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•Original article•

Cytotoxic lignans from the roots and rhizomes of *Diphylleia sinensis*, a China's endemic plant species

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[ABSTRACT] Eight novel arylnaphthalide lactone lignans, designated as diphylignan A−H (**1**−**8**), and a new dibenzyltyrolactone lignan, designated as diphylignan I (**9**), were isolated from the roots and rhizomes of *Diphylleia sinensis*, along with two additional novel natural products (**11** and **14**) and four known metabolites (**10**, **12**, **13**, **15**). The structural and stereochemical characterization of these compounds was accomplished using NMR spectroscopy and electronic circular dichroism (ECD) analysis. The cytotoxic activities of all isolated compounds were assessed against A-549 and SMMC-7721 cell lines. Notably, compound **2** demonstrated the most significant cytotoxicity, with IC_{50} values of 10.27 and 11.58 μmol·L^{-1} against A-549 and SMMC-7721 cell lines, respectively, exhibiting greater potency than the positive control, cisplatin.

[KEY WORDS] *Diphylleia sinensis*; Dibenzyltyrolactone; Arylnaphthalide; Cytotoxic
[CLC Number] R284.1 **[Document code]** A **[Article ID]** 2095-6975(2024)09-0831-11

Introduction

The genus *Diphylleia* comprises three species: *D. cymosa* from North America, *D. grayi* from Japan, and *D. sinensis* from China [1]. *D. sinensis*, endemic to China, is predominantly found in the provinces of Shanxi, Gansu, Sichuan, Hubei, and Yunnan. In traditional Chinese medicine, its dried roots and rhizomes, known as "Woerqi", are used to treat snake bites, rheumatic arthralgia, fall injuries, abdominal pain, abnormal menstruation, tendon and bone pain, tracheitis, and traumatic swelling [2]. Previous phytochemical studies have shown that *D. sinensis* is particularly rich in arylnaphthalide lactone lignans [2]. Nearly 60 naturally occurring arylnaphthalide lactone lignans and their glycosylated derivatives have been isolated from dietary and medicinal plants, exhibiting a range of biological activities including antibacterial, insecticidal, antiplatelet, antiangiogenic, anti-inflammatory, antiviral, cytotoxic, antineoplastic, and antitumor properties. Notably, compounds such as podophyllotoxin and its derivatives demonstrate potent cytotoxic activity by inhibiting microtubule assembly or topoisomerase II enzymes. Synthetic derivatives of podophyllotoxin, such as etoposide, teniposide, and etopophos, are crucial chemotherapeutic agents for various cancers [3, 4]. Thus, structurally diverse arylnaphthalide lactone lignans are significant sources of natural anticancer agents. In our quest for cytotoxic natural products, we isolated eight new arylnaphthalide lactone lignans, named diphylignan A−H (**1**−**8**), one new dibenzyltyrolactone lignan, named diphylignan I (**9**), two new natural products (**11** and **14**), and four known metabolites (**10**, **12**, **13**, **15**) from the roots and rhizomes of *D. sinensis* (Fig. 1). This report details their isolation, structural elucidation, and cytotoxic activities.

Results and Discussion

Compound **1** was isolated as a white amorphous powder. Its molecular formula was determined to be $C_{27}H_{30}O_{12}$ based on high-resolution electrospray ionization mass spectrometry

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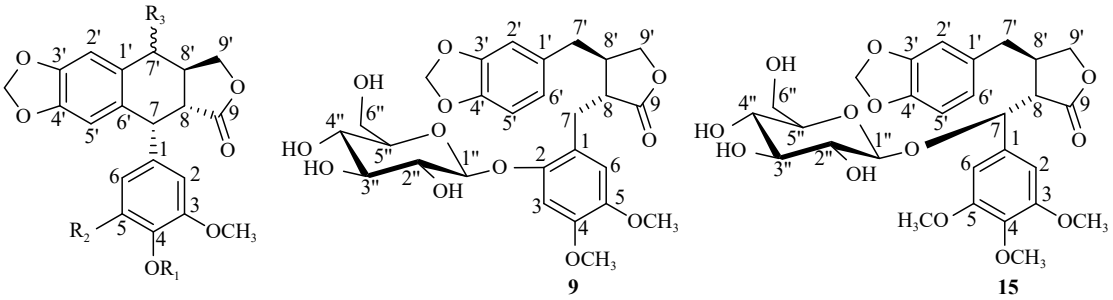
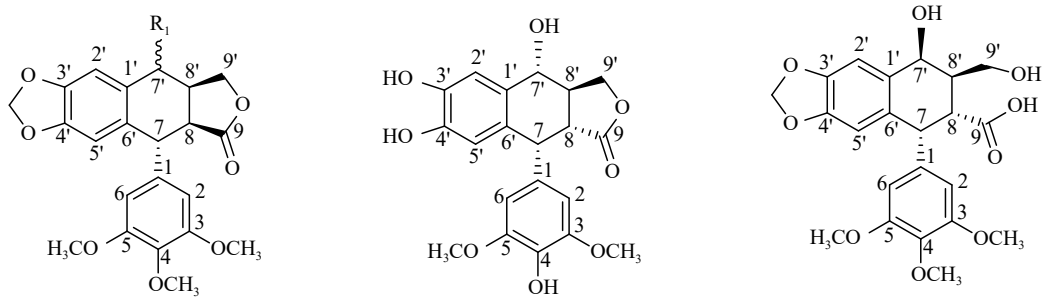
These authors have no conflict of interest to declare.



9 **15**

1: R_1 = CH_3, R_2 = H, R_3 = *α*-*O*-*β*-D-glc
2: R_1 = R_2 = H, R_3 = *β*-*O*-*β*-D-glc
3: R_1 = H, R_2 = OCH_3, R_3 = *α*-(2‴*R**)-6″-(2‴-hydroxypropionyl)-*O*-*β*-D-glc
4: R_1 = H, R_2 = OCH_3, R_3 = *α*-(2‴*S**)-6″-(2‴-hydroxypropionyl)-*O*-*β*-D-glc
5: R_1 = CH_3, R_2 = OCH_3, R_3 = *α*-6″-(4‴-methoxy-4‴-oxobutyryl)-*O*-*β*-D-glc
6: R_1 = CH_3, R_2 = OCH_3, R_3 = *α*-*O*-*α*-L-ara-(1→3)-*β*-D-glc
8: R_1 = CH_3, R_2 = OCH_3, R_3 = *α*-*O*-*α*-D-glc-(1→4)-*α*-D-glc-(1→4)-*β*-D-glc
13: R_1 = CH_3, R_2 = OCH_3, R_3 = *α*-6″-aetyl-*O*-*β*-D-glc



12 **14**

7: R_1 = *α*-*O*-*β*-D-glc-(1→4)[*α*-D-glc-(1→6)]-*β*-D-glc
10: R_1 = *β*-OH
11: R_1 = *β*-OCH_3

Fig. 1 Chemical structures of compounds 1−15.

(HR-ESI-MS) data (*m*/*z* 569.1631 $[M + Na]^+$, Calcd. for $C_{27}H_{30}O_{12}Na$, 569.1635). The ^1H nuclear magnetic resonance (NMR) spectrum (Table 1) exhibited signals corresponding to two methoxy groups at *δ* 3.68 (3H, s) and 3.69 (3H, s), five aromatic protons at *δ* 7.34 (1H, s), 6.48 (1H, s), 6.87 (1H, d, *J* = 1.6 Hz), 6.29 (1H, dd, *J* = 8.4, 1.6 Hz), and 6.80 (1H, d, *J* = 8.4 Hz), a methylenedioxy group at *δ* 5.97 (1H, s) and 5.98 (1H, s), and an anomeric proton of a glucopyranosyl moiety at *δ* 4.27 (1H, d, *J* = 7.7 Hz). The ^{13}C NMR spectrum (Table 2) revealed a structure consistent with an aryltetralin lactone lignan. Key signals included a carbonyl group at *δ* 174.6, twelve aromatic carbons, and five aliphatic carbons at *δ* 42.6, 44.2, 78.3, 38.6, and 70.9. Additional signals were observed for two methoxy groups at *δ* 55.4 (× 2), a methylenedioxy group at *δ* 101.1, and a glucopyranosyl moiety at *δ* 102.1, 73.4, 76.7, 70.0, 77.1, and 63.9. Comparative analysis of the NMR spectra of compound **1** with those of podophyllotoxin 7′-*O*-*β*-D-glucopyranoside [5] suggested that compound **1** is a demethoxylated derivative of podophyllotoxin 7′-*O*-*β*-D-glucopyranoside. This conclusion was further supported by HR-ESI-MS data, showing a mass difference of 30 units less than that of podophyllotoxin 7′-*O*-*β*-D-glucopyranoside. Heteronuclear multiple bond correlations (HMBCs) provided further insights into the structure. The methoxy group at *δ* 3.69 (3H, s) correlated with C-3 (*δ* 147.7), and the methoxy group at *δ* 3.68 (3H, s) correlated with C-4 (*δ* 147.6). Correlations of the aromatic protons at *δ* 6.87 (1H, d, *J* = 1.6 Hz, H-2) and *δ* 6.29 (1H, dd, *J* = 8.4, 1.6 Hz, H-6) with C-7 (*δ* 42.6) indicated that the 3,4-dimethoxyphenyl group was attached to C-7. Additionally, the HMBC cross-peak (Fig. 2) of the anomeric proton at *δ* 4.27 (1H, d, *J* = 7.7 Hz) with C-7′ (*δ* 78.3) indicated glycosylation of the 7′-OH group with glucose. The ^{13}C NMR chemical shift at *δ* 102.1 and the spin-spin coupling constant of 7.7 Hz confirmed the presence of a *β*-glucopyranosyl moiety [5]. The identity of the D-glucose was confirmed by microhydrolysis, followed by HPLC analysis [6].

The relative configurations of compound **1** were determined using the chemical shift of C-9, ^1H coupling constants (*J* values), and nuclear Overhauser enhancement spectroscopy (NOESY) experiments. For lactones with a *cis*-orientation at C-8′ and C-8, the C-9 signal appears around *δ* 178.0, whereas for a *trans*-orientation, the signal shifts upfield to around *δ* 175.0 [5]. In compound **1**, the C-9 signal at *δ* 174.6 indicates a *trans*-orientation of H-8/H-8′. Coupling constant (*J*) values provided additional support. A cis-configuration of H-7/H-8 typically exhibits a $J_{H\text{-}7/H\text{-}8}$ value of 3−5 Hz, while a *trans*-configuration shows 7−9 Hz. For H-7′/H-8′, a *cis*-configura-

Table 1 ^{1}H NMR (500 MHz) data (DMSO-d_6) for **1**–**5** and **9**

No.	1	2	3	4	5	9
2/3	6.87, d (1.6)	6.73, d (1.9)	6.26, s	6.26, s	6.30, s	6.93, s
5	6.80, d (8.4)	6.58, d (8.2)				
6	6.29, dd (8.4, 1.6)	6.12, dd (8.2, 1.9)	6.26, s	6.26, s	6.30, s	6.78, s
7	4.50, d (4.5)	4.49, d (5.4)	4.48, d (4.6)	4.47, d (4.6)	4.53, d (4.8)	2.16, dd (4.1, 13.0) 2.80, dd (7.0, 13.0)
8	3.13, dd (4.5, 14.8)	3.37, dd (5.4, 14.8)	3.13, dd (4.6, 14.3)	3.12, dd (4.6, 14.2)	3.16, dd (4.8, 14.6)	2.77, m
2′	7.34, s	7.05, s	7.33, s	7.31, s	7.32, s	6.75, d (1.5)
5′	6.48, s	6.52, s	6.51, s	6.51, s	6.53, s	6.74, d (8.0)
6′						6.61, dd (8.0, 1.5)
7′	4.96, d (9.8)	5.02, d (3.5)	4.95, d (10.1)	4.93, d (10.0)	4.96, d (10.0)	2.38, m; 2.75, m
8′	2.86, m	2.83, m	2.79, m	2.80, m	2.78, m	2.44, m
9′	4.20, dd (10.2, 8.6); 4.56, t (7.8)	4.36, dd (10.6, 8.6); 4.31, t (8.1)	4.15, dd (10.1, 8.8); 4.49, ovelapped	4.17, dd (10.0, 9.0); 4.46, m	4.16, dd (10.2, 8.6); 4.47, t (7.7)	3.90, t (8.1); 3.81, d (8.7)
OCH_2O	5.97, s; 5.98, s	6.019, s; 6.017, s	5.97, s; 6.00, s	5.96, s; 6.00, s	5.97, s; 6.00, s	5.941, s; 5.942, s
3/5-OCH_3	3.69, s	3.68, s	3.62, s	3.62, s	3.63, s	3.65, s
4-OCH_3/OH	3.68, s	8.84, s			3.62, s	3.72, s
1″	4.27, d (7.7)	4.26, d (7.7)	4.30, d (7.6)	4.35, d (7.8)	4.30, d (7.5)	4.72, d (7.3)
2″	3.07, m	3.01, m	3.12, m	3.08, m	3.09, m	3.28, m
3″	3.19, m	3.12, m	3.17, m	3.19, m	3.17, m	3.37, m
4″	3.13, m	3.03, m	3.14, m	3.13, m	3.10, m	3.07, m
5″	3.15, m	3.12, m	3.24, m	3.38, m	3.27, m	3.36, m
6″	3.49, dd (11.4, 4.8); 3.67, ovelapped	3.47, dd (10.4, 7.3); 3.77, dd (10.4, 3.3)	4.42, d (10.4); 3.95, m	4.33, d (11.8); 4.08, dd (11.8, 7.4)	4.32, dd (11.7, 1.7); 4.06, dd (11.7, 7.8)	3.80, d (12.3); 3.49, dd (12.3, 6.4)
2‴			3.95, m	3.97, m	2.24, m	
3‴			0.93, d (6.9)	1.07, d (6.9)	2.42, m	
4‴-OCH_3					3.56, s	

tion has a J value of about 3 Hz, and a *trans*-configuration shows 9−10 Hz. Compound **1** exhibited a $J_{\text{H-7/H-8}}$ value of 4.5 Hz, indicating a *cis*-configuration, and a $J_{\text{H-7′/H-8′}}$ value of 9.8 Hz, indicating a *trans*-configuration. The NOESY experiment further confirmed these findings. NOE correlations between H-7/H-8 and H-8/H-7′ indicated that H-7, H-8, and H-7′ were on the same face. Analysis of ORD and electronic circular dichroism (ECD) curves of 7-aryltetralin lignans showed that 7*β* (*S*)-aryl compounds exhibit negative Cotton effects around 280−290 nm, while 7*α* (*R*)-aryl compounds show positive Cotton effects [5]. The ECD spectrum of compound **1** displayed a positive Cotton effect at 293 nm, indicating an absolute configuration of *R* at C-7. Therefore, compound **1** was identified as 5-demethoxypodophyllotoxin 7′-*O*-*β*-D-glucopyranoside and was named diphylignan A.

Compound **2** was isolated as a white amorphous powder. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) of compound **2** closely resembled those of compound **1**, with key differences. In compound **2**, a phenolic hydroxy group was observed at *δ* 8.84 (1H, s) and an aliphatic proton at *δ* 5.02 (1H, d, *J* = 3.5 Hz, H-7′), replacing the methoxy group at *δ* 3.68 (3H, s) and the aliphatic proton at *δ* 4.96 (1H, d, *J* = 9.8 Hz, H-8′) seen in compound **1**. This was supported by HR-ESI-MS data, showing an $[M + Na]^+$ quasi-molecular ion peak at *m/z* 555.1478, matching the calculated value of 555.1478 and indicating a difference of 14 mass units less than compound **1**. HMBCs (Fig. 2) provided further structural elucidation. The methoxy group at *δ* 3.68 (3H, s) correlated with C-3 (*δ* 146.0), and the aromatic protons of the 1,3,4-tri-substituted benzene ring at *δ* 6.73 (1H, d, *J* = 1.9 Hz, H-2) and *δ* 6.12 (1H, dd, *J* = 8.2, 1.9 Hz, H-6) correlated with C-7 (*δ* 42.6). These correlations suggested that the 3-methoxy-4-hydroxyphenyl group was attached to C-7. The coupling constant $J_{\text{H-7′/H-8′}}$ = 3.5 Hz indicated a *cis*-configuration of H-7′/H-8′. Therefore, compound **2** was identified as 4-demethyl-5-demethoxylepipodophyllotoxin 7′-*O*-*β*-D-glucopyranoside and named diphylignan B.

Compounds **3** and **4** were isolated as white amorphous powders. Their ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) were similar to those of compound **2** but with notable differences. Both compounds **3** and **4** exhibited a methoxy group

Table 2 ^{13}C NMR (125 MHz) data (DMSO-d_6) for **1**–**5** and **9**

No.	1	2	3	4	5	9
1	131.3, C	131.4, C	132.5, C	132.3, C	136.5, C	117.8, C
2	114.9, CH	115.3, CH	108.7, CH	108.7, CH	108.3, CH	150.1, C
3	147.7, C	146.0, C	147.2, C	147.2, C	152.0, C	101.4, CH
4	147.6, C	145.3, C	135.0, C	134.9, C	136.3, C	143.4, C
5	111.1, CH	114.7, CH	147.2, C	147.2, C	152.0, C	148.1, C
6	122.4, CH	122.3, CH	108.7, CH	108.7, CH	108.3, CH	114.8, CH
7	42.6, CH	42.6, CH	43.0, CH	43.0, CH	43.2, CH	26.8, CH_2
8	44.2, CH	40.4, CH	44.6, CH	44.5, CH	44.1, CH	45.3, CH
9	174.6, C	175.0, C	174.6, C	174.7, C	174.4, C	178.5, C
3/5-OCH_3	55.4	55.5	56.1	56.1	55.8	56.0
4-OCH_3	55.4				59.9	55.4
1′	132.2, C	133.3, C	130.7, C	130.7, C	132.0, C	132.8, C
2′	108.1, CH	110.1, CH	107.9, CH	108.0, CH	107.9, CH	108.8, CH
3′	146.9, C	147.7, C	146.9, C	146.9, C	146.7, C	147.3, C
4′	146.5, C	146.6, C	146.6, C	146.6, C	146.9, C	145.6, C
5′	109.1, CH	110.0, CH	109.2, CH	109.2, CH	109.2, CH	108.0, CH
6′	133.2, C	128.2, C	130.9, C	131.0, C	130.9, C	121.5, C
7′	78.3, CH	70.4, CH	78.4, CH	79.0, CH	78.6, CH	37.1, CH_2
8′	38.6, CH	37.3, CH	38.1, CH	38.5, CH	38.4, CH	40.6, CH
9′	70.9, CH_2	67.7, CH_2	71.0, CH_2	70.8, CH_2	70.1, CH_2	70.3, CH_2
OCH_2O	101.1	101.2	101.0	101.0	101.3	100.7
1″	102.1, CH	99.8, CH	101.1, CH	101.8, CH	101.1, CH	102.3, CH
2″	73.4, CH	73.6, CH	73.2, CH	73.3, CH	73.3, CH	73.4, CH
3″	76.7, CH	76.6, CH	76.4, CH	76.2, CH	76.6, CH	76.8, CH
4″	70.0, CH	70.5, CH	70.0, CH	70.0, CH	71.0, CH	70.6, CH
5″	77.1, CH	77.1, CH	73.9, CH	73.9, CH	73.8, CH	77.6, CH
6″	63.9, CH_2	61.3, CH_2	63.9, CH_2	64.2, CH_2	63.9, CH_2	61.1, CH_2
1‴			174.5, C	174.7, C	172.2, C	
2‴			65.6, CH	65.8, CH	28.1, CH_2	
3‴			20.0, CH_3	20.2, CH_3	28.2, CH_2	
4‴					171.7, C	
4‴-OCH_3					51.4, CH_3	

[δ 3.62 (3H, s) in both compounds**]** and a 2-hydroxypropionyl group [δ 0.93 (3H, d, J = 6.9 Hz), 3.95 (1H, m), δ 20.0, 65.6, 174.5 in compound **3**; δ 1.07 (3H, d, J = 6.9 Hz), 3.97 (1H, m), δ 20.2, 65.8, 174.7 in compound **4**] [7]. This was further confirmed by HR-ESI-MS, which showed $[M + Na]^+$ quasi-molecular ion peaks at *m/z* 657.1796 (calculated for 657.1795) for both compounds, indicating an increase of 102 mass units compared to compound **2**. HMBCs (Fig. 2) provided further structural insights. The oxygenated methine proton [δ 3.95 (1H, m) in compound **3** and δ 3.97 (1H, m) in compound **4**] correlated with C-6″ (δ 63.9 in compound **3** and δ 64.2 in compound **4**), suggesting the 2-hydroxypropionyl group was attached to C-6″ of the glucopyranosyl moiety. The methoxy groups at C-3 and C-5 were confirmed by HMBCs, with signals at δ 3.62 (6H, s) correlating with C-3 and C-5 [both at δ 147.2]. The differences in the chemical shifts of the methyl groups [δ 0.93 (3H, d, J = 6.9 Hz) in compound **3** and δ 1.07 (3H, d, J = 6.9 Hz) in compound **4**] indicated dif-

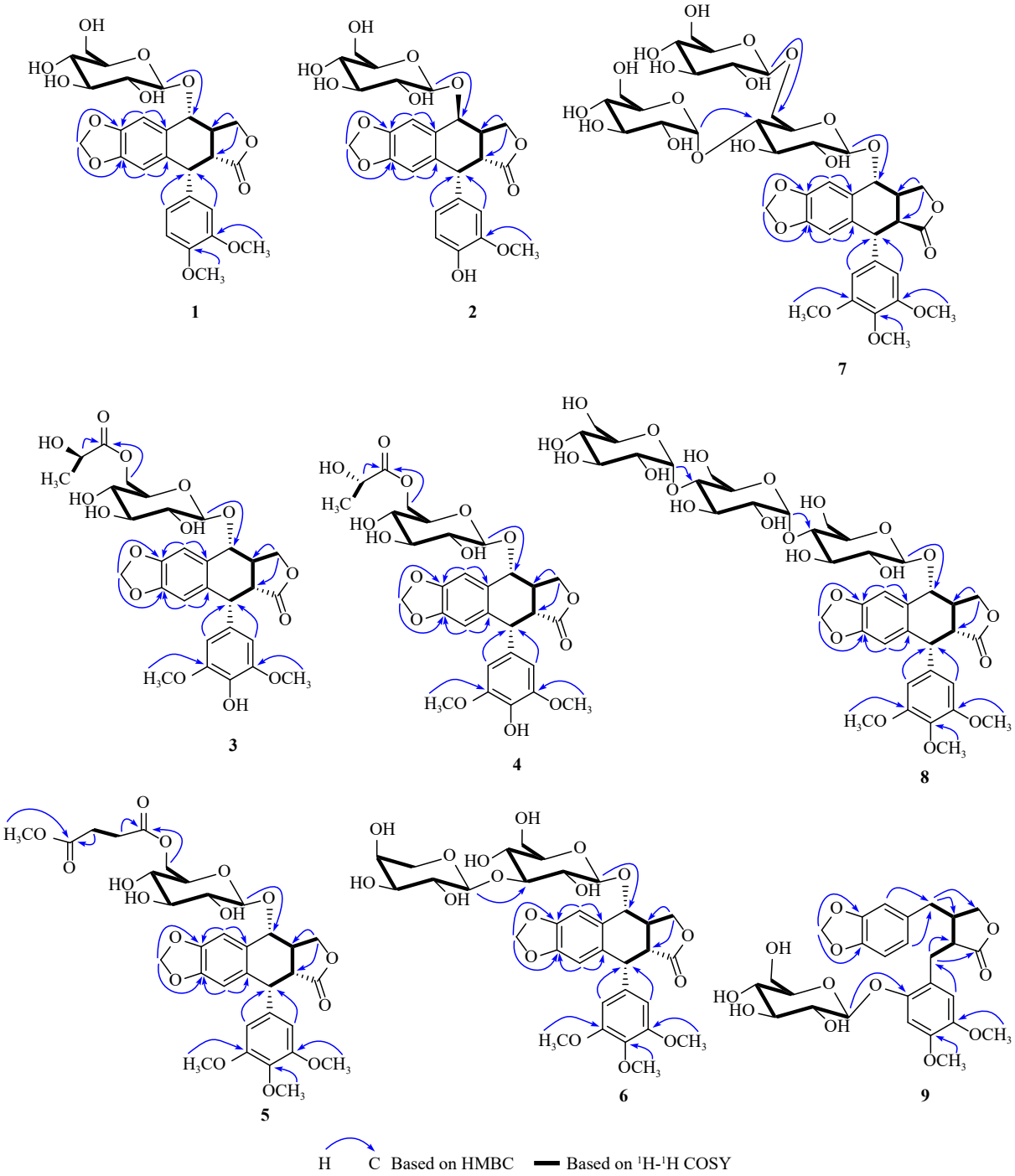


Fig. 2 Key ^{1}H-^{1}H COSY and HMBCs of compounds 1−9.

ferent absolute configurations of the 2-hydroxypropionyl group. Consequently, compounds **3** and **4** were identified as (2‴*R**)-6″-*O*-(2‴-hydroxypropionyl)-4-demethylpodophyllotoxin 7′-*O*-*β*-D-glucopyranoside and (2‴*S**)-6″-*O*-(2‴-hydroxypropionyl)-4-demethylpodophyllotoxin 7′-*O*-*β*-D-glucopyranoside, respectively. These compounds were named diphylignan C and diphylignan D.

Compound **5** was isolated as a white amorphous powder. Its ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) closely resembled those of compounds **3** and **4**, with a notable exception. Compound **5** featured a 4-methoxy-4-oxobutyryl group [*δ* 3.56 (3H, s), 2.42 (2H, m), 2.24 (2H, m), *δ* 28.1, 28.2, 171.7, 51.4] [8], replacing the 2-hydroxypropionyl group present in compounds **3** and **4**. This was corroborated by HR-ESI-MS, which showed an $[M + Na]^{+}$ quasi-molecular ion peak at *m/z* 713.2053 (calculated for 713.2058), indicating an additional 56 mass units compared to compounds **3** and **4**. Further structural elucidation was provided by HMBCs (Fig. 2). The methylene group signal at *δ* 2.24 (2H, m) correlated with C-6″ (*δ* 63.9), indicating that the 4-methoxy-4-ox-

obutyryl group was attached at C-6″ of the glucopyranosyl moiety. The presence of a methoxy group at C-4 was confirmed by the HMBC of the methoxy signal at *δ* 3.62 (3H, s) with C-4 (*δ* 136.3). Thus, compound **5** was identified as 6″-*O*-(4‴-methoxy-4‴-oxobutyryl)-podophyllotoxin 7′-*O*-*β*-D-glucopyranoside and was named diphylignan E.

Compound **6** was isolated as a white amorphous powder, with its molecular formula determined to be $C_{33}H_{40}O_{17}$ based on HR-ESI-MS analysis (*m/z* 731.2163 $[M + Na]^+$, Calcd. for $C_{33}H_{40}O_{17}Na$, 731.2163). The ^{1}H NMR spectrum (Table 3) showed three methoxy groups at *δ* 3.64 (6H, s) and 3.62 (3H, s), four aromatic protons at *δ* 7.35 (1H, s), 6.52 (1H, s), and 6.32 (2H, s), a methylenedioxy group at *δ* 6.00 (1H, s) and 5.98 (1H, s), and anomeric protons of two pyranose moieties at *δ* 4.39 (1H, d, *J* = 7.7 Hz) and 4.46 (1H, d, *J* = 6.4 Hz). The ^{13}C NMR spectrum (Table 4) revealed a structure consistent with aryltetralin lactone lignans, showing signals for a carbonyl group at *δ* 174.4, twelve aromatic carbons, five aliphatic carbons at *δ* 43.1, 44.3, 78.1, 38.7, and 71.0, three methoxy groups at *δ* 55.9 (× 2) and 60.0, a glucopyranosyl group at *δ* 101.2, 72.3, 85.2, 68.2, 76.7, and 60.9, an arabinopyranosyl group at *δ* 103.8, 71.0, 72.7, 67.2, and 65.3 [6], and one methylenedioxy group at *δ* 101.1. Comparative analysis of the NMR spectra of compound **6** with those of podophyllotoxin suggested that compound **6** is a glycosylation derivative of podophyllotoxin. The coupling constants (7.7 Hz and 6.4 Hz) of the anomeric protons indicated the presence of one *β*-glucopyranosyl group and one *α*-arabinopyranosyl group. The identities of D-glucose and L-arabinose were confirmed through microhydrolysis and HPLC analysis [6]. Further structural elucidation was provided by HMBCs (Fig. 2). The anomeric proton at *δ* 4.39 (1H, d, *J* = 7.7 Hz, H-1″) correlated with C-7′ (*δ* 78.1), and the anomeric proton at *δ* 4.46 (1H, d, *J* = 6.4 Hz, H-1‴) correlated with C-3″ (*δ* 85.2), indicating that the glucopyranosyl group was attached to C-7′ of the aglycone and the arabinopyranosyl group was attached at C-3″ of the glucopyranosyl moiety.

The $J_{H\text{-}8/H\text{-}8'}$ (14.5 Hz), $J_{H\text{-}7'/H\text{-}8'}$ (10.3 Hz) and $J_{H\text{-}7/H\text{-}8}$ (4.9 Hz) values and the NOE correlations of H-7/H-8 and H-8/H-7′ indicated that **6** possessed the same relative configuration as podophyllotoxin [5]. Additionally, the ECD spectrum of compound **6** displayed a positive Cotton effect at 290 nm, confirming the absolute configuration of C-7 as *R*. Consequently, compound **6** was determined to be podophyllotoxin 7′-*O*-*α*-L-arabinopyranosyl-(1→3)-*O*-*β*-D-glucopyranoside, and was named diphylignan F.

Compound **7** was isolated as a white amorphous powder, with its molecular formula determined to be $C_{40}H_{52}O_{23}$ based on HR-ESI-MS analysis (*m/z* 923.2805 $[M + Na]^+$, Calcd. for $C_{40}H_{52}O_{23}Na$, 923.2797). The ^{1}H NMR spectrum (Table 3) revealed the presence of three methoxy groups at *δ* 3.75 (6H, s) and 3.68 (3H, s), four aromatic protons at *δ* 7.18 (1H, s), 6.64 (2H, s), and 5.96 (1H, s), a methylenedioxy group at *δ* 5.96 (1H, s) and 5.83 (1H, s), and anomeric protons of three pyranose moieties at *δ* 4.54 (1H, d, *J* = 7.7 Hz), 5.04 (1H, d, *J* = 3.6 Hz), and 4.14 (1H, d, *J* = 7.8 Hz). The ^{13}C NMR spectrum (Table 4) indicated a structure consistent with aryltetralin lactone lignans, displaying signals for a carbonyl group at *δ* 177.8, twelve aromatic carbons, and five aliphatic carbons at *δ* 43.5, 43.1, 76.6, 42.1, and 68.9. Additional signals included three methoxy groups at *δ* 55.9 (× 2) and 60.0, a methylenedioxy group at *δ* 100.7, and three sets of glucopyranosyl groups at *δ* 103.8, 73.4, 76.3, 80.2, 75.3, 67.5; 101.0, 72.4, 73.5, 69.8, 73.2, 60.8; and 103.1, 73.2, 76.2, 69.9, 76.6, 60.9. The aglycone was identified as picropodophyllotoxin by comparing its NMR data with those reported in the literature [9], as well as correlations observed in the NOESY, heteronuclear single quantum coherence spectroscopy (HSQC), and HMBC spectra. The spin-spin coupling constants (7.7 Hz, 7.8 Hz, 3.6 Hz) of the anomeric protons identified two *β*-glucopyranosyl groups and one *α*-glucopyranosyl group [10]. The presence of D-glucose as the only monosaccharide was confirmed by microhydrolysis and HPLC analysis [6]. Further structural details were elucidated using HMBC cross-peaks (Fig. 2). The anomeric proton at *δ* 4.54 (1H, d, *J* = 7.7 Hz, H-1″) correlated with C-7′ (*δ* 76.6), the anomeric proton at *δ* 5.04 (1H, d, *J* = 3.6 Hz, H-1‴) correlated with C-4″ (*δ* 80.2), and the anomeric proton at *δ* 4.14 (1H, d, *J* = 7.8 Hz, H-1⁗) correlated with C-6″ (*δ* 67.5). These correlations indicated that one *β*-glucopyranosyl group was attached at C-7′ of the aglycone, while one *α*-glucopyranosyl group and another *β*-glucopyranosyl group were attached at C-4″ and C-6″ of the inner glucose, respectively.

The $J_{H\text{-}7/H\text{-}8}$ (8.2 Hz), $J_{H\text{-}7'/H\text{-}8'}$ (9.9 Hz) and $J_{H\text{-}8/H\text{-}8'}$ (11.2 Hz) values, combined with the NOE correlations of H-7/H-7′ and H-8/H-8′, determined the relative configuration of 7,8-*trans*-7′,8′-*trans*-8,8′-cis. The ECD spectrum of compound **7** showed positive Cotton effects at 289 nm, confirming the absolute configuration of C-7 as *R*. Therefore, compound **7** was characterized as picropodophyllotoxin 7′-*O*-*β*-D-glucopyranosyl-(1→4)[*α*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside, and was named diphylignan G.

Compound **8** was isolated as a white amorphous powder. Its molecular formula was determined to be $C_{40}H_{52}O_{23}$ based on HR-ESI-MS analysis (*m/z* 923.2804 $[M + Na]^+$, calculated for $C_{40}H_{52}O_{23}Na$, 923.2797). The ^{1}H NMR spectrum (Table 3) revealed the presence of three methoxy groups at *δ* 3.64 (6H, s) and 3.62 (3H, s), four aromatic protons at *δ* 7.31 (1H, s), 6.52 (1H, s), and 6.32 (2H, s), a methylenedioxy group at *δ* 6.00 (1H, s) and 5.98 (1H, s), and anomeric protons of three pyranose moieties at *δ* 4.33 (1H, d, *J* = 7.7 Hz), 5.00 (1H, d, *J* = 3.8 Hz), and 5.05 (1H, d, *J* = 3.8 Hz). The ^{13}C NMR spectrum (Table 4) indicated a structure characteristic of aryltetralin lactone lignans, with signals for a carbonyl group at *δ* 174.4, twelve aromatic carbons, and five aliphatic carbons at *δ* 43.1, 44.2, 78.1, 38.8, and 71.0. Additional signals included three methoxy groups at *δ* 55.9 (× 2) and 60.0, a methylenedioxy group at *δ* 100.9, and three sets of glucopyranosyl groups at *δ* 100.6, 73.3, 75.4, 79.5, 76.2, 60.3; 101.1, 72.6, 72.0, 79.7, 71.8, 60.7; and 101.8, 72.9,

Table 3 ^{1}H NMR (500 MHz) data (DMSO-d_6) for **6**–**8**

No.	6	7	8	No.	6	7	8
2	6.32, s	6.64, s	6.32, s	4″	3.23, m	3.38, m	3.34, m
6	6.32, s	6.64, s	6.32, s	5″	3.24, m	3.57, m	3.47, m
7	4.51, d (4.9)	3.92, d (8.2)	4.51, d (4.8)	6″	3.70, m; 3.50, m	4.00, d (11.8); 3.62, m	3.62, m; 3.66, m
8	3.19, dd (4.9, 14.5)	3.48, dd (8.2, 11.2)	3.20, dd (4.8, 14.4)	1‴	4.46, d (6.4)	5.04, d (3.6)	5.00, d (3.8)
2′	7.35, s	7.18, s	7.31, s	2‴	3.49, m	3.24, m	3.24, m
5′	6.52, s	5.96, s	6.52, s	3‴	3.32, m	3.46, m	3.32, m
7′	4.99, d (10.3)	4.64, d (9.9)	4.97, d (9.6)	4‴	3.66, m	2.94, m	3.39, m
8′	2.80, m	2.78, m	2.79, m	5‴	3.45, m; 3.77, dd (12.2, 3.8)	3.37, m	3.57, m
9′	4.19, dd (10.1, 9.2); 4.60, m	4.59, d (9.5); 4.42, m	4.20, dd (10.3, 8.8); 4.59, m	6‴		3.47, m; 3.62, m	3.62, m; 3.72, m
OCH_2O	5.98, s; 6.00, s	5.83, s; 5.96, s	5.98, s; 6.00, s	1⁗		4.14, d (7.8)	5.05, d (3.8)
3,5-OCH_3	3.64, s	3.75, s	3.64, s	2⁗		3.25, m	3.13, m
4-OCH_3	3.62, s	3.68, s	3.62, s	3⁗		2.66, m	3.48, m
1″	4.39, d (7.7)	4.54, d (7.7)	4.33, d (7.7)	4⁗		3.08, m	3.08, m
2″	3.42, m	2.84, m	3.37, m	5⁗		3.49, m	3.69, m
3″	3.43, m	2.44, m	3.31, m	6⁗		3.56, m; 3.36, m	3.61, m; 3.46, m

Table 4 ^{13}C NMR (125 MHz) data (DMSO-d_6) of **6**–**8**

No.	6	7	8	No.	6	7	8
1	136.3, C	136.1, C	136.2, C	OCH_2O	101.1	100.7	100.9
2	108.3, CH	106.5, CH	108.3, CH	1″	101.2, CH	103.8, CH	100.6, CH
3	152.0, C	152.9, C	152.0, C	2″	72.3, CH	73.4, CH	73.3, CH
4	136.5, C	137.7, C	136.5, C	3″	85.2, CH	76.3, CH	75.4, CH
5	152.0, C	152.9, C	152.0, C	4″	68.2, CH	80.2, CH	79.5, CH
6	108.3, CH	106.5, CH	108.3, CH	5″	76.7, CH	75.3, CH	76.2, CH
7	43.1, CH	43.5, CH	43.1, CH	6″	60.9, CH_2	67.5, CH_2	60.3, CH_2
8	44.3, CH	43.1, CH	44.2, CH	1‴	103.8, CH	101.0, CH	101.1, CH
9	174.4, C	177.8, C	174.4, C	2‴	71.0, CH	72.4, CH	72.6, CH
3/5-OCH_3	55.9	55.9	55.9	3‴	72.7, CH	73.5, CH	72.0, CH
4-OCH_3	60.0	60.0	60.0	4‴	67.2, CH	69.8, CH	79.7, CH
1′	131.2, C	132.1, C	131.2, C	5‴	65.3, CH_2	73.2, CH	71.8, CH
2′	108.2, CH	105.9, CH	108.0, CH	6‴		60.8, CH_2	60.7, CH_2
3′	146.6, C	145.5, C	146.6, C	1⁗		103.1, CH	101.8, CH
4′	146.9, C	146.2, C	146.9, C	2⁗		73.2, CH	72.9, CH
5′	109.1, CH	107.3, CH	109.0, CH	3⁗		76.2, CH	73.5, CH
6′	131.9, C	132.2, C	131.8, C	4⁗		69.9, CH	69.9, CH
7′	78.1, CH	76.6, CH	78.1, CH	5⁗		76.6, CH	73.2, CH
8′	38.7, CH	42.1, CH	38.8, CH	6⁗		60.9, CH_2	60.8, CH_2
9′	71.0, CH_2	68.9, CH_2	71.0, CH_2				

73.5, 69.9, 73.2, 60.8. The aglycone was identified as podophyllotoxin by comparing its NMR data with those reported in the literature [5], combined with correlations observed in the NOESY, HSQC, and HMBC spectra. The spin-spin coupling constants (7.7 Hz, 3.8 Hz, 3.8 Hz) of the three anomeric protons identified one *β*-glucopyranosyl group and two *α*-glucopyranosyl groups [10]. The presence of D-glucose was confirmed using the same methods as for compound **7**. Further structural elucidation was provided by HMBC cross-peaks (Fig. 2): the anomeric proton at *δ* 4.33 (1H, d, *J* = 7.7 Hz, H-1″) correlated with C-7′ (*δ* 78.1), the anomeric proton at *δ* 5.00 (1H, d, *J* = 3.8 Hz, H-1‴) correlated with C-4″ (*δ* 79.5), and the anomeric proton at *δ* 5.05 (1H, d, *J* = 3.8 Hz, H-1⁗) correlated with C-4‴ (*δ* 79.7). These correlations indicated that the *α*-D-glucopyranosyl-(1→4)-*α*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside fragment was linked to C-7′ of the aglycone.

The $J_{H\text{-}8/H\text{-}8'}$ (14.4 Hz), $J_{H\text{-}7'/H\text{-}8'}$ (9.6 Hz) and $J_{H\text{-}7/H\text{-}8}$ (4.8 Hz) values and the NOE correlations of H-7/H-7′ and H-8/H-7′ indicated that **8** possessed the same relative configuration as podophyllotoxin [5]. The ECD spectrum of compound **8** exhibited a positive Cotton effect at 289 nm, establishing the absolute configuration of C-7 as *R*. Thus, compound **8** was identified as podophyllotoxin 7′-*O*-*α*-D-glucopyranosyl-(1→4)-*α*-D-glucopyranosyl-(1→4)-*O*-*β*-D-glucopyranoside, and it was named diphylignan H.

Compound **9** was isolated as a white amorphous powder, and its molecular formula was determined to be $C_{27}H_{32}O_{12}$ based on HR-ESI-MS analysis (*m/z* 571.1785 $[M + Na]^+$, Calcd. for $C_{27}H_{32}O_{12}Na$, 571.1791). The ^1H NMR spectrum (Table 1) revealed two methoxy groups at *δ* 3.72 (3H, s) and 3.65 (3H, s), a 1,2,4,5-tetrasubstituted benzene ring at *δ* 6.78 (1H, s) and 6.93 (1H, s), a 1,3,4-trisubstituted benzene ring at *δ* 6.75 (1H, d, *J* = 1.5 Hz), 6.74 (1H, d, *J* = 8.0 Hz), and 6.61 (1H, dd, *J* = 8.0, 1.5 Hz), and a methylenedioxy group at *δ* 5.941 (1H, s) and 5.942 (1H, s). The ^{13}C NMR spectrum (Table 2) displayed signals for a carbonyl group at *δ* 178.5, twelve aromatic carbons, and five aliphatic carbons at *δ* 26.8, 45.3, 37.1, 40.6, and 70.3. Additionally, it showed signals for two methoxy groups at *δ* 56.0 and 55.4, a methylenedioxy group at *δ* 100.7, and a glucopyranosyl group at *δ* 102.3, 73.4, 76.8, 70.6, 77.6, and 61.1. The aglycone was identified as bursehernin by comparing its NMR and ECD data with those reported in the literature [11], supported by data from HSQC, HMBC, ^1H-^1H correlation spectroscopy (COSY), and NOESY experiments. The ^{13}C NMR chemical shift at *δ* 102.3 and the spin-spin coupling constant (7.3 Hz) of the anomeric proton confirmed the presence of a *β*-glucopyranosyl moiety. The presence of D-glucose was verified by microhydrolysis and HPLC analysis [6]. The HMBC cross-peak (Fig. 2) of the anomeric proton at *δ* 4.72 (1H, d, *J* = 7.3 Hz, H-1″) with C-2 (*δ* 150.1) indicated that the *β*-D-glucopyranosyl group was attached to C-2 of the aglycone.

The relative configuration was established through NOESY, with NOE correlations between H-7 (*δ* 2.80) and H-8' (*δ* 2.44) indicating a trans relationship for H-8/H-8'. The ECD spectrum of compound **9** was consistent with that of the 8*R*, 8′*R*-isomer wenchuanensin [12], thereby assigning the 8*R*, 8′*R*-configurations to compound **9**. Thus, compound **9** was identified as bursehernin 2-*O*-*β*-D-glucopyranoside and was named diphylignan I.

The known metabolites were identified by comparing their physical and spectroscopic data with literature values: epipicropodophyllotoxin (**10**) [9], 7-*O*-methylepipicropodophyllotoxin (**11**) [13], 3′,4′-demethylene-4-demethylpodopodophyllotoxin (**12**), sinolignan A (**13**) [5], Naphtho[2,3,d]-1,3-dioxole-6-carboxylic acid (**14**) [14], and podorhizol-7-*O*-*β*-D-glucopyranoside (**15**) [15].

All isolated compounds were evaluated for their *in vitro* cytotoxic activities against A-549 and SMMC-7721 cell lines using the MTS assay [16], with cisplatin, paclitaxel, and podophyllotoxin serving as positive controls. The IC_{50} values are summarized in Table 5. Most of the tested compounds exhibited moderate cytotoxic activities against both A-549 and SMMC-7721 cells. Notably, compounds **1**−**6**, **8**, **11**, and **13** demonstrated greater potency than cisplatin against the A-549 cell line, with compound **2** showing the highest activity against the SMMC-7721 cell line. Compounds with a *trans*-fusion between the tetralin and lactone moieties (**3**−**6**, **8**, and **13**) exhibited higher cytotoxicity compared to the corresponding *cis*-fusion analog (**7**). Furthermore, the ring E-opened compound (**14**) was found to be less potent than its lactone analog (**10**). Glycosylation of the 7′*α*-hydroxy group (as observed in compounds **5**, **6**, and **8**) significantly reduced

Table 5 Cytotoxicities of compounds 1−15 against A549 and SMMC-7721 cell lines (IC_{50}, μmol·L^{-1}, mean ± SD, *n* = 3)

No.	A549	SMMC-7721	No.	A549	SMMC-7721
1	15.52 ± 1.33	19.61 ± 1.78	**10**	35.27 ± 2.29	26.18 ± 2.50
2	10.27 ± 0.91	11.58 ± 1.36	**11**	25.99 ± 2.16	23.47 ± 2.01
3	13.74 ± 1.24	15.30 ± 1.41	**13**	12.40 ± 1.15	14.98 ± 0.92
4	14.58 ± 1.15	16.22 ± 1.37	**7, 9, 12, 14, 15**	> 40	> 40
5	14.39 ± 1.26	18.73 ± 1.69	Cisplatin	30.08 ± 0.95	12.15 ± 0.82
6	13.85 ± 0.96	17.52 ± 1.48	Paclitaxel	< 0.008	0.616 ± 0.060
8	17.58 ± 1.46	20.42 ± 2.05	Podophyllotoxin	0.137 ± 0.011	0.0092 ± 0.0005

cytotoxic activity. A preliminary structure-activity relationship (SAR) analysis suggested that the *trans*-fusion between the tetralin and lactone moieties, the absence of glycosylation at the 7′*α*-hydroxy group, and the presence of an intact lactone ring E are critical structural features for maintaining the cytotoxicity of podophyllotoxin analogs.

Experimental

General experimental procedures

Optical rotations were measured using a Rudolph AP-IV polarimeter (Rudolph, Hackettstown, NJ, USA), while ECD spectra were recorded with an Applied Photophysics Chirascanq CD spectropolarimeter (Applied Photophysics, Leatherhead, Surrey, UK). UV and IR spectra were obtained using a Thermo EVO 300 spectrometer and a Thermo Nicolet IS 10 spectrometer (Thermo, Waltham, MA, USA), respectively. NMR spectra were acquired on a Bruker Avance III 500 spectrometer, and mass spectra were recorded on a Bruker maXisHD mass spectrometer (Bruker, Germany). Preparative HPLC separations were performed on a SEP system (Beijing Sepuruisi Scientific Co., Ltd., China) equipped with a variable-wavelength UV detector, utilizing a YMC-Pack ODS-A column (250 mm × 20 mm, 5 μm). Chromatographic materials, including ODS (50 μm), Sephadex LH-20 (40−70 μm), and silica gel (200−300 mesh), were sourced from YMC Co., Ltd. (Kyoto, Japan), Amersham Pharmacia Biotech AB (Uppsala, Sweden), and Marine Chemical Industry (Qingdao, China), respectively. MCI gel CHP-20 was obtained from Mitsubishi Chemical Corp. (Tokyo, Japan), and AB-8 macroporous resin was acquired from Qinshi Science and Technology Ltd. (Zhengzhou, China). All chemical reagents used for isolation were of analytical grade and purchased from Tianjin Siyou Co., Ltd. (Tianjin, China). Biological reagents were sourced from Sigma-Aldrich (St. Louis, MO, USA).

Plant material

The roots and rhizomes of *Diphylleia sinensis* were collected in Wenchuan, Sichuan Province, China, in July 2019. The plant material was identified by Prof. DONG Chengming from the School of Pharmacy at Henan University of Chinese Medicine. A voucher specimen (DS 20190728) has been deposited at the herbarium of the same institution.

Extraction and Isolation

The powdered roots and rhizomes of *Diphylleia sinensis* (40 kg) were subjected to reflux extraction using 95% ethanol (120 L × 3, 1.5 h each) followed by 50% ethanol (120 L × 3, 1.5 h each) at 95 °C. The combined filtrates were evaporated under reduced pressure to yield a dark brown residue (5.2 kg). This residue was adsorbed onto siliceous earth and sequentially eluted with CH_2Cl_2, EtOAc, and MeOH.

The MeOH extract (1808 g) was fractionated using silica gel column chromatography (CC) with a gradient elution of CH_2Cl_2−MeOH (100 ∶ 0, 100 ∶ 1, 100 ∶ 3, 100 ∶ 5, 100 ∶ 7, 100 ∶ 10, 100 ∶ 30, 100 ∶ 50, 0 ∶ 100). Seven fractions (M1−M7) were collected based on TLC monitoring results. Fraction M4 (117.5 g) was further separated by Sephadex LH-20 CC using methanol, yielding subfractions M4-1−M4-3. Subfraction M4-2 (39.2 g) was subjected to silica gel CC with a CH_2Cl_2−MeOH gradient (100 ∶ 1, 50 ∶ 1, 30 ∶ 1, 20 ∶ 1, 10 ∶ 1, 3 ∶ 1), resulting in subfractions M4-2-1−M4-2-3. Subfraction M4-2-2 (4.6 g) was purified by preparative HPLC (MeOH ∶ H_2O, 52 ∶ 48) at a flow rate of 6 mL·min^{-1}, yielding subfractions M4-2-2-1 (t_R 8.8−10.3 min) and M4-2-2-2 (t_R 25.7 min). Subfraction M4-2-2-1 (1.8 g) was further purified by preparative HPLC (MeOH ∶ H_2O, 15 ∶ 85) at a flow rate of 6 mL·min^{-1}, yielding compounds **3** (t_R 42.8 min, 3.6 mg) and **4** (t_R 44.4 min, 3.6 mg). Subfraction M4-2-3 (1.4 g) was purified by preparative HPLC (MeOH ∶ H_2O, 48 ∶ 52) at a flow rate of 6 mL·min^{-1}, yielding compound **6** (t_R 46.2 min, 8.0 mg). Fraction M5 (120.6 g) was processed using AB-8 macroporous resin CC and eluted with an EtOH−H_2O gradient (0%, 10%, 30%, 50%, 60%, 70%, 90%), resulting in subfractions M5-1−M5-4. Subfraction M5-2 (32.8 g) was separated by MCI gel CC, eluted with MeOH−H_2O (10%, 30%, 50%, 70%), yielding subfractions M5-2-1−M5-2-3. Subfraction M5-2-2 (3.5 g) was purified by preparative HPLC (MeOH ∶ H_2O, 50 ∶ 50) at a flow rate of 6 mL·min^{-1}, yielding subfractions M5-2-2-1 (t_R 10.8 min), M5-2-2-2 (t_R 15.0 min), M5-2-2-3 (t_R 22.0 min), and compound **8** (t_R 32.6 min, 20.0 mg). Subfraction M5-2-2-2 (0.6 g) was further purified by preparative HPLC (MeOH ∶ H_2O, 40 ∶ 60) at a flow rate of 6 mL·min^{-1}, yielding compound **7** (t_R 28.1 min, 3.5 mg). Fraction M5-3 (27.1 g) was subjected to Sephadex LH-20 CC using methanol, yielding subfractions M5-3-1 and M5-3-2. Subfraction M5-3-2 (3.9 g) was purified by preparative HPLC (MeOH ∶ H_2O, 58 ∶ 42) at a flow rate of 6 mL·min^{-1}, yielding compounds **10** (t_R 24.5 min, 4.5 mg), **11** (t_R 54.5 min, 3.0 mg), and **14** (t_R 21.8 min, 3.7 mg).

The EtOAc extract (690 g) was subjected to silica gel CC using a gradient elution of CH_2Cl_2−MeOH (100 ∶ 0, 100 ∶ 1, 100 ∶ 3, 100 ∶ 5, 100 ∶ 7, 100 ∶ 10, 100 ∶ 30, 100 ∶ 50, 100 ∶ 100), resulting in fractions E1−E5. Fraction E3 (100.6 g) was further separated by ODS CC with a MeOH−H_2O gradient (10%, 30%, 50%, 70%), yielding subfractions E3-1−E3-3. Subfraction E3-1 (42.9 g) was subjected to Sephadex LH-20 CC eluted with methanol, producing subfractions E3-1-1−E3-1-3. Subfraction E3-1-1 (25.3 g) was further fractionated by silica gel CC using a PE-EtOAc gradient (100 ∶ 3, 100 ∶ 5, 100 ∶ 7, 100 ∶ 10, 100 ∶ 30, 100 ∶ 50, 100 ∶ 100, 0 ∶ 100), resulting in subfractions E3-1-1-1−E3-1-1-5. Subfraction E3-1-1-3 (4.9 g) was isolated by silica gel CC with a CH_2Cl_2−MeOH gradient (100 ∶ 1, 100 ∶ 3, 100 ∶ 5, 100 ∶ 7, 100 ∶ 10, 100 ∶ 30), producing subfractions E3-1-1-3-1−E3-1-1-3-8. Subfraction E3-1-1-3-4 (0.8 g) was purified by preparative HPLC (MeOH ∶ H_2O, 30 ∶ 70) at a flow rate of 6 mL·min^{-1}, yielding compound **12** (t_R 32.5 min, 2.2 mg). Subfraction E3-1-1-4 (0.7 g) was purified by preparative HPLC (MeOH ∶ H_2O, 50 ∶ 50) at a flow rate of 6 mL·min^{-1}, yielding subfraction E3-1-1-4-1 (t_R 13.9 min). Subfraction E3-1-1-4-1 (95.1 mg) was further purified by preparative HPLC (MeOH ∶ H_2O, 44 ∶ 56) at a flow rate of

6 mL·min^{-1}, yielding compound **2** (t_R 18.9 min, 3.0 mg). Subfraction E3-3 (24.0 g) was subjected to silica gel CC with a PE-EtOAc gradient (100 ∶ 3, 100 ∶ 5, 100 ∶ 7, 100 ∶ 10, 100 ∶ 30, 100 ∶ 50, 100 ∶ 100), resulting in subfractions E3-3-1−E3-3-7. Subfraction E3-3-4 (5.5 g) was further separated by Sephadex LH-20 CC eluted with methanol, yielding subfractions E3-3-4-1−E3-3-4-6. Subfraction E3-3-4-1 (1.3 g) was purified by preparative HPLC (MeOH : H_2O, 70 ∶ 30) at a flow rate of 3 mL·min^{-1}, yielding compounds **9** (t_R 16.9 min, 5.0 mg), **15** (t_R 20.8 min, 7.6 mg), **13** (t_R 26.7 min, 21.0 mg), and **5** (t_R 33.4 min, 3.6 mg). Subfraction E3-3-5 (2.8 g) was subjected to Sephadex LH-20 CC eluted with methanol, yielding subfractions E3-3-5-1−E3-3-5-7. Subfraction E3-3-5-3 (95.8 mg) was purified by preparative HPLC (MeOH : H_2O, 62 ∶ 38) at a flow rate of 3 mL·min^{-1}, yielding compound **1** (t_R 8.4 min, 35.3 mg).

Identification of new compounds

Diphylignan A (**1**): white, amorphous powder; $[\alpha]_D^{20}$ −67.2 (*c* 0.02, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 238 (+0.2), 272 (−0.1), 293 (+0.1) nm; UV (MeOH) λ_{max} (log ε) 203 (3.54), 242 (2.81), 283 (2.72) nm; IR (iTR) ν_{max} 3397, 2945, 2837, 1772, 1593, 1515, 1482, 1270, 1238, 1176, 1122, 1038 cm^{-1}; HR-ESI-MS (positive) *m/z* 569.1631 $[M + Na]^+$ (Calcd. for $C_{27}H_{30}O_{12}Na$, 569.1635); NMR data (DMSO-d_6) (Tables 1 and 2).

Diphylignan B (**2**): white, amorphous powder; $[\alpha]_D^{20}$ −64.2 (*c* 0.02, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 235 (+0.7), 277 (−0.6), 293 (+0.1) nm; UV (MeOH) λ_{max} (log ε) 203 (4.20), 238 (3.35), 284 (3.29) nm; IR (iTR) ν_{max} 3388, 2946, 2832, 1766, 1515, 1485, 1275, 1238, 1082, 1035 cm^{-1}; HR-ESI-MS (positive) *m/z* 555.1478 $[M + Na]^+$ (Calcd. for $C_{26}H_{28}O_{12}Na$, 555.1478); NMR data (DMSO-d_6) (Tables 1 and 2).

Diphylignan C (**3**): white, amorphous powder; $[\alpha]_D^{20}$ −38.5 (c 0.16, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 237 (+1.4), 275 (−1.2), 290 (+0.7) nm; UV (MeOH) λ_{max} (log ε) 206 (4.05), 242 (3.95), 284 (3.37) nm; IR (iTR) ν_{max} 3381, 2909, 1742, 1606, 1515, 1482, 1459, 1424, 1373, 1329, 1309, 1220, 1112, 1080, 1032 cm^{-1}; HR-ESI-MS (positive) *m/z* 657.1796 $[M + Na]^+$ (Calcd. for $C_{30}H_{34}O_{15}Na$, 657.1795), *m/z* 673.1528 $[M + K]^+$ (Calcd. for $C_{30}H_{34}O_{15}K$, 673.1535); NMR data (DMSO-d_6) (Tables 1 and 2).

Diphylignan D (**4**): white, amorphous powder; $[\alpha]_D^{20}$ −35.5 (*c* 0.14, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 236 (+1.7), 274 (−1.7), 290 (+1.1) nm; UV (MeOH) λ_{max} (log ε) 206 (4.67), 242 (4.09), 284 (3.56) nm; IR (iTR) ν_{max} 3386, 2905, 1742, 1606, 1515, 1482, 1459, 1424, 1373, 1330, 1220, 1115, 1081, 1037 cm^{-1}; HR-ESI-MS (positive) *m/z* 657.1796 $[M + Na]^+$ (Calcd. for $C_{30}H_{34}O_{15}Na$, 657.1795), *m/z* 673.1533 $[M + K]^+$ (Calcd. for $C_{30}H_{34}O_{15}K$, 673.1535); NMR data (DMSO-d_6) (Tables 1 and 2).

Diphylignan E (**5**): white, amorphous powder; $[\alpha]_D^{20}$ −67.8 (*c* 0.16, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 218 (+0.9), 273 (−0.2), 288 (+0.2) nm; UV (MeOH) λ_{max} (log ε) 203 (4.30), 239 (3.52), 284 (3.41) nm; IR (iTR) ν_{max} 3393, 2941, 2839, 1770, 1736, 1590, 1505, 1482, 1462, 1421, 1373, 1329, 1239, 1171, 1126, 1081, 1036 cm^{-1}; HR-ESI-MS (positive) *m/z* 713.2053 $[M + Na]^+$ (Calcd. for $C_{33}H_{38}O_{16}Na$, 713.2058); NMR data (DMSO-d_6) (Tables 1 and 2).

Diphylignan F (**6**): white, amorphous powder; $[\alpha]_D^{20}$ −38.5 (*c* 0.16, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 219 (+3.8), 273 (−1.1), 290 (+0.1) nm; UV (MeOH) λ_{max} (log ε) 206 (4.49), 243 (3.58), 291 (3.32) nm; IR (iTR) ν_{max} 3378, 2915, 2837, 1768, 1646, 1589, 1504, 1482, 1459, 1420, 1375, 1153, 1124, 1078, 1031 cm^{-1}; HR-ESI-MS (positive) *m/z* 731.2163 $[M + Na]^+$ (Calcd. for $C_{33}H_{40}O_{17}Na$, 731.2163), *m/z* 747.1893 $[M + K]^+$ (Calcd. for $C_{33}H_{40}O_{17}K$, 747.1903); NMR data (DMSO-d_6) (Tables 3 and 4).

Diphylignan G (**7**): white, amorphous powder; $[\alpha]_D^{20}$ +5.6 (*c* 0.02, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 211 (+5.1), 239 (+0.4), 289 (+1.2) nm; UV (MeOH) λ_{max} (log ε) 203 (3.85), 242 (2.75), 288 (3.32) nm; IR (iTR) ν_{max} 3383, 2920, 2851, 1767, 1593, 1505, 1464, 1424, 1377, 1240, 1126, 1078, 1031 cm^{-1}; HR-ESI-MS (positive) *m/z* 923.2805 $[M + Na]^+$ (Calcd. for $C_{40}H_{52}O_{23}Na$, 923.2797); NMR data (DMSO-d_6) (Tables 3 and 4).

Diphylignan H (**8**): white, amorphous powder; $[\alpha]_D^{20}$ +16.1 (*c* 0.11, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 203 (−17.2), 220 (+2.8), 273 (−0.6), 289 (+0.2) nm; UV (MeOH) λ_{max} (log ε) 205 (4.78), 241 (3.68), 291 (3.57) nm; IR (iTR) ν_{max} 3347, 2921, 2834, 1768, 1589, 1504, 1482, 1458, 1419, 1373, 1330, 1237, 1194, 1149, 1122, 1075, 1019 cm^{-1}; HR-ESI-MS (positive) *m/z* 923.2804 $[M + Na]^+$ (Calcd. for $C_{40}H_{52}O_{23}Na$, 923.2797); NMR data (DMSO-d_6) (Tables 3 and 4).

Diphylignan I (**9**) white, amorphous powder; $[\alpha]_D^{20}$ −71.6 (c 0.05, MeOH); ECD (MeOH) λmax ($\Delta\varepsilon$) 207 (– 4.2), 233 (– 3.2), 284 (– 0.5) nm; UV (MeOH) λmax (log ε) 202 (4.72), 231 (3.94), 286 (3.72) nm; IR (iTR)ν_{max} 3386, 2937, 2909, 2834, 1756, 1611, 1515, 1490, 1445, 1245, 1221, 1220, 1100, 1076, 1037 cm^{-1}; HR-ESI-MS (positive) *m/z* 571.1785 $[M + Na]^+$ (Calcd. for $C_{27}H_{32}O_{12}Na$, 571.1791); NMR data (DMSO-d_6) (Tables 1 and 2).

Acid hydrolysis and sugar determination

Compound **6** (1.5 mg) was dissolved in 0.05 mol·L^{-1} H_2SO_4 (1.0 mL) and heated under reflux at 90 °C for 3 h. After cooling, the reaction mixture was diluted with 5.0 mL of water and extracted with 10.0 mL of CH_2Cl_2. The aqueous layer was neutralized with a saturated $Ba(OH)_2$ solution, and the resulting precipitate was filtered off. The filtrate was evaporated to dryness, and the residue was dissolved in 1.0 mL of pyridine. This solution was heated with L-cysteine methyl ester (1.5 mg) at 60 °C for 1.5 h. Subsequently, *O*-tolyl isothiocyanate (15.0 μL) was added to the mixture and heated at 60 °C for an additional 1.5 h. The reaction mixture was then analyzed by HPLC using an Asahipak NH_2P-50 4E column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of CH_3CN−0.2% CF_3COOH (30 ∶ 70), with a flow rate of 3 mL·min^{-1}. UV detection was performed at 250 nm. Peaks at retention times of 18.63 and 21.70 min were identified as D-glucose and L-arabinose, respectively. Compounds

1−**5** and **7**−**9** were also hydrolyzed, and the presence of D-glucose was confirmed by comparing the retention times with those of authentic D-glucose samples [6].

Cytotoxicity assay

Tumor cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. The cytotoxic activities of compounds **1**−**15** were assessed against human lung cancer (A-549) and hepatocellular carcinoma (SMMC-7721) cell lines using the MTS assay as previously described [16]. IC_{50} values were calculated using the Reed-Muench method. All experiments were conducted in triplicate.

Supporting Information

Supporting information of this paper can be requested by sending E-mails to the corresponding authors.

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