Cytotoxic Diterpenoids from *Croton argyrophylloides*

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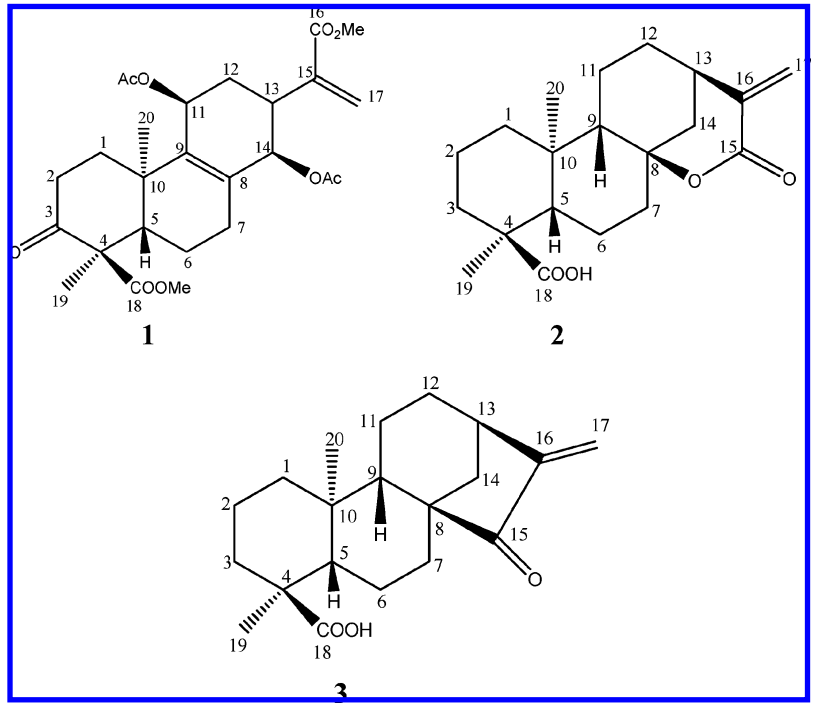
Two new diterpenes, **1** and **2**, together with the known *ent*-15-oxo-kaur-16-en-18-oic acid (**3**), were isolated from the bark of *Croton argyrophylloides*. The structural characterization of **1** and **2** was determined on the basis of spectroscopic data interpretation. The cytotoxicity of each compound was evaluated against HL-60 (leukemia), MDAMB-435 (melanoma), SF-295 (glioblastoma), and HCT-8 (colon carcinoma) human tumor cell lines and against human peripheral blood mononuclear cells. The hemolytic potential in mouse erythrocytes was also tested for **1**−**3**.

The genus *Croton* in the plant family Euphorbiaceae is widespread in northeastern Brazil. Popular medicine using this genus includes treatment of cancer, constipation, diabetes, digestive problems, dysentery, external wounds, fever, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria, pain, ulcers, and weight-loss.[1] Previous phytochemical investigations have shown that plants in this genus produce alkaloids,[2,3] flavonoids,[4−6] triterpenoids, steroids,[7,8] and a large number of diterpenoids.[9−13] *Croton argyrophylloides* Muell. Arg. is a small shrub very common in the hinterland of the semiarid regions. Previous work on the bark of *C. argyrophylloides* has yielded *ent*-kaurene diterpene acids and clerodane diterpenes.[14] The present paper reports the isolation of an abietane (**1**) and two *ent*-kaurene diterpenes (**2** and **3**) and the evaluation of the cytotoxicity of these diterpenes against several human tumor cell lines and human peripheral blood mononuclear cells (PBMC). The hemolytic potential in mouse erythrocytes was also determined.

The new diterpene **1** was isolated as a white solid with the molecular formula $C_{26}H_{34}O_9$, as deduced by HRESIMS at *m/z* 513.2079 $[M + Na]^+$, in combination with the ^1H and ^{13}C NMR spectra. Its IR spectrum showed bands indicating the presence of ester (ν_{max} 1739 and 1250 cm^{-1}), ketone (ν_{max} 1707 cm^{-1}), and olefin (ν_{max} 1632 cm^{-1}) functionalities. The ^1H NMR spectrum (Table 1) of **1** displayed signals due to two methoxy [δ_H 3.77 (s, 3H) and 3.74 (s, 3H)], two acetoxy [δ_H 2.04 (s, 3H) and 2.01 (s, 3H)], one terminal methylene [δ_H 6.30 (s, 1H) and 5.55 (s, 1H)], and two tertiary methyl [δ_H 1.39 (s, 3H) and 1.22 (s, 3H)] groups. An analysis of the $\{^1H\}^{13}C$ NMR (Table 1) spectrum of **1** with the aid of DEPT 135 and HMQC experiments revealed the signals of 26 carbons: five carbonyl [δ_C 170.0 and 169.0 (acetates), 167.0 and 173.4 (methyl esters), and 209.9 (ketone)], four olefinic [δ_C 135.8 (C), 139.1 (C), 139.0 (C), and 126.1 (CH_2)], and six methyl [δ_C 52.8 (H_3CO), 52.2 (H_3CO), 21.7 (H_3CCO), 21.1 (H_3CCO), 21.0 (H_3C), and 16.8 (H_3C)] as well as five simple methylenes, four methines, and two quaternary sp^3 carbons.



1 **2** **3**

Since 10 degrees of unsaturation were indicated by the molecular formula, three rings were inferred. Two ^1H NMR signals at δ_H 5.63 and 5.33 and two ^{13}C NMR resonances at δ_C 68.0 and 71.8 indicated

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Table 1. NMR Spectroscopic Data for **1**−**3** (^{1}H, 500 MHz; ^{13}C, 125 MHz; in $CDCl_3$)[a]

	1		**2**		**3**	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	2.06, 1.79, m	34.2	1.95, 1.10, m	39.9	1.80, 0.89 m	38.9
2	2.61, ddd (3.3, 2.8, 6.2), 2.44, m	34.9	1.63, m	17.9	1.65, 1.48 m	18.0
3		209.9	1.80, 1.65, m	36.7	1.78, 1.47 m	36.8
4		61.8		47.2		47.6
5	2.78, dd (2.5)	46.5	1.90, m	49.2	1.80, m	49.5
6	1.79, 1.34, m	20.8	1.48, 1.35, m	22.0	1.50, 1.26, m	21.7
7	2.41, 2.18, m	30.2	2.00, 1.90, m	41.0	2.05, dt (13.4, 4.3), 1.26, m	33.2
8		135.8		85.5		52.7
9		139.1	1.70, m	52.2	1.45, m	52.6
10		37.7		39.0		39.5
11	5.63, t (6.6)	68.0	1.80, 1.65, m	16.2	1.63, 1.58, m	17.8
12	1.94 ddd (6.1, 12.0, 13.1), 2.20, m,	29.4	1.98, 1.55, m	30.6	1.89, 1.68, m	32.5
13	3.01, td (12.0, 2.5)	37.4	2.92, br s	34.7	3.05, s	38.2
14	5.33, d (2.5)	71.8	2.35, 1.75, m	34.1	2.39, d (11.8), 1.68, m	36.7
15		139.0		166.7		210.4
16		167.0		139.5		149.5
17	6.30, 5.55, s	126.1	6.54, 5.54, s	127.1	5.95, 5.25, s	114.8
18		173.4		183.8		184.9
19	1.39, s	16.8	1.17, s	16.7	1.13, s	18.1
20	1.22, s	21.0	1.07, s	18.0	1.17, s	16.3
MeO-16	3.77, s	52.2				
MeO-18	3.74, s	52.8				
AcO-11	2.04, s	21.7				
AcO-14	2.01, s	21.1				

[a] Number of hydrogens bound to each carbon atom was deduced by comparative analysis of {^{1}H}- and DEPT-^{13}C NMR spectra. Chemical shifts (δ values) and coupling constants [J (Hz), in parentheses)] were obtained from the 1D ^{1}H NMR spectrum.

that the two secondary carbons bear the acetoxy groups. In the HMBC spectrum of **1**, the proton signal at δ_H 5.63 (t, 6.6) and 5.33 (d, 2.5) showed long-range ^{1}H−^{13}C correlations with both the olefinic carbons at δ_C 135.8 and 139.1 (tetrasubstituted double bond). Additionally, the resonance at δ_H 5.33, as well as those at δ_H 6.30 and 5.55 (geminal methylene), exhibited, long-range ^{1}H−^{13}C correlations with the carbon at δ_C 139.0 (C-15). These observations permitted the acetyl groups to be placed at C-11 and C-14, with a double bond between C-8 and C-9 and a 1-carbomethoxyethenyl moiety [δ_C 167.0/52.2 (CO_2CH_3), 139.0/126.1 (C═CH_2)] at C-13 of a tricyclic diterpene. The relative configuration of **1** was determined from the coupling constants of relevant hydrogens and from the observed ^{1}H−^{1}H-NOESY spectrum. The NOESY spectrum of **1** showed cross-peaks assigned to dipolar interactions (spatial proximity) of H_3-20 (δ_H 1.22, s, axial position attached to C-10) and both H-11 (axial linked to C-11) and H_3-19 (δ_H 1.39, s, axial position attached to C-4), which must consequently lie on the same side of the molecular plane. These were used to postulate the relative configurations of three other stereocenters (C-4, C-10, and CH-11) in **1**. The chemical shift at δ_C 46.5 attributed to the methine carbon CH-5 revealed the γ-effect (shielding) of the oxygen atoms of the carbomethoxy group (*cis* with H-5) at C-4. (In the absence of this γ-effect, the chemical shift of CH-5 appears about δ_C 55.) The values corresponding to vicinal interaction ($^3J_{H,H}$) between the hydrogens H-14eq [δ_H 5.33 (d, J = 2.5 Hz)] and H-13ax [δ_H 3.01, td, J = 2.5 (axial−equatorial coupling with H-14eq) and 12.0 Hz (axial−axial coupling with H-12ax, δ_H 1.94, ddd, J = 6.1, 12.0, 13.1 Hz)] are consistent with the relative configuration shown in **1** (H-14equatorial and H-13axial). Thus, the chemical structure of diterpene **1** was assigned as *rel*-(1*R*,4a*R*,5*R*,8*R*)-methyl-7-(1-(methoxycarbonyl)vinyl)-5,8-diacetoxy-1,2,3,4a,5,6,7,8,9,10,10a-dodecahydro-1,4a-dimethyl-2-oxophenanthrene-1-carboxylate.

Compound **2**, isolated as a white solid, gave a molecular formula of $C_{20}H_{28}O_4$ by HRESIMS at m/z 355.2005 $[M + Na]^+$. The IR spectrum showed absorption bands suggesting the presence of hydroxy (ν_{max} 3163) and carbonyl (ν_{max} 1712 and 1673 cm^{-1}) groups and a conjugated double bond (ν_{max} 1626 cm^{-1}). The ^{1}H NMR spectrum of **2** exhibited singlet signals for two tertiary methyls at δ_H 1.17 and 1.07 and for two vinyl hydrogens at δ_H 6.54 (brs, 1H) and 5.54 (brs, 1H), characteristic of an exomethylene group. The resonances at δ_H 6.54 and 5.54 showed correlation peaks with a methylene carbon at δ_C 127.1 and with a carbonyl carbon at δ_C 166.7 in the HMQC and HMBC spectra, respectively. The {^{1}H}^{13}C NMR spectrum (Table 1) of **2**, with the aid of DEPT 135 and HMQC experiments, indicated counts of 20 carbons and 27 hydrogens. The carbon signals were exhibited by DEPT as two methyls, eight methylenes, one terminal methylene, three methines, two sp^3 quaternary carbons, a oxygenated quaternary carbon, a sp^2 quaternary carbon, and two carbonyl carbons. These assignments were consistent with the empirical formula, supporting the presence of one hydroxy group and seven degrees of unsaturation/rings. The presence of one double bond and two carbonyl signals suggested **2** to be a tetracyclic diterpene bearing a carboxyl (δ_C 183.8) and containing an α,β-unsaturated lactone [δ_C 166.7 (C═O) and 139.5/127.1 (C═CH_2)] ring. The position of the carboxyl group at C-4 could be determined using the HMBC experiment, in which the H_3-19 (δ_H 1.17) signal showed a two-bond correlation to a C-4 (δ_C 47.2) and a three-bond coupling to C-3 (δ_C 36.7), C-5 (δ_C 49.2), and C-18 (δ_C 183.8), which, in turn, correlated with H_2-3 (δ_H 1.80 and 1.65) and H-5 (δ_H 1.90). The relative configuration of **2**, particularly at the ring junction, was assigned on the basis of a NOESY experiment, which showed clearly cross-peaks assigned to dipolar interactions (NOE, spatial proximity) between H_3-20 (δ_H 1.07) and H_3-19 (δ_H 1.17), H-11a (δ_H 1.98), H-12a (δ_H 2.20), and H-14a (δ_H 2.35). Thus, the structure of diterpene **2** was assigned as *rel*-(1*S*,4a*S*,7*S*,8a*S*)-7-(1-vinyl)-tetradecahydro-1,4a-dimethylphenanthrene-7,8a-carbolactone-1-carboxylic acid. Previously, a similar diterpene was isolated from *Hyalis argentea*.[15] However, comparison of the physical and spectroscopic data for hyalic acid and diterpene **2** indicates that **2** has the opposite configuration at C-4, with the latter compound identified as 14-*epi*-hyalic acid.

The identification of compound **3** as *ent*-15-oxo-kaur-16-en-18-oic acid was made on the basis of comparison of its physical and spectroscopic (IR, NMR, and MS) data with published values.[16]

The cytotoxic activity of diterpenes **1**−**3** is shown in Table 2. Among the compounds tested, diterpene **3** exhibited the most potent cytotoxic activity (IC_{50} values from 1.5 ± 0.06 to 4.2 ± 1.08 μM). Against PMBC, it was inactve. Previously, diterpene **3** was tested against six other cell lines, namely, PC-3,[17]A549, MCF-7, KB, and

Table 2. Effects of **1**−**3** on Human Tumor Cell Lines and Human Peripheral Blood Mononuclear Cells[a]

compound	HL-60	MDAMB-435	SF-295	HCT-8	PBMC
1	NA[b]	NA	NA	NA	NA
2	8.2	NA	NA	5.2	NA
3	2.0	4.2	3.7	1.5	NA
doxorubicin[c]	0.05	1.67	0.37	0.09	0.50

[a] Results are expressed as means of IC_{50} values (the concentration that reduced cell growth by 50%) in μM, and data were obtained from triplicate experiments. [b] NA = no activity (IC_{50} > 10 μM). [c] Positive control.

KB-VIN.[18] Regarding the hemolytic potential, none of the compounds showed activity even at a high concentration (200 μg/mL).

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. IR spectra were recorded using a Perkin-Elmer 1000 spectrophotometer. ^{1}H and ^{13}C NMR were recorded on a Bruker Avance DRX-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C); chemical shifts are given in ppm (δ_C and δ_H), relative to residual $CHCl_3$ (7.24 and 77.0 ppm). Mass spectra were registered using Shimadzu LCMS-IT-TOF and Shimadzu QP5050A mass spectrometers. Silica gel 60 (230−400 mesh, Merck) was used for analytical TLC. Silica gel 60 (70−230 mesh, Merck) was used for column chromatography. All compounds were visualized by TLC using vanillin−perchloric acid−EtOH followed by heating.

Plant Material. The bark of *Croton argyrophylloides* was collected in January 2005, in Crato, Ceará State, Brazil. The plant material was identified by Dr. Edson Paula Nunes at the Herbário Prisco Bezerra (EAC), Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, CE, Brazil, where a voucher specimen (No. 27600) was deposited.

Extraction and Isolation. The bark (1.0 kg) of *C. argyrophylloides* was powdered and extracted with ethanol (10 L × 3, at room temperature, for four weeks). The solvent was removed under reduced pressure to give an EtOH extract. The EtOH extract (66.0 g) was fractionated coarsely on a silica gel column by elution with *n*-hexane (fractions 1−5), $CHCl_3$ (fractions 6−27), EtOAc (fractions 28−35), and EtOH (fractions 36−46), affording a total of 46 fractions of 100 mL each. The $CHCl_3$ fractions (40.0 g) were pooled and fractionated on a silica gel column using *n*-hexane (fractions 1′−3′), *n*-hexane/EtOAc (9:1 F4′−8′; 8:2 F9′−12′; 6:4 F13′−17′; 4:6 F18′−22′; 2:8 F23′, 24′), EtOAc (F25′, 26′), and EtOH (F27′), providing 27 fractions of 100 mL each. Fractions 15′, 16′ and 9′, obtained with *n*-hexane/EtOAc (6:4 and 8:2), yielded diterpenes **2** (3.0 g, 7.5%) and **3** (90.0 mg, 0.22%), respectively. Flash chromatography of subfractions 18′−22′ (2.9 g), using *n*-hexane−EtOAc (7:3), afforded 47 fractions of 50 mL each. Fraction F42′ yielded diterpene **1** (70 mg, 0.18%).

***rel*-(1*R*,4a*R*,5*R*,8*R*)-Methyl-7-(1-(methoxycarbonyl)vinyl)-5,8-diacetoxy-1,2,3,4a,5,6,7,8,9,10,10a-dodecahydro-1,4a-dimethyl-2-oxophenanthrene-1-carboxylate (1):** white, amorphous powder; $[\alpha]^{20}_D$ +25 (*c* 0.06, $CHCl_3$); IR (KBr) ν_{max} 1731, 1666, 1632, 1435, 1373, 1248, 1157 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1; HRESIMS *m*/*z* 513.2079 $[M + Na]^+$ (calcd for $C_{26}H_{34}O_9Na$, 513.2100).

***rel*-(1*S*,4a*S*,7*S*,8a*S*)-7-(1-Vinyl)tetradecahydro-1,4a-dimethylphenanthrene-7,8a-carbolactone-1-carboxylic acid (2):** white, amorphous powder; $[\alpha]^{20}_D$ −136 (*c* 0.38, $CHCl_3$); IR (KBr) ν_{max} 3163, 1712, 1673, 1626, 1232, 1186 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1; HRESIMS *m*/*z* 355.2005 $[M + Na]^+$ (calcd for $C_{20}H_{28}O_4Na$, 355.1885).

MTT Cytotoxicity Assay. Cytotoxic potential was evaluated by the MTT assay[19] against four human tumor cell lines: HL-60 (promyelocytic leukemia), SF-295 (glyoblastoma), HCT-8 (colon cancer), and MDAMB-435 (melanoma), all obtained from the U.S. National Cancer Institute (Bethesda, MD). The cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37 °C with 5% CO_2. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a purple formazan product. Briefly, cells were plated in 96-well plates (0.7×10^5 cells/well for adherent cells and 0.3×10^6 cells/well for suspended cells), and test compounds (0.078−5 μg/mL), dissolved in DMSO, were added to each plate well. Control groups received the same amount of vehicle. After 69 h of incubation, the supernatant was replaced by fresh medium containing MTT (0.5 mg/mL). Three hours later, the MTT formazan product was dissolved in 150 μL of DMSO, and absorbance was measured at 595 nm (DTX-880, Beckman Coulter). The final concentration of DMSO in the culture medium was kept constant, below 0.1% (v/v). All cell treatments were carried out with three replicates, and doxorubicin was used as positive control.

Alamar Blue Assay. In order to investigate the selectivity of test compounds toward normal proliferating cells, the Alamar Blue assay was performed with human peripheral blood mononuclear cells (PBMC) after 72 h drug exposure.[20] Heparinized blood (from healthy, nonsmoker donors who had not taken any drugs at least 15 days prior to sampling) was collected, and PBMC were isolated by a standard method of density-gradient centrifugation over Ficoll-Hypaque. PBMC were washed and resuspended at a concentration of 3×10^5 cells/mL in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. Phytohemagglutinin (3%) was added at the beginning the process. After 24 h, each test compound (0.078−5 μg/mL), dissolved in DMSO, was added to a plate well and incubated for 72 h. Control groups received the same amount of vehicle, and doxorubicin was used as positive control. The final concentration of DMSO in the culture medium was kept constant, below 0.1% (v/v). Twenty-four hours before the end of the incubation, 10 μL of stock solution (0.312 mg/mL) of the Alamar Blue (Resazurin, Sigma-Aldrich) was added to each well. The absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter), and the drug effect was quantified as the percentage of control absorbance at 570 and 595 nm. The absorbance of Alamar Blue in the culture medium was measured at a higher wavelength and a lower wavelength. The absorbance of the medium was also measured at the higher and lower wavelengths. The absorbance of the medium alone was subtracted from the absorbance of medium plus Alamar Blue at the higher wavelength (AO_{HW}). The absorbance of the medium alone was subtracted from the absorbance of medium plus Alamar Blue at the lower wavelength (AO_{LW}). A correction factor R_0 was calculated from AO_{HW} and AO_{LW}, where $R_0 = AO_{LW}/AO_{HW}$. The percent Alamar Blue reduced was expressed as follows: % reduced = $A_{LW} - (A_{HW} \times R_0) \times 100$.

Hemolytic Assay. A hemolysis test was performed in a 96-well plate.[21] Each well received 50 μL of 0.85% NaCl solution containing 10 mM $CaCl_2$. DMSO (1%) was used as the negative control. Compounds were tested at concentrations ranging from 0.78 to 200 μg/mL. The last well was administered 50 μL of 0.2% Triton X-100 (in 0.85% saline) to obtain 100% hemolysis. Next, each well received 50 μL of a 2% suspension of mouse erythrocytes in 0.85% NaCl containing 10 mM $CaCl_2$. After incubation at room temperature for 1 h and centrifugation, the supernatant was removed and the hemoglobin released was measured spectrophotometrically at an absorbance of 540 nM.

Statistical Analysis. Data are expressed as means ± SEM. The IC_{50} or EC_{50} values and their 95% confidence intervals were obtained by nonlinear regression using the GraphPad program (Intuitive Software for Science, San Diego, CA).

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Supporting Information Available: 1D, 2D NMR, IR, and MS spectra of **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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