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

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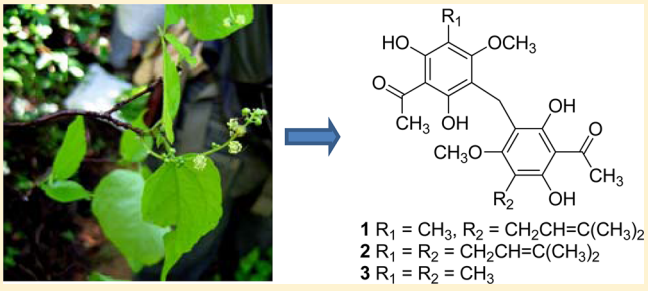
Supporting Information

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

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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	

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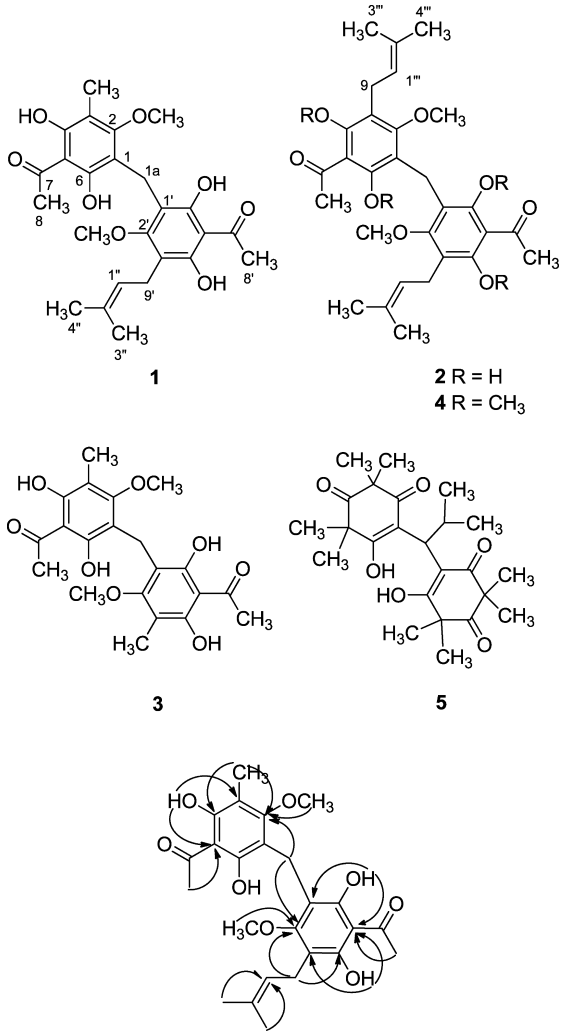

Figure 1. Important HMBC correlations observed in **1**.

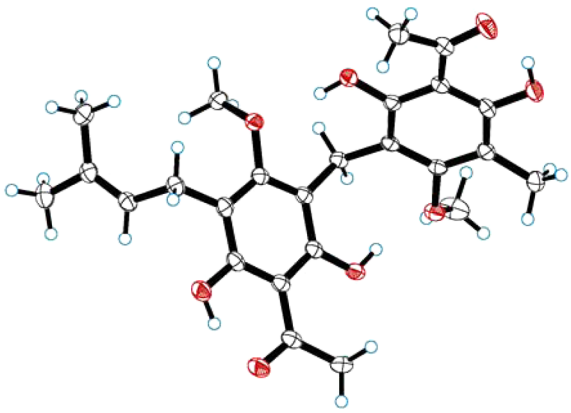

Figure 2. Anisotropic displacement ellipsoid drawing (50%) of **1**.

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.

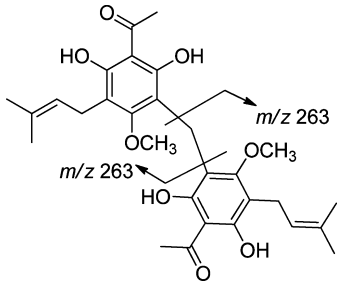

Figure 3. Mass fragmentation observed for **2**.

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.

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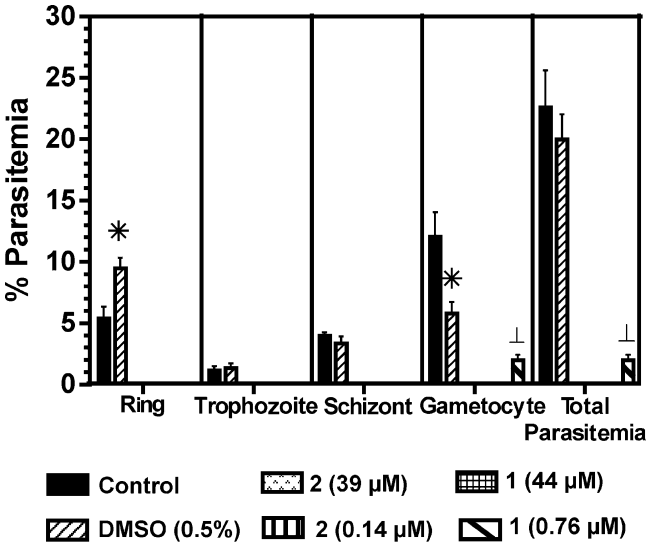

Figure 4. *P. falciparum* blood stage distribution after 13 days of treatment at the indicated concentrations to assess antimalarial activity against early-stage gametocytes. The control is untreated parasites. DMSO (drug vehicle at 0.5%) was included as a second control since it affects the blood-stage 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.

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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.

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DEDICATION

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REFERENCES

(1) Biodiversity Conservation and Drug Discovery in Madagascar, Part 53. For Part 52, see: Dai, Y.; Harinantenaina, L.; Brodie, P. J. Birkinshaw, C.; Randrianasolo, S.; Ratsimbason, M.; Rasamison, V. E.; Shen, Y.; TenDyke, K.; Kingston D. G. I. *Chem. Biodiversity*, accepted for publication.

(2) Radcliffe-Smith, A. *Genera Euphorbiacearum*; Royal Botanic Gardens: Kew, UK, 2001.

(3) Yoder, B. J.; Cao, S.; Norris, A.; Miller, J. S.; Ratovoson, F.; Razafitsalama, J.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I. *J. Nat. Prod.* **2007**, *70*, 342−346.

(4) Beutler, J. A.; Shoemaker, R. H.; Johnson, T.; Boyd, M. R. *J. Nat. Prod.* **1998**, *61*, 1509−1512.

(5) McGookin, A.; Reed, F. P.; Robertson, A. *J. Chem. Soc.* **1937**, 748−755.

(6) Liao, Y.-F.; Hung, Y.-C.; Chang, W.-H.; Tsay, G. J.; Hour, T.-C.; Hung, H.-C.; Liu, G.-Y. *Life Sci.* **2005**, *77*, 707−719.

(7) Sharma, V. *J. Plant Biochem. Biotechnol.* **2011**, *20*, 190−195.

(8) Valacchi, G.; Pecorelli, A.; Sticozzi, C.; Torricelli, C.; Muscettola, M.; Aldinucci, C.; Maioli, E. *Chem. Biol. Drug Des.* **2011**, *77*, 460−470.

(9) Kingston, D. G. I. *J. Nat. Prod.* **2011**, *74*, 496−511.

(10) Adekunle, A. A.; Ikumapayi, A. M. *West Indian Med. J.* **2006**, *55*, 219−223.

(11) Okwu, D. E.; Ekei, O. *Global J. Pure Appl. Sci.* **2003**, *9*, 235−238.

(12) Arisawa, M.; Fujita, A.; Hayashi, T.; Morita, N.; Kawano, N.; Koshimura, S. *J. Nat. Prod.* **1985**, *48*, 455−459.

(13) Owing to symmetry considerations, the assignment of the 3,3-dimethylallyl group to the C-3′ position as opposed to the C-3 position is arbitrary.

(14) Arisawa, M.; Fujita, A.; Morita, N.; Okuyama, T.; Nishino, H. *J. Nat. Prod.* **1991**, *54*, 1409−1412.

(15) Paguio, M. F.; Bogle, K. L.; Roepe, P. D. *Mol. Biochem. Parasitol.* **2011**, *178*, 1−6.

(16) Lelievre, J.; Almela, M. J.; Lozano, S.; Miguel, C.; Franco, V.; Leroy, D.; Herreros, E. *PLoS One* **2012**, *7*, e35019.

(17) Peatey, C. L.; Leroy, D.; Gardiner, D. L.; Trenholme, K. R. *Malar. J.* **2012**, *11*, 34−37.

(18) Singh, I. P.; Bharate, S. B. *Nat. Prod. Rep.* **2006**, *23*, 558−591.

(19) Costa, E. S.; Hiruma-Lima, C. A.; Lima, E. O.; Sucupira, G. C.; Bertolin, A. O.; Lolis, S. F.; Andrade, F. D. P.; Vilegas, W.; Souza-Brito, A. R. M. *Phytother. Res.* **2008**, *22*, 705−707.

(20) Cottiglia, F.; Casu, L.; Leonti, M.; Caboni, P.; Floris, C.; Busonera, B.; Farci, P.; Ouhtit, A.; Sanna, G. *J. Nat. Prod.* **2012**, *75*, 225−229.

(21) Farombi, E. O.; Ogundipe, O. O.; Uhunwangho, E. S.; Adeyanju, M. A.; Moody, J. O. *Phytother. Res.* **2003**, *17*, 713−716.

(22) Mavar-Manga, H.; Haddad, M.; Pieters, L.; Baccelli, C.; Penge, A.; Quetin-Leclercq, J. *J. Ethnopharmacol.* **2008**, *115*, 25−29.

(23) Verotta, L. *Phytochem. Rev.* **2002**, *1*, 389−407.

(24) Carroll, A. R.; Avery, V. M.; Duffy, S.; Forster, P. I.; Guymer, G. P. *Org. Biomol. Chem.* **2013**, *11*, 453−458.

(25) Arisawa, M.; Fujita, A.; Hayashi, T.; Hayashi, K.; Ochiai, H.; Morita, N. *Chem. Pharm. Bull.* **1990**, *38*, 1624−1628.

(26) Arisawa, M.; Fujita, A.; Morita, N. *J. Nat. Prod.* **1990**, *53*, 638−643.

(27) Ishii, R.; Horie, M.; Saito, K.; Arisawa, M.; Kitanaka, S. *Biochim. Biophys. Acta* **2003**, *1620*, 108−118.

(28) Nakane, H.; Arisawa, M.; Fujita, A.; Koshimura, S.; Ono, K. *FEBS Lett.* **1991**, *286*, 83−85.

(29) Cao, S.; Brodie, P. J.; Miller, J. S.; Randrianaivo, R.; Ratovoson, F.; Birkinshaw, C.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I. *J. Nat. Prod.* **2007**, *70*, 679−681.

(30) Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F. *Cancer Res.* **1985**, *45*, 2110−2115.

(31) Bennett, T. N.; Paguio, M.; Gligorijevic, B.; Seudieu, C.; Kosar, A. D.; Davidson, E.; Roepe, P. D. *Antimicrob. Agents Chemother.* **2004**, *48*, 1807−1810.

(32) Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J. X.; Wilairat, P.; Riscoe, M. *Antimicrob. Agents Chemother.* **2004**, *48*, 1803−1806.

(33) Tanaka, T. Q.; Williamson, K. C. *Mol. Biochem. Parasitol.* **2011**, *177*, 160−163.

(34) Lambros, C.; Vanderberg, J. P. *J. Parasitol.* **1979**, *65*, 418−420.

(35) *CrysAlisPro Software System*, v1.171.35.11; Agilent Technologies UK Ltd: Oxford, UK,: 2011.

(36) Sheldrick, G. M. *Acta Crystallogr.* **2008**, *A64*, 112−122.

(37) Dolomanov, O. V.; Bourhis, L. J.; Gildea, R. J.; Howard, J. A. K.; Puschmann, H. *J. Appl. Crystallogr.* **2009**, *42*, 339−341.

(38) Crystallographic data for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).