH_2Cl_2−MeOH (1:1), to afford phyllanthusmin C (**4**, 9.5 mg). To isolate the polar analogues of **4**, the EtOAc-soluble extract (9.0 g) was subjected to silica gel column chromatography (4.5 × 45 cm), eluted with a gradient of CH_2Cl_2−MeOH. Fractions were pooled by TLC analysis to give five combined fractions (D3F1−D3F5). Of these, D3F1 and D3F2 were combined, further chromatographed over a silica gel column (2.5 × 20 cm), eluted with a gradient of CH_2Cl_2−MeOH, and purified by passage over a Sephadex LH-20 column, eluted with a mixture of CH_2Cl_2−MeOH (1:1), to afford cleistanthin B (**5**, 1.5 mg).

*Phyllanthusmin D (**1**):* colorless, fine needles (*n*-hexane−acetone), showing a blue color under UV light at 365 nm; mp 210−211 °C; $[\alpha]^{20}_D$ −3 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 260 (4.54) nm; ECD (MeOH, nm) λ_{max} ($\Delta\varepsilon$) 292 (−3.65); IR (dried film) ν_{max} 3446, 1747, 1619, 1507, 1481, 767 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; positive-ion HRESIMS *m*/*z* 619.1444, calcd for $C_{30}H_{28}O_{13}Na$, 619.1422.

*Phyllanthusmin E (**2**):* amorphous, colorless powder showing a blue color under UV light at 365 nm; $[\alpha]^{20}_D$ −4 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 260 (4.58) nm; ECD (MeOH, nm) λ_{max} ($\Delta\varepsilon$) 296 (−4.15); IR (dried film) ν_{max} 3419, 1738, 1622, 1506, 1481, 770 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; positive-ion HRESIMS *m*/*z* 577.1319, calcd for $C_{28}H_{26}O_{12}Na$, 577.1316.

X-ray Crystal Structure Analysis of Phyllanthusmin D (1). Intensity data for a small, colorless needle (mp 210−211 °C; molecular formula $C_{30}H_{28}O_{13}$, MW = 596.52, hexagonal, space group $P6_122$, *a* = 21.4292(6) Å, *c* = 21.4162(6) Å, *V* = 8517.0(4) $Å^3$, *Z* = 12, density(calculated) = 1.396 mg/m^3, size 0.01 × 0.01 × 0.20 mm^3) from **1** were collected at 150 K on a D8 goniostat equipped with a Bruker APEXII CCD detector at Beamline 11.3.1 using synchrotron radiation tuned to λ = 1.2399 Å at the Advanced Light Source at Lawrence Berkeley National Laboratory. For data collection, frames were measured for a duration of 1 s for low-angle data and 4 s for high-angle data at 0.3° intervals of ω with a maximum 2θ value of around 91°. The data frames were collected using the program APEX2 and processed using the program SAINT within APEX2, and the data were corrected for absorption and beam corrections based on the multiscan technique as implemented in SADABS.[51]

The structure was solved by direct methods in SIR-2004.[52] Full-matrix least-squares refinements based on F^2 were performed in SHELXL-97,[53] as incorporated in the WinGX package.[54] The benzodioxole group of this molecule is disordered over two sites. During refinement it was necessary to apply distance restraints for this group along with restraints on the anisotropic displacement parameters (SIMU and DELU). For each methyl group, the hydrogen atoms were added at calculated positions using a riding model with U(H) = 1.5U_{eq}(bonded carbon atom). The torsion angle, which defines the orientation of the methyl group about the C−C or O−C bond, was refined. The hydroxy group hydrogen atom bonded to O(8) was refined isotropically and is involved in an intermolecular hydrogen bond with atom O(2). The rest of the hydrogen atoms were included in the model at calculated positions using a riding model with U(H) = 1.2U_{eq}(bonded atom). The final refinement cycle was based on 4507 intensities, 191 restraints, and 478 variables and resulted in agreement factors of $R_1(F)$ = 0.050 and $wR_2(F^2)$ = 0.089. For the subset of data with $I > 2\sigma(I)$, the $R_1(F)$ value is 0.038 for 3850 reflections. The final difference electron density map contains maximum and minimum peak heights of 0.12 and −0.16 e/$Å^3$. Neutral atom scattering factors were used and included terms for anomalous dispersion.[55] The CIF file of the X-ray data of **1** has been deposited in the Cambridge Crystallographic Data Centre (deposition no. CCDC 981532).

Acetylation of Phyllanthusmins C (4) and D (1) to 7-*O*-[(2,3,4-Tri-*O*-acetyl)-α-L-arabinopyranosyl)]diphyllin (7). To a dried 25 mL flask equipped with a magnetic stirrer, containing 3.0 mg of phyllanthusmin D (**1**), were added 5 μL of acetic anhydride and 1 mL of pyridine. After the mixture was stirred at 40 °C for 1 h, it was cooled to room temperature. Then, 5 mL of $CHCl_3$ was transferred into the flask, and the solution was extracted with distilled H_2O. The organic layer was washed with distilled H_2O and evaporated at reduced pressure. The residue was purified by silica gel column chromatography, using *n*-hexane−acetone (5:1 → 1:1), to afford 7-*O*-[(2,3,4-tri-*O*-acetyl)-α-L-arabinopyranosyl)]diphyllin {**7** (1.0 mg, $[\alpha]^{20}_D$ −12 (*c* 0.1, $CHCl_3$)}. Using the same protocol, 5.0 mg of phyllanthusmin C (**4**) was reacted with 10 μL of acetic anhydride and 2 mL of pyridine at 60 °C for 1 h and yielded 1.0 mg of **7** {$[\alpha]^{20}_D$ −12 (*c* 0.1, $CHCl_3$)}. These values are very close to that of {$[\alpha]^{20}_D$ −13 (*c* 0.3, $CHCl_3$)} reported for synthetic **7**.[32]

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Acid Hydrolysis of Phyllanthusmin C (4) to Diphyllin (8). To a dried 25 mL flask equipped with a water condenser and magnetic stirrer containing phyllanthusmin C (**4**, 5.0 mg dissolved in 1 mL of MeOH), was transferred 5 mL of 37% hydrochloric acid (HCl) into the flask. After the mixture was stirred at 70 °C for 30 min, the mixture was cooled to room temperature and diluted by 0.1 N NaOH to pH 7.0. Then, 5 mL of $CHCl_3$ was transferred into the flask, and the solution was extracted with distilled H_2O. The organic layer was washed with distilled H_2O and evaporated under reduced pressure. The residue was separated by silica gel column chromatography, using *n*-hexane−acetone (3:1), to afford diphyllin (**8**, 1.5 mg; data obtained are shown in the Supporting Information).

Cytotoxicity against HT-29 Cells. The cytotoxicity of the test compounds was screened against HT-29 cells by a previously reported procedure.[38] Paclitaxel and etoposide were used as positive controls.

Cytotoxicity against CCD-112CoN Cells. Following a previous procedure[39] and the method for screening cytotoxicity toward HT-29 cells mentioned above, the cytotoxicity of the samples was screened against CCD-112CoN normal human colon cells.

In Vivo Hollow Fiber Assay. The hollow fiber assay is an excellent method for evaluating the potential of natural products for activity in vivo. The human colon cancer cell line HT-29 was used to evaluate **1** using procedures previously described in detail by our group.[40,41] Eight- to nine-week-old immunodeficient NCr *nu/nu* mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in microisolation cages at room temperature and a relative humidity of 50−60% under 12:12 h light−dark cycle. All animal work was approved by University of Illinois at Chicago Animal Care and Use Committee (protocol number 13-057), and the mice were treated in accordance with the institutional guidelines for animal care. Phyllanthusmin D (**1**) was dissolved initially in DMSO and subsequently diluted with Cremophor. The mixture was diluted with distilled water to 13% DMSO and 25% Cremophor. The mice were injected ip once daily for 4 days with **1** or the positive control (paclitaxel). Each mouse was weighed daily during the study. Animals showed no signs of toxicity even at the highest concentration of **1**, and all the remaining mice were sacrificed on day 7. The fibers were retrieved, and viable cell mass was evaluated by a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The percentage of the net growth for the cells in each treatment group was calculated by subtracting the day 0 absorbance from the day 7 absorbance and dividing this difference by the net growth in the vehicle control (minus the value between day 7 and day 0). Data were compared by Student's *t* test, and a *p* value less than 0.05 was considered statistically significant (Figure 3).

Topoisomerase II Assay. Topo II-DNA covalent complexes induced by topo II poisons such as etoposide may be trapped by rapidly denaturing the complexed enzyme with sodium dodecyl sulfate (SDS), digesting the enzyme, and releasing the cleaved DNA as linear DNA. The formation of linear DNA was detected by separating the SDS-treated reaction products using ethidium bromide gel electrophoresis by a modification of a previously described procedure.[42,43] In this system, topo II-mediated catalytic conversion of supercoiled pBR322 DNA to the "relaxed" form of plasmid DNA can also be observed. A 20 μL cleavage assay reaction mixture contained 250 ng of topo IIα protein, 160 ng of pBR322 plasmid DNA (NEB, Ipswich, MA, USA), 1.0 mM ATP in assay buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 50 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5% glycerol], and 100 μM test compounds or DMSO solvent, as indicated. Assay buffer (17 μL) and test compound/DMSO (1 μL) were mixed and allowed to sit at room temperature for 30 min, after which 2 μL of topo IIα was added to initiate the reaction. Tubes were incubated at 37 °C for 15 min and then quenched with 1% (v/v) SDS−10 mM disodium EDTA−200 mM NaCl. The mixture was treated subsequently with 0.77 mg/mL proteinase K (Sigma) at 55 °C for 60 min to digest the protein, and DNA bands were separated by electrophoresis (18 h at 2 V/cm) on an agarose gel (1.3% w/v) containing 0.7 μg/mL ethidium bromide in TAE buffer pH 8.0 (40 mM Tris base, 0.114% (v/v) glacial acetic acid, 2 mM EDTA). The DNA in the gel was imaged by its fluorescence on a Chemi-Doc XRS+ imager (Bio-Rad, Hercules, CA, USA). Linear DNA was quantified by accounting for the relationship between fluorescence and relative band intensity for open circular, linear (LNR), supercoiled, and relaxed configurations of DNA,[43] then calculating the percent of LNR from the total DNA content in each lane. Results are shown for etoposide, phyllanthusmins C (**4**) and D (**1**), and 7-*O*-[(2,3,4-tri-*O*-acetyl)-α-L-arabinopyranosyl)]diphyllin (**7**) in replicate experiments performed on separate days (Figure 4).

Annexin V Staining Method. As described in previous studies,[48] HT-29 cells were treated with the vehicle control or etoposide (1 or 5 μM) or **1** (1 or 5 μM) for 72 h. The cells were washed with annexin V binding buffer, centrifuged at 300*g* for 10 min, and suspended (1×10^6) in 100 μL of 1× annexin V binding buffer. Then, 10 μL of annexin V-APC was added to the suspension. After the suspension was mixed and incubated in a dark room at room temperature for 15 min, the cells were centrifuged, and the cell pellet was resuspended in 500 μL of 1× annexin V binding buffer. Then, 5 μg/mL of 7-AAD solution was added in the suspension, and flow cytometry was conducted immediately.

Western Blot Analysis. After a 24 h treatment, HT-29 cells were harvested, washed once with ice-cold PBS, and lysed (10^8 cells/mL lysis buffer) in hypertonic buffer {1% NP-40, 10 mM HEPES (pH 7.5), 0.5 M NaCl, 10% glycerol supplemented with protease and phosphatase inhibitors [(1 mM phenylmethylsulfonylfluoride, 1 mM Na_3VO_4, 50 mM NaF, 10 mM β-glycerol phosphate, 1 mM EDTA], and protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN, USA]}. Cell lysates, adding 4× or 2× laemmli buffer (Bio-Rad) by supplementing with 2.5% β-mercaptoethanol to give 1× SDS sample buffer, were boiled for 5 min and subjected to immunoblot analysis, as described previously.[49] Protein samples were resolved on 4−15% SDS-PAGE (Bio-Rad), and immunoblot analysis was performed using antibodies against the indicated signaling molecules. The antibodies used were rabbit monoclonal cleaved caspase-3 (Asp175) (Cell Signaling Technology, Beverly, MA, USA) and goat polyclonal β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

■ ASSOCIATED CONTENT

Supporting Information

Mass and NMR spectra of compounds **1**−**8**; ECD spectra of compounds **1** and **2**; diagrams of COSY, the key HMBC, and NOESY correlations of compounds **2**−**8**; assignments of the ^1H and ^{13}C NMR data of the known compounds **3**−**8**; analytical data of the known compounds obtained from *P. poilanei*; and the CIF file of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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