APPENDIX 1: PAPER PUBLISHED FROM THESIS

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Bioactive Diterpenes and Other Constituents of *Croton steenkampianus*

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A new indanone derivative (**1**) and two new diterpenoids (**2** and **3**), together with three known flavonoids, have been isolated from an ethanol extract of the leaves of *Croton steenkampianus*. The structure of **2** was solved by single-crystal X-ray diffraction analysis, whereas those of **1** and **3** were established mainly by 1D and 2D NMR spectroscopic methods. The isolated compounds were tested for their antiplasmodial activity and cytotoxicity. Antiplasmodial assays against chloroquine-susceptible strains (D10 and D6) and the chloroquine-resistant strains (Dd2 and W2) of *Plasmodium falciparum* showed that compound **2** gave moderate activities at 9.1−15.8 μM, while none of the compounds were cytotoxic against Vero cells.

Croton steenkampianus Gerstner (Euphorbiaceae), commonly known as "Marsh Fever-berry" and "Tonga Croton", is a shrub or tree endemic to restricted areas of central Africa and eastern parts of southern Africa.[1] Various medicinal uses of the genus *Croton* are reported in countries all over the world, and many species are used to treat bleeding, bleeding gums, chest complaints, coughs, fever, indigestion, malaria, and rheumatism.[1] Chemically, the genus contains very diverse compound types including alkaloids, flavonoids, and terpenoids.[2,3] However, no phytochemical reports have appeared for the species *C. steenkampianus*. Medicinally, the vapor from the fresh leaves is used to relieve aches.[1] In this paper, we describe the isolation of six compounds, namely, three known flavonoids, quercetin (**5**), taxmarixetin, and eriodictyol,[4–6] a new indane derivative (**1**), and two new diterpenoids (**2** and **3**), from an ethanol extract of leaves of *C. steenkampianus*. The structures of **1**−**3** were elucidated mainly by 1D and 2D NMR spectroscopic means and, in the case of **2**, by single-crystal X-ray diffraction analysis. In addition, we also report on antiplasmodial activity and cytotoxicity of the isolated compounds.

Repeated chromatographic processes on the EtOH extract of the dried leaves of *C. steenkampianus* allowed the isolation of three flavonoids (quercetin (**5**), taxmarixetin, and eriodictyol) together with the indane derivative **1** and the diterpenoids **2** and **3** (steenkrotins A and B, respectively). The flavonoids were identified by comparison of their data with published values.[4–6]

Low-resolution mass spectrometry and combustion analysis indicated a molecular formula of $C_{12}H_{12}O_3$ for **1**, and its NMR spectroscopic data were in agreement with the structure of 2,6-dimethyl-1-oxo-4-indanecarboxylic acid (**1**). In particular, the HMBC correlations observed for **1** (C-1 with H-2, H_2-3, H-7, and Me-10; C-12 with H-5 and H-7; C-9 with H-2, H_2-3, H-5, and H-7; C-11 with H-5; and C-12 with H-5 and H-7) established a 1-indanone skeleton and a 2,6-dimethyl-4-carboxyl substitution pattern for this new substance. Since **1** did not show any optical rotation between 589 and 365 nm, it was evident that it is a racemic mixture of the two enantiomers at the C-2 asymmetric center, which can be easily racemized through the enolic form of the 1-indanone.

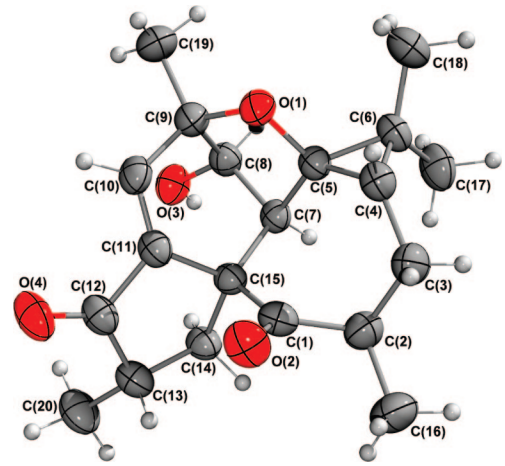

Figure 1. ORTEP diagram for compound **2**.

The structure of steenkrotin A (**2**, $C_{20}H_{26}O_4$) was established from X-ray diffraction studies. Figure 1 is a perspective view of the molecule of **2** showing its relative configuration. Moreover, the 1D and 2D NMR spectroscopic data of **2** (Table 1 and Tables S1 and S2, Supporting Information) were in complete agreement with the proposed structure.

Steenkrotin B (**3**, $C_{20}H_{28}O_7$) showed ^{1}H and ^{13}C NMR spectra very similar to those of **2** (Table 1). The observed differences were consistent with the presence in **3** of a hydroxyisopropyl group [δ_H 1.45 and 1.34 (Me-16 and Me-17); δ_C 22.3 and 25.7 (C-16 and C-17), and 87.6, qC, C-15] and a hemiacetal carbon (δ 110.1, qC, C-14) instead of the pentasubstituted cyclopropane of **2**. This structural difference was also supported by the HMBC spectra (Table S1, Supporting Information), because **2** showed correlations through three bonds between C-15 and H-8 and between C-14 and Me-16 and Me-17 that were not observed for **3**. Three of the seven oxygen atoms of **3** were involved in two ketones [δ 206.5 (C-3) and 212.4 (C-10)] and in a secondary hydroxyl group [δ_H 4.30; $\delta\mu_C$ 75.6, CH (C-7)], as in **2**, and two other oxygens must be placed at C-14 and C-15 as one of the hemiacetal oxygens and as a tertiary hydroxyl group, respectively (see above). The remaining two oxygens of **3** must be part of an endoperoxide moiety between C-6 (δ 68.6, qC) and the hemiacetalic C-14 carbon. The presence of this endoperoxide was also in agreement with molecular formula

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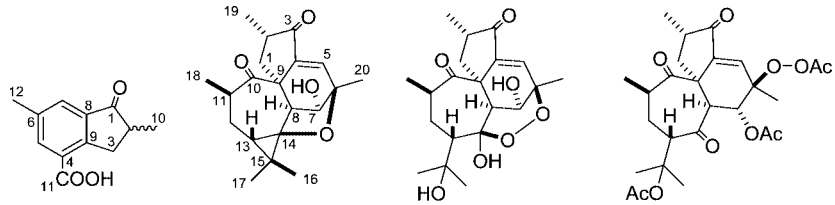
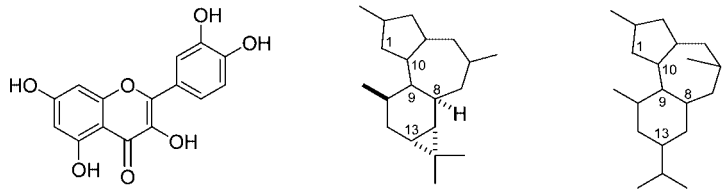
Table 1. NMR Spectroscopic Data [400 (^{1}H) and 100 (^{13}C) MHz, $CDCl_3$] for Compounds **2**−**4**[a]

	2		**3**		**4**	
position	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1[b]	35.9, CH_2	1.98, dd (12.5, 11.4) 2.20, dd (12.5, 8.4)	39.5, CH_2	2.13, dd (13.1, 12.5) 2.63, dd (13.1, 7.2)	38.8, CH_2	1.72, dd (12.8, 11.3) 2.15, dd (12.8, 8.4)
2	39.73, CH	2.31, ddq (11.4, 8.4, 7.0)	39.2, CH	2.27, ddq (12.5, 7.2, 7.0)	38.7, CH	2.52, ddq (11.3, 8.4, 7.0)
3	206.5 qC		206.5, qC		204.7, qC	
4	143.7, qC		144.6, qC		142.7, qC	
5	134.5, CH	6.50, s	129.7, CH	6.36, d (0.8)	130.4, CH	6.69, d (1.0)
6	75.1, qC		68.6, qC		74.9, qC	
7	79.2, CH	4.22, d (3.9)	75.6, CH	4.30, dd (2.5, 0.8)	71.4, CH	5.60, dd (2.3, 1.0)
8	40.8, CH	2.69, d (3.9)	36.5, CH	3.05, d (2.5)	44.6, CH	4.44, dd (2.3, 0.8)
9	62.6, qC		55.6, qC		52.8, qC	
10	213.1, qC		212.4, qC		211.3, qC	
11	39.73, CH	2.95, ddq (12.5, 6.8, 6.4)	44.5, CH	3.08, ddq (7.4, 7.1, 3.4)	43.1, CH	3.79, ddq (10.7, 2.4, 6.7)
12[b]	29.6, CH_2	1.67, ddd (14.3, 6.8, 2.4) 1.83, ddd (14.3, 12.5, 10.1)	29.4, CH_2	1.93, ddd (15.7, 8.9, 3.4) 1.79, ddd (15.7, 7.1, 4.9)	29.3, CH_2	2.22, ddd (16.3, 4.5, 2.4) 2.07, ddd (16.3, 6.7, 4.5)
13	25.3, CH	0.68, dd (10.1, 2.4)	60.3, CH	2.93, dd (8.9, 4.9)	61.2, CH	2.66, td (4.5, 0.8)
14	74.1, qC		110.1, qC		208.2, qC	
15	22.8, qC		87.6, qC		87.4, qC	
16	20.7, CH_3	1.03, s	22.3, CH_3[c]	1.45, s[c]	24.4, CH_3[c]	1.48, s[c]
17	16.5, CH_3	1.07, s	25.74, CH_3[c]	1.34, s[c]	24.7, CH_3[c]	1.44, s[c]
18	17.0, CH_3	1.06, d (6.4)	18.0, CH_3	1.27, d (7.4)	18.3, CH_3	1.20, d (6.7)
19	14.9, CH_3	1.04, d (7.0)	14.7, CH_3	1.07, d (7.0)	15.6, CH_3	1.06, d (7.0)
20	21.8, CH_3	1.43, s	25.70, CH_3	1.47, s	20.9, CH_3	1.52, s
OOAc-6β				[d]	167.0, qC 17.3, CH_3	2.08, s
OAc-7α				[d]	169.9, qC 21.1, CH_3	2.05, s
OAc-15				[d]	168.5, qC 21.0, CH_3	1.87, s

[a] Assignments were in agreement with COSY and HSQC spectra and with 1D NOESY experiments. [b] For methylene groups, the first reported δ value belongs to the α-proton and the second δ value is assigned to the β-proton. [c] Interchangeable assignments. [d] Hydroxyl protons at δ 5.89 br s, 3.56 br, and 2.16 s.



1 **2** **3** **4**



5 **6** **7**

requirements (seven unsaturations) and with the observed loss of O_2 (ion at m/z 348) from the molecular ion (m/z 380) in the EIMS of **3**.[7]

Treatment of **3** with acetic anhydride−pyridine yielded the triacetyl derivative, **4** ($C_{26}H_{34}O_{10}$), the IR spectrum of which was devoid of hydroxyl absorptions. In its ^{13}C NMR spectrum, the methyl carbon of one of the acetates (δ_C 167.0, qC and 17.3, CH_3; δ_H 2.08) appeared unusually shifted (δ_C 17.3), indicating the presence of a peroxyacetate function,[8,9] which is also in agreement with molecular formula requirements. Thus, apart from the esterification of the hydroxyl groups at C-7 and C-15, the acetylation of **3** caused the hydrolysis of the 14-hemiacetal and subsequent acetylation of the resulting 6-hydroperoxide, as was also evidenced by the appearance of an additional ketone at C-14 (δ_C 208.2, HMBC correlated with H-7, H-8, H_2-12, and H-13, Table S1, Supporting Information) in the ^{13}C NMR spectrum of **4**.

The relative stereochemistry of **3** and **4** must be identical to that of **2** for the following reasons: NOE experiments (Table S2, Supporting Information) established that H-8 and H-11 are in a *cis* spatial relationship in **2** and **4**, because a strong NOE enhancement was observed in H-11 when H-8 was irradiated and vice versa. Consequently, Me-18 must be *trans* (β-oriented) with respect to H-8. Moreover, the NOE observed between H-13 and Me-18 in the 1D NOESY spectra of **2**−**4** was in agreement with a β-orientation of H-13. In the case of **2**, a *cis* arrangement between H-8α and the 1-methylene protons was supported by weak NOE enhancements, but no NOEs between these protons were observed for **3** and **4**. However, the similar ^{1}H NMR chemical shifts and the almost identical coupling constant values for H_2-1, H-2, and Me-19 in **2**−**4** (Table 1), together with the absence of NOE between Me-18 and Me-19, supported an α-orientation for the 1-methylene and Me-19 groups in **3** and **4**, as was established for **2** by X-ray crystallographic

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Table 2. Antiplasmodial Activities and Cytotoxicity (μM) of Isolates against Chloroquine-Susceptible Strains (D10 and D6) and the Chloroquine-Resistant Strains (Dd2 and W2) of *Plasmodium falciparum*

	antiplasmodial activity (IC_{50})				
sample	D6	W2	D10	Dd2	cytotoxicity (IC_{50})
ethanol extract[a]	nt[b]	nt[b]	8.60	5.80	45.0
1	>49.0	>49.0	>49.0	>49.0	1216.7
2	>30.0			9.40	107.0
3	>26.3	9.1	15.8	>26.30	805.0
quercetin (**5**)	nt[b]	>26.3	>26.3		110.0
tamarixetin	nt[b]			2.40	170.3
eriodictyol	nt[b]	nt[b]	1.10	nt[b]	nt[b]
chloroquine[c]		nt[b]	nt[b]	nt[b]	
	0.03	0.06	0.02	0.14	48.5

[a] Activity recorded in μg/mL. [b] nt = not tested. [c] Positive control substance.

analysis. In addition, one of the H_2-1 protons of **3** (δ 2.63) showed a NOE with the proximal Me-18 group, and this proton must be the β-oriented one, which in turn displayed a strong NOE with H-2 and no NOE with Me-19, thus establishing an α-orientation for Me-19. This conclusion was also supported by the 1D NOESY behavior of **4**, which showed a weak NOE in H-2 (+1%) and a medium NOE in Me-19 (+3.4%) when H-1α (δ 1.72) was irradiated. For structural requirements, the α-orientation of H-8 in **2** requires a 6β, 14β closure of its cyclic ether, and **3** must also have the same stereochemistry for its endoperoxide, because steenkrotin B (**3**) also has its H-8 proton α-oriented (see above). Consequently, the 6-peroxyacetate of **4** must be β-oriented.[10]

The cyclohexene ring of **3** and **4** adopts an envelope conformation with the flap at C-7, like in **2** (Figure 1), and H-7β and OH-7α (or OAc-7α in **4**) are equatorial and axial substituents, respectively, thus explaining the similar $J_{7\beta,8\alpha}$ values (Table 1) and the NOE behavior of these compounds (Table S2, Supporting Information).

NOE experiments also allowed the assignment of the Me-16 and Me-17 groups in **2**, as it is shown in the formula and Table S2 (Supporting Information). Irradiation at δ 1.43 (Me-20) caused NOE enhancement only in one of the two C-15 methyls (δ 1.03, Me-16), whereas the signal of the other one (δ 1.07, Me-17) was enhanced when H-8α was irradiated. The assignment of the configuration for both methylene protons at C-12 in **2**−**4** (Table 1) was also supported by NOE results (Table S2, Supporting Information).

To the best of our knowledge, diterpenoids **2** and **3** possess new carbon skeletons that may be derived from the tigliane (**6**) and daphnane (**7**) types, respectively, by an 8(9→10)-*abeo* rearrangement.[11] It is of interest that diterpenes belonging to **6** and **7** carbon frameworks have repeatedly been found in several *Croton* species and in other Euphorbiaceae genera.[12]

Antiplasmodial testing (Table 2) showed that the ethanolic extract of *C. steenkampianus* demonstrated an IC_{50} activity against the chloroquine-susceptible strain D10 (8.6 μg/mL) and the chloroquine-resistant strain Dd2 (5.8 μg/mL) of *Plasmodium falciparum*. Activity was more pronounced against the resistant strain. Compound **2** displayed antiplasmodial activities of 15.8 (D10), 9.1 (W2), and 9.4 (Dd2) μM. Quercetin (**5**) was active against the D10 and Dd2 strains. The cytotoxicity values for the isolated compounds on Vero cells using MTT assays were all >30 μM, showing that the substances tested (Table 2) are not potent cytotoxic agents.

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler block and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. UV spectra were recorded on a Perkin-Elmer Lambda 2 UV/vis spectrophotometer. IR spectra were obtained on a Perkin-Elmer Spectrum One spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution on a Varian INOVA 400 spectrometer at 400 and 100 MHz, respectively. Chemical shifts are reported relative to the residual $CHCl_3$ (δ 7.25) for protons and to the solvent (δ_{CDCl3} 77.00) for carbons. All the assignments for protons and carbons were in agreement with 2D COSY, gHSQC, gHMBC, and 1D NOESY spectra. Mass spectra were registered in the positive EI mode on a Hewlett-Packard 5973 instrument (70 eV) and in the positive or negative ESI mode on a Hewlett-Packard Series 1100 MSD spectrometer. Elemental analyses were conducted on a Leco CHNS-932 apparatus. Merck Si gel (70−230 and 230−400 mesh, for gravity flow and flash chromatography, respectively) was used for column chromatography. Merck 5554 Kieselgel 60 F254 sheets were used for TLC analysis. Petroleum ether (bp 50−70 °C) was used for column chromatography.

Plant Material. Leaves of *Croton steenkampianus* were collected in April 2003, at Tember Elephant Park in northern KwaZulu-Natal, South Africa, and voucher specimens (registry number 92520) are identified and preserved at the H.G.W.J. Schweickerdt Herbarium (PRU) at the University of Pretoria.

Extraction and Isolation. The dried leaves (2 kg) were extracted with ethanol (0.2 kg leaves/L solvent) for 3 days at room temperature. The extract was filtered and concentrated with a rotary evaporator at 40 °C. The extraction procedures were repeated twice. The total extract (80.0 g) was applied to a flash silica gel column, developed with a solvent gradient of hexane−ethyl acetate mixtures of increasing polarity. Similar fractions were combined together, to yield four main fractions, A1−A4. Fraction A2 (10 g) was applied to a silica gel column developed with hexane− ethyl acetate (95:5) to yield pure compounds **1** (148 mg) and **2** (250 mg) and eriodictyol (5 mg). Fraction A4 (3.0 mg) was applied to a silica gel column developed with a solvent gradient of hexane−ethyl acetate (90:10). Subfraction 2 (350 mg) was further chromatographed over Sephadex LH-20, using ethanol as eluent, to give quercetin (**5**, 10 mg), tamarixetin (5 mg), and compound **3** (50 mg).

2,6-Dimethyl-1-oxo-4-indanecarboxylic acid (1): amorphous, white solid; $[\alpha]_D^{20}$ 0, $[\alpha]_{578}^{20}$ 0, $[\alpha]_{546}^{20}$ 0, $[\alpha]_{436}^{20}$ 0, $[\alpha]_{365}^{20}$ 0 (*c* 0.26, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 215 (5.56), 248 (4.02), 310 (3.66) nm; IR (KBr) ν_{max} 3400−2300, 2924, 1709, 1580, 1459, 1423, 1250, 1182, 1133, 927, 836, 693 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31, (3H, d, J = 7.0 Hz, H-10), 2.45, (3H, s, H-12), 2.72, (1H, ddq, J = 7.9, 7.0, 3.7 Hz, H-2), 3.02, () (H, dd, J = 18.6, 3.7 Hz, H_2-3), 3.77 (1H, dd J = 18.6, 7.9 Hz, H_1-3), 7.79 (1H, d, J = 2.0 Hz, H-7), 8.18 (1H, d J = 2.0 Hz, H-5), 8.60 (1H, br, H-11); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.2 (CH_3, C-10), 20.9 (CH_3, C-12), 36.1 (CH_2, C-3), 42.0 (CH, C-2), 127.1 (qC, C-4), 129.4 (CH, C-7), 137.9 (qC, C-8), 138.0 (CH, C-5), 138.1 (qC, C-6), 153.1 (qC, C-9), 171.3 (qC, C-11), 209.1 (qC, C-1); EIMS *m/z* 204 $[M]^+$ (100), 189 (86), 176 (21), 161 (44), 159 (19), 148 (44), 131 (26), 115 (37), 91 (21), 77 (18), 63 (13), 51 (14); *anal.* C 70.67%, H 5.81%, calcd for $C_{12}H_{12}O_3$, C 70.58%, H 5.92%.

Steenkrotin A (2): colorless needles (EtOAc−petroleum ether), mp 219−221 °C; $[\alpha]_D^{20}$ +188.6 (*c* 0.32, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 227 (3.96) 250 shoulder (3.68) nm; IR (KBr) ν_{max} 3359, 3050, 3020, 2971, 2940, 1702, 1649, 1454, 1375, 1286, 1231, 1112, 1055, 1019, 905, 743, 650 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 330 $[M]^+$ (3), 315 (0.5), 312 (2), 297 (0.5), 262 (60), 244 (27), 227 (14), 187 (18), 177 (62), 161 (30), 160 (26), 145 (16), 121 (29), 97 (35), 91 (14), 83 (13), 77 (14), 69 (100), 55 (29); *anal.* C 72.43%, H 8.25%, calcd for $C_{20}H_{26}O_4$, C 72.70%, H 7.93%.

X-ray Structure Determination of 2.[13] The crystallographic data set was collected at 20 °C on a Siemens P4 diffractometer fitted with a Bruker 1K CCD detector and SMART control software[14] using graphite-monochromated Mo Kα radiation by means of a combination of phi and omega scans. Data reduction was performed using SAINT+,[18] and the intensities were corrected for absorption using SADABS.[18] The structure was solved by direct methods using SHELXTS[14] and refined by full-matrix least-squares using SHELXTL[14] and SHELXL-97.[15] All hydrogen atoms for the structure of **2** were located experimentally. In the refinement, the hydrogen atoms were refined without any positional constraints. Isotropic displacement parameters for the non-methyl hydrogen atoms were calculated as 1.2 × U_{eq} of the atom to which they were attached, and the corresponding value for methyl hydrogen atoms was 1.5 × U_{eq} of the carbon atom to which they were attached. All non-hydrogen atoms were refined with anisotropic displacement parameters. Drawings of the structure were

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produced using ORTEP-3 for Windows,[16] Mercury,[17] and POV-Ray for Windows.[18]

Steenkrotin B (3): colorless fine needles (MeOH), mp 130−132 °C (dec); $[\alpha]_D^{20}$ +24.4 (*c* 0.12, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 233 (3.98) nm; IR (KBr) ν_{max} 3435, 2976, 2932, 1731, 1691, 1676, 1457, 1372, 1194, 1078, 901 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; positive mode ESIMS *m/z* 403 $[M + Na]^+$, 783 $[2M + Na]^+$; negative mode ESIMS *m/z* 379 $[M - H]^-$; EIMS *m/z* 380 $[M]^+$ (0.3), 362 (1), 348 (1), 338 (25), 305 (19), 264 (27), 245 (21), 219 (27), 203 (38), 187 (45), 177 (56), 161 (55), 152 (39), 141 (43), 121 (40), 95 (31), 91 (41), 85 (41), 83 (45), 71 (57), 69 (54), 55 (100); *anal.* C 63.31%, H 7.36%, calcd for $C_{20}H_{28}O_7$, C 63.14%, H 7.42%.

Acetylation of Steenkrotin B (3). Treatment of **3** (15.0 mg) with Ac_2O−pyridine (1:1, 4.0 mL) for 24 h at room temperature, followed with evaporation of the volatiles at 50 °C under reduced pressure, yielded a residue, which was chromatographed on a silica gel column using 10% ethyl acetate in hexane to give **4** (8 mg, 53.0% yield): amorphous, white solid; $[\alpha]_D^{20}$ −99.3 (*c* 0.29, $CHCl_3$); IR (KBr) ν_{max} 2929, 1745, 1736, 1706, 1694, 1457, 1369, 1225, 1102, 1076, 1039, 945, 922, 849 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; positive mode ESIMS *m/z* 529 $[M + Na]^+$, 1035 $[2M + Na]^+$; *anal.* C 61.83%, H 6.80%, calcd for $C_{26}H_{34}O_{10}$, C 61.65%, H 6.77%.

Antiplasmodial Bioassay. The antiplasmodial activity against strains of *Plasmodium falciparum* (Table 2) was determined as previously described.[19–21]

Cytotoxicity. The cytotoxicity was determined as previously described.[22]

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Supporting Information Available: Tables of 2D NMR data for **1**−**4** and crystallographic data of **2**. This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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