Antiproliferative Compounds from *Cleistanthus boivinianus* from the Madagascar Dry Forest[1]

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ABSTRACT: The two new lignans 3α-O-(β-D-glucopyranosyl)-desoxypodophyllotoxin (**1**) and 4-*O*-(β-D-glucopyranosyl)-dehydropodophyllotoxin (**2**) were isolated from *Cleistanthus boivinianus*, together with the known lignans deoxypicropodophyllotoxin (**3**), (±)-β-apopicropodophyllin (**4**), (-)-desoxypodophyllotoxin (**5**), (-)-yatein (**6**), and β-peltatin-5-*O*-β-D-glucopyranoside (**7**). The structures of all compounds were characterized by spectroscopic techniques. Compounds **1**, **4**, and **5** showed potent antiproliferative activities against the A2780 ovarian cancer cell line, with IC_{50} values of 33.0 ± 3.6, 63.1 ± 6.7, and 230 ± 1 nM, respectively. Compounds **2** and **7** showed only modest A2780 activities, with IC_{50} values of 2.1 ± 0.3 and 4.9 ± 0.1 μM, respectively, while compounds **3** and **6** had IC_{50} values >10 μM. Compound **1** also had potent antiproliferative activity against the HCT-116 human colon carcinoma cell line, with an IC_{50} value of 20.5 nM, and compound **4** exhibited modest antiproliferative activity against the A2058 human caucasian metastatic melanoma and MES-SA human uterine sarcoma cell lines, with IC_{50} values of 4.6 and 4.0 μM, respectively.

In the course of work as part of the Madagascar International Cooperative Biodiversity Group (ICBG) program,[2,3] an ethanol extract of the stems of *Cleistanthus boivinianus* (Baill.) Müll. Arg. (Phyllanthaceae) was found to have moderate antiproliferative activity against the A2780 ovarian cancer cell line (IC_{50} 4.5 μg/mL), and was thus selected for evaluation of the presence of novel antiproliferative agents. The genus *Cleistanthus* is native to Africa, India, and Australia,[4] and comprises approximately 140 species, but only five of these have been investigated chemically: *C. collinus*, *C. patulus*, *C. schlechteri var. schlechteri*, *C. gracilis*,[4] and *C. indochinensis*.[5] Arylnaphthalide lignans are major constituents of the genus, while arytetralin, furofuranoid, and dibenzylbutane lignans and terpenoids have also been isolated from them and are known for their cytotoxic properties.[4-12]

RESULTS AND DISCUSSION

Dereplication of an active EtOAc-soluble fraction obtained from liquid-liquid partition of the extract (100 mg) as previously described[13] indicated that it contained at least one new bioactive compound, so a larger sample was investigated. Fractionation of this extract yielded an antiproliferative EtOAc fraction, which was further subjected to size-exclusion column chromatography on Sephadex LH-20 followed by reversed-phase solid-phase extraction (SPE). The most active fractions from the C_{18} SPE were subjected to C_{18} HPLC to yield compounds **3**, **5**, and **6** and three semi-pure active fractions. Further purification of these fractions by silica gel or diol HPLC furnished compounds **1**, **2**, **4**, and **7**.

Compound **1** was isolated as a white solid with the molecular formula $C_{28}H_{32}O_{13}$ based its HRESIMS data. Its ^{1}H NMR spectrum contained three singlet aromatic signals together with signals for a methylenedioxy group and three aromatic methoxy groups. Signals suggestive of a sugar unit were also present. Analysis of COSY, HSQC, and HMBC data assigned the sugar as a β-glucopyranosyl unit. The remaining aliphatic signals were assigned by HSQC and COSY analysis to two isolated methylene groups, one of which was oxygenated, and to two adjacent methines. The above data indicated that **1** has an aryltetralin lignan skeleton. This was supported by the HMBC data (Figure 1), with cross-peaks from H-1 to C-8 and to C-2'/C-6', supporting the linkage of C-1 to both the A- and B-rings. HMBC cross-peaks between H-5 and C-4 and between H_2-4 and C-8a established the C-4a/C-4 connectivity, and the presence of the lactone ring was confirmed by cross-peaks between the methylene protons H_2-3a and the ester carbonyl carbon C-2a. Correlations of the methylenedioxy protons with C-6 and C-7 indicated the position of the methylenedioxy group. Finally, the positions of the methoxy groups were assigned based on cross-peaks from the two methoxy signals at δ_H 3.75 to C-3' and C-5', and for the other methoxy signal to C-4'.

The 2.3 Hz *J*-value between H-1 and H-2 was suggestive of a *cis*-relationship, which was confirmed by the NOESY interaction of H-1 and H-2. The *trans*-configuration of the D-ring was determined by the NOESY correlation between H-2 and H_2-3a. The 1*R* absolute configuration was established by the electronic circular dichroism (ECD) spectrum of **1**, which gave a positive Cotton effect at 288 nm (Δε +1.02), consistent with the positive effect for other 1*R* lignans.[14-19] These data combined

with the relative configuration determined above allowed the *R* and *S* configurations to be assigned for the C-2 and C-3 chiral centers, respectively.

Mild acid hydrolysis of **1** gave (+)-*β*-apopicropodophyllin (**4a**) and a sugar that was identified as D-glucose by TLC and optical rotation comparison with a standard sample. Placement of the glucose unit at C-3 was evident from the HMBC correlation between the anomeric proton at δ_H 4.18 and the oxygenated quaternary carbon at δ_C 83.6 (C-3). The complete assignment of all protons and carbons of **1** (Table 1) was accomplished by analysis of the COSY, HSQC, HMBC, and NOESY spectra. Thus, compound **1** was assigned as (1*R*,2*R*,3*S*)-3-*O*-(*β*-D-glucopyranosyl)-3',4',5'-trimethoxy-6,7-methylenedioxy-1,9-cyclolignan-11,12-olide, and named 3*α*-O-(*β*-D-glucopyranosyl)-desoxypodophyllotoxin.

As indicated by the experiment described above, compound **1** is prone to conversion to its *α*,*β*-unsaturated derivative **4a**, and a sample of **1** left in MeOH/$CHCl_3$ for a few days underwent decomposition, most likely caused by traces of HCl in the $CHCl_3$. Attempted enzymatic hydrolysis of **1** did not proceed under normal conditions, so the aglycone could not be isolated.

Compound **2** had the molecular formula of $C_{28}H_{29}O_{13}$, based on its HRESIMS data. Its ^{1}H NMR spectrum was similar to that obtained for **1**, and its UV absorption maxima at 260, 315, and 351 nm indicated the presence of a naphthalene nucleus,[20] suggesting that **2** is an arylnaphthalide lignan. The ^{1}H NMR spectrum of **2** exhibited signals for two aromatic protons at δ_H 8.04 and 6.95 (s, each 1H, H-5 and H-8) and for

an oxymethylene group at δ_H 5.75 and 5.53 (J = 15.4 Hz, each 1H, H_2-3a), but lacked the signals for the two methine protons at C-1 and C-2 and the methylene protons at C-4 observed in **1**. These facts confirmed the presence of a naphthalene unit in **2**. In the HMBC spectrum, the correlation from H-5 (δ_H 8.04) and H_2-3a (δ_H 5.75 and 5.53) to δ_C 144.3 (C-4) confirmed the linkage of C-4 to both the B- and D-rings, while the correlations of δ_H 6.59 (s, 1H, H-2' or/and H-6') and δ_H 6.95 (H-8) to δ_C 128.6 (C-1) connected the A- and B-rings to C-1. A comparison of the NMR spectra of **2** with those of dehydropodophyllotoxin (**2a**) suggested that compound **2** is a glycosylated derivative of **2a**.[21] The HMBC correlation between the anomeric proton signal at δ_H 4.89 (H-1") and δ_C 144.3 (C-4) confirmed that the sugar is located at C-4 of the aglycone. Acid hydrolysis of **2** gave dehydropodophyllotoxin (**2a**) as the aglycon, and a sugar that was identified as D-glucose by ^{1}H NMR, TLC, and optical rotation comparison with a standard sample. The complete assignments of all protons and carbons of **2** (Table 1) were accomplished by analysis of the HSQC and HMBC spectra. Compound **2** was thus assigned as 4-*O*-(*β*-D-glucopyranosyl)-dehydropodophyllotoxin.

The five known aryltetralin lignans deoxypicropodophyllotoxin (**3**),[22] (±)-*β*-apopicropodophyllin (**4**),[20,23,24] (-)-desoxypodophyllotoxin (**5**),[25] (-)-yatein (**6**),[26] and *β*-peltatin-5-*O*-*β*-D-glucopyranoside (**7**)[27] were also isolated. Their structures were determined by comparison of their ^{1}H NMR spectroscopic, mass spectrometric, and optical rotation values with the data reported in the literature, except for the case of *β*-apopicropodophyllin (**4**), which was optically inactive. The isolation of racemic **4** is noteworthy, since all previous isolates have been of the dextrorotatory isomer, and

indicates that it is not formed by elimination of glucose from **1**.

All isolated compounds were evaluated for their antiproliferative activity against the A2780 human ovarian cancer cell line, and some were evaluated in other cell lines and for antimalarial activity (Table 2). Compound **1** showed the highest antiproliferative activity (IC_{50} 33.0 ± 3.6 nM) against the A2780 cell line, followed by **4** (63.1 ± 6.7 nM) and **5** (230 ± 1 nM). The potency of **1** is similar to that of the anticancer drug paclitaxel, which has an IC_{50} value of 73 nM in this assay. Compounds **2** and **7** showed only modest antiproliferative activities, with IC_{50} values of 2.1 ± 0.3 and 4.9 ± 0.1 μM, respectively. The glycosylated compound **1** had slightly improved activity compared with the racemic compound **4**. The unsaturated D-ring of **4** increased the activity about four-fold compared with the *trans* dihydro D-ring analogue **5**, while the *cis* D-ring analogue **3** was much less potent than **4**, consistent with previous studies indicating the significance of the trans-fused lactone for activity.[28,29] A glucose moiety at C-5 or C-4 and the aromatization of the C-ring reduced activity, as shown by the fact that compounds **2** and **7** were about ten- and twenty-fold less potent than **5**, respectively. Furthermore, although good antiproliferative activity has been observed in other cell lines for (-)-yatein (**6**),[20] it was only weakly active against the A2780 cell line. Compound **1** also displayed potent antiproliferative activity against the HCT-116 human colon carcinoma cell line with an IC_{50} value of 20.5 nM, and weak antimalarial activity against *Plasmodium falciparum* with an IC_{50} value of 12.6 ± 3.2 μM. Compound **4** displayed moderate antiproliferative activity against A2058 human caucasian metastatic melanoma and MES-SA human uterine sarcoma cells, with IC_{50}

values of 4.6 and 4.0 μM, respectively.

In summary, compound **1** is a new lignan with potent antiproliferative activity against the A2780 cell line. It is also the first reported C-3 substituted podophyllotoxin analogue. It would be an attractive substrate for further studies to explore its mechanism of action were it not for its lability under acidic conditions, which suggests that it would not be stable enough for drug use.

■ EXPERIMENTAL SECTION

General Experimental Procedures. IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer in CD_3OD (with CD_3OD as reference) and $CDCl_3$ (with $CDCl_3$ as reference). Mass spectra were obtained on an Agilent 6220 mass spectrometer. Open column chromatography was performed using Sephadex LH-20, and solid-phase extraction was performed using C_{18} cartridges. Semi-preparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a semi-preparative Phenomenex C_{18} column (5 μm, 250 × 10 mm), a Shimadzu SPD M10A diode array detector, and a SCL-10A system controller. All isolated compounds were purified to 95% purity or better, as judged by HPLC (both UV and ELSD detection) before determining bioactivity.

Plant Material. Leaves of *Cleistanthus boivinianus* (collection: Stéphan Rakotonandrasana et al. 1036) were obtained at an elevation of 51 m from a 3 m tall

tree with yellow flowers. Collection was made 5 km north-east of the village of Marivorahona, PK 123, Andohanantsohihy, in a mosaic of dry forest and savanna trees; coordinates 13°06'37"S 049°09'39"E. Collection was made by Stéphan Rakotonandrasana with assistance from R. Randrianaivo, R. Rakotonandrasana, C. Claude, V. Benjara, and M. Modeste. Duplicate voucher specimens are deposited at the Centre National d'Application des Recherches Pharmaceutiques (CNARP), the Herbarium of the Parc Botanique et Zoologique de Tsimbazaza, Antananarivo, Madagascar (TAN), the Missouri Botanical Garden, St. Louis, Missouri (MO), and the Museum National d'Histoire Naturelle in Paris, France (P).

Extraction and Isolation. A ground sample of *Cleistanthus boivinianus* leaves (250 g) was extracted with EtOH at room temperature to yield 33.4 g of crude EtOH extract designated MG4031. A total of 5.88 g of this extract was made available to Virginia Tech. An active EtOAc-soluble fraction obtained from liquid-liquid partition of the extract (100 mg) was subjected to dereplication studies using size-exclusion chromatography, reversed-phase HPLC coupled with bioassay, high-resolution ESIMS, ^{1}H NMR spectroscopy, and a database search using the online *Dictionary of Natural Products* (DNP). The results indicated the extract to contain at least one new bioactive compound, and so a 3.0 g sample was investigated. The crude EtOH extract was dissolved in 90% aq. MeOH (300 mL), and extracted with hexanes (3 × 200 mL), and extracted with hexanes (3 × 200 mL). Evaporation of the hexane-soluble fraction afforded 271 mg of residue. The 90% aq. MeOH layer was then evaporated, suspended in H_2O (300 mL), and extracted with EtOAc (3 × 200 mL) to yield 640 mg of an EtOAc-

soluble fraction with an IC_{50} value of 0.19 µg/mL. The EtOAc fraction was subjected to Sephadex LH-20 open column chromatography (CH_2Cl_2-MeOH, 1:1) to give six fractions. The most active fraction, Fr 3 (IC_{50} 0.11 µg/mL), was then divided into three sub-fractions by C_{18} solid-phase extraction by using 40% aq. MeOH (Fr 3-1), 70% aq. MeOH (Fr 3-2), and 100% MeOH (Fr 3-3). Further purification of the most active sub-fraction, Fr 3-3 (IC_{50} 0.11 µg/mL), by HPLC on a C_{18} column with a solvent gradient from H_2O-CH_3CN, 60:40 to 50:50 from 0 to 17 min, to 40:60 from 17 to 22 min, to 40:60 from 22 to 27 mins and ending with 100% CH_3CN from 27 to 35 min, yielded compounds **3** (1.2 mg, t_R 23.5 min), **5** (1.5 mg, t_R 24.5 min), and **6** (2.1 mg, t_R 26.5 min). Purification of the sub-fraction Fr 3-2 (IC_{50} 0.1 µg/mL) by C_{18} HPLC furnished eight fractions (solvent gradient from H_2O-CH_3CN, 70:30 to 62:38 from 0 to 15 min, and ending with 100% CH_3CN from 15 to 24 min), among which the 3rd (t_R 12 min), 6th (t_R 14.5 mins), and 7th (t_R 15.5 mins) fractions were active with IC_{50} value of 1.5, 0.1, and 1.2 µg/mL, respectively. Further purification of the 3rd fraction by HPLC on a silica gel column with a solvent gradient from $CHCl_3$-MeOH, 95:5 to 90:10 from 0 to 5 min, to 88:12 from 5 to 12 min, and ending with a 100% MeOH wash from 13 to 20 min, yielded compound **7** (2.8 mg, t_R 13.5 min). Further purification of the 6th fraction by HPLC on a silica gel column with a solvent gradient from $CHCl_3$-MeOH, 95:5 to 90:10 from 0 to 5 min, to 80:20 from 5 to 10 min, to 70:30 from 10 to 15 min, and ending with a 100% MeOH wash from 16 to 25 min, yielded compound **4** (5 mg, t_R 6 min) and **1** (19 mg, t_R 14 min). Further purification of the 7th fraction by HPLC on a diol column with a solvent gradient from $CHCl_3$-MeOH, 100:0 to 95:5 from 0 to 5 min, to 90:10

from 5 to 15 min, to 87.5:12.5 from 15 to 20 min, and ending with a 100% MeOH wash from 20 to 25 min, yielded compound **2** (3 mg, t_R 22.5 min).

*3α-O-(β-D-Glucopyranosyl)-desoxypodophyllotoxin (**1**):* amorphous powder; $[\alpha]^{25}_D$ +120 (*c* 0.1, MeOH); CD (*c* 0.031, MeOH) λ_{max} ($\Delta\varepsilon$) 210 (+22.59), 234 (+5.98), 248 (-1.33), 288 (+1.02) nm; UV (MeOH) λ_{max}(log ε) 291 (3.19), 245 (3.7), 208 (4.4) nm; IR (film) ν_{max} 3402, 2912, 1760, 1590, 1053 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* $[M+Na]^+$ 599.1741 (calcd for $C_{28}H_{32}NaO_{13}^+$, 599.1735)

*4-O-(β-D-Glucopyranosyl)-dehydropodophyllotoxin (**2**):* amorphous powder, $[\alpha]^{25}_D$ -14 (*c* 0.22, MeOH); UV (MeOH) λ_{max} (log ε) 351 (3.69), 315 (4.0), 260 (4.7) nm; IR (film) ν_{max}: 3400, 2920, 1734, 1645, 1070 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* $[M+H]^+$ 573.1635 (calcd for $C_{28}H_{29}O_{13}^+$, 599.1603)

*(±)-β-Apopicropodophyllin (**4**):* amorphous powder, $[\alpha]^{25}_D$ 0 (*c* 0.5, $CHCl_3$). The ^1H and ^{13}C NMR spectra were identical with literature data.[20,23,24]

Acid Hydrolysis of Compounds 1 and 2. Compound **1** (2 mg) was dissolved in dilute citrate/phosphate buffer (pH 7.0, 4 mL) and β-D-glucosidase (4 mg) was added. The mixture was incubated for 2 weeks at 37 °C; no reaction was observed. The reaction mixture was then adjusted to pH 5.0 with dilute HCl and stirred at 37 °C overnight. The mixture was then briefly heated to boiling, extracted with EtOAc (3x10 mL), and both the organic and the water layers were evaporated to dryness under reduced pressure. The structure of the white powder (1.2 mg) derived from the organic

layer was determined to be **4a** by its ^{1}H NMR spectrum and optical rotation ($[\alpha]^{25}_{D}$ +86, *c* 1.2, $CHCl_3$); lit.[20] +96.8). The semi-solid carbohydrate from the water layer (0.5 mg) was dissolved in 2 mL of water and kept overnight before determination of its optical rotation. Compound **2** (1 mg) was dissolved in dilute HCl (pH 5.0, 2 mL) and stirred at 37 °C overnight. The reaction mixture was extracted with EtOAc (3x10 mL) and both the organic and the water layers were evaporated to dryness under reduced pressure. The structure of the dried powder from the organic layer was determined to be **2a** by its ^{1}H NMR spectrum. TLC of the sugar from both **1** and **2** on a silica gel plate with $CHCl_3$-MeOH-H_2O (15:6:1) showed that it had an identical R_f value (0.23) to glucose. The isolated glucose had $[\alpha]^{25}_{D}$ (*c* 0.5, H_2O) values of +41.9 and +43.2, respectively.

Antiproliferative Bioassay. The A2780 ovarian cancer cell line antiproliferative bioassay was performed at Virginia Tech as previously reported.[30,31] The A2780 cell line is a drug-sensitive ovarian cancer cell line.[32] Paclitaxel was used as the positive control and gave an IC_{50} value of 0.073 ± 0.015 μM.

Antimalarial Bioassay. The effect of each compound on parasite growth of the Dd2 strain of *P. falciparum* was measured after 72 h treatment using the malaria SYBR green I-based fluorescence assay as described previously.[2,33,34] Artemisinin was used as the positive control and it had an IC_{50} value of 8 ± 1 nM.

■ ASSOCIATED CONTENT

***S Supporting Information**

^{1}H NMR spectra of compounds **1** – **7**. This material is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.xxxxxx

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Notes

The authors declare no competing financial interest.

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Table 1. ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) Chemical Shift Data (δ, ppm) for Compounds 1 and 2.

	1		2	
position	δ_H (*J* in Hz)	δ_C (DEPT)	δ_H (*J* in Hz)	δ_C (DEPT)
1	4.47 d (2.3)	47.1 (CH)		128.6 (C)
2	3.76 d (2.3)	52.8 (CH)		119.7 (C)
2a		178.4 (C)		170.4 (C)
3a	4.58 d (10.2) 3.98 d (10.2)	76.9 (CH_2)	5.75 d (15.3) 5.53 d (15.3)	67.3 (CH_2)
3		83.6 (C)		130.4 (C)
4	3.35 d (15.1) 2.91 d (15.1)	37.0 (CH_2)		144.3 (C)
4a		127.3 (C)		136.5 (C)
5	6.78 s	108.9 (CH)	8.04 s	98.6 (CH)
6		147.3 (C)		149.6 (C)
7		147.3 (C)		149.6 (C)
8	6.72 s	105.7 (CH)	6.95 s	102.8 (CH)
8a		130.5 (C)		131.7 (C)
1'		130.5 (C)		131.1 (C)
2'	6.55 s	104.9 (CH)	6.59 s	107.6 (CH)
3'		154.4 (C)		152.9 (C)
4'		136.2 (C)		137.2 (C)
5'		154.4 (C)	6.59 s	152.9 (C)
6'	6.55 s	104.9 (CH)		107.6 (CH)
OCH_2O	5.94 s	101.1 (CH_2)	6.11 s	101.6 (CH_2)
OMe-3'	3.79 s	59.8	3.82 s	55.4
OMe-4'	3.75 s	55.3	3.88 s	59.9
OMe-5'	3.79 s	59.8	3.82 s	55.4
Glc				
1"	4.18 d (7.7)	99.2 (CH)	4.89[a]	104.8 (CH)
2"	3.14-3.09	73.6 (CH)	3.63 dd (9.0, 7.8)	74.2 (CH)
3"	3.24-3.17	76.4 (CH)	3.48 t (9.0)	76.7 (CH)
4"	3.24-3.17	69.8 (CH)	3.44 m	70.1 (CH)
5"	3.02 ddd (9.2, 8.5, 2.4)	76.7 (CH)	3.33[a]	77.0 (CH)
6"	3.79 dd (11.8, 2.4) 3.59 dd (11.8, 2.4)	61.3 (CH_2)	3.90[b] 3.71 dd (11.9, 6.0)	61.3 (CH_2)

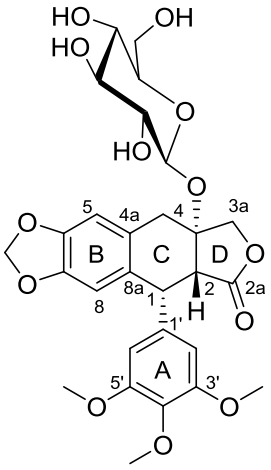
[a]Peaks covered by the CH_3OH peak; chemical shift assigned from HSQC and HMBC spectra.

[b]Peak covered by an OMe peak.

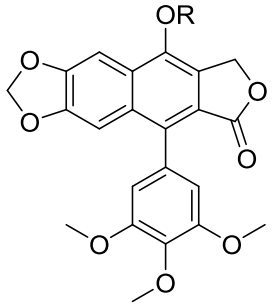
Glc: *β*-D-glucopyranosyl

Table 2. Antiproliferative and Antimalarial Activities of the Isolated Compounds (IC_{50} Values, μM)

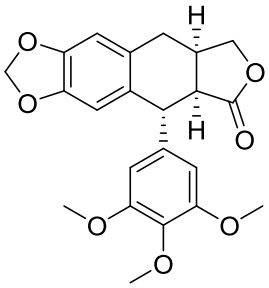
cell line	paclitaxel	**1**	**2**	**3**	**4**	**5**	**6**	**7**
A2780	0.073 ± 0.015	0.033 ± 0.0036	2.1 ± 0.3	> 10	0.063 ± 0.0067	0.23 ± 0.001	> 10	4.9 ± 0.1
HCT-116	NT	0.0205	NT	NT	NT	NT	NT	NT
A2058	NT	NT	NT	NT	4.6	NT	NT	NT
MES-SA	NT	NT	NT	NT	4.0	NT	NT	NT
P. falciparum Dd2	NT	12.6 ± 3.2	NT	NT	NT	NT	NT	NT



1



2 R = β-D-glucopyranose
2a R = H



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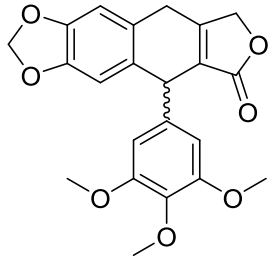
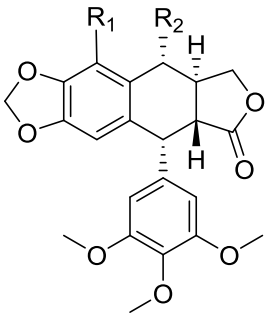
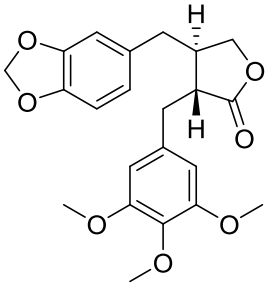


4 racemate
4a (+) isomer





5 R_1 = R_2 = H
6
7 R_1 = *O*-β-D-glucopyranose R_2 = H
8 R_1 = H, R_2 = OH

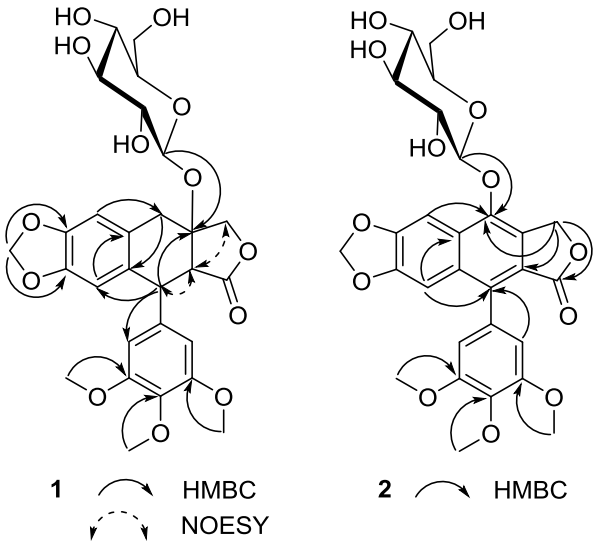


Figure 1. Key HMBC and NOESY correlations of **1** and key HMBC correlations of **2**.

GRAPHICAL ABSTRACT

Photograph by R. Rakotondrajaona