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Phenylpropanoids from *Phragmipedium calurum* and their antiproliferative activity

Courtney M. Starks[*], **Russell B. Williams**, **Vanessa L. Norman**, **Julie A. Lawrence**, **Mark O'Neil-Johnson**, and **Gary R. Eldridge**
Lead Discovery and Rapid Structure Elucidation Group, Sequoia Sciences, Inc., 1912 Innerbelt Business Center Drive, St. Louis, MO 63114, United States

Abstract

Two new and five known stilbenes and one new alkylresorcinol were isolated from the orchid *Phragmipedium calurum* during a screen for new anticancer compounds. The compounds were evaluated for antiproliferative activity against multiple human cancer cell lines. Two of the compounds (**1** and **7**) displayed moderate activity against several cell lines.

Keywords

Phragmipedium calurum; Orchidaceae; stilbene; alkylresorcinol; cytotoxicity; cancer

1. Introduction

Cancer comprises over 100 different diseases and affects more than 13 million people in the US alone (Malakoff, 2011; Mariotto et al., 2011). Great strides have been made in combating cancer over the last 40 years, and newer targeted therapies have extended survival times and avoided the toxicity of earlier chemotherapy drugs. Unfortunately, many types of cancer do not yet have adequate treatments, and many tumors that initially respond to targeted therapy eventually become resistant (Kaiser, 2011). Thus, it is clear that there is still a need for new cancer drugs.

As part of an effort to discover new compounds as leads for cancer drugs, we screened a portion of our existing library of purified plant natural products for antiproliferative activity against NCI-H460 lung cancer cells. One of the hits from this screen was the stilbene **7**, which we had previously isolated from orchid species of the genus *Phragmipedium* (Garo et al., 2007). A related compound that had been isolated from *Phragmipedium* at the same time was also found to be active in this assay, but lack of material prohibited full structure determination. In order to further examine the bioactivity of these compounds, we carried out a larger scale isolation. This larger isolation, along with more sensitive NMR technology, allowed us to establish the structure of the minor compound as the new compound **1**. The re-isolation also yielded two more new compounds (**2**, **3**) as well as

[*]Corresponding author. Tel.: +001 314 373 5181 ext. 107; fax: +001 314 373 5186. cstarks@sequoiasciences.com.

additional material for compounds **4**–**8**. Structure determination of the new compounds and assessment of all the compounds' antiproliferative activity is presented here.

2. Results and discussion

The molecular formula of **1** was determined to be $C_{22}H_{20}O_3$ based on HRESIMS. The ^{1}H NMR spectrum of **1** (Table 1) matched that of the previously isolated active compound, confirming the re-isolation. Like its isomer **5** (Garo et al., 2007), it displayed resonances for an unsubstituted phenyl ring (δ_H 7.52, 7.33, 7.22), a 4-hydroxybenzyl moiety (δ_H 7.06, 6.61, 3.84), two *meta*-coupled aromatic protons (δ_H 6.66, 6.67), a methoxyl group (δ_H 3.83), and a *trans*-double bond (δ_H 7.04, 7.08; J = 16 Hz). Compound **1** thus appeared to have the same chemical moieties as **5** but with different connectivity. Because only a small amount of **1** (120 μg) was available, obtaining a complete 2D NMR data set was facilitated by using the Bruker BioSpin TCI 1.7 mm MicroCryoProbe. HMBC correlations from the H-1″ methylene protons (δ_H 3.84) to two oxygenated carbons C-5′ (δ_C 157.0) and C-3′ (δ_C 160.0) placed the 4-hydroxybenzyl group at the 4′-position on the central aromatic ring. Chemical shifts and COSY and HMBC correlations (Table 1) indicated that the rest of the molecule was the same as **5**. In particular, the chemical shifts of C-2′ (δ_C 101.8) and C-6′ (δ_C 107.4) indicated that each of these carbons was adjacent to an oxygenated carbon. HMBC correlations from H-2′ and H-6′ to C-8 (δ_C 130.0) supported the placement of the stilbene linkage. Chemical shifts of C-3′ and C-5′ were assigned based on HMBC correlations from H-2′ to C-3′ (δ_C 160.0) and from H-6′ to C-5′ (δ_C 157.0). The methoxyl group was then placed at C-3′ based on an HMBC correlation from the methoxyl protons to C-3′.

HRESIMS indicated a molecular formula of $C_{23}H_{22}O_4$ for **2**. Its ^{1}H NMR spectrum (Table 1) also resembled that of **5**. The most notable difference was the loss of symmetry in the phenyl ring; the coupling pattern for the protons of this ring indicated substitution at the 2-position. HMBC correlations from H-3 (δ_H 6.79), H-6 (δ_H 7.35), and H-7 (δ_H 7.26) to C-2 (δ_C 156.0) confirmed oxygenation at this position. HMBC correlations from H-1′ (δ_H 3.99) to C-1′ and C-3′ indicated attachment of the 4-hydroxybenzyl group to C-2′ in the central ring, between the stilbene linker and an oxygenated position. The *meta*-coupling of H-4′ and H-6′ (J = 2.5 Hz) and the chemical shifts of C-4′ (δ_C 95.5) and C-6′ (δ_C 102.2) required that the remaining oxygen on the central ring be placed at C-5′. Finally, the ^{1}H NMR spectrum indicated that **2** contains two methoxyl groups (δ_H 3.81, 3.85). These were placed at C-3′ and C-5′, respectively, based on HMBC correlations from the methoxyl protons to C-3′ (δ_C 159.8) and C-5′ (δ_C 160.2).

The ^{1}H NMR spectrum of **3** (Table 2) was markedly different than those of the other compounds. The aromatic region contained only two signals: δ_H 6.15 (2H, H-4 and H-6) and δ_H 6.12 (1H, H-2). The chemical shifts, integration, and coupling constants (J = 2.1 Hz) of these protons, as well as the chemical shifts of the corresponding carbons (δ_C 108.8 and 101.5, respectively) indicated a symmetric phenyl ring oxygenated at positions 3 and 5 (according to the atom numbering shown for **3**). The ^{1}H spectrum also indicated the presence of an alkyl chain, an oxygenated methine (δ_H 5.00, H-2′), and an acetate (δ_H 1.98). HMBC correlations from the H-1′ methylene protons at δ_H 2.63 and 2.69 to C-5 and C-4/6 placed this methylene adjacent to the phenyl ring. The COSY and HMBC NMR spectra were used to trace the remainder of the alkyl chain, and placed the oxygenated carbon at C-2′. Placement of the acetate at C-2′ was confirmed by HMBC correlations from the acetate protons and H-2′ to the ester carbonyl at δ_C 172.6. The length of the alkyl chain is consistent with the molecular formula of $C_{17}H_{26}O_4$ established by HRESIMS.

Compound **3** is classified as an alkylresorcinol. Alkylresorcinols have been found in bacteria, fungi, and plants, and have been shown to have antimicrobial, antiparasitic, and cytotoxic activities (Kozubek and Tyman, 1999). Plant alkylresorcinols tend to have longer aliphatic chains (13–27 carbons) with one or more unsaturations (Baerson et al., 2010); however, short, saturated alkylresorcinols similar to **3** have been identified from *Ononis natrix* (Fabaceae) (Cañedo et al., 1997). Alkylresorcinol synthases (ARS) and stilbene synthases (STS) are closely related Type III polyketide synthase enzymes (Cook et al., 2010). Each catalyzes the successive addition of multiple two-carbon units to a starter molecule, followed by intramolecular cyclization and aromatization of the resulting ketide chain. ARS and STS differ in their starting molecules; STS uses *p*-coumaroyl-CoA as a starter, while ARS enzymes use a fatty acid-derived CoA starter. The structure of **3** is consistent with a heptanoyl-CoA starter condensed with five two-carbon units and then cyclized; in this scenario, the oxygen at the 2′-position is derived from the ketone of the first two-carbon unit added (Funa et al., 2007).

Each of the isolated compounds was assayed for antiproliferative activity against a small panel of human cancer cell lines. The resulting IC_{50} values are listed in Table 3. Compounds **1** and **7** displayed moderate activity against several of the cell lines.

2.1. Concluding remarks

In this study, we explored the antiproliferative activity of seven stilbenes and one alkylresorcinol from the orchid *Phragmipedium calurum*. Five of the stilbenes are decorated with 4-hydroxybenzyl moieies, a somewhat unusual substituent for stilbenes (Garo et al., 2007). Compounds **1** and **7** were moderately active against one or more human cancer cell lines, and provide a starting point for medicinal chemistry efforts and mechanistic studies.

3. Experimental

3.1. General experimental procedures

NMR spectra were acquired at 600 MHz on a Bruker Avance 600 MHz spectrometer equipped with a Bruker BioSpin TCI 1.7 mm MicroCryoProbe. For each compound, ^{1}H, gCOSY, ROESY, gHSQC, and gHMBC spectra were acquired; ^{13}C chemical shifts were obtained from the HSQC and HMBC spectra. Spectra were recorded in CD_3OD, and chemical shifts are reported with respect to the residual non-deuterated solvent signal. HRESIMS was done on a Waters LCT time-of-flight mass spectrometer with an electrospray interface and polyethylene glycol as the internal standard. The amount of each compound isolated was determined using HPLC/ELSD as previously described (Hu et al., 2005). Semipreparative HPLC was performed on a Beckman HPLC system including a Beckman 168 diode array UV detector, a Sedere Sedex 75 ELSD detector, and an ISCO Foxy Jr. fraction collector. A splitter was used to divert 10% of the eluent to the ELSD detector, while the rest passed through a diode array UV detector and was then collected. UV λ_{max} values were taken from the diode array detector during semipreparative HPLC purification in 45–50% CH_3CN with 0.05% TFA. Optical rotation was measured on Jasco P-1010 polarimeter using a 100 μL cell with a 0.1 dm path length.

3.2. Plant material

P. calurum was grown in the greenhouse at the Missouri Botanical Garden (USA), and sent frozen to Sequoia, where it was lyophilized. A voucher specimen of *P. calurum* (Naibi 23) is kept at the Missouri Botanical Garden.

3.3 Extraction and isolation

P. calurum (60 g dry weight) was extracted with EtOH/EtOAc (1:1, v/v) to obtain 8 g of dry extract. The extract was then subjected to silica gel CC (5 × 28 cm) with the following eluents (500 ml each): hexane-EtOAc (3:1), hexane-EtOAc (1:1), EtOAc, EtOAc-MeOH (7:3), and EtOAc-MeOH (1:1). Three to four fractions were collected during elution with each solvent. The second fraction from the hexane-EtOAc (1:1) elution was dried down and then further purified using a C18 solid phase extraction (SPE) column (5 g) equilibrated in 50% MeOH and eluted with MeOH (30 ml). The SPE product (250 mg) was then further fractionated using preparative HPLC on a Betasil C_{18} column (Thermo Scientific, 21.2 × 100 mm, 5 μm, 3 × 83 mg injections) with a flow rate of 20 ml/min. The column was eluted with 60% CH_3CN for 2 min, followed by a 34 min gradient to 85% CH_3CN, then 85% CH_3CN for 6 minutes; the HPLC solvents contained 0.05% TFA. Fractions (one minute each) were collected from 2–42 min.

Preparative HPLC fraction 3 from the three runs was pooled and fractionated by semipreparative HPLC on an Atlantis C_{18} column (Waters, 10 × 250 mm, 5 μm) eluted at 3 ml/min with 45% CH_3CN for 5 min, followed by a gradient to 50% CH_3CN over 60 min. Twenty-five injections afforded 5.4 mg of **4** (t_R = 44.3 min), 2.1 mg of **6** (t_R = 48.1 min), 152 μg of **7** (t_R = 56.2 min), and 169 μg of **2** (t_R = 57.3 min). Preparative HPLC fraction 4 from the three runs was pooled and fractionated using the same semipreparative HPLC column using an isocratic elution with 47% CH_3CN for 64 min. Seven injections afforded 476 μg of **8** (t_R = 40.4 min), 202 μg of **5** (t_R = 44.0 min), 347 μg of **3** (t_R = 47.7 min), and 120 μg of **1** (t_R = 59.2 min). All semipreparative HPLC solvents contained 0.05% (v/v) TFA.

3.4 Cytotoxicity assay

NCI-H460 (large cell lung carcinoma), NCI-H226 (lung squamous cell carcinoma), NCI-H522 (non-small lung adenocarcinoma), and PC-3 (prostate adenocarcinoma) cells were obtained from ATCC. M14 (amelanotic melanoma) and A549 (non-small lung adenocarcinoma) cells were obtained from the National Cancer Institute. Cells were grown in RPMI-1640 with 10% FBS supplemented with L-glutamine and HEPES. Cells were seeded into 96-well plates at 5×10^2 to 5×10^4 cells/well and allowed to adhere overnight; the medium was then removed. A stock solution of test compound in DMSO was diluted in medium to generate a series of working solutions. Aliquots (100 μl) of the working solutions were added to the appropriate test wells to expose cells to the final concentrations of compound in a total volume of 100 μl. Eight different concentrations were tested, with 2–5 wells per concentration.

Camptothecin was used as a positive control; wells containing vehicle without compound were used as negative controls. Plates were kept for 72 h in a 37° C, 5% CO_2 incubator. After incubation, viable cells were detected with the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega). Dose-response curves were generated and IC_{50} values were determined using GraphPad Prism 5 software.

3.5 (E)-5′-hydroxy-4′-(4-hydroxybenzyl)-3′-methoxystilbene (1)

UV λ_{max} 223, 317 nm; ^1H and ^{13}C NMR: see Table 1; HR-ESIMS *m/z* 333.1502 $[M+H]^+$ (calcd for $C_{22}H_{21}O_3$: 333.1491).

3.6 (E)-2-hydroxy-2′-(4-hydroxybenzyl)-3′,5′-dimethoxystilbene (2)

UV λ_{max} 217, 235, 286, 324 nm; ^1H and ^{13}C NMR: see Table 1; HR-ESIMS *m/z* 363.1567 $[M+H]^+$ (calcd for $C_{23}H_{23}O_4$: 363.1596).

3.7 5-(2-acetoxynonyl)resorcinol (3)

$[\alpha]_D^{25}$ −223° (*c* 0.10, $CHCl_3$); UV λ_{max} 209, 223, 279 nm; ^1H and ^{13}C NMR: see Table 2; HR-ESIMS *m/z* 295.1916 $[M+H]^+$ (calcd for $C_{17}H_{27}O_4$: 295.1909).

Acknowledgments

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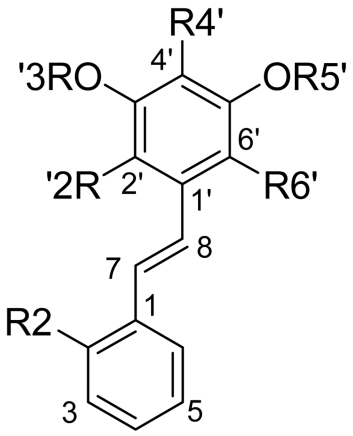
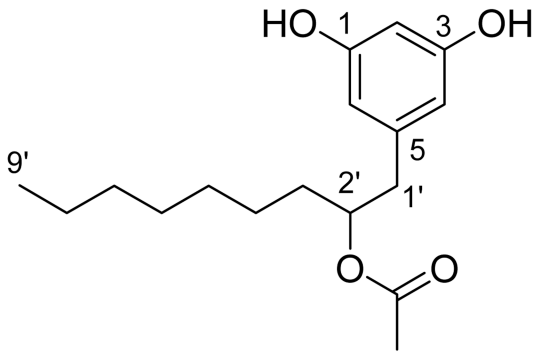
Highlights

- Two stilbenes and an alkylresorcinol were isolated from *Phragmipedium calurum*.
- These compounds were found during a screen for new anticancer agents.
- Two of the compounds (**1** and **7**) displayed moderate antiproliferative activity.

Structure Block

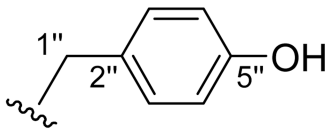




3

1 R2 = R2' = R5' = R6' = H; R3' = CH_3; R4' = **A**
2 R2 = OH; R2' = **A**; R3' = R5' = CH_3; R4' = R6'= H
4 R2 = R2' = R4' = R5' = R6' = H; R3' = CH_3
5 R2 = R2' = R4' = R5' = H; R3' = CH_3; R6' = **A**
6 R2 = R4' = R5' = R6' = H; R2' = **A**; R3' = CH_3
7 R2 = R4' = R5' = H; R2' = R6' = **A;** R3' = CH_3
8 R2 = OH; R2' = R4' = R6' = H; R3' = R5' = CH_3

A =



1. .

Table 1

NMR Spectroscopic Data (600 MHz, CD3OD) for **1** and 2

	1			**2**		
position	**δ_C**	**δ_H (*mult*, *J* in Hz)**	**HMBC[a]**	**δ_C**	**δ_H (*mult*, *J* in Hz)**	**HMBC[a]**
1	138.5		3/5, 8	125.5		8
2	127.4	7.52 (*d*, 7.7)	4, 7	156.0		4, 6, 7
3	129.6	7.33 (*t*, 7.5)	3/5	116.3	6.79 (*d*)[b]	5
4	128.4	7.22 (*t*, 7.3)	2/6	129.0	7.05 (*td*, 7.8, 1.3)	6
5	129.6	7.33 (*t*, 7.5)	3/5	120.4	6.79 (*t*)[b]	3
6	127.4	7.52 (*d*, 7.7)	4, 7	127.4	7.35 (*dd*, 8.1, 1.3)	4, 7
7	129.5	7.08 (*d*, 16.0)	2/6	126.4	7.26 (*d*, 16.2)	6
8	130.0	7.05 (*d*, 16.0)	2•, 6•	127.2	7.41 (*d*, 16.2)	7, 6•
1•	138.5		7	139.9		7, 1••
2•	101.8	6.67 (*br s*)	6•	121.4		8, 4•, 6•, 1••
3•	160.0		2•, 1••, OCH_3-3•	159.8		1••, OCH3-3•
4•	117.7		2•, 6•, 1••	98.5	6.50 (*d*, 2.5)	6•
5•	157.0		6•, 1••	160.2		6•, OCH3-5•
6•	107.5	6.66 (*br s*)	8, 2•	102.2	6.83 (*d*, 2.5)	8, 4•
1••	28.6	3.84 (*s*)	3••/7••	30.3	3.99 (*s*)	3••/7••
2••	133.9		1••, 4••/6••	133.7		1••, 4••/6••
3••/7••	130.5	7.06[b]	1••, 3••/7••	129.8	6.97 (*d*, 8.4)	1••, 3••/7••
4••/6••	115.5	6.61 (*d*, 8.5)	4••/6••	115.5	6.63 (*d*, 8.4)	4••/6••
5••	155.6		3••/7••	155.8		3••/7••
OCH3-3•	56.0	3.83 (*s*)		55.7	3.81 (*s*)	
OCH3-5•				55.3	3.85 (*s*)	

[a] HMBC correlations are from the proton(s) listed to the indicated carbon.

[b] Signal partially obscured.

Table 2

NMR Spectroscopic Data (600 MHz, CD_3OD) for 3

	3		
position	**δ_C**	**δ_H (*mult*, *J* in Hz)**	**HMBC[a]**
1/3	159.0		2, 4/6
2	101.5	6.12 (*t*, 2.1)	4/6
4/6	108.8	6.15 (*d*, 2.1)	2, 4/6, 1•
5	141.1	1•	
1•	41.3	2.63 (*dd*, 13.5, 6.2)	2•, 3•
		2.69 (*dd*, 13.5, 7.0)	
2•	76.2	5.00 (*m*)	1•, 3•
3•	34.3	1.54 (*m*)	1•, 2•
4•	25.9	1.30 (*m*) [b]	2•, 3•
5•	30.0	1.30 (*m*) [b]	
6•	30.0	1.30 (*m*) [b]	
7•	32.7	1.30 (*m*) [b]	9•
8•	23.5	1.27 (*m*) [b]	9•
9•	14.2	0.89 (*t*, 7.0)	
OAc-2•	20.7	1.98 (*s*)	
	172.6		2•, OAc-2•

[a] HMBC correlations are from the proton(s) listed to the indicated carbon.

[b] Signal partially obscured.

Table 3

IC_{50}s against human cancer cell lines (μM)

Compound	NCI-H460	NCI-H226	NCI-H522	A549	M14	PC3
1	1.3 ± 0.7	8.5 ± 0.8	8.5 ± 0.9	5.5 ± 0.4	2.2 ± 0.2	4.1 ± 0.4
2	> 10	> 10	9 ± 1	> 10	> 10	> 10
3	> 10	> 10	> 10	9 ± 1	> 10	> 10
4	> 10	> 10	> 10	>10	> 10	> 10
5	7.8 ± 0.4	> 10	> 10	> 10	> 10	> 10
6	> 10	> 10	> 10	> 10	> 10	> 10
7	2.0 ± 0.8	> 10	> 10	> 10	3 ± 1	5 ± 2
8	8.1 ± 0.7	> 10	> 10	> 10	> 10	> 10
camptothecin	0.003	0.05	0.2	0.01	0.01	0.05