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Cytotoxic and Other Compounds from *Didymochlaena truncatula* from the Madagascar Rain Forest

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Bioassay-guided fractionation of the EtOH extracts obtained from a plant identified as *Didymochlaena truncatula* led to the isolation of two cytotoxic alkaloids, camptothecin and 9-methoxycamptothecin. A second plant collection yielded three lignan derivatives, didymochlaenone A (**1**), didymochlaenone B (**2**) and (–)-wikstromol, one stilbene, (*E*)-3-methoxy-5-hydroxystilbene, and two stigmaterol derivatives, stigmast-4-en-3 β -ol and stigmast-4-en-3-one, but no camptothecins, and it is probable that a coding error led to a mistaken identification of the original extract. The structures of the new compounds **1** and **2** were established on the basis of extensive interpretation of one and two dimensional NMR spectroscopic data.

In our continuing search for bioactive molecules from the Madagascar rainforests as part of an International Cooperative Biodiversity Group (ICBG) program,² we obtained a cytotoxic extract (MG 1736A) from the roots of a plant collected in forest adjacent to the Zahamena National Park and identified as *Didymochlaena truncatula* (Sw.) J. Sm (Dryopteridaceae) collected in Madagascar. This extract, with an IC₅₀ value of 3.9 μ g/mL, was selected for bioassay-guided fractionation based on its cytotoxicity against the A2780 human ovarian cancer cell line, and also on the absence of any previous detailed phytochemical studies on the genus. The crude extract furnished the two known active alkaloids camptothecin³ and 9-methoxycamptothecin^{3d} as the active constituents. A large recollection (MG 1736B) of *D. truncatula* was then obtained in an attempt to isolate other cytotoxic alkaloids analogous to camptothecin and 9-methoxycamptothecin, but surprisingly this collection did not contain detectable levels of camptothecins. Instead, four aromatic compounds and two stigmaterol derivatives were isolated as the weakly active constituents of this collection.

The second collected sample (MG 1736B) of *D. truncatula* was subjected to liquid-liquid partitioning to give a moderately active CH₂Cl₂ fraction with an IC₅₀ value of 16 μ g/mL in the A2780 assay. Activity-guided purification of this fraction by passage over a C18 open column and subjection of active fractions to further purification using C18 HPLC and PTLC led to the isolation of the two new compounds **1** and **2** and the four known compounds (–)-wikstromol,⁴ (*E*)-3-methoxy-5-hydroxystilbene,⁵ and the two stigmaterol derivatives, stigmast-4-en-3 β -ol⁶ and stigmast-4-en-3-one.⁷ Here we report the structures of the two new

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Supporting Information Available: ¹H NMR spectra for compounds **1**–**2** and the structures of all isolated compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

components, didymochlaenone A (**1**), didymochlaenone B (**2**), as well as the activity of the isolates.

Didymochlaenone A (**1**) was obtained as a colorless oil. Its positive HRFABMS revealed a pseudo-molecular ion $[(M+H)^+]$ consistent with the molecular formula $C_{20}H_{22}O_5$, requiring ten double-bond equivalents. The IR spectrum for **1** displayed a characteristic absorbance for an α,β -unsaturated ketone moiety (1653 cm^{-1}),⁸ while the UV spectrum exhibited absorbances compatible with an oxygenated benzene ring and/or an α,β -unsaturated ketone moiety (283 and 246 nm).⁸ ^1H and ^{13}C NMR spectra (Table 1) and DEPT data indicated the presence of three aromatic protons, four methines (three olefinic and one aliphatic), six methylenes (two olefinic, one di-oxygenated, and three aliphatic), one methoxy, and six quaternary carbons. The homonuclear 2D NMR COSY (Fig. 1) and TOCSY data for **1** identified connectivity sequences indicative of three coupling systems: subunit B: $\text{H}_2\text{-3}$ through H-2 , $\text{H}_2\text{-7}$, and H-8 to $\text{H}_2\text{-9}$ ($\text{CH}_2=\text{CH-CH}_2\text{-CH-CH}_2$); subunit D: H-12 through H-13 to H-15 (a 1,2,4-trisubstituted benzene ring); subunit E: $\text{H}_2\text{-17}$ through H-18 to $\text{H}_2\text{-19}$ (an allyl group). Heteronuclear 2D NMR correlations [HSQCED and HMBC (Fig. 1)] permitted connection of subunits A-C (H-3_e to C-1, C-5, C-7; H-6 to C-2, C-4; $\text{H}_2\text{-10}$ to C-4, C-5), and subunits D-F (H-12 to C-14, C-16; $\text{H}_2\text{-17}$ to C-13, C-14, C-15; $\text{H}_3\text{-20}$ to C-16). Using this, two fragments, I and II, could be proposed for didymochlaenone A (**1**). Based on its molecular formula, it was concluded that fragments I and II were connected *via* an oxygen bridge between C-4 and C-11, which was confirmed by a ROESY correlation between H-12 and H-3_e .

The α,β -unsaturated cyclohexanone ring had the same conformation as that of (2*R*)-illicinone F,⁹ a hemichair. This was evidenced by the fact that H-3_e appeared as a broad doublet ($J_{3e,3a} = 14.4\text{ Hz}$), indicating a small dihedral angle, while the coupling constant between H-2 and H-3_a ($J_{2,3a} = 8\text{ Hz}$) was consistent with a large dihedral angle, and hence H-2 was axial. Molecular modeling using the MM2 subprogram in Chem3D showed the dihedral angles $\text{H(2)-C(2)-C(3)-H(3}_a)$ and $\text{H(2)-C(2)-C(3)-H(3}_e)$ to be -177° and -60° respectively, consistent with the observed $J_{2,3a}$ of 8 Hz. The only possible alternate structure, with a *syn* orientation of the C-2 and C-4 substituents, was calculated to have dihedral angles of -45° and 69° , respectively; these values would require a much smaller $J_{2,3a}$ coupling constant. In addition, both H-2 and H-12 showed ROESY correlations to H-3_e , but not H-3_a , confirming the *anti* orientation of the C-2 and C-4—O—C-11 substituents. The CD spectrum of **1** showed similar positive Cotton effects at 240.9 and 314.3 nm, similar to those at 236 and 308 nm in (2*R*)-illicinone F,¹⁰ confirming that the absolute stereochemistry of **1** is (2*R*,4*S*).

Didymochlaenone B (**2**) was also obtained as a colorless oil, and its molecular formula was determined as $C_{20}H_{20}O_6$ by HRFABMS and ^{13}C NMR spectroscopy. Its ^1H NMR spectrum was very similar to that of **1**, but it contained resonances of an additional methylenedioxy instead of a methoxy group and a 1,2,4,5-tetrasubstituted benzene ring instead of the 1,2,4-trisubstituted aromatic moiety in **1**. The dioxymethylene group in fragment II of **2** was fused to the benzene ring at C-13/C-14, as $\text{H}_2\text{-20}$ exhibited HMBC correlations with C-13 and C-14. The allyl group in fragment II of **2** was located at the 16-position since $\text{H}_2\text{-17}$ showed a ROESY correlation with H-15 . ROESY crosspeaks between H-3_e and H-12 , and between H-3_e and H-2 were also observed, demonstrating the *anti* orientation of the C-2 and C-4—O—C-11 substituents. Hence, the structure of **2** was determined as shown.

The optical rotation and circular dichroism spectrum of **2** were approximately the opposite of those of **1**, indicating that **2** most likely has the opposite stereochemistry to that of **1** at the 2 and 4 positions.¹⁰ Although it is unusual for a plant to produce two similar compounds with opposite stereochemistry, it is not unprecedented; thus, various *Pinus* sp. produce both (+) and (–)- α -pinene, and (+) and (–)-limonene both occur in peppermint.¹¹

Camptothecin and 9-methoxycamptothecin isolated from the first collection (MG 1736A) were strongly cytotoxic, with IC₅₀ values of 0.07 and 0.02 µg/mL, respectively, against the A2780 ovarian cancer cell line. All the isolated compounds from the second collection (MG 1736B) were evaluated for their cytotoxicity against the A2780 human ovarian cancer cell line. It was found that only (*E*)-3-methoxy-5-hydroxystilbene was active, with an IC₅₀ value of 10 µg/mL.

As noted in the introduction, the bioactive compounds isolated from the first collection (MG 1736A) were not present in the second plant collection (MG 1736B). In the light of this surprising result, the two plant collections and the resulting two extracts were carefully compared. The herbarium specimens of each collection were compared and were confirmed to be of the same species. The two extracts did however show some major differences; not only were the camptothecins found in the first extract absent from the second extract, but also the major compounds found in the second extract were absent from the first extract. We thus concluded that a possible coding error may have occurred during the extraction process, and that the first extract could in fact be from another plant. The records of the extraction laboratory were used to identify plants which had been extracted at the same time as the original collection, and six extracts were identified as possible candidates for a coding error. These six extracts were all compared by HPLC analysis with the second and authentic *D. truncatula* extract, and extract MG 1726 (*Coffea* sp.) was found to correspond most closely with this extract. It is thus probable that the original extract that was coded as *D. truncatula* was in actuality a *Coffea* sp., and that this was the source of the camptothecins isolated. Unfortunately our collection work in the Zahamena region had ceased when this discovery was made, and it was not feasible to recollect the *Coffea* sp. to confirm this conclusion. It can be noted, however, that camptothecins have recently been shown to be produced by a fungus designated RJMEF001 isolated from *Nothapodytes foetida*,¹² and it is thus very possible that the true source of these alkaloids is a fungal one.

Experimental Section

General Experimental Procedures

Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. CD analysis was performed on a JASCO-720 spectropolarimeter. NMR spectra were obtained on a JEOL Eclipse 500 and an Inova 400 spectrometer in CDCl₃. The chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. Mass spectra were obtained on a JEOL JMS-HX-110 instrument, in the positive ion mode. HPLC was performed on a Shimadzu LC-10AT instrument with a semi-preparative C18 and phenyl Varian Dynamax column (5 µm, 250 × 10 mm) and a preparative C18 Varian Dynamax column (8 µm, 250 × 21.4 mm). Finnigan LTQ LC/MS with a C18 Hypersil column (5 µm, 100 × 2.1 mm) was also used for crude sample analysis.

Cytotoxicity Bioassays

Cytotoxicity measurements were performed at Virginia Polytechnic Institute and State University against the A2780 ovarian cancer cell line as previously described.^{13,14} The A2780 cell line is a drug - sensitive ovarian cancer cell line.¹³

Plant Material

Roots of *Didymochlaena truncatula* (Sw.) J. Sm (Dryopteridaceae) were collected by Stephan Rakotonandrasana et al. 665 on December 2002, and Andrianjafy Mamisoa et al. 414 on 24 April 2004, 1 km north west of the village of Antenina, forest adjacent to the Zahamena National Park, in Toamasina Province, Madagascar (17. 30 25S; 48. 46. 03E, elevation ca. 860 m). Duplicate voucher specimens were deposited at Centre National d'Application des

Recherches Pharmaceutiques (CNARP) and Direction des Recherches Forestieres et Piscicoles Herbarium in Antananarivo, Madagascar (TEF), Missouri Botanical Garden, St. Louis, Missouri (MO), and Museum National d'Histoire Naturelle in Paris, France (P).

Extraction and Isolation

Field-dried plant was ground in a hammer mill, then extracted by overnight percolation at room temperature with EtOH to give the crude extracts MG 1736A (first collection) and MG 1736B (second collection). MG 1736A (1 mg) was injected into a C18 Varian Dynamax column (5 μ m; 250 \times 10 mm; 2 mL/min 70% H₂O to 100% MeCN for 30 min, then 100% MeCN for 10 min), and forty fractions were collected into a 96-well block. The block was evaporated (GenVac EZ-2), and the 40 dried fractions were tested in the A2780 assay. Only fractions 24 and 26 showed activity. Then 100 mg crude extract was loaded on a C18 Varian Dynamax column (8 μ m, 250 \times 21.4 mm, 10 mL/min 70% H₂O to 100% MeCN for 30 min, then 100% MeCN for 10 min), and five fractions were collected. Fractions II and IV, corresponding to fractions 24 and 26 of the initial column respectively, were further purified using phenyl HPLC (65% MeOH) to furnish camptothecin (0.4 mg, t_R 20 min) and 9-methoxycamptothecin (0.3 mg, t_R 27 min). The structures of the camptothecins were confirmed by comparison of their spectroscopic data (NMR and MS) with those of the same compounds previously isolated by this group.^{3d}

MG 1736B (23 g) was suspended in aqueous MeOH (MeOH-H₂O, 9:1, 1000 mL) and extracted with hexane (3 \times 1000 mL portions). The aqueous layer was then diluted to 50% MeOH (v/v) with H₂O and extracted with CH₂Cl₂ (3 \times 1000 mL portions). Both the hexane and the CH₂Cl₂ extracts were evaporated *in vacuo* to leave 86 mg and 1 g of residues (IC₅₀: 12 and 16 μ g/mL, respectively). The aqueous MeOH extract was inactive. The CH₂Cl₂ extract was selected due to its relatively greater quantity than the hexane extract, and this was fractionated by flash chromatography over 40 g of C18 reverse phase column using H₂O-MeOH [(50 – 100%, in 10% increments, then 90% MeOH/CH₂Cl₂, 200 mL \times 7)] to furnish seven fractions (I, II, III, IV, V, VI and VII with IC₅₀ values of 19, 17, 13, 14, 15, 1000, 1000 μ g/mL, respectively). Fraction V yielded an inseparable mixture of **1** and **2** (t_R 15.5 min) over C18 HPLC using 80% MeOH, which could not be separated using phenyl HPLC with 75% MeCN (t_R 9.3 min) either. But the mixture from fraction V could be separated by preparative TLC over Si gel developed with CH₂Cl₂ to afford **1** (1 mg, R_f 0.4) and **2** (1 mg, R_f 0.3). Further purification of fractions I and III was carried out by C18 HPLC with 50% and 70% MeOH as eluents to yield (–)-wikstromol (5 mg, t_R 13.5 min) and (*E*)-3-methoxy-5-hydroxystilbene (3 mg, t_R 23 min). Fraction II and the hexane extract were combined and chromatographed on a Si gel column eluted with hexane-CH₂Cl₂-MeOH (100:0:0; 100:1:0; 100:2:0; 100:3:0; 100:4:0; 100:5:0; 100:10:0; 0:100:0; 0:100:10). Fraction 9 of this separation yielded stigmast-4-en-3 β -ol (2 mg, R_f 0.2) and stigmast-4-en-3-one (8 mg, R_f 0.3) after separation by preparative TLC developed with CH₂Cl₂.

Didymochlaenone A (**1**)

colorless oil; $[\alpha]_D^{22} +112.5^\circ$ (c 0.08, EtOH); IR (film) ν_{\max} 2917, 1653, 1507, 1350, 1264, 1185, 1126, 1037, 1023, 918; UV (EtOH) λ_{\max} (log ϵ) 246 (3.97), 283 (3.46) nm; CD (EtOH, c 0.04) $[\theta]_{225.0} -21.0$, $[\theta]_{240.9} +43.3$, $[\theta]_{314.3} +9.9$; ¹H NMR (500 MHz, CDCl₃) and ¹³C NMR (125 MHz, CDCl₃) see Table 1; HRFABMS m/z 343.1557 (calcd for C₂₀H₂₃O₅, 343.1546).

Didymochlaenone B (**2**)

colorless oil; $[\alpha]_D^{22} -27.5^\circ$ (c 0.08, EtOH); IR (film) ν_{\max} 2921, 1653, 1502, 1482, 1345, 1259, 1185, 1037, 918; UV (EtOH) λ_{\max} (log ϵ) 243 (4.08), 298 (3.59) nm; CD (EtOH, c 0.04) $[\theta]_{236.4} +38.2$, $[\theta]_{247.4} -12.3$, $[\theta]_{316.2} -2.3$; ¹H NMR (500 MHz, CDCl₃) and ¹³C NMR (125 MHz, CDCl₃) see Table 1; HRFABMS m/z 357.1356 (calcd for C₂₀H₂₁O₆, 357.1338).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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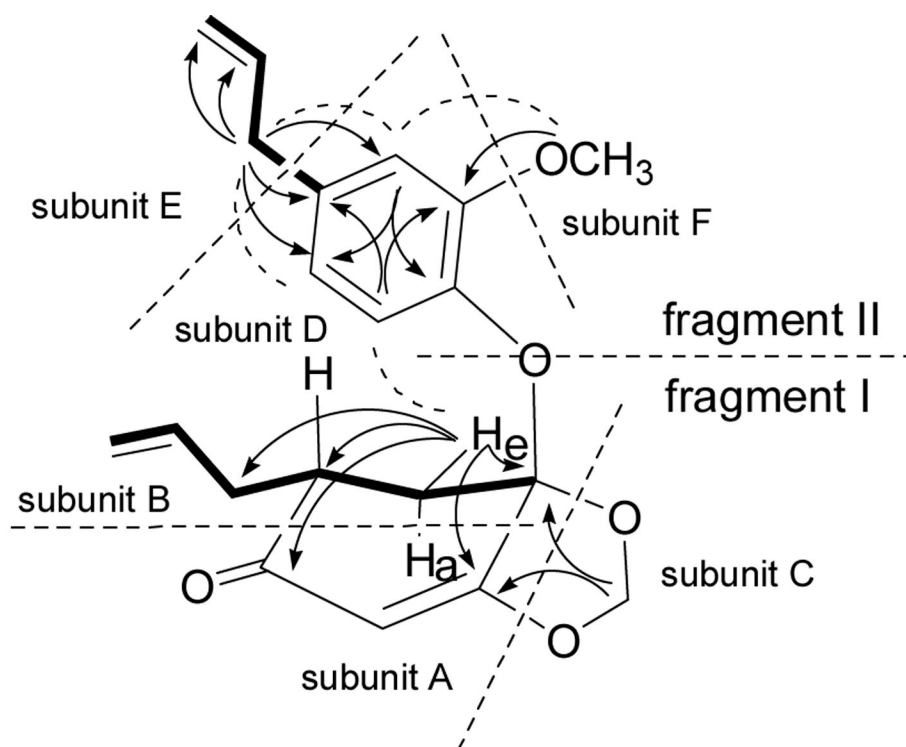


Figure 1.
COSY (bold), key HMBC (arrows) and ROESY (dashed arc) correlations of **1**

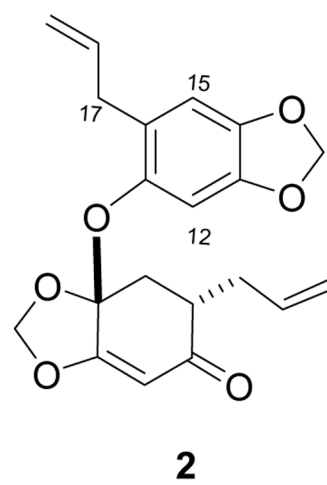
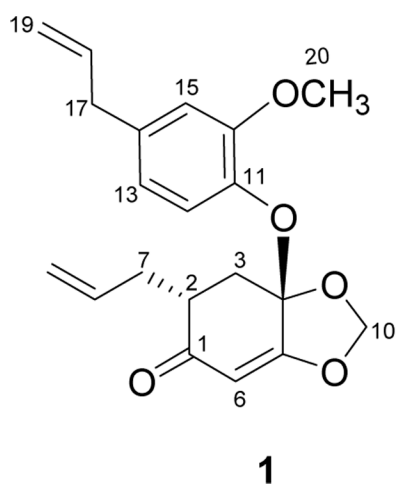


Table 1
 ^1H and ^{13}C NMR Data (δ) for Compounds **1** and **2** in CDCl_3

no	1		2	
	^1H	^{13}C	^1H	^{13}C
1		199.7		199.4
2	2.47 m	43.4	2.52 m	43.2
3	3.05 br d (14.4)	29.7	3.05 br d (14.7)	30.2
	2.17 dd (14.4, 8.0)		2.20 dd (14.7, 8.5)	
4		103.5		103.1
5		168.7		169.0
6	5.57 br s	101.0	5.60 br s	100.7
7	2.60 m	35.6	2.60 m	36.2
	2.40 m		2.28 m	
8	5.54 m	136.6	5.63 m	136.2
9	4.82 br d (10.1)	116.9	4.92 br d (10.5)	117.6
	4.46 br d (17.0)		4.56 br d (16.3)	
10	5.56 br s	98.5	5.52 br s	98.8
	5.49 br s		5.22 br s	
11		140.9		143.2
12	7.30 d (8.1)	119.5	6.86 br s	100.7
13	6.70 dd (8.1, 2.2)	120.4		146.0
14		136.1		144.8
15	6.74 d (2.2)	112.9	6.68 br s	109.8
16		150.8		123.6
17	3.35 d (6.4)	39.9	3.38 dd (15.2, 5.3)	34.3
			3.17 dd (15.2, 6.0)	
18	5.94 m	137.3	5.84 m	136.4
19	5.09 br s	115.9	5.04 br s	116.1
	5.07 br s		5.02 br s	
20	3.77 s	55.6	5.93 br s	101.3
			5.91 br s	