Two New Antiprotozoal 5-Methylcoumarins from *Vernonia brachycalyx*

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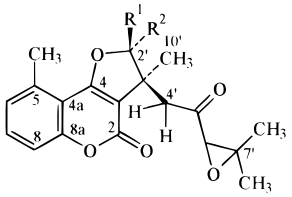
Two new isomeric 5-methylcoumarins, 2′-epicycloisobrachycoumarinone epoxide (**1**) and cycloisobrachycoumarinone epoxide (**2**), have been isolated from the roots of *Vernonia brachycalyx* by means of bioactivity-guided fractionation. The structures were elucidated by MS and NMR spectroscopic methods. Compounds **1** and **2** showed *in vitro* activity against *Leishmania major* promastigotes and against *Plasmodium falciparum* schizonts and demonstrated an inhibition on the proliferation of human lymphocytes, which was significantly weaker than the antiparasitic effects.

The large genus *Vernonia* comprises more than 1000 species found all over the world. In Kenya, more than 30 species have been described.[1] The phytochemistry of this genus has been extensively studied.[2] Characteristic are sesquiterpene lactones of the glaucolide, the hirsutinolide, and the elemanolide types.[2] However, besides the cytotoxicity of vernolepin and related sesquiterpene lactones toward human carcinoma of the nasopharynx (KB)[3,4] and the antiparasitic effects of some sesquiterpene lactones and some steroids,[5] little is reported on the biological properties of secondary metabolites from this genus. *Vernonia brachycalyx* Hoffm. (Asteraceae) is a herb 1−4 m tall, commonly found in dry forest edges, in semideciduous clump bushland, on riversides, and on roadsides. Our interest on this plant stemmed from the reported use of an infusion of the leaves by the Maasai, the Kipsigis, and other tribes in East Africa for treatment of parasitic diseases.[1] The roots are also chewed for stomachache and as a purgative.[6,7]

In this paper, we report two new isomeric 5-methylcoumarins, 2′-epicycloisobrachycoumarinone epoxide (**1**) and cycloisobrachycoumarinone epoxide (**2**), with some antiplasmodial and antileishmanial activities that have been isolated from *V. brachycalyx* root extracts through bioactivity-guided fractionation.

Results and Discussion

The comminuted plant material was divided into two parts. One portion was defatted with petroleum ether and extracted successively with two different solvents [$CHCl_3$−EtOAc (1:1) and MeOH] in order of increasing polarity, and from the other an aqueous extract was prepared. The residues of the three extracts were tested for antiplasmodial activity against chloroquine-susceptible (K39) and chloroquine-resistant strains (V1/S) strains of *Plasmodium falciparum*. The residue from the $CHCl_3$−EtOAc (1:1) extract showed the highest antiplasmodial activity and was therefore subjected to bioactivity-guided fractionation, which led to the isolation of two active coumarins **1** and **2**. Compounds **1** and **2** were initially isolated as a mixture in the ratio of 3:2 as evidenced by the ^{1}H-NMR spectrum. The two compounds were finally separated by reversed-phase HPLC over silanized (RP-8) silica gel. The MS of the two compounds both gave a molecular ion at *m*/*z* 342, and high-resolution MS provided an empirical formula of $C_{20}H_{22}O_5$. In the ^{1}H-NMR spectrum of compound **1** (Table 1), a three-proton singlet at δ 2.67 and three signals originating from aromatic protons δ 7.03 (d, J = 8.4 Hz), 7.20 (d, J = 7.5 Hz), and 7.39 (dd, J = 8.4, 7.5 Hz) indicated that the compound was a 4-oxygenated 3-substituted 5-methylcoumarin.[8,9] The presence of a 5-methylcoumarin moiety was further confirmed by the presence of 10 signals in the ^{13}C-NMR spectrum, which could be assigned to this heterocyclic nucleus by comparison with literature data[10−12] and from the DEPT spectrum. The presence of a coumarin skeleton was also supported by the IR spectrum, which showed the expected bands at 1700, 1610, and 1580 cm^{-1}. 4-Oxygenated 3-substituted 5-methylcoumarins have previously been isolated from a number of other *Vernonia* species.[13] In addition to the 10 carbons of the 5-meth-



1 $R^1 = CH_3$, $R^2 = H$
2 $R^1 = H$, $R^2 = CH_3$

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Table 1. ^{1}H-NMR Data for Compounds **1** and **2** in $CDCl_3$, 400 MHz

H	compd **1**	compd **2**
6	7.03 d, 1H, J = 8.4 Hz	7.03 d, J = 7.5 Hz
7	7.39 dd, 1H, J = 7.5, 8.4 Hz	7.39 dd, J = 7.5, 8.3 Hz
8	7.20 d, 1H, J = 7.5 Hz	7.18 d, 1H, J = 8.0 Hz
9	2.67 s, 3H	2.65 s, 3H
1′	1.33 d, 3H, J = 6.5 Hz	1.56 d, 3H, J = 6.6 Hz
2′	5.05 q, 1H, J = 6.5 Hz	5.14 q, 1H, J = 6.6 Hz
$4'_1$	2.91 d, 1H, J = 18.7 Hz	3.11 d, 1H, J = 16.7 Hz
$4'_2$	3.66 d, 1H, J = 18.7 Hz	3.21 d, 1H, J = 16.7 Hz
6′	3.49 s, 1H	3.50 s, 1H
8′	1.23 s, 3H	1.14 s, 1H
9′	1.42 s, 3H	1.36 s, 3H
10′	1.49 s, 3H	1.39 s, 3H

Table 2. ^{13}C-NMR Data of Compound **1** and **2** in $CDCl_3$, 50 MHz

C	compd **1**	compd **2**	C	compd **1**	compd **2**
2	166.7	166.8	1′	16.2	15.6
3	108.0	108.1	2′	91.1	89.6
4	160.2	160.0	3′	44.8	47.9
4a	112.1	111.9	4′	44.0	44.0
5	136.6	136.7	5′	204.3	204.8
6	126.3	126.4	6′	61.5	61.9
7	131.7	131.8	7′	65.2	65.5
8	114.8	114.8	8′	24.6	24.5
8a	155.9	155.9	9′	21.4	21.3
9	24.6	19.3	10′	18.1	17.8

ylcoumarin part of **1**, the empirical formula proved the presence of 10 additional carbons, which were tentatively assigned to a monoterpenoid moiety. The ^{13}C-NMR spectrum of **1** (Table 2) revealed that one of the remaining carbons was a nonconjugated ketone (δ 204.3), as was also evidenced by the presence of a band at 1730 cm^{-1} in the IR spectrum. The shifts of two oxygenated carbons, a methine group resonating at δ 61.5 and a quaternary carbon resonating at δ 65.2, indicated the presence of an epoxide, which was supported by IR bands at 1230, 900, and 830 cm^{-1}.[14] In addition, the ^{13}C-NMR spectrum revealed the presence of four methyl groups (δ 16.2, 18.1, 21.4, 24.6), one methylene group (δ 44.0), one oxygenated methine group (δ 91.1), and one quaternary carbon (δ 44.8). In the ^{1}H NMR spectrum of **1** there was a vicinal coupling between one of the methyl groups (δ 1.33) and the proton of the oxygenated methine group (δ 5.05) and a geminal coupling between the two protons of the methylene group (δ 2.91 and 3.66). The large coupling constant for the geminal coupling (J = 18.7 Hz) of the two protons of the methylene group is consistent with the methylene being adjacent to the ketone group. These two isolated coupling systems were observed in a COSY experiment. The only structure that could account for the observed NMR data is that given in formula **1**, which possesses a regular terpenoid skeleton (3,7-dimethyloctane) in the side chain. The presence of a dihydrofuran residue in structure **1** was supported by the high δ value (5.05) of the proton of the oxygenated methine group. Similar δ values have been observed for analogous protons in related structures.[8,15] The fragmentation pattern in the EIMS spectrum supported the proposed branching at C-3′. The base peak at m/z 229 $[M-C_6H_9O_2]^+$ could be explained by a cleavage between C-3′ and C-4′. The relative configuration at C-2′ and C-3′ was determined by means of a NOESY spectrum, which revealed a strong interaction between H-2′ and the methyl group at C-3′, but no interaction between the two methyl groups at C-2′ and C-3′, indicating that H-2′ was *cis* disposed to the methyl group at C-3′. Consequently, compound **1** must possess the structure *cis*-2,3-dihydro-2,3,9-trimethyl-3-(2-keto-3,4-epoxy-4-methylpentyl)-4*H*-furo[3,2-*c*][1]benzopyran-4-one. Since compound **1** only differs from 2′-epicycloisobrachycoumarin[15] by the presence of a keto group and an epoxy group instead af an alkene we suggest the name 2′-epicycloisobrachycoumarinone epoxide.

Table 3. IC_{50} Values for Coumarin **1** and Coumarin **2** against a Chloroquine-Susceptible (3D7) and a Chloroquine-Resistant (Dd2) Strain of *Plasmodium falciparum*, against *Leishmania major* Promastigotes (*L. maj.*), and against Phytohemagglutinin A-induced Proliferation of Human Lymphocytes (Lymp)

	IC_{50} μM ± sd (number of experiments)		
organism	coumarin **1**	coumarin **2**	chloroquine
3D7	160 ± 15 (3)	111 ± 17 (3)	0.11
Dd2	54 ± 8 (7)	54 ± 5 (10)	0.15
L. maj.	37 ± 1.8 (7)	13.4 ± 1.4 (6)	
Lymp	313 ± 150 (5)	1810 ± 1220 (5)	

The ^{13}C-NMR spectrum for compound **2** was almost identical to that of **1**, with the only major differences being the signal of C-3′, which appeared 3.1 ppm downfield of the corresponding signal in **1**, and that of C-2′, which appeared upfield of the corresponding signal of **1** by 1.5 ppm. In the ^{1}H-NMR spectrum major differences were noted for the chemical shifts of the protons of the methylene group at C-4′, the proton at C-2′, and the signal for the methyl group at C-3′ (Table 1). These differences indicated that the two isomers have different stereochemistry at the dihydrofuran ring. This assumption was supported by the NOESY spectrum, which showed no correlation between H-2′ (δ 5.14) and the methyl at C-3′ (δ 1.39), but in contrast a strong correlation between the methyl groups at C-2′ (δ 1.56) and C-3′ (δ 1.39). Thus, compound **2** must possess the structure *trans*-2,3-dihydro-2,3,9-trimethyl-3-(2-keto-3,4-epoxy-4-methylpentyl)-4*H*-furo[3,2-*c*][1]benzopyran-4-one. The presence of the carbon skeleton also found in cycloisobrachycoumarin[15] makes us suggest the name cycloisobrachycoumarinone epoxide.

The absolute configuration and the relative configuration at C-6′ has not been investigated in either of the two compounds.

Compounds **1** and **2** were tested *in vitro* for antiplasmodial activity against a chloroquine-susceptible strain (3D7, IC_{50} = 0.11 μM) and chloroquine-resistant strain (Dd2, IC_{50} = 0.15 μM) of *Plasmodium falciparum*, as well as against promastigotes of *L. major*. As a measure of their suppression of the human immune system the compounds were tested for the ability to inhibit phytohemagglutinin A (PHA)-induced proliferation of human lymphocytes. Both compounds inhibited the growth of *P. falciparum* and *L. major* in a concentration-dependent manner. Although both substances seemed to show a higher inhibition of the growth of the chloroquine-resistant strain (Dd2) than the chloroquine-susceptible strain (3D7), this difference was not significant (Table 3). In the antileishmanial tests, both compounds again showed about the same level of growth inhibition with IC_{50} values of 37.1 μM equals 12.7 μg/mL and 39.2 μM equals 13.4 μg/mL for **1** and **2,** respectively. These values are significantly lower than that of pentostam (IC_{50} 67.3 μg/mL) in the same test.

In the lymphocyte proliferation assay, the compounds did not inhibit lymphocyte proliferation at concentrations below 300 μM, indicating that the two coumarins will not suppress the immune system in concentrations that affect the growth of parasites. However, there was a significant difference ($P < 0.01$) between the concentration that inhibited 50% growth of the lymphocytes found at about 1.8 mM and 290 μM for **1** and **2**, respectively. This might be attributed to the stereochemical differences between the two compounds.

Although many biological properties of coumarins have been described,[16] only three reports have found on antiplasmodial activity,[17−19] and none of the mentioned coumarins belong to the 5-methylcoumarin series. No antileishmanial activity by coumarins has been reported before.

This study has revealed that the two 5-methylcoumarin isomers inhibit the growth of *P. falciparum* and promastigotes of *L. major* in concentrations that do not significantly inhibit human lymphocyte proliferation. Thus, these compounds appear to have a selective toxicity against parasites worth further investigation.

Experimental Section

General Experimental Procedures. LC was performed on silica gel 60 (0.0063−0.200 mm) and LPLC on silica gel (0.040−0.063 mm) or on LiChroprep RP-18 (40−60 μm). TLC was performed on Merck TLC aluminum sheets precoated with silica gel 60 F_{254} or on Merck TLC glass plates precoated with RP-18 or RP-8. Zones on TLC plates were visualized under UV light at 254/366 nm and sprayed with phosphomolybdic acid in (5% in EtOH) followed by heating at 100 °C for 10 min. Analytical HPLC over RP-18 (Spherisorb ODS-2, 5 μm) was performed on a Waters liquid chromatograph equipped with Model 501 pumps and a Model 484 UV variable detector or on a system consisting of a Waters 6000A pump and a Shimadzu SPD-6 A UV-Vis detector. LPLC was performed using glass columns fitted with stainless steel and Teflon tubings (Separo, Sweden) and FMI lab pump Model QD. NMR spectra were recorded on a 200 or 400 MHz (Bruker) spectrometer with TMS as internal standard. UV spectra were recorded on a Perkin-Elmer 265 UV/vis spectrophotometer, and IR were recorded on a IR Perkin-Elmer 781 spectrophotometer. Mass spectrometry was performed on a JEOL AX505W mass spectrometer, and HRMS was performed in the FAB^+ mode.

Plant Material. The roots of *V. brachycalyx* were collected during the dry season in August 1995 from Kithembe hill in Machakos District, Kenya. A voucher specimen has been deposited at the East African Herbarium at the National Museums of Kenya, Nairobi, where identification of the plant was confirmed as *V. brachycalyx* Hoffm. by the taxonomist Onesmus Mwangangi (voucher no. HO-016).

Extraction and Isolation. Air-dried powdered root (850 g) was defatted with petroleum ether bp 40−60 °C and extracted successively with $CHCl_3$−EtOAc (1:1) and MeOH to give three fractions VBR-0 (10 g), VBR-1 (16.63 g), and VBR-3 (18 g). Residues from these fractions were screened for antiplasmodial activity as described in the section for biological activity testing, and the highest activity occurred in VBR-1. This was subjected to bioactivity-guided fractionation using antiplasmodial tests against *P. falciparum* (strain K39 and V1/S) as a guide to select active fractions to be purified further.

Fraction VBR-1 (9.9 g) was chromatographed over silica gel eluting with petroleum ether bp 40−60 °C containing increasing amounts of $CHCl_3$ and EtOAc. Fractions were monitored by TLC [petroleum ether−EtOAc (10:1), (9:2), and (7:3)] and zones on the TLC plates visualized under UV light (254/366 nm) and by spraying with phosphomolybdic acid (5% in EtOH) followed by heating at 60 °C for 10 min. Fractions with similar TLC profiles were pooled and further purified by LPLC over RP-18 using MeOH−H_2O mixtures (6:4−1:1) as eluents. The fraction showing a single spot on TLC was analyzed by HPLC using MeOH−H_2O (1:1) with detection at 220 nm and found to contain three compounds appearing at t_R 2.0, 4.4, and 11.6 min. Further purification of this fraction was achieved by preparative HPLC on RP-8 using MeOH−H_2O (6:4) as an eluent to give the two compounds with t_R 2.0 min and t_R 4.4 min, respectively.

Compound **1** (85 mg) was obtained as a colorless oil: $[\alpha]^{25}_{589}$ −51°, $[\alpha]^{25}_{578}$ −54°, $[\alpha]^{25}_{546}$ −64°, $[\alpha]^{25}_{436}$ −142° (*c* 0.86, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ), 298 (4.03), 312 (3.94), 327 (3.75) nm; IR (KBr) ν_{max} 1720 (C=O), 1630, 1605, 1570 (aromatic), 1230, 990, 850 (epoxide) cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; HRFABMS *m/z* 343.1575 (calcd for $C_{20}H_{22}O_5$, 343.1546); EIMS (70 eV) *m/z* $[M]^+$ 342 (8), 341 (5) 283 (4), 243 (4), 229 (100), 227 (12), 201 (5), 157 (7), 135 (8), 109 (5), 43 (5).

Compound **2** (68 mg) was obtained as a colorless oil: $[\alpha]^{25}_{589}$ −3.0°, $[\alpha]^{25}_{578}$ −4°, $[\alpha]^{25}_{546}$ −28°, $[\alpha]^{25}_{436}$ −161° (*c* 0.39, $CHCl_3$); UV(MeOH) λ_{max} (log ϵ), 298 (3.84), 313 (3.75), 327 (3.56) nm; IR (KBr) ν_{max} 1720 (C=O), 1630, 1605, 1570 (aromatic), 1230, 990, 850 (epoxide); ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; HR-FABMS *m/z* 343.1580 (calcd for $C_{20}H_{22}O_5$ 343.1546); EIMS (70 eV) *m/z* $[M]^+$ 342 (8), 341 (3) 283 (3), 271 (4), 229 (100), 228 (18), 201 (5), 185 (4), 157 (7), 135 (10), 134 (7), 109 (7), 83 (7), 57 (7), 43 (10).

***In Vitro* Biological Activity. Antileishmanial Activity**. A WHO reference vaccine strain of *L. major* originally isolated from a patient in Iran and kindly provided by R. Behin, WHO Immunology Research and Training Center, Lausanne, Switzerland, was used in the tests. The promastigotes were cultured in medium 199 containing 0.02 mg/mL of gentamycin, 25 mM *N*-(2-hydroxyphenyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 4 mM L-glutamine, and 10% heat-inactivated (56 °C, 30 min) fetal calf serum (HFCS). The parasites were incubated at 26 °C.

The effect of different plant extracts and pure compounds on the growth of promastigotes was assessed by monitoring the inhibition of ^{3}[H]thymidine uptake as previously described.[20] A parasite concentration of 1−3 $\times$ 10^6/mL from a 4-day-old culture was used in the tests. Parasites (180 μL) were incubated in the presence of different concentrations of the extracts, compounds, or the medium alone. A stock solution of 1 mg/50 μL in dimethyl sulfoxide (DMSO) or EtOH (70%) was diluted with medium 199 containing 2% HFCS to achieve final well concentrations of 100, 50, 10, and 5 μg/mL for the crude extracts and in the range of 100−1.5 μg/mL for compounds **1** and **2**. The final concentration of DMSO

in the test solution was shown to have no effect on the parasite growth by including solvent controls alongside the tests. Each concentration was tested in triplicate. The plates were incubated at 26 °C for 2 h in 5%−9% CO_2, after which 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added to each well. The promastigotes were further incubated under the same conditions, harvested 18 h later on filter paper using a cell harvester (Skatron, Lierdyen, Norway), and counted in a scintillation counter (Minaxi Ti-Carb 4000, United Technologies, Packard, Downers Grove, IL).

Antiplasmodial Activity. A chloroquine-susceptible strain of *P. falciparum* (3D7, K39) and a chloroquine-resistant strain (Dd2 and V1/S) were used in the tests. Strains K39 and V1/s were obtained from the Wellcome Trust Research Laboratory, Nairobi, and were used during bioactivity-guided fractionation. Strains 3D7 and Dd2 were provided by D. Walliker (Edinburgh, Scotland). The parasites were kept in continuous culture by a modification of the method of Trager and Jensen[21] as described Chen *et al.*[22] A modification of Desjardin's radioisotopic method[23] for measuring parasite growth, as previously described,[22] was adopted for the *in vitro* antiplasmodial tests. The test compounds were prepared as described in the section for antileishmanial testing.

Lymphocyte Proliferation Assay. The effects of the crude extracts and compounds **1** and **2** on the proliferation of phytohemagglutinin A-stimulated human peripheral blood mononuclear cells was assessed by monitoring the uptake of radiolabeled thymidine as previously described.[24] Compounds **1** and **2** were tested at concentrations ranging from 600 to 9.0 μM.

Data Analysis. The percentage inhibition was determined using the following formula: inhibition = [(cpm control − cpm test compound)/(cpm control − cpm background)] 100, where cpm test compound = cpm for test compound, cpm control = cpm for the nontreated parasite/lymphocytes in the experiment, and cpm background = cpm for empty wells (no parasite/lymphocytes) with medium alone. The percentage inhibition data were used to derive the drug concentration causing 50% inhibition of [^{3}H]hypoxanthine incorporation into nucleic acids (IC_{50}) using GraFit 3.0,[25] whereby the data were fitted to the equation

$$y = [a/[(1 + 1/IC_{50}c)x^c]] + d$$

where a is the maximum y range, d is the background y value, and c is a slope factor. Where necessary, statistical differences between mean IC_{50} were examined by the Student's t-test.

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