JOURNAL OF NATURAL PRODUCTS

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Antiproliferative and Antiplasmodial Dimeric Phloroglucinols from *Mallotus oppositifolius* from the Madagascar Dry Forest[1]

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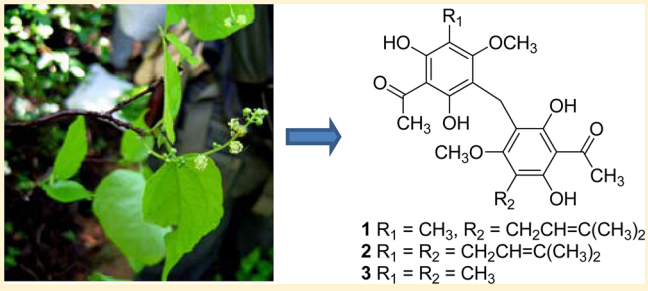
Supporting Information

ABSTRACT: Bioassay-guided fractionation of an ethanol extract of the leaves and inflorescence of *Mallotus oppositifolius* collected in Madagascar led to the isolation of the two new bioactive dimeric phloroglucinols mallotojaponins B (**1**) and C (**2**), together with the known mallotophenone (**3**). The structures of the new compounds were determined on the basis of spectroscopic evidence, including their 1D- and 2D-NMR spectra, mass spectrometry, and an X-ray crystal structure. Compounds **1** and **2** showed potent antimalarial activity against chloroquine-resistant *Plasmodium falciparum*, with IC_{50} values of 0.75 ± 0.30 and 0.14 ± 0.04 μM, while **3** was inactive in this assay. Compounds **1**−**3** also displayed strong antiproliferative activity against the A2780 human ovarian cancer cell line (IC_{50} 1.10 ± 0.05, 1.3 ± 0.1 and 6.3 ± 0.4 μM, respectively).



The tropical genus *Mallotus*, a member of the family Euphorbiaceae, contains about 150 species of trees and shrubs.[2] It shares membership in the tribe Acalypheae with the genus *Macaranga*,[2] a genus that has afforded several promising bioactive compounds.[3,4] *Mallotus philippinensis* is the source of rottlerin, a natural product that first appears to have been isolated in 1855[5] and has been the subject of numerous biological investigations.[6−8]

Our ongoing screening of extracts from plants collected in Madagascar as part of the Madagascar International Cooperative Biodiversity Group (ICBG) program for antiproliferative activity toward the A2780 ovarian cancer cell line[9] has recently been supplemented with screening for antiplasmodial activity against the malaria parasite *Plasmodium falciparum*. An ethanol extract of *Mallotus oppositifolius* (Geiseler) Müll. Arg. (Euphorbiaceae) was found to display strong activity against *P. falciparum* as well as antiproliferative activity against the A2780 cell line, and this extract was thus selected for further investigation to isolate the active metabolite(s) responsible for the observed activities. *M. oppositifolius* has been used as a chewing stick in Nigeria,[10] and its aqueous and ethanol extracts have been reported to have antifungal activity,[11] but no previous work on its constituents has been reported.

RESULTS AND DISCUSSION

Isolation of Bioactive Constituents. Initial dereplication studies using size-exclusion chromatography and HPLC on a small amount of an active hexanes-soluble fraction obtained from the liquid−liquid partition of the active extract indicated the presence of unknown antiproliferative phloroglucinols. Scale-up of the isolation to 1 g of extract yielded an antiproliferative hexanes fraction (IC_{50} 6.7 μg/mL), which was subjected to further size-exclusion column chromatography (Sephadex LH-20) to furnish two active fractions, with IC_{50} values of 1.6 and 2.3 μg/mL, and the known phloroglucinol mallotophenone (**3**, IC_{50} 6.3 ± 0.4 μM). The most active fractions were subjected to HPLC and silica gel column

Special Issue: Special Issue in Honor of Lester A. Mitscher

Received: October 30, 2012
Published: January 3, 2013

chromatography to yield the two new bioactive phloroglucinols **1** and **2**.

Structure Elucidation. Mallotophenone (**3**) was identified by single-crystal X-ray analysis and by comparison of its spectroscopic data with values reported in the literature.[12]

Compound **1** was obtained as yellowish crystals and gave the molecular formula $C_{25}H_{30}O_8$, as indicated by high-resolution ESIMS analysis, which gave a protonated molecular ion peak at m/z 459.2023 $[M+H]^+$. Its IR spectrum showed an absorption characteristic of a conjugated hydrogen-bonded carbonyl group (1620 cm^{-1}). The UV spectrum of **1** was very similar to that of **3**, suggesting that the two compounds share the same chromophore. The ^1H NMR spectroscopic data of **1** (Table 1) displayed resonances due to an aromatic methyl (δ 2.13, s, 3H), a 3,3-dimethylallyl group (δ 1.68, s, 3H and 1.77, s, 3H; δ 5.21, tq, J = 6.5, 1.4 Hz, 1H, and δ 3.31, d, J = 6.5 Hz, 2H), two methoxy groups (δ 3.98, s, 6H), two acyl methyl groups (δ 2.71, s, 6H), and one methylene group at δ 3.68 (s, 2H), together with signals for two hydroxy groups, one of which was hydrogen bonded (δ 8.97, s, 1H and 13.64, s, 1H). The ^{13}C NMR data of **1** (Table 2) exhibited 25 carbon signals that were identical with those of **3** except for the replacement of the signal of an aromatic methyl carbon with signals for the carbons of a 3,3-dimethylallyl unit (δ 18.0, 22.9, 25.8, 122.7, 132.2). On comparison of the ^{13}C NMR data of **1** with those of **3**, the deshielding of the signal for C-3′ (δ 114.2 instead of 109.1 in **3**) suggested that the 3,3-dimethylallyl group is attached at this position, which is methylated in **3**.[13] The locations of the methyl, methoxy, methylene, carboxyl, hydroxy, and the 3,3-dimethylallyl groups were confirmed by interpretation of the 1D- and 2D-NMR spectroscopic data of **1**, including COSY, HSQC, HMBC, and nuclear Overhauser effect spectroscopy experiments. The attachment of the 3,3-dimethylallyl group at C-3′ was confirmed by the observation of the HMBC long-range correlation between the two geminal methyls at δ 1.68 and 1.77 to C-1″ (δ 122.7) and from the methine proton at δ 5.21 to C-3′ (δ114.2). The methoxy groups were assigned to C-2 and C-2′ due to the long-range correlations (Figure 1) observed between the signals at δ 3.98 and those at δ 157.2 (C-2) and 157.7 (C-2′), between the C-1a methylene proton signal (δ 3.68) and C-2 and C-2′, and those observed between the aromatic methyl protons at δ 2.13 and C-2 and between the methylene protons signals at δ 3.31 and C-2′. In the same manner, the acyl group was assigned to C-5 and C-5′ from the HMBC cross-peaks between the methyl protons at δ 2.71 (CH_3-8 and 8′) and C-5 and C-5′. The two hydroxy groups must be located at C-4, C-4′ and C-6, C-6′, as indicated by the presence of two hydrogen-bonded hydroxy protons and the HMBC long-range correlations between the hydroxy group at δ 8.97 and C-3, C-3′, C-5, and C-5′. Moreover, NOESY correlations were observed between the methoxy protons and H-1″, H-1a, and CH_3-9.

The structure of **1** was confirmed by single-crystal X-ray diffraction (Figure 2). Compound **1** was thus assigned as 3′-(3,3-dimethylallyl)-1′-(5-acetyl-6-hydroxy-3-methyl-2-methoxybenzyl)-2′-methoxyphloracetophenone and has been named mallotojaponin B based on its relationship to mallotojaponin, seen here as mallotojaponin A.[14]

Compound **2**, named mallotojaponin C, gave the molecular formula $C_{29}H_{36}O_8$, as determined by positive ion HRESIMS (m/z 513.2499, $[M + H]^+$, required for $C_{29}H_{37}O_8$, 513.2488). Similarly to **1**, the IR and UV spectra of **2** were indicative of the presence of a prenylated phloroglucinol. The ^1H and ^{13}C NMR spectroscopic data of **2** were superposable upon those of **1** (Tables 1 and 2), except for the replacement of the signal due to the aromatic methyl group with the signals for a second 3,3-dimethylallyl group. Thus, the signal due to the methine of the 3,3-dimethylallyl group (δ 5.21, brt, J = 6.0 Hz) integrated for two protons (H-1″ and H-1‴), while the signal at δ 3.68 (s, H_2-1a) also integrated for two protons. In addition, the broad triplet observed for the ^1H NMR signal of the methine protons at δ 5.21 suggested the presence of two overlapping signals (H-1″ and H-1‴). These data coupled with the high-resolution mass spectra allowed the conclusion to be made that

Table 1. ^1H NMR Data of Compounds 1−3 (500 MHz, $CDCl_3$)

position	1	2	3
1a	3.68 s	3.68 s	3.66 s
8	2.71 s	2.70 s	2.70 s
9	2.13 s	3.31 d (6.3)	2.11 s
8′	2.71 s	2.70 s	2.70 s
9′	3.31 d (6.5)	3.31 d (6.3)	2.11 s
OCH_3	3.98 s, 3.98 s	3.98 s, 3.98 s	3.97 s, 3.97 s
1″	5.21 (tq, 6.5, 1.4)	5.21 (brt, 6.0)	
3″	1.68 s	1.68 s	
4″	1.77 s	1.77 s	
1‴		5.21 (brt, 6.0)	
3‴		1.68 s	
4‴		1.77 s	
OH	8.97 s, 13.64 s	9.05 s, 13.48 s	8.99, 13.66

Table 2. ^{13}C NMR Data for Compounds 1−3 (125 MHz, $CDCl_3$)

carbon	1	2	3
1	108.3	108.5	108.4
1a	18.1	17.9	18.1
2	157.2	157.5	157.1
3	109.2	114.2	109.1
4	162.9	162.8	163.0
5	110.0	109.2	110.2
6	159.8	159.6	159.9
7	205.4	205.4	205.6
8	33.8	33.8	34.1
9	8.9	22.9	9.2
1′	108.5	108.5	108.4
2′	157.7	157.5	157.1
3′	114.2	114.2	109.1
4′	162.9	162.8	163.0
5′	109.2	109.2	110.2
6′	159.6	159.6	159.9
7′	205.4	205.4	205.6
8′	33.8	33.8	34.1
9′	22.9	22.9	9.2
OCH_3	62.1 63.0	63.0 63.0	62.2 62.2
1″	122.7	122.7	
2″	132.2	132.2	
3″	18.0	17.9	
4″	25.8	25.8	
1‴		122.7	
2‴		132.2	
3‴		17.9	
4‴		25.8	

dx.doi.org/10.1021/np300750q | J. Nat. Prod. 2013, 76, 388−393

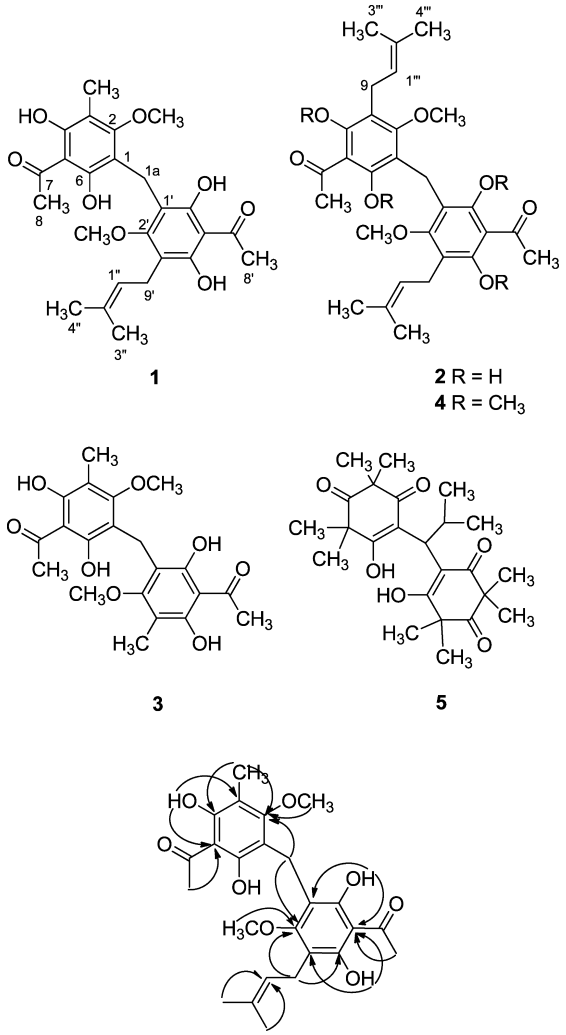


Figure 1. Important HMBC correlations observed in **1**.

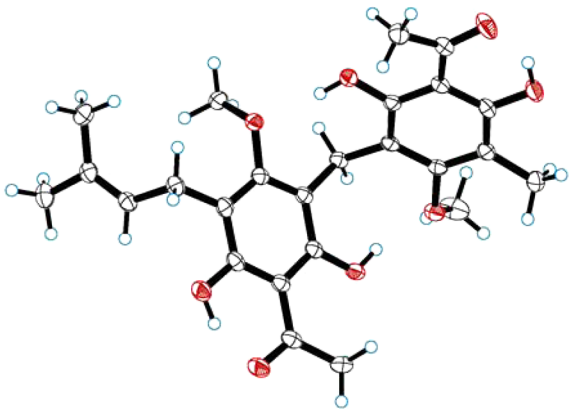


Figure 2. Anisotropic displacement ellipsoid drawing (50%) of **1**.

compound **2** was a symmetrical dimer with two 3,3-dimethylallyl units, one each at C-3 and C-3′. This was also confirmed by the observation of the base peak at *m*/*z* 263 in its mass spectrum (Figure 3). Comparison of the ^{13}C NMR spectroscopic data of **2** with those of **1** demonstrated that the methyl group at C-3 of **1** is replaced by a 3,3-dimethylallyl unit in **2**. Also, HMBC long-range correlations were observed from H-1″ and H-1‴ to C-3 and C-3′, respectively, and from H-9 and H-9′ to C-2 and C-4 and to C-2′ and C-4′, respectively.

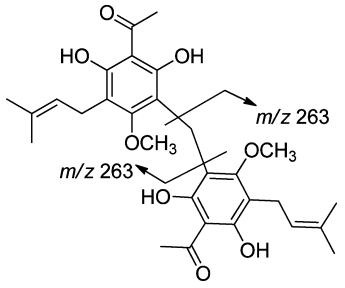


Figure 3. Mass fragmentation observed for **2**.

The locations of the hydroxy groups at C-4 (C-4′) and C-6 (C-6′), the methoxyl groups at C-2 (C-2′), the acetyl group at C-5 (C-5′), and the methylene at C-1 (C-1′) were elucidated in the same manner as for **1**. These data led to the assignment of the structure of **2** as 1-methylene-bis-4-methoxy-6-hydroxy-3-(3,3-dimethylallyl)-2-methoxyacetophenone.

Biological Activities. Compounds **1**−**3** were evaluated for their activity against *P. falciparum* Dd2 (a chloroquine/mefloquine-resistant strain). Compounds **1** and **2** showed submicromolar activity with half-maximum inhibitory concentration (IC_{50}) values of 0.75 ± 0.30 and 0.14 ± 0.04 μM, respectively, while compound **3** was not active. In addition to their cytostatic activity in inhibiting the growth of *P. falciparum*, compounds **1** and **2** also showed cytocidal activity vs *P. falciparum*. Using a newly developed rapid assay for determination of cytocidal activity,[15] compound **1** was found to have median lethal dose (LD_{50}) values of 14.6 ± 0.7 and 6.7 ± 0.2 μM vs the drug-sensitive HB3 strain and the drug-resistant Dd2 strain, respectively, while compound **2** had LD_{50} values of 0.81 ± 0.05 and 0.80 ± 0.02 μM vs the same two strains. In these same assays, chloroquine exhibited LD_{50} values of 0.10 ± 0.01 and 15.3 ± 0.9 μM (HB3 vs Dd2), so for the drug-resistant Dd2 strain, compound **2**, in particular, is significantly more cytocidally potent than chloroquine.

Compounds **1** and **2** were further evaluated for their gametocytocidal activity against late-stage gametocytes (the stage responsible for malaria transmission) using the chloroquine-sensitive NF54 strain to generate gametocytes. This strain was used because it forms gametocytes in culture much better than the chloroquine-sensitive HB3 strain. Only compound **2** showed gametocytocidal activity, with an IC_{50} value of 3.6 ± 0.2 μM. This activity is comparable to the current antimalarial drug artesunate (IC_{50} value of 2.3 μM) and to NPC1161B, an antimalarial drug currently in the development pipeline (IC_{50} value of 3.8 μM).[16,17] The IC_{50} value determined for compound **2** against asexual stages in the NF54 strain was 0.07 ± 0.01 μM.

In order to address if compounds **1** and **2** were able also to prevent gametocytogenesis, *P. falciparum* in vitro cultures were treated with 0.76 μM (~IC_{50}) or 44 μM (~IC_{100}) of compound **1** and 0.14 μM (~IC_{50}) or 39 μM (~IC_{100}) of compound **2** for 13 days, as described in the Experimental Section. On day 13, cultures were recovered and smeared for microscopic examination. Neither asexual intraerythrocytic stages nor gametocytes were observed in cultures treated with 0.14 μM of compound **2** (Figure 4). Compound **1** cleared asexual intraerythrocytic stages in vitro at 0.76 μM, and the presence of mature gametocytes was reduced 80% as compared to untreated parasites (control). This is the first report on mallotojaponin derivatives showing antimalarial activity.

dx.doi.org/10.1021/np300750q | *J. Nat. Prod.* 2013, 76, 388−393

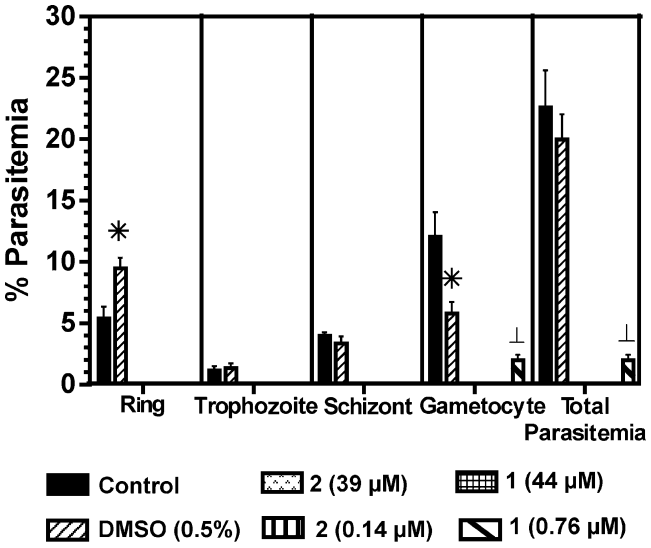


Figure 4. *P. falciparum* blood stage distribution after 13 days of treatment at the indicated concentrations to assess antimalarial activity against early-stage gametocytes. The control is untreated parasites. DMSO (drug vehicle at 0.5%) was included as a second control since it affects the blood-stage distribution but does not affect the total parasitemia. No parasite growth was observed at doses of **2** of 0.14 and 39 μM and of **1** of 44 μM. Results are presented as means of two independent experiments ± SEM, (*) $p \leq 0.02$ and (⊥) $p \leq 0.005$ compared with the control group.

Compounds **1** and **2** also displayed strong antiproliferative activity against the A2780 human ovarian cancer cell line with IC_{50} values of 1.10 ± 0.05 and 1.3 ± 0.1 μM, respectively. Since mallotophenone (**3**) showed weaker activity (IC_{50} 6.3 ± 0.4 μM), it appears that the presence of the 3,3-dimethylallyl group in the molecule enhances the activity; however, this effect was more pronounced in the malaria parasite. Phloroglucinols have been reported to have a wide range of biological activities.[14,18−22] Previous investigation showed that 3′-(3,3-dimethylallyl)-1′-(5-acetyl-6-hydroxy-3-methyl-2-methoxybenzyl)-2′-hydroxyphloracetophenone, a 2′-hydroxylated derivative of mallotojaponin B (**1**), displayed cytotoxic activities against KB and mouse leukemia L-5178Y (ED_{50} 0.58 and 0.74 μg/mL, respectively).[12]

The discovery of the potent antimalarial and gametocytocidal activities of compound **2** raised the question of its possible mechanism of action. It has been proposed that acylphloroglucinols can function as antimalarial agents by acting as radical-generating species or by inhibiting hemozoin formation.[23] The latter may occur through binding to precrystalline forms of heme via $\pi-\pi$ interactions (given their electron-rich structures) and/or coordination with the Fe^{3+} center of hematin (such as an Fe−O interaction with the phenolic moiety of **2**).[23] As a test of the importance of the phenolic hydroxy groups of **2**, the compound was converted to its tetramethyl ether **4** by treatment with potassium carbonate and excess methyl iodide. Compound **4** was active but was 15 times less potent than its parent compound **2** against *P. falciparum*, with IC_{50} values of 2.2 ± 0.5 and 2.5 ± 0.5 μM to the drug-sensitive NF54 cell line and the drug-resistant Dd2 cell line, respectively. It thus appears that the phenolic hydroxy groups of **2** are important for its antimalarial activity, supporting the idea that coordination to Fe^{3+} and/or radical generation plays an important role in the antimalarial activity of the phloroglucinols in general and of compound **2** in particular. This conclusion is also supported by the recent report of the modest antiplasmodial activity of watsonianone A (**5**), a related compound lacking phenolic hydroxyl groups isolated from the Australian tree *Corymbia watsoniana*.[24]

Bioactive phloroglucinols have been detected in the Aspidiaceae, Cannabinaceae, Clusiaceae, Compositae, Crassulaceae, Euphorbiaceae, Fagaceae, Guttiferae, Lauraceae, Myrtaceae, Rosaceae, and Rutaceae families.[18] From the present study, it may be concluded that *Mallotus japonicus* and *M. oppositifolius* are two Euphorbiaceous species that produce dimeric phloroglucinols such as mallotophenone (**3**) and related compounds.[12,25,26] It is also noteworthy that an extract of *M. japonicus* inhibited production of pro-inflammatory cytokines during macrophage activation, and mallotojaponin was one of the bioactive phloroglucinol derivatives isolated from this extract that showed this effect.[27] Mallotojaponin was also shown to inhibit HIV-reverse transcriptase activity.[28]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a JASCO P-2000 polarimeter. IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. ^1H and ^{13}C NMR spectra were recorded on a JEOL Eclipse 500 spectrometer in $CDCl_3$ with TMS as internal standard. Mass spectra were obtained on a JEOL JMS-HX-110 and an Agilent 6220 LC-TOF-MS. Preparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a semipreparative Varian Dynamax C_{18} column (5 μm, 250 × 10 mm), a Shimadzu SPD M10A diode array detector, and a SCL-10A system controller.

Plant Material. Leaves and inflorescences of *Mallotus oppositifolius* (Geiseler) Müll. Arg. (collection: Richard Randrianaivo et al. 1425) were collected at an elevation of 137 m in December 2006 near the village of Befarafara in the dry forest of Solanampilana, 35 km north of Daraina, Antsiranana, Sava region, 13°05′42″ S 049°34′57″ E, northern Madagascar. The sample collected was from a shrub 3 m tall, with white flowers. The plant was determined by Dr. Gordon McPherson (Missouri Botanical Garden). Duplicate voucher specimens were deposited at the Centre National d'Application des Recherches Pharmaceutiques, the Herbarium of the Parc Botanique et Zoologique de Tsimbazaza, Antananarivo, Madagascar, the Missouri Botanical Garden, St. Louis, Missouri, and the Museum National d'Histoire Naturelle in Paris, France.

Antiproliferative Bioassay. The A2780 ovarian cancer cell line assay was performed at Virginia Tech as previously reported.[29] The A2780 cell line is a drug-sensitive ovarian cancer cell line.[30]

Intraerythrocytic Stage Antimalarial Bioassay. The effect of each fraction and pure compound on parasite growth of the Dd2 strain was measured in a 72 h growth assay in the presence of drug as described previously with minor modifications.[31,32] Briefly, ring stage parasite cultures (200 μL per well, with 1% hematocrit and 1% parasitemia) were then grown for 72 h in the presence of increasing concentrations of the drug in a 5.05% CO_2, 4.93% O_2, and 90.2% N_2 gas mixture at 37 °C. After 72 h in culture, parasite viability was determined by DNA quantitation using SYBR Green I (50 μL of SYBR Green I in lysis buffer at 0.4 μL of SYBR Green I/mL of lysis buffer).[32] The half-maximum inhibitory concentration (IC_{50}) calculation was performed with GraFit software using a nonlinear regression curve fitting. IC_{50} values are the average of three independent determinations with each determination in duplicate and are expressed ± SEM.

Intraerythrocytic Stage Cytocidal Antimalarial Bioassay. The effectiveness of compounds **1** and **2** at killing intraerythrocytic stages of the chloroquine-sensitive HB3 and chloroquine-resistant Dd2 strains of *P. falciparum* was assessed as previously reported.[15] LD_{50} values are the average of three replicate determinations and are expressed ± SEM.

dx.doi.org/10.1021/np300750q | J. Nat. Prod. 2013, 76, 388−393

Late Gametocyte Stage Antimalarial Bioassay. To test compounds for their effectiveness in killing late-stage (stage V) gametocytes, late-stage gametocytes were generated using a combination of established methods.[16,33] Initial gametocyte cultures were developed from the *P. falciparum* NF54 strain (chloroquine-sensitive strain). All cultures were maintained in 75 cm^2 culture flasks in a reduced oxygen environment (5% O_2, 5% CO_2, 95% N_2) at 37 °C. Parasitaemia was calculated by counting the percentage of infected RBCs by Giemsa staining of thin smears and light microscopy. Asexual stages were synchronized by sorbitol treatment at least two days before setting gametocyte cultures.[34] Thin blood smears were made and stained with Giemsa to check parasite development on days 4, 8, 12, and 13 after the initial subculture. On day nine of the gametocyte cultures, parasites were treated with 5% sorbitol for 10 min at 37 °C to start removing asexual stages. Sorbitol treatment was performed for four consecutive days, which effectively removes >99% of asexual parasites. Gametocyte recovery and concentration was achieved on day 13 using a NycoPrep 1.077 cushion,[16] and the number of gametocytes was calculated using a Neubauer chamber. About 30 000 to 50 000 gametocytes per well were added to the black flat-bottom half-area 96-well plates containing drug candidates in a 100 μL final volume. The plate was incubated in a humidified chamber at 37 °C and low oxygen conditions (5% O_2, 5% CO_2, 95% N_2) for 72 h. Alamar Blue was added on day 16 postinduction at 10% of the well volume.[33] The plate was returned to the chamber for an additional 24 h and then was read in a microplate reader at 585 nm after excitation at 540 nm. IC_{50} values were calculated using a dose−response curve fitting with GraFit. IC_{50} values are the average of two independent determinations, each determination in duplicate and are expressed ± SEM.

Early Gametocyte Stage Antimalarial Bioassay. To test efficacy in preventing gametocytogenesis, 24-well plates were set at 0.75% parasitemia (NF54 strain) and 1% hematocrit and cultured for 13 days with or without the presence of compounds **1** and **2**. The plate was incubated in a humidified chamber at 37 °C and low oxygen conditions for the duration of the experiment. Medium or medium supplemented with drug was replaced on days 4, 6, 8, and 9−12. On day 13, each well was recovered and the parasitaemia (both asexual and sexual) was calculated from Giemsa-stained smears.

Extraction and Isolation. A ground sample of *M. oppositifolius* leaves and inflorescences (137 g) was extracted with ethanol at room temperature to yield 6.0 g of crude ethanol extract, designated MG 4129. A total of 1.8 g of this extract was made available to Virginia Polytechnic Institute and State University. In order to locate the biological activity and to have an idea about the types of metabolites responsible for the activity of the active fraction, 100 mg of the crude ethanol extract of *M. oppositifolius* was subjected to a liquid−liquid partition using hexanes, EtOAc, and H_2O to afford 42.5 mg of an active hexanes fraction (IC_{50} 6.7 μg/mL). Size-exclusion chromatography on Sephadex LH-20 of the hexanes fraction eluted with MeOH−CH_2Cl_2 gave mallotophenone (**3**, IC_{50} 6.3 ± 0.4 μM) and two active fractions (Fr. 3, 13 mg, IC_{50} 2.3 μg/mL, and Fr. 4, 8.4 mg, IC_{50} 1.6 μg/mL). High-performance liquid chromatography (HPLC) on a C_{18} column with a solvent gradient from water−MeOH (system I) 40:60 to 30:70 for 10 min, to 20:80 from 10 to 15 min, to 15:85 from 15 to 20 min, maintained at 15:85 for 5 min, to 10:90 from 25 to 30 min, and to 0:100 from 30 to 35 min, ending with 100% MeOH for 50 min of fractions 3 and 4 showed the presence of two major and active phloroglucinols (t_R 39.79 min; IC_{50} 0.5 μg/mL and t_R 44.15 min; IC_{50} 0.61 μg/mL). To isolate more material for structure elucidation and for bioctivity evaluations, the isolation was scaled up by starting with 1 g of ethanol extract. Liquid−liquid partion (hexanes, 3 × 200 mL) followed by Sephadex LH-20 of the hexanes fraction (407 mg) afforded two active fractions (Fr. 3, 125.2 mg, IC_{50} 2.4 μg/mL and Fr. 4, 137.1 mg, IC_{50} 2.2 μg/mL). Mallotophenone (**3**) was obtained from fractions 3 and 4 by precipitation. The mother liquid of Fr. 3 was subjected to silica gel column chromatography to give compounds **1** (8.3 mg) and **2** (6 mg). Also, HPLC of the mother liquid of Fr. 4 on a C_{18} column using isocratic 100% MeOH gave two active peaks (t_R 39.79 and 44.15 min), which were purified by silica gel CC to yield compounds **1** (3.1 mg) and **2** (5 mg).

*Mallotojaponin B (**1**):* colorless prisms (EtOAc−hexanes); mp 175 ± 1 °C; UV (MeOH) λ_{max} (log ε) 283 (4.06) nm; IR (film) ν_{max} 3450, 3223, 1620, 1596, 1405, 1283, 1128 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Tables 1 and 2; positive HRESIMS *m/z* 459.2023 $[M + H]^+$ (calcd for $C_{25}H_{31}O_8$, 459.2019).

X-ray Crystallography of 1. A colorless prism of **1** was centered on the goniometer of an Oxford Diffraction SuperNova A diffractometer operating with Cu Kα radiation. The data collection routine, unit cell refinement, and data processing were carried out with the program CrysAlisPro.[35] The Laue symmetry and systematic absences were consistent with the monoclinic space group $P2_1/c$. The structure was solved using SHELXS-97[36] and refined using SHELXL-97[36] via OLEX2.[37] The final refinement model involved anisotropic displacement parameters for non-hydrogen atoms. A riding model was used for the aromatic and alkyl hydrogens. The −OH hydrogen positions were located from the residual electron density map and refined independently.

Crystal data: colorless prism; $C_{25}H_{30}O_8$, M_r = 458.49, monoclinic, $P2_1/c$, a = 12.88614(12) Å, b = 12.34409(7) Å, c = 14.84644(13) Å, β = 110.4761(10)°, V = 2212.38(3) $Å^3$, 37 837 reflections, 317 parameters; crystal size 0.4149 × 0.1458 × 0.1287 mm^3. The final indices were R_1 = 0.0367, wR_2 = 0.0973 [$I > 2\sigma(I)$]. Crystallographic data for compound **1** have been deposited as Supporting Information at the Cambridge Crystallographic Data Centre (deposition no. CCDC 874720).[38]

*Mallotojaponin C (**2**):* amorphous powder; UV (MeOH) λ_{max} (log ε) 283 (4.12) nm; IR (film) ν_{max} 3440, 3220, 1620, 1595, 1434, 1405, 1280, 1121 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Tables 1 and 2; positive HRESIMS *m/z* 513.2499 $[M + H]^+$ (calcd for $C_{29}H_{37}O_8$, 513.2488).

*Mallotophenone (**3**).* The structure of **3** was identified by single-crystal X-ray analysis and by comparison of its spectroscopic data with values reported in the literature.[12] Crystallographic data for compound **3** has been deposited as Supporting Information at the Cambridge Crystallographic Data Centre (deposition no. CCDC 874719).[38]

*Methylation of Mallotojaponin B (**2**).* Compound **2** (0.9 mg) was dissolved in acetone (1.5 mL) and treated with K_2CO_3 (120 mg) and methyl iodide (100 μL). The mixture was stirred at room temperature for 17 h. The reaction mixture was evaporated, dissolved in water, and extracted with EtOAc to give compound **4** (1 mg). The structure of **4** was confirmed by interpretation of its ^1H NMR spectrum and by HRESIMS (see Supporting Information).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR spectra of compounds **1** and **2**, ^1H NMR spectrum of **4**, and an ORTEP drawing of **3**. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This project was supported by the Fogarty International Center, the National Cancer Institute, the National Science Foundation, the National Heart, Lung and Blood Institute, the National Institute of Mental Health, the Office of Dietary Supplements, and the Office of the Director of NIH, under Cooperative Agreement U01 TW000313 with the International Cooperative Biodiversity Groups. This project was also supported by the National Research Initiative of the Cooperative State Research, Education and Extension Service,

USDA, Grant 2008-35621-04732, and by the National Center for Complementary and Alternative Medicine under Cooperative Agreement U01 TW000313-19S1. This work was also supported by the National Science Foundation under Grant No. CHE-0619382 for purchase of the Bruker Avance 600 NMR spectrometer and Grant No. CHE-0722638 for the purchase of the Agilent 6220 mass spectrometer. We thank Mr. B. Bebout for obtaining the mass spectra and Dr. Hugo Azurmendi for assistance with the NMR spectra. Field work essential for this project was conducted under a collaborative agreement between the Missouri Botanical Garden and the Parc Botanique et Zoologique de Tsimbazaza and a multilateral agreement between the ICBG partners, including the Centre National d'Applications des Recherches Pharmaceutiques. We gratefully acknowledge courtesies extended by the Government of Madagascar (Ministère des Eaux et Forêts). All support is gratefully acknowledged.

DEDICATION

Dedicated to Dr. Lester A. Mitscher, of the University of Kansas, for his pioneering work on the discovery of bioactive natural products and their derivatives.

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NPC Natural Product Communications

2013
Vol. 8
No. 9
1201 - 1203

A New Bioactive Diterpene Glycoside from *Molinaea retusa* from the Madagascar Dry Forest

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Received: April 23rd, 2013; Accepted: June 21st, 2013

In a continuing collaboration in a search for new antiproliferative compounds in Madagascar as part of an International Cooperative Biodiversity Group (ICBG), an ethanol extract of *Molinaea retusa* Radlk. (Sapindaceae) was investigated on the basis of its moderate antiproliferative activity against the A2780 human ovarian cancer cell line (IC_{50} 16 μg/mL). One new compound, 2",3",4",6'-de-*O*-acetylcupacinoside (**1**, IC_{50} 15.4 μM) and two known compounds, cupacinoside (**2**, IC_{50} 9.5 μM) and 6-de-*O*-acetylcupacinoside (**3**, IC_{50} 10.9 μM), were isolated by bioassay-directed fractionation using liquid-liquid partitioning, column chromatography, and HPLC. Compounds **2** and **3** also had moderate antiplasmodial activities, with IC_{50} values of 4.0 and 6.4 μM, respectively, against *Plasmodium falciparum,* Dd2 strain. The structures were determined using spectroscopic methods.

Keywords: Antiproliferative, Antiplasmodial, *Molinaea retusa*, Diterpene, Saponin.

As a part of the Madagascar International Cooperative Biodiversity Group (ICBG) program [1] an ethanol extract of *Molinaea retusa* Radlk. was found to have moderate antiproliferative activity (IC_{50} 16 μg/mL) against the A2780 cell line, and the extract was selected for evaluation. *Molinaea retusa* is a member of the Sapindaceae family which contains approximately 135 genera and 1,600 species [2]. While plants of the Sapindaceae family have been the subject of multiple phytochemical studies, the genus *Molinaea* has not previously been investigated.

Plants of the Sapindaceae family are known to contain terpenoids, flavonoids, and ceramides, with varying types of activity, including antiproliferative and antiplasmodial activities [3]. *Molinaea retusa* is one of ten members of the *Molinaea* genus, all of which are endemic to Madagascar [2]. During this study, one unreported compound, 2",3",4",6'-de-*O*-acetylcupacinoside (**1**, IC_{50} 15.4 μM) (Figure 1) and two known compounds, cupacinoside (**2**, IC_{50} 9.5 μM, 4.0 μM) and 6'-de-*O*-acetylcupacinoside (**3**, IC_{50} 10.9 μM, 6.4 μM) (Figure 1) were isolated (IC_{50} values against the A2780 ovarian cancer cell line and *Plasmodium falciparum* strain Dd2, respectively) [3a].

Compounds **2** and **3** were previously isolated from *Cupania cinerea* (Sapindaceae) with activities of 1.3 μM (**2**) and 2.1 μM (**3**) against *P. falciparum* (K1 strain) and activities of 11.6 μM (**2**) and 8.7 μM (**3**) against rat skeletal myoblast cells (L-6 cell line) [3a].

Liquid/liquid partitioning of an EtOH extract of *M. retusa* (IC_{50} 16 μg/mL) yielded an active EtOAc fraction (IC_{50} 10 μg/mL). Further bioassay-guided isolation, including the use of C_{18} reverse phase chromatography and amino normal phase chromatography, yielded three bioactive compounds with moderate antiproliferative

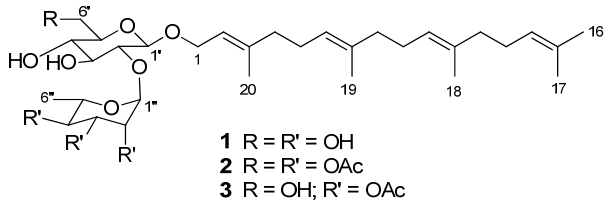

Figure 1: Structure of 2",3",4",6'-de-*O*-acetylcupacinoside (**1**), cupacinoside (**2**), and 6'-de-*O*-acetylcupacinoside (**3**).

activity exhibited against the A2780 ovarian cancer cell line. Two of the compounds, cupacinoside (**2**) and 6'-de-*O*-acetylcupacinoside (**3**) have previously been reported and were identified through comparison of ^{1}H NMR, ^{13}C NMR, and HRESIMS spectroscopic data with previously published data [3a].

The previously unreported compound **1** (IC_{50} 15.4 μM) was also isolated. The structure of compound **1** was found to be very similar to **2** and **3** based on comparison of their ^{1}H NMR and ^{13}C NMR spectroscopic data. The ^{1}H NMR and ^{13}C NMR showed that five methyl groups (δ 1.60, 6H, s; δ 1.60, 3H, s; δ 1.61, 3H, s; δ 1.67, 3H, s; and δ 1.70, 3H, s) together with a broad triplet at δ 5.39 were present, consistent with the presence of a geranylgeraniol group as found in **2** and **3** [3a]. The ^{1}H and ^{13}C NMR spectra exhibited two anomeric protons, suggesting the presence of two sugar units. The signal in the ^{1}H NMR spectrum at δ 4.35 (d, J = 7.7 Hz) is indicative of the anomeric proton on a *β*-glucopyranosyl unit. The remaining carbon and proton signals for the glucopyranosyl moiety were assigned through HSQC and HMBC correlations and have shifts corresponding to glucopyranosyl based on comparison with published literature data [3a]. The doublet (J = 6.2 Hz) signal at δ 1.21 (H-6") is characteristic of the methyl group present in a

Table 1: NMR data of 2",3",4",6'-de-*O*-acetylcupacinoside (**1**).

	Position	δ_H	*J* (Hz)	δ_C		Position	δ_H	*J* (Hz)	δ_C
Isoprene Chain	1 (CH_2)	4.37 dd	11.7, 6.6	66.4		17 (CH_3)	1.67	-	25.9
		4.18 dd	11.7, 7.5	-		18 (CH_3)	1.61[e]	-	16.1[e]
	2 (CH)	5.39 bt	-	121.5		19 (CH_3)	1.60[e]	-	16.1[e]
	3 (C)	-	-	141.8		20 (CH_3)	1.70	-	16.6
	4 (CH_2)	1.94-2.10[f]	-	40.7[a]	β-Glucopyranosyl	1' (CH)	4.35 d	7.7	101.6
	5 (CH_2)	2.01-2.17[f]	-	27.8[b]		2' (CH)	3.35 t	8.5	79.9
	6 (CH)	5.11[f]	-	125.3[c]		3' (CH)	3.44 t	9.0	79.2
	7 (C)	-	-	135.9[d]		4' (CH)	3.28 t	8.8	71.8
	8 (CH_2)	1.94-2.10[f]	-	40.8[a]		5' (CH)	3.21 ddd	9.7, 5.7, 2.3	77.8
	9 (CH_2)	2.01-2.17[f]	-	27.6[b]		6' (CH_2)	3.86 dd	12.0, 2.3	62.8
	10 (CH)	5.11[f]	-	125.4[c]			3.67 dd	12.0, 5.8	-
	11 (C)	-	-	136.2[d]	α-Rhamnopyranosyl	1" (CH)	5.12[f]	-	102.6
	12 (CH_2)	1.94-2.10[f]	-	40.9[a]		2" (CH)	3.92 dd	3.4, 1.7	72.2
	13 (CH_2)	2.01-2.17[f]	-	27.5[b]		3" (CH)	3.64 dd	9.6, 3.4	72.4
	14 (CH)	5.11[f]	-	125.5[c]		4" (CH)	3.37 t	9.6	73.9
	15 (C)	-	-	132.0		5" (CH)	4.01 dq	6.2, 9.6	69.8
	16 (CH_3)	1.60	-	17.8		6" (CH_3)	1.21 d	6.2	18.0

[a, b, c, d, e] – Interchangeable signals. [f] – Overlapping signals.

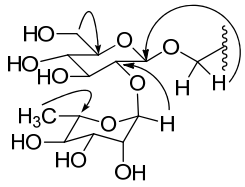

Figure 2: Key HMBC correlations of 2'',3'',4'',6'-de-*O*-acetylcupacinoside (**1**).

rhamnopyranosyl unit. The anomeric proton signal was assigned as δ 5.12 through use of HMBC and HMQC correlations. The coupling constant could not be directly obtained, due to overlap of signals in the ^{1}H NMR spectrum, but could be inferred through the coupling constant present for the H-2" signal (dd, *J* = 3.4, 1.7 Hz). Since 3.4 Hz represents the coupling of H-2" to H-3", the coupling of 1.7 Hz represents of coupling of H-1" to H-2". Furthermore, the chemical shift of C-5" (δ 69.8) is consistent with an α-rhamnopyranosyl group [4].

Assignments of protons and carbons for the α-rhamnopyranosyl unit were made based on HMBC and HSQC correlations and coupling constants present in the ^{1}H NMR spectrum. A correlation in the HMBC from H-1 to C-1' indicated that *β*-glucopyranosyl was attached to the aglycone, and a correlation from H-1" to C-2' indicated the α-rhamnopyranosyl is connected to the *β*-glucopyranosyl unit at the 2' position (Figure 2). The aglycone connectivity and sugar linkage were thus confirmed to be the same in **1** as in **2** and **3**, as expected due to the similarity of their spectroscopic data.

In comparison to **2** and **3**, H-2", H-3", and H-4" of **1** were shifted upfield (*ca.* – 1.6 ppm); conversely, C-2", C-3", and C- 4" were shifted downfield (*ca*. +2.7 ppm) which is in agreement with the lack of acetylation. The ^{13}C NMR spectrum further indicated a lack of acetylation due to the absence of peaks downfield of δ 160. The molecular formula ($C_{32}H_{54}O_{10}$) was established using HRESIMS which yielded an ion at *m/z* 643.3727 corresponding to $[M+HCOO]^-$ (calcd. 643.3694 for $C_{33}H_{55}O_{12}$). Compound **1** was thus determined to be 2",3",4",6'-de-*O*-acetylcupacinoside

Antiproliferative activities of compounds **1** – **3** against a human ovarian cancer cell line (A2780) were comparable to published activities against a rat skeletal myoblast cell line (L-6). Likewise antimalarial activities against *P. falciparum* (Dd2 strain) were comparable to previously reported activities against *P. falciparum* (K1 strain) [3a].

Table 2: Activities of isolated compounds against the A2780 cell line and *Plasmodium falciparum*

Compound	Antiproliferative activity against A2780 IC_{50}, μM	Antiplasmodial activity against *P. falciparum* Dd2 strain, IC_{50}, μM
1, 2",3",4",6'-de-*O*-Acetylcupacinoside	15.4	Not determined
2, Cupacinocide	9.5	4.0 ± 0.6
3, 6'-de-*O*-Acetylcupacinoside	10.9	6.4 ± 0.5

Experimental

General: Optical rotations were recorded on a JASCO P-2000 polarimeter. UV spectrum was measured using a Shimadzu UV-1201 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Advance 500 spectrometer in CD_3OD. Mass spectra were obtained with an Agilent 6220 LC-TOF-MS. Preparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a Sedex 75 Evaporative Light Scattering Detector (ELSD), a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and a Varian Dynamax NH_2 column (250 x 10 mm). Flash chromatography collection was performed using a Biotage Horizon Pump coupled with a Biotage Horizon Flash Collector and a Biotage Horizon UV Detector.

Plant Material: Roots of *Molinaea retusa* Radlk. (Sapindaceae) (collection: Roland Rakotondrajaona et al. 366) were collected at an elevation of 275 m in November 2005, 2 km northwest of the village of Ankijabe in the Bemôsy forest, Vohemar, Daraina, Antsiranana, Sava region, 13°14'41" S 049°37'53" E, northern Madagascar. The sample was collected from a bush 4 m tall with orange fruits and black seeds.

Extraction and Isolation: Dried and powdered *M. retusa* roots (275 g) were extracted with EtOH for 24 h to yield 28.6 g of EtOH extract, of which 8.1 g was made available to Virginia Tech. Liquid/liquid partition of this extract (2 g) yielded an active EtOAc fraction (253 mg, IC_{50} 12 μg/mL). Purification of the EtOAc fraction was performed on a C-18 column (MeOH/H_2O gradient) using a flash chromatography fraction collector, and yielded one active sub-fraction (140 mg, IC_{50} 9 μg/mL). The sub-fraction was fractionated further on a flash chromatography fraction collector utilizing a silica gel column with a hexanes/EtOAc gradient yielding compound **3** (25 mg, IC_{50} 10.9 μM) and one other active fraction (55 mg, IC_{50} 9 μg/mL). The active fraction was fractionated further through the use of an open silica gel column (EtOAc mobile phase) yielding compound **2** (7 mg, IC_{50} 9.5 μM). A second fractionation was carried out to isolate a minor active compound detected during

the first fractionation. Liquid/liquid partitioning of another 2 g of EtOH extract yielded the active EtOAc fraction (480 mg, IC_{50} 11 μg/mL) which was separated by a C-18 column ($MeOH/H_2O$ gradient) to yield one major active fraction (160 mg, IC_{50} 9 μg/mL). Compound **1** (17 mg, IC_{50} 15.4 μM) was obtained using an NH_2 open column (85:15 EtOAc/MeOH mobile phase) followed by fractionation using a silica gel open column (EtOAc mobile phase), followed by purification using high pressure liquid chromatography (HPLC) utilizing a silica gel column with a hexanes/EtOAc gradient.

Antiproliferative Bioassay: Assay was performed at Virginia Tech according to specifications previously described [5]. The A2780 cell line is a drug-sensitive ovarian cancer cell line [6].

Antimalarial Bioassay: Assay was performed at Virginia Tech as previously described [7].

2'',3'',4'',6'-de-*O*-acetylcupacinoside (**1**)

$[\alpha]_D^{23}$: –22.0 (*c* 0.84, MeOH).
UV/Vis λ_{max} (MeOH) nm (log ε): 203 (3.22).
^{1}H NMR (500 MHz, CD_3OD): Table 1.
^{13}C NMR (125 MHz, CD_3OD): Table 1.
HRESIMS: *m/z* $[M + HCOO]^-$ calcd. for $C_{33}H_{55}O_{12}$: 643.3694; found: 643.3727. *m/z* $[M + Cl]^-$ calcd. for $C_{32}H_{54}O_{10}Cl$: 633.4306; found: 633.4326. *m/z* $[M-H]^-$ calcd. for $C_{32}H_{53}O_{10}$: 597.3639; found: 597.3652.

Supplementary data: ^{1}H-NMR, ^{13}C-NMR, and ESIMS data is available in electronic form on the publishers website for 2'',3'',4'',6'-de-*O*-acetylcupacinoside (**1**).

Acknowledgments - This International Cooperative Biodiversity Group project was supported by the Fogarty International Center, the National Cancer Institute, the National Institute of Allergy and Infectious Diseases, the National Institute of Mental Health, the National Institute on Drug Abuse, the National Heart Lung and Blood Institute, the National Center for Complementary and Alternative Medicine, the Office of Dietary Supplements, the National Institute of General Medical Sciences, the Biological Sciences Directorate of the National Science Foundation, and the Office of Biological and Environmental Research of the U.S. Department of Energy under Cooperative Agreement U01 TW00313 with the International Cooperative Biodiversity Groups. This project was also supported by the National Research Initiative of the Cooperative State Research, Education and Extension Service, USDA, Grant #2008-35621-04732. Funds from the Fralin Life Science Institute, The Thomas F & Kate Miller Jeffress Memorial Trust (J-1058) and Commonwealth Health Research Board (208-03-12) to provided funding to MBC, and JDB is recipient of a scholarship from the National Science Foundation S-STEM project (DUE-0850198). These supports are gratefully acknowledged. Work at Virginia Tech was supported by the National Science Foundation under Grant CHE-0722638 for the purchase of the Agilent 6220 mass spectrometer. We thank Mr. B. Bebout for obtaining the mass spectra. Fieldwork essential for this project was conducted under a collaborative agreement between the Missouri Botanical Garden and the Parc Botanique et Zoologique de Tsimbazaza and a multilateral agreement between the ICBG partners, including the Centre National d'Application des Recherches Pharmaceutiques. We gratefully acknowledge courtesies extended by the Government of Madagascar (Ministère des Eaux et Forêts).

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Two Antiproliferative Triterpene Saponins from *Nematostylis anthophylla* from the Highlands of Central Madagascar[1])

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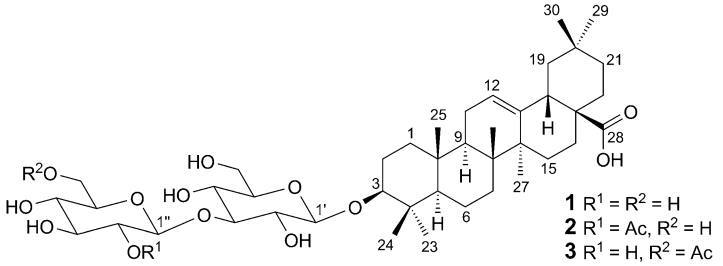
Investigation of the endemic Madagascan plant *Nematostylis anthophylla* (Rubiaceae) for antiproliferative activity against the A2780 ovarian cancer cell line led to the isolation of the known triterpene saponin randianin (**1**), and the two new bioactive triterpene saponins 2″-*O*-acetylrandianin (**2**) and 6″-*O*-acetylrandianin (**3**). The structures of the two new compounds were elucidated based on analysis of their 1D- and 2D-NMR spectra, and mass spectrometric data. The three isolated triterpene saponins displayed moderate but selective antiproliferative activities, with IC_{50} values of 1.2, 1.7, and 2.2 μM, respectively, against the A2780 ovarian cancer, but only weak inhibitions of the proliferation of A2058 melanoma and the H522 lung cancer cell lines.

Introduction. – As part of our engagement in an *International Cooperative Biodiversity Group* (*ICBG*) program, we are focusing on the search for antiproliferative natural products from a diversity of vegetation types in Madagascar [1–3]. The A2780 human ovarian cancer cell line is used as the primary screen, because it is a stable and yet relatively drug-sensitive cell line and gives reproducible results. As a part of this research, an EtOH extract from the roots of *Nematostylis anthophylla* (Rubiaceae) from the Highlands of Central Madagascar was investigated and found to exhibit antiproliferative activity against the A2780 cell line, with an IC_{50} value of 6.9 μg/ml. The Rubiaceae is a large family of 630 genera and *ca.* 13,000 species found worldwide [4]. This family is a rich source of indole alkaloids, terpenoids, and anthraquinones, all of which are well-known for their broad range of bioactivity, including antimicrobial, antimalarial, antidiabetic, vasorelaxant, cytotoxic, antioxidant, and anti-inflammatory activities among others [5–9]. Since *Nematostylis* is one of the many genera of the Rubiaceae that have not been systematically investigated for their phytochemical composition, the EtOH extract of *N. anthophylla* was selected for bioassay-guided fractionation to isolate its active components.

[1]) 'Biodiversity Conservation and Drug Discovery in Madagascar', Part 52. For Part 51, see [1].

Results and Discussion. – *Isolation of Bioactive Compounds.* An EtOH extract of the roots of *N. anthophylla* was subjected to liquid–liquid partitioning to give an active BuOH fraction with an IC_{50} value of 2.2 μg/ml. Bioassay-guided separation, including *LH-20* size-exclusion, *HP-20 Diaion*, and silica-gel normal-phase chromatography, was used to obtain three bioactive compounds comprising the known triterpene saponin randianin (**1**), and the two new related glycosides 2″-*O*-acetylrandianin (**2**) and 6″-*O*-acetylrandianin (**3**). All three compounds had moderate antiproliferative activities against A2780 ovarian cancer cells, with IC_{50} values of 2.2, 1.2, and 1.7 μM, respectively. Herein, we report the structure elucidation and antiproliferative properties of the two new isolates.



Identification of Compounds **1** *and* **2**. Compound **1** was identified as randianin (=oleanolic acid 3-*O*-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranoside) by comparison of its chemical and spectroscopic data with those reported in the literature for the aglycone [10] and the glycoside [11].

Compound **2**, $[\alpha]_D^{21} = +12$ ($c = 1.2$, MeOH), was isolated as a light-yellow solid. Its positive-ion HR-ESI-MS exhibited cationized molecular-ion peaks at *m*/*z* 845.4692 ($[M+Na]^+$) and 861.4618 ($[M+K]^+$), corresponding to the molecular formula $C_{44}H_{70}O_{14}$. The observation of a C=O absorption at 1734 cm^{-1} in the IR spectrum, a ^{13}C-NMR resonance at δ(C) 170.7 ppm, and a *singlet* signal at δ(H) 1.98 ppm in the ^{1}H-NMR spectrum (*Table 1*) suggested the presence of an Ac group. Meanwhile, its glycosidic nature was corroborated by the presence of two anomeric H-atom signals at δ(H) 4.83 and 5.43 ppm. In addition to the Me and C=O C-atoms of the Ac group, there were 42 C-atom signals in the ^{13}C-NMR spectrum, among which 30 C-atom signals were assigned to a triterpenoid aglycone and the remaining 12 C-atoms to a disaccharide moiety. The ^{1}H-NMR spectrum of **2** indicated that the aglycone had seven Me groups corresponding to 3-H *singlet*s at δ(H) 0.80, 0.89, 0.97, 1.00, 1.03, 1.27 and 1.33, and one olefinic H-atom signal appeared at δ(H) 5.49. Correspondingly, signals for seven Me C-atoms at δ(C) 15.8, 17.2, 17.8, 24.1, 26.6, 28.5 and 33.7 ppm, and for two olefinic C-atoms at δ(C) 122.9 and 145.2 ppm were observed in the ^{13}C-NMR spectrum. The presence of a C=O absorption at 1689 and a broad OH absorption at 3453 cm^{-1} in its IR spectrum, together with a ^{13}C-NMR resonance at δ(C) 180.6 ppm, supported the presence of a carboxylic acid group.

Inspection of the ^{1}H- and ^{13}C-NMR spectra of compound **2** indicated that it had the same oleanolic acid aglycone as compound **1**. The HMB correlation between H–C(18) (*dd*, $J = 4.1$, 14.0) and C(28) confirmed that the carboxylic C-atom was connected to C(17) [12]. HMBCs between the anomeric H–C(1′) and C(3), as well as between

Table 1. *^{1}H- and ^{13}C-NMR Data for* **1**–**3**. Recorded in (D_5)pyridine at 500 and 125 MHz, resp.; δ in ppm, J in Hz.

Position	**1**		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	1.21–1.25 (*m*), 1.39–1.42 (*m*)	39.0	1.21–1.25 (*m*), 1.37–1.40 (*m*)	39.0	1.22–1.26 (*m*), 1.39–1.42 (*m*)	39.0
2	1.75–1.78 (*m*), 2.14–2.18 (*m*)	26.8	1.76–1.79 (*m*), 2.15–2.19 (*m*)	26.9	1.74–1.77 (*m*), 2.14–2.18 (*m*)	26.8
3	3.36 (*dd*, J=4.4, 11.9)	89.3	3.36 (*dd*, J=4.4, 11.9)	89.3	3.36 (*dd*, J=4.4, 11.7)	89.4
4	–	40.1	–	40.1	–	40.1
5	0.76–0.80 (*m*)	56.1	0.76–0.79 (*m*)	56.1	0.78–0.82 (*m*)	56.1
6	1.21–1.25 (*m*), 1.45–1.49 (*m*)	18.8	1.22–1.25 (*m*), 1.46–1.50 (*m*)	18.8	1.23–1.26 (*m*), 1.46–1.50 (*m*)	18.8
7	1.78–1.82 (*m*), 1.85–1.87 (*m*)	33.6	1.78–1.82 (*m*), 1.85–1.87 (*m*)	33.6	1.78–1.82 (*m*), 1.85–1.87 (*m*)	33.6
8	–	39.8	–	39.8	–	39.8
9	1.65 (br. *t*, J=8.9)	48.3	1.64 (br. *t*, J=8.9)	48.4	1.65 (br. *t*, J=8.9)	48.3
10	–	37.3	–	37.3	–	37.3
11	1.88–1.92 (*m*)	24.1	1.88–1.92 (*m*)	24.1	1.88–1.92 (*m*)	24.1
12	5.50 (*t*, J=3.3)	122.8	5.49 (*t*, J=3.3)	122.9	5.50 (*t*, J=3.3)	122.8
13	–	145.3	–	145.2	–	145.3
14	–	42.5	–	42.6	–	42.5
15	1.18–1.21 (*m*), 2.02–2.05 (*m*)	28.7	1.18–1.21 (*m*), 2.02–2.05 (*m*)	28.7	1.18–1.21 (*m*), 2.02–2.05 (*m*)	28.7
16	1.76–1.79 (*m*), 2.18–2.21 (*m*)	24.1	1.75–1.78 (*m*), 2.17–2.20 (*m*)	24.1	1.76–1.79 (*m*), 2.18–2.21 (*m*)	24.1
17	–	47.0	–	47.1	–	47.0
18	3.32 (*dd*, J=4.1, 14.0)	42.4	3.32 (*dd*, J=4.1, 14.0)	42.4	3.32 (*dd*, J=4.0, 13.9)	42.4
19	1.28–1.31 (*m*), 1.82–1.84 (*m*)	46.9	1.28–1.31 (*m*), 1.82–1.84 (*m*)	46.9	1.28–1.31 (*m*), 1.82–1.84 (*m*)	46.9
20	–	31.3	–	31.3	–	31.3
21	1.49–1.52 (*m*), 1.82–1.84 (*m*)	34.6	1.49–1.52 (*m*), 1.82–1.84 (*m*)	34.6	1.49–1.52 (*m*), 1.82–1.84 (*m*)	34.6
22	1.45–1.49 (*m*), 2.05–2.08 (*m*)	33.6	1.45–1.49 (*m*), 2.05–2.08 (*m*)	33.6	1.46–1.50 (*m*), 2.05–2.08 (*m*)	33.5
23	1.27 (*s*)	17.8	1.27 (*s*)	17.8	1.32 (*s*)	17.8
24	0.89 (*s*)	28.5	0.89 (*s*)	28.5	1.01 (*s*)	28.5
25	0.82 (*s*)	15.8	0.80 (*s*)	15.8	0.82 (*s*)	15.8
26	1.00 (*s*)	17.4	1.00 (*s*)	17.2	1.00 (*s*)	17.3
27	1.33 (*s*)	26.5	1.33 (*s*)	26.6	1.33 (*s*)	26.5
28	–	180.7	–	180.6	–	180.7
29	1.03 (*s*)	24.1	1.03 (*s*)	24.1	1.02 (*s*)	24.1
30	0.97 (*s*)	33.7	0.97 (*s*)	33.7	0.97 (*s*)	33.6
3-*O*-Glucosyl						
1′	4.91 (*d*, J=7.8)	106.7	4.83 (*d*, J=7.8)	107.1	4.91 (*d*, J=7.6)	106.7
2′	4.09–4.11 (*m*)	74.8	3.96–4.02 (*m*)	74.4	4.05–4.08 (*m*)	74.7
3′	4.23 (*t*, J=8.8)	89.3	4.15 (*t*, J=8.8)	89.3	4.18 (*t*, J=8.9)	89.7
4′	4.11–4.14 (*m*)	70.2	4.04–4.08 (*m*)	70.7	4.11 (*t*, J=9.3)	70.0
5′	3.92–3.98 (*m*)	78.3	3.89–3.93 (*m*)	78.1	3.92–3.98 (*m*)	78.3
6′	4.32 (*d*, J=11.3), 4.51 (*d*, J=11.0)	62.9	4.26 (*d*, J=11.4), 4.48 (*dd*, J=2.1, 11.5)	63.1	4.32 (*dd*, J=6.2, 11.8), 4.52 (*dd*, J=2.2, 11.8)	62.9

Table 1 (cont.)

Position	**1**		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
3′-*O*-Glucosyl						
1″	5.32 (*d*, *J*=7.8)	106.3	5.43 (*d*, *J*=8.1)	103.7	5.25 (*d*, *J*=7.9)	106.3
2″	4.02–4.05 (*m*)	75.9	5.66 (*dd*, *J*=8.1, 9.1)	75.7	4.03–4.05 (*m*)	75.7
3″	4.26 (*t*, *J*=9.1)	75.8	4.31 (*t*, *J*=9.1)	76.6	4.22 (*t*, *J*=9.1)	75.7
4″	4.20 (*t*, *J*=9.2)	72.0	4.20 (*t*, *J*=9.2)	72.3	4.01 (*t*, *J*=9.1)	71.9
5″	4.07–4.09 (*m*)	79.1	4.07–4.10 (*m*)	79.1	4.23–4.26 (*m*)	78.3
6″	4.34 (*d*, *J*=11.1), 4.56 (*d*, *J*=10.7)	62.8	4.28 (*d*, *J*=11.1), 4.58 (*dd*, *J*=2.1, 11.5)	62.7	4.67 (*dd*, *J*=6.8, 11.7), 4.95 (*dd*, *J*=2.2, 11.8)	64.9
2″-AcO						
CO				170.7		
Me			1.98 (*s*)	21.5		
6″-AcO						
CO						171.2
Me					2.00 (*s*)	21.0

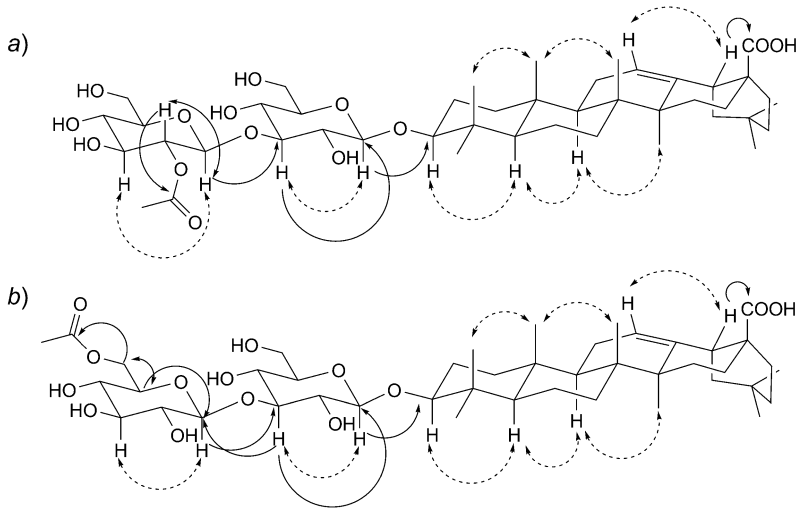


Figure. *HMBC* (H→C), *COSY* (H↔H), *and NOESY* (H← – →H) *correlations of* **2** (*a*) *and* **3** (*b*)

H–C(3) and the anomeric C(1′), confirmed that the disaccharide moiety was connected to C(3) (*Fig.*, *a*).

Both sugar molecules, which were represented by the of two sets of anomeric atom signals at δ(H) 4.83/δ(C) 107.1 ppm and δ(H) 5.43/δ(C) 103.7 ppm, respectively, were identified as glucose, based on the similarity of their ^{13}C-NMR chemical shifts with those of the sugar moiety of **1**. The linkage between the two glucopyranosyl units was determined as 1→3 on the basis of HMBCs between H–C(3′) and the two anomeric C-atoms C(1′) and C(1″), as well as the cross-peak between H–C(3′) and H–C(2′) in the

COSY spectrum (*Fig.*, *a*). The coupling constants between H–C(1′) and H–C(2′), and H–C(1″) and H–C(2″) ($J = 7.8$ and 8.1, resp.) indicated their axial–axial conformation, and thus the β-configuration of the two sugar units. The AcO group was deduced to be located at C(2″) of a glucopyranosyl residue, based on the comparison of the ^{1}H- and ^{13}C-NMR spectra of **2** with those of **1**. Due to this acetylation, the chemical shift of H–C(2″) of **2** was δ(H) 5.66 as compared to δ(H) 4.02–4.05 for **1**, while other H-atoms in the distal glucose had chemical shifts similar to those of compound **1**. The position of the Ac group was confirmed by the COSY cross-peak between the downfield H–C(2″) and the corresponding anomeric H–C(1″), and a three-bond HMBC between H–C(2″) and the C=O C-atom of the Ac group (*Fig.*, *a*).

To determine the absolute configuration of the two glucose moieties and to confirm the overall structure assignment, compound **2** was hydrolyzed with 6M NH_4OH to yield a product identified as randianin (**1**) by its ^{1}H- and ^{13}C-NMR spectra. Further hydrolysis of **1** with 3M HCl yielded oleanolic acid, identified by its ^{1}H- and ^{13}C-NMR spectra, and a single monosaccharide, identified as D-glucose on the basis of a single TLC spot observed with the same R_f value as a D-glucose standard. Its absolute configuration was determined as D based on its positive optical rotation.

Based on these evidences, the structure of **2** was elucidated as oleanolic acid 3-*O*-β-D-glucopyranosyl-(1 → 3)-(2″-*O*-acetyl)-β-D-glucopyranoside, or 2″-*O*-acetylrandianin.

Identification of Compound **3**. Compound **3**, isolated as light-yellow solid, $[\alpha]_D^{21} = +17$ ($c = 1.2$, MeOH), had the same molecular formula as compound **2** as determined by HR-ESI-MS (*m*/*z* 845.4643 ($[M+Na]^+$) and 861.4569 ($[M+K]^+$)), corresponding to the molecular formula of $C_{44}H_{70}O_{14}$. Due to the similarity of its NMR data with those of compounds **1** and **2**, the aglycone portion of **3** was also assigned as oleanolic acid, with the disaccharide moiety connected to C(3) of the aglycone.

As in compound **2**, the presence of two sugar moieties was evidenced by the NMR spectra, which showed two sets of anomeric-atom signals at δ(H) 4.91/δ(C) 106.7 and δ(H) 5.25/δ(C) 106.3, respectively. The two sugar moieties were determined as glucosyls, as corroborated by the similarity of the ^{13}C-NMR chemical shifts of all C-atoms compared to those of compound **1**. The linkage between the two glucopyranosyl units was determined as 1 → 3 on the basis of the HMBCs between H–C(3′) and two anomeric C-atoms (C(1′) and C(1″)), as well as the cross-peak between H–C(3′) and H–C(2′) in the COSY spectrum (*Fig.*, *b*). The coupling constants between H–C(1′) and H–C(2′), and H–C(1″) and H–C(2″) ($J = 7.8$ and 7.8, resp.) indicated their axial–axial orientation and thus the β-configuration of the two sugar units. The presence of an Ac group was evidenced by a C=O absorption at 1727 cm^{-1} in its IR spectrum, ^{13}C-NMR resonances at δ(C) 171.2 ppm, and a *singlet* signal at 2.00 ppm in its ^{1}H-NMR spectrum. The 6″-OH group of the outer glucose moiety of **3** was acetylated, insted of the 2″-OH group of **2**. This was established by comparing the NMR data of the outer glucose moiety of **3** with those of **1**. The chemical shift of the two diastereotopic H-atoms, CH_2(6″), of **1** were shifted from δ(H) 4.34 and 4.56 ppm to δ(H) 4.67 and 4.95 ppm in **3**, while the resonances of the other H-atoms of the outer glucose moiety were similar to those of compound **1**. Furthermore, the location of the AcO group at C(6″) was confirmed by the COSY cross-peak between CH_2(6″) and H–C(5″), and a three-bond HMBCs between CH_2(6″) and the Ac C=O C-atom at 171.2 ppm, and between H–C(5″) and the anomeric C(1″) (*Fig.*, *b*).

As with compound **2**, the absolute configuration of the two glucose units and the overall structure assignment were confirmed by successive basic hydrolysis of **3** to randianin, followed by acidic hydrolysis to oleanolic acid and D-glucose. Based on this evidence, the structure of **3** was elucidated as oleanolic acid 3-*O*-*β*-D-glucopyranosyl-(1→3)-(6″-*O*-acetyl)-*β*-D-glucopyranoside, or 6″-*O*-acetylrandianin.

Biological Evaluation. Compounds **1**–**3** were tested for their antiproliferative activities against the A2780 ovarian cancer, the A2058 melanoma, and the H522 lung cancer cell lines. All three compounds showed modest inhibitions of the proliferation of A2780 ovarian cancer cells, with IC_{50} values in the low micromolar range. However, they showed only weak inhibition of the proliferation of A2058 melanoma and the H522 lung cancer cell lines (*Table 2*). Several hundred cytotoxic triterpene saponins have been identified from plants, but only a few of them showed selective antiproliferative activity [13]. 2″-*O*-Acetylrandianin (**2**) and 6″-*O*-acetylrandianin (**3**) are examples of compounds that selectively inhibit the proliferation of A2780 ovarian cancer cells. Furthermore, in the A2780 assay, the cytotoxicities of the two acetylated saponins are stronger than that of randianin (**1**), which has no Ac group in its structure. This suggests that the increase in activity on acetylation may be due to an increase in lipophilicity, facilitating cellular uptake [14].

Table 2. *Antiproliferative Activities* (IC_{50} [μM]) *of Compounds* **1**–**3**

Compound	A2780[a])	A2058	H522
1	2.2±0.2	7.63	7.32
2	1.7±0.1	>3.3, <10	>10
3	1.2±0.3	>3.3, <10	>10
Paclitaxel	0.028±0.003	ND[b])	ND
Vinblastine	ND	0.004	0.009

[a]) Mean of three replicates. [b]) ND, Not determined.

This project was supported by the *Fogarty International Center*, the *National Cancer Institute*, the *National Science Foundation*, the *National Heart, Lung and Blood Institute*, the *National Institute of Mental Health*, the *Office of Dietary Supplements*, and the *Office of the Director of NIH*, under Cooperative Agreement U01 TW000313 with the *International Cooperative Biodiversity Groups*. This project was also supported by the *National Research Initiative of the Cooperative State Research, Education and Extension Service, USDA*, Grant #2008-35621-04732. Funds for the plant collection and extraction were provided by *Dow AgroSciences* and *Eisai Inc*. This support is gratefully acknowledged. This work was also supported by the *National Science Foundation* under Grant No. CHE-0619382 for purchase of the *Bruker-Avance 500* NMR spectrometer and Grant No. CHE-0722638 for the purchase of the *Agilent 6220* mass spectrometer. We thank Mr. *Bill Bebout* for recording the mass spectra, Dr. *Hugo Azurmendi* for assistance with the NMR spectra, and Drs. *Donald Hahn* (*Dow AgroSciences*) and *Edward Suh* (*Eisai Inc.*) for their encouragement and support. Field work essential for this project was conducted under a collaborative agreement between the *Missouri Botanical Garden* and the *Parc Botanique et Zoologique de Tsimbazaza*, and a multilateral agreement between the ICBG partners, including the *Centre National d'Applications des Recherches Pharmaceutiques*. We gratefully acknowledge courtesies extended by the Government of Madagascar (Ministère des Eaux et Forêts).

Experimental Part

General. Optical rotations: *Jasco P-2000* polarimeter. IR Spectra: *MIDAC M-series* FT-IR spectrophotometer as a film; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: in (D_5)pyridine on a *Bruker Avance 500* spectrometer; chemical shifts δ in ppm, and coupling constants J in Hz. MS: *Agilent 6220 LC-TOF-MS* in the pos.-ion mode; m/z.

Antiproliferative Bioassays. Antiproliferative activities were evaluated at Virginia Polytechnic Institute and State University against the drug-sensitive A2780 human ovarian cancer cell line as described in [15]. The values reported are the mean of three replicates. Antiproliferative activities against the A2058 melanoma and the H522 lung cancer cell lines were determined at *Eisai Inc.* by similar procedures to those used for the H460 cell line [16].

Plant Materials. A sample of the roots of *Nematostylis anthophylla* (A.RICH.) BAILL. was collected in March 2011. The sample was a shrub of 60 cm with red flowers and succulent leaves, growing in rocky habitat on Ibity Massif in the Vakinakaratra region of the Antsirabe II district, Madagascar at an elevation of 1650 m, and coordinates 20°03′59″S 047°00′01″E (−20.0663889, 47.0002778). Duplicate voucher specimens (*Richard Randrianaivo et al. 1803)* have been deposited with the Parc Botanique et Zoologique de Tsimbazaza (TAN), the Centre National d'Application des Recherches Pharmaceutiques in Antananarivo, Madagascar (CNARP), the Missouri Botanical Garden in St. Louis, Missouri (MO), and the Muséum National d' Histoire Naturelle in Paris, France (P).

Extraction and Isolation. Dried root parts of *N. anthophylla* (273 g) were ground in a hammer mill, then extracted with EtOH by percolation for 24 h at r.t. to give the crude extract MG 4657 (12.4 g), of which 3.2 g was shipped to Virginia Tech for bioassay-guided isolation. A 1.1-g sample of MG 4657 (IC_{50} 6.9 μg/ml) was suspended in aq. MeOH (MeOH/H_2O 9:1; 100 ml), and extracted with hexane (3 × 100 ml). The aq. layer was then diluted to 60% MeOH (*v*/*v*) with H_2O and extracted with CH_2Cl_2 (3 × 150 ml). The remaining aq. layer was further extracted with BuOH (3 × 100 ml). The hexane fraction was evaporated *in vacuo* to leave 131.2 mg of material with $IC_{50} > 20$ μg/ml. The residue from the CH_2Cl_2 fraction (166.1 mg) had an IC_{50} value of 7.7 μg/ml, the residue from the BuOH fraction (248.6 mg) had an IC_{50} value of 2.5 μg/ml and the remaining aq. MeOH fraction had an IC_{50} value of 20 μg/ml. Chromatography of the CH_2Cl_2 fraction over a *Sephadex® LH-20* size-exclusion column with CH_2Cl_2/MeOH 1:1 was used to obtain six fractions, of which the most active fraction (40.3 mg) had an IC_{50} value of 2.0 μg/ml. This fraction was then applied to a silica-gel column with $CHCl_3$/MeOH 9:1 to give fourteen fractions, of which *Fr. 11* (4.8 mg) was the most active (IC_{50} 1.0 μg/ml) and yielded compound **3**. The BuOH fraction was applied to an open column of *Diaion HP-20* resin and eluted with a step MeOH/H_2O gradient of 40, 70, and 100% MeOH. The 100% MeOH fraction was the most active fraction (100 mg) with an IC_{50} value of 2.2 μg/ml. This fraction was applied to a silica-gel column and eluted with $CHCl_3$/MeOH 6:1 to give thirteen fractions, of which *Fr. 4* (1.8 mg) yielded compound **2**, with an IC_{50} value of 1.5 μg/ml, and *Fr. 7* (6.3 mg) yielded compound **1**, with an IC_{50} value of 1.9 μg/ml.

*2″-*O*-Acetylrandianin* (*=(3β)-3-{[3-*O*-(2-*O*-Acetyl-β-*D*-glucopyranosyl)-β-*D*-glucopyranosyl]oxy}-olean-12-en-28-oic Acid*; **2**). Light-yellow solid. $[\alpha]_D^{21} = +12$ ($c = 1.2$, MeOH). IR: 3453, 2935, 1734, 1689, 1027. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 845.4692 ($[M+Na]^+$, $C_{44}H_{70}NaO_{14}^+$; calc. 845.4663).

*6″-*O*-Acetylrandianin* (*=(3β)-3-{[3-*O*-(6-*O*-Acetyl-β-*D*-glucopyranosyl)-β-*D*-glucopyranosyl]oxy}-olean-12-en-28-oic Acid*; **3**). Light-yellow solid. $[\alpha]_D^{21} = +17$ ($c = 1.2$, MeOH). IR: 3439, 2935, 1727, 1689, 1027. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 845.4643 ($[M+Na]^+$, $C_{44}H_{70}NaO_{14}^+$; calc. 845.4663).

Hydrolysis of Compounds **2** *and* **3**. Compound **3** (3.0 mg) was hydrolyzed with 6M NH_4OH for 2 h at 110°. The soln. was evaporated to dryness under reduced pressure, and then the residue was dissolved in H_2O and extracted with BuOH (3 ×) [17][18]. The BuOH extract was evaporated to dryness and yielded a light-yellow powder (2.6 mg) identified as compound **1** by its ^{1}H- and ^{13}C-NMR spectra. The light-yellow powder was further hydrolyzed with 3M HCl for 4 h at 100°. The soln. was extracted with AcOEt (3 ×), and both the org. and the aq. layers were evaporated to dryness under reduced pressure. The structure of the white powder (1.4 mg) obtained from the org. layer was determined to be oleanolic acid by ^{1}H- and ^{13}C-NMR spectroscopy. The semisolid carbohydrate mixture from the aq. layer (0.9 mg) was

dissolved in 2 ml of H_2O and kept overnight before TLC analysis and determination of its optical rotation. The same procedure was also applied to compound **2**. The sugars from both **2** and **3** had R_f values identical to that of glucose by TLC on a silica-gel plate with $CHCl_3$/MeOH/H_2O 15 : 6 : 1, and had $[\alpha]_D^{21}$ values of +13.9 and +14.2, resp. ($c = 0.1$, H_2O).

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Received May 7, 2012