

# Englerin A, a Selective Inhibitor of Renal Cancer Cell Growth, from *Phyllanthus engleri*

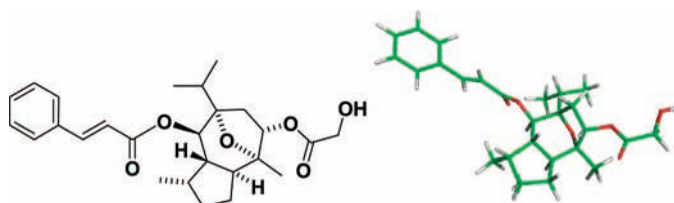
Ranjala Ratnayake,<sup>†</sup> David Covell,<sup>‡</sup> Tanya T. Ransom,<sup>†</sup> Kirk R. Gustafson,<sup>†</sup> and John A. Beutler<sup>\*,†</sup>

Molecular Targets Development Program, Center for Cancer Research, and Screening Technologies Branch, Developmental Therapeutics Program, National Cancer Institute, NCI–Frederick, Frederick, Maryland 21702

beutlerj@mail.nih.gov

Received October 9, 2008

## ABSTRACT



An extract from *Phyllanthus engleri* was identified in a bioinformatic analysis of NCI 60-cell natural product extract screening data that selectively inhibited the growth of renal cancer cell lines. Bioassay-guided fractionation yielded two new guaianes sesquiterpenes, englerins A (1) and B (2). Englerin A showed 1000-fold selectivity against six of eight renal cancer cell lines with GI<sub>50</sub> values ranging from 1–87 nM. The structures of 1 and 2 and their relative stereochemistry were established by spectroscopic methods.

Kidney cancer affects an estimated 39000 individuals in the USA yearly and is a major cause of morbidity and mortality in adults.<sup>1</sup> Currently approved drugs such as bevacizumab, sunitinib, and sorafenib offer benefit to patients with metastatic renal cancer but do not produce complete responses, require long-term administration for continued disease control, and have serious adverse side effects.<sup>1</sup> Thus, the search for new agents which display specific activity against renal cancers is of great interest.

With this in mind, an extensive study was carried out to identify natural product extracts that exhibited a preferential selectivity toward renal tumor cells in the NCI 60-cell panel when compared to the other eight organ panels in the screen (leukemia, nonsmall cell lung cancer, colon, CNS, melanoma,

ovarian, prostate, and breast). This analysis for renal panel selectivity identified an initial 34 natural product extracts (see the Supporting Information). The extract of *Phyllanthus engleri* was chosen because of its excellent selectivity and potency, relative to the other extracts, against the renal panel.

The genus *Phyllanthus* is one of the largest genera of the Euphorbiaceae, containing approximately 700 species. Like most other members of the Euphorbiaceae, *Phyllanthus* is a source of biologically active compounds, with numerous species being widely used in various traditional medicine systems.<sup>2–4</sup> *P. engleri* is found in East Africa, particularly Tanzania and Zimbabwe, and has not been subjected to chemical study in recent years. The present investigation was

<sup>†</sup> Center for Cancer Research.

<sup>‡</sup> Developmental Therapeutics Program.

(1) Atkins, B. A.; Ernstoff, M. S.; Figlin, R. A.; Flaherty, K. T.; George, D. J.; Kaelin, W. G.; Kwon, E. D.; Libermann, T. A.; Linehan, W. M.; McDermott, D. F.; Ochoa, A. C.; Pantuck, A. J.; Rini, B. I.; Rosen, M. A.; Sosman, J. A.; Sukhatme, V. P.; Vieweg, J. W.; Wood, C. G.; King, L. *Clin. Cancer Res.* **2007**, *13* (suppl 2), 667s.

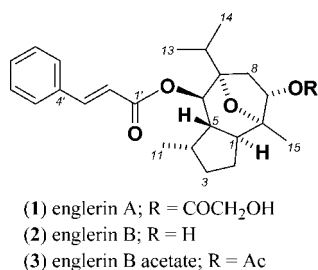
(2) Calixto, J. B.; Santos, A. R. S.; Filbo, V. C.; Yunes, R. A. *Med. Res. Rev.* **1998**, *18*, 225.

(3) Bagalkotkar, G.; Saad, M. S. S.; Stanslas, J. J. *Pharm. Pharmacol.* **2006**, *58*, 1559.

(4) (a) Webster, G. L.; Blumberg, B. S. *J. Ethnopharmacol.* **1990**, *30*, 233. (b) Webster, G. L.; Blumberg, B. S. *J. Ethnopharmacol.* **1991**, *34*, 97. (c) Webster, G. L.; Blumberg, B. S. *J. Ethnopharmacol.* **1992**, *36*, 103. (d) Webster, G. L.; Blumberg, B. S. *J. Ethnopharmacol.* **1995**, *45*, 1.

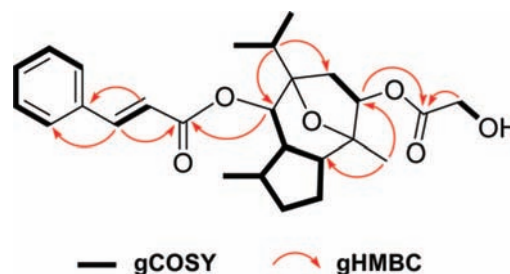
carried out on extracts from a collection made in Tanzania, where the roots and bark of this plant are reported to be lethal.<sup>5,6</sup> The only previously characterized compound isolated from *P. engleri* is the triterpene phyllanthol,<sup>7</sup> which has not been shown to possess biological activity. A poorly characterized toxic glycoside was also found by other early investigators.<sup>5</sup>

We report here the isolation of two new guaiane sesquiterpenes, englerins A (**1**) and B (**2**), isolated from the 1:1 CH<sub>2</sub>Cl<sub>2</sub>–MeOH extractives of the stem bark of *P. engleri*. Employing a two-cell assay consisting of one sensitive (A498 or UO-31) and one resistant (SF-295) cell line, we carried out bioassay-guided fractionation of the stem bark extract using an initial diol SPE separation. This identified the active components as being present in the CH<sub>2</sub>Cl<sub>2</sub> solubles. Successive fractionation using silica gel chromatography and C<sub>18</sub> HPLC led to the isolation of **1** as the major active compound (see the Supporting Information).



Englerin A (**1**) was isolated as a white solid with a pseudomolecular ion  $[M + H]^+$  consistent with a molecular formula (C<sub>26</sub>H<sub>35</sub>O<sub>6</sub>,  $\Delta = 1.7$  ppm) that required 10 double bond equivalents (DBE). COSY NMR data for **1** identified six isolated spin systems (Figure 1). The <sup>13</sup>C NMR spectrum (Table 1) revealed ten sp<sup>2</sup> carbons ( $\delta_C$  117.8–145.2) corresponding to four carbon–carbon double bonds and two carbonyl carbons. The deshielded resonances at  $\delta_H$  6.59 and 7.68 with a diagnostic coupling ( $J_{2',3'} = 16.0$  Hz) and  $\delta_H$  7.72 (2H, brd, 7.5 Hz) and  $\delta_H$  7.43 (3H, m) in the <sup>1</sup>H NMR spectrum were indicative of a *trans* double bond and a monosubstituted benzene ring, respectively. These signals together with the two additional deshielded sp<sup>2</sup> carbons ( $\delta_C$  172.4 and 165.2), consistent with ester carbonyls, accounted for 7 DBE, requiring the remaining elements to constitute a tricyclic system. A set of HMBC correlations from the *trans* double bond methines to the benzene ring and C-1' carbonyl ( $\delta_C$  165.2) confirmed the presence of a cinnamate side chain, while an HMBC correlation from the oxymethine signal at  $\delta_H$  4.97 to the C-1' carbonyl confirmed its substitution at C-6. An additional HMBC correlation from an hydroxymethylene ( $\delta_H$  4.04, brs) to the C-1'' carbonyl ( $\delta_C$  172.4) confirmed the glycolate fragment as shown in Figure 1.

Two of the three remaining COSY spin systems accounted for an isopropyl group and a methylene (H-8,  $\delta_{Ha}$  2.64 and  $\delta_{Hb}$  1.73) attached to an oxymethine (H-9,  $\delta_H$  5.15 and  $\delta_C$



**Figure 1.** Some key 2D correlations for englerin A (**1**).

74.4). The remaining COSY spin system involved three adjacent sp<sup>3</sup> methines connected via two methylenes to form a five-membered (H-1 to H-5) ring (Figure 1). Additional COSY correlations suggested the methyl substitution at H-4 and extended H-5 to the H-6 oxymethine ( $\delta_H$  4.97 and  $\delta_C$  72.6). Strong HMBC correlations from a methyl singlet to C-1 and C-9 and, likewise, from H-12 to C-6 and C-8 via two quaternary carbons ( $\delta_C$  84.4 and 84.8) defined the guaiane sesquiterpene core<sup>8</sup> incorporating the cinnamate and glycolate substituents as shown. The above data accounted for all but one oxygen atom and included nine of the 10 DBEs, necessitating the C-7 and C-10 connection through the unassigned oxygen atom to complete the planar structure of englerin A (**1**).

The <sup>1</sup>H and <sup>13</sup>C NMR data (Table 1) for englerin B (**2**) were very similar to those of **1** with common structure fragments confirmed by a sequence of diagnostic 2D NMR correlations. The key differences were observed in the upfield shift of the <sup>1</sup>H NMR resonance for H-9 ( $\delta_H$  3.92,  $\Delta = 1.2$  ppm) and in the absence of a methylene singlet around ~4.00 ppm with lack of a corresponding resonance in the <sup>13</sup>C NMR spectrum for one carbonyl resonance. HRMS analysis of compound **2** revealed a pseudomolecular ion  $[M + H]^+$  consistent with a molecular formula (C<sub>24</sub>H<sub>33</sub>O<sub>4</sub>,  $\Delta = 1.5$  ppm) requiring 9 DBE. These data supported englerin B lacking glycolate substitution at C-9. Acetylation of englerin B (**2**) with pyridine/acetic anhydride, followed by subsequent NMR analysis of **3**, revealed a methyl singlet ( $\delta_H$  2.05) and the expected downfield shift for H-9 ( $\delta_H$  5.09) in the <sup>1</sup>H NMR spectrum, further supporting a free hydroxyl group at this position in the parent molecule (**2**).

The vicinal coupling data along with selective 1D NOESY experiments for englerin A (**1**) allowed the relative stereochemistry to be assigned as follows. The coupling constant for H-6 ( $J_{5,6} = 10.0$  Hz) was indicative of a pseudodiaxial relationship between H-5 and H-6. Selective 1D NOESY experiments revealed strong correlations from H-4, H-8a, and H-9 to H-5, suggesting that they were on the same face of the molecule (Figure 2). The absence of any NOESY correlations from H-6 to H-4, H-5, H-8a, and H-9 in three

(5) Breyer-Brandwijk, M. G. *J. Pharm. Pharmacol.* **1934**, 7, 167.  
 (6) Verdourt, B.; Trump, E. C. In *Common Poisonous Plants of East Africa*; Collins: London, 1969; pp 58–59.  
 (7) Alberman, K. B.; Kipping, F. B. *J. Chem. Soc.* **1951**, 2296.

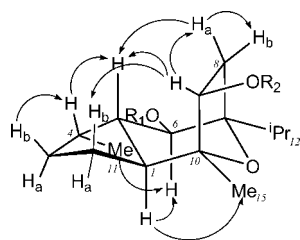
(8) (a) Ferreira, M. J. P.; Oliveira, F. C.; Rodrigues, G. V.; Emerenciano, V. P. *Internet Elec. J. Mol. Design* **2004**, 3, 737. (b) Stierle, A. A.; Stierle, D. B.; Goldstein, E.; Parker, K.; Bugni, T.; Baarson, C.; Gress, J.; Blake, D. *J. Nat. Prod.* **2003**, 66, 1097. (c) Peng, G.-P.; Tian, G.; Huang, X.-F.; Lou, F.-C. *Phytochemistry* **2003**, 63, 877.

**Table 1.** NMR (DMSO-*d*<sub>6</sub>) Data for Englerins A (**1**) and B (**2**)

no.	$\delta_{\text{H}}$ (m, <i>J</i> (Hz))	$\delta_{\text{C}}$	COSY	HMBC ( $^1\text{H}-^{13}\text{C}$ )	$\delta_{\text{H}}$ (m, <i>J</i> (Hz))	$\delta_{\text{C}}$
(1)				(2)		
1	1.66 (m)	47.1	H-5, H-2b	C-15, C-10, C-9, C-5, C-4, C-2	1.60 (m)	47.3
2a	1.62 (m)	24.2	H-3b, H-2b, H-1	C-4, C-1	1.60 (m)	24.4
2b	1.23 (m)		H-3a, H-3b, H-2a, H-1	C-15, C-10, C-9, C-3, C-1	1.12 (m)	
3a	1.93 (m)	30.6	H-4, H-3b, H-2b	C-11, C-4, C-2, C-1	1.87 (m)	30.7
3b	1.16 (m)		H-3a, H-2a	C-11, C-5, C-4, C-2	1.12 (m)	
4	2.04 (m)	30.7	H-11, H-5, H-3a	C-11, C-3, C-2, C-1	1.99 (m)	30.6
5	1.62 (m)	46.0	H-6, H-4, H-1	C-11, C-10, C-6, C-4, C-2, C-1	1.50 (m)	45.8
6	4.97 (d, 10.0)	70.6	H-5	C-12, C-8, C-7, C-5, C-1'	4.91 (d, 10.0)	71.1
7		84.8				84.1
8a	2.64 (dd, 14.5, 8.0)	39.5	H-9, H-8b	C-10, C-6	2.49 (dd, 13.5, 7.5)	42.5
8b	1.73 (dd, 14.5, 2.5)		H-9, H-8a	C-12, C-9, C-7, C-6	1.57 (m)	
9	5.15 (dd, 8.0, 2.5)	74.4	H-8a, H-8b	C-7, C-6	3.92 (brs)	70.8
10		84.4				85.0
11	0.85 (d, 7.5)	16.7	H-4	C-5, C-3	0.82 (d, 7.0)	16.8
12	1.79 (m)	32.7	H-14, H-13	C-14, C-13, C-8, C-7, C-6	1.75 (m)	32.9
13	0.94 (d, 7.0)	17.3	H-12	C-14, C-12, C-7	0.92 (d, 7.0)	17.4
14	0.88 (d, 7.0)	18.1	H-12	C-13, C-12, C-7	0.86 (d, 7.0)	18.3
15	1.10 (s)	18.8		C-10, C-9, C-1	1.11 (s)	19.4
1'		165.2				165.3
2'	6.59 (d, 16.0)	117.8	H-3'	C-9'/C-5', C-4', C-3', C-1'	6.57 (d, 16.0)	118.0
3'	7.68 (d, 16.0)	145.2	H-2'	C-9'/C-5', C-4', C-2', C-1'	7.66 (d, 16.0)	145.0
4'		133.9				134.0
5'	7.72 (brd, 7.5)	128.5	H-6'	C-9', C-7', C-4', C-3'	7.71 (m)	128.5
6'	7.43 (m)	129.0	H-5'	C-8', C-5', C-4', C-3'	7.42 (brdd, 3.5, 3.0)	129.0
7'	7.43 (m)	130.7	<sup>a</sup>	C-8'/C-6', C-9'/C-5'	7.42 (brdd, 3.5, 3.0)	130.6
8'	7.43 (m)	129.0	H-9	C-9', C-6', C-4', C-3'	7.42 (brdd, 3.5, 3.0)	129.0
9'	7.72 (brd, 7.5)	128.5	H-8'	C-7', C-5', C-4', C-3'	7.71 (m)	128.5
1''		172.4				
2''	4.04 (brs)	59.8		C-1''		
OH	5.36 (brs)				4.66 (brd, 4.0)	

<sup>a</sup> Overlapping signals prevented unambiguous assignments.

different solvents for both compounds **1** and **2** supported an *anti* relationship between H-5 and H-6 as proposed. However, the resolution of H-5, H-1, and H-2a was insufficient to unambiguously assign NOESY correlations from H-6. Fortunately, a strong NOESY correlation from H-9 to H-2b suggested that these protons were on the same face. The overlapping  $^1\text{H}$  NMR signals for H-1 and H-2a notwithstanding, a strong correlation from H-6 to the multiplet at  $\delta_{\text{H}}$  1.62–1.67 was in favor of H-2a/H-1 (Table 1) being in an  $\alpha$  orientation. Therefore with H-5 eliminated from (see above) the correlation observed was preferably for H-2a/H-

**Figure 2.** Some key selective 1D NOESY correlations observed for englerin A (**1**).

1. An energy minimized 3D model clearly favored a 1D NOESY correlation from H-6 to H-1 (2.7 Å) in preference to H-2a (4.9 Å) thereby supporting a *trans* ring junction with H-1 and H-5 both in pseudoaxial orientations.

Although the chemical shift for H-5 in DMSO-*d*<sub>6</sub> (Table 1) and methanol-*d*<sub>4</sub> (Table S1, Supporting Information) partly overlapped with H-1 and H-2a, the H-5 resonance was resolved ( $\delta_{\text{H}}$  1.60, ddd, *J* = 10.0, 7.0, 3.0 Hz) in deuterated methylene chloride. This provided a means to observe unambiguous correlations in the 1D NOESY experiments. The vicinal coupling ( $J_{1,5}$  = 7.0 Hz) was consistent with the proposed pseudodiaxial relationship between H-5 and H-1 protons. Similar patterns of 1D NOESY correlations were also observed for englerin B (**2**) in both deuterated DMSO and methylene chloride. Hence, we propose the relative stereochemistry for the englerins as depicted.

Englerin A demonstrated excellent selectivity for the renal cancer cell line panel, with 5 of 8 renal lines having  $\text{GI}_{50}$  values under 20 nM (Table 2, Figure 3), while for most other cell lines the  $\text{GI}_{50}$  values ranged from 10–20  $\mu\text{M}$  (see Supporting Information). The low activity and selectivity of the structural analogue englerin B (**2**) (see the Supporting Information), suggests that substitution at the C-9 position by the glycolate ester may be important for the observed

**Table 2.** Renal Cancer Cell Growth Inhibition Data (Mean GI<sub>50</sub> in  $\mu$ M) for Englerin A (**1**), Compared to Average Values for Taxol

renal cell line	<b>1</b>	Taxol
786-0	<0.01	0.034
A498	<0.01	0.10
ACHN	<0.01	0.65
CAKI-1	15.5	0.35
RXF-393	0.011	0.041
SN12C	0.087	0.018
TK-10	15.5	0.11
UO-31	<0.01	0.45

potency and selectivity. It is well-known that glycolic acid (GA), an important metabolite of ethylene glycol, causes acute renal toxicity in mammals.<sup>9</sup> Hydroxy acid containing natural products are relatively rare; some of the known GA-containing metabolites include pleuromutilin,<sup>10</sup> saframycin R,<sup>11</sup> and an ecdysteroid from a Caribbean sponge.<sup>12</sup> The NCI 60-cell data<sup>13</sup> for pleuromutilin indicated no significant cytotoxicity for this compound, while saframycin R, although quite potent, did not show renal selectivity in the NCI 60-cell panel.<sup>13</sup> Therefore, it appears that glycolate substitution alone cannot account for the renal selectivity of **1**. While we have not yet systematically determined which ester substitutions will enhance or reduce englerin activity, in a 2-cell assay englerin B acetate (**3**) showed an approximately 400-fold selectivity against the renal cell line (A498); 60-cell testing is pending to confirm this result. Encouraged by this observation, it is our intention to prepare a series of englerin analogues with different ester substitution at both C-6 and C-9 for biological evaluation.

(9) (a) Carney, E. W.; Freshour, N. L.; Dittenber, D. A.; Dryzga, M. D. *Toxicol. Sci.* **1999**, *50*, 117. (b) Bove, K. E. *Amer. J. Clin. Pathol.* **1966**, *45*, 46.

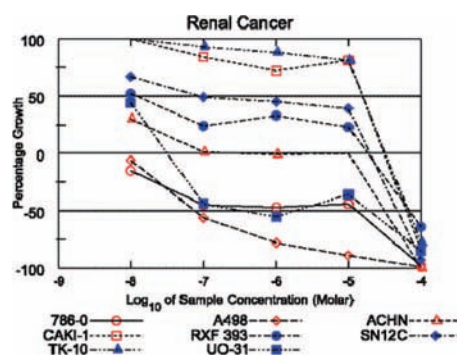
(10) Knauseder, F.; Brandl, E. *J. Antibiot.* **1976**, *29*, 125.

(11) Saito, N.; Kameyama, N.; Kubo, A. *Tetrahedron* **2000**, *56*, 9937.

(12) Costantino, V.; Dell'Aversano, C.; Fattorusso, E.; Mangoni, A. *Steroids* **2000**, *65*, 138.

(13) <http://dtp.nci.nih.gov/dtpstandard/cancerscreeningdata/index.jsp>.

(14) Paull, K. D.; Shoemaker, R. H.; Hodes, L.; Monks, A.; Scudiero, D. A.; Rubinstein, L.; Plowman, J.; Boyd, M. R. *J. Natl. Cancer Inst.* **1989**, *81*, 1088.



**Figure 3.** Dose-response curves for cytotoxic activity of englerin A (**1**) against the renal cancer cell lines in the NCI 60-cell panel.

A COMPARE analysis of the englerin 60 cell data against the NCI standard agents did not suggest a known mechanism of action. Preliminary mouse toxicity studies with **1** identified a maximum tolerated dose of 5 mg/kg i.p. in mice. Mouse xenograft studies of **1** are underway.

**Acknowledgment.** We thank the Missouri Botanical Garden collectors (Roy Gereau, James Lovett, C. O. Ky-alawa, and Z. H. Mbwambo) for the plant collection, Thomas McCloud (Natural Products Support Group, NCI-Frederick) for plant extraction, Melinda Hollingshead (Biological Testing Branch, DTP) for mouse toxicity studies, and Sergey Tarasov and Marzena Dyba (Biophysics Resource Core, Structural Biophysics Laboratory, CCR) for assistance with high-resolution mass spectrometry. This research was supported jointly by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research, and by the Developmental Therapeutics Program in the Division of Cancer Treatment and Diagnosis of the National Cancer Institute.

**Supporting Information Available:** Full details on the subpanel sensitivity selection of *P. engleri*, plant collection, isolation and spectral data, two-cell assay methodology, and NCI60 cell data for **1** and **2**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

OL802339W