Cytotoxic 3,4-*seco*-Atisane Diterpenoids from *Croton barorum* and *Croton goudotii*

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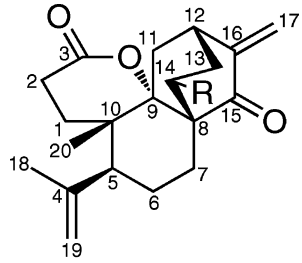
In a screening program directed to the discovery of new anticancer agents from Madagascan plants, ethyl acetate extracts of *Croton barorum* and *C. goudotii* showed strong cytotoxic activity, with 100% inhibition at 10 μg/mL in a primary screen using the murine lymphocytic leukemia P388 cell line. Bioassay-guided fractionation led to the isolation of two new 3,4-*seco*-atisane diterpenoids, crotobarin (**1**) and crotogoudin (**2**). Their structures were elucidated by spectroscopic data interpretation. Compounds **1** and **2** produced a net progression in the number of cells arrested at the G2/M growth stage in the cell cycle of the K562 human leukemia cell line at 4 μM.

The first inventory of the Madagascan *Croton* (Euphorbiaceae) was published in 1939,[1] but this has not been subsequently subjected to an updated species revision. According to the "efloras" database on the vascular plants of Madagascar,[2] the genus *Croton* is represented by 139 species distributed in various ecological regions ranging from the eastern rainforests to the semiarid region of the south. Despite their ethnomedical uses, only five Madagascan species in the genus have been investigated phytochemically, namely, *C. mongue, C. geayi, C. hovarum, C. antanosiensis,* and *C. sakamaliensis*.[3−7]

In the course of a continuing search for biologically active compounds from plants of Madagascar, nine *Croton* species in different regions of the island were collected. Ethyl acetate extracts of two species, namely, *C. barorum* Leandri and *C. goudotii* Baill., showed strong cytotoxic activities with 100% inhibition at 10 μg/mL against the P388 murine lymphocytic leukemia cell line. *C. barorum* is used for the treatment of cough and malaria, but some toxicity has been reported.[6] *C. goudotii* is used to treat chronic blennorrhea and cough and is also claimed to be an aphrodisiac.[6] Bioassay-guided fractionation of these plants led to the isolation of two new, closely related diterpenes belonging to the 3,4-*seco*-atisane compound class, crotobarin (**1**) and crotogoudin (**2**). Prior to our work on these species, no previous phytochemical or biological studies have been reported.



1: R = $OCOCH_3$
2: R = H

The IR data of compound **1** (crotobarin) showed a strong lactone band at 1730 cm^{-1} and another ester band at 1238 cm^{-1}. The molecular weight was determined as *m/z* 372 as a result of observation of a sodium-cationized molecular ion at *m/z* 395.1849 in the positive electrospray FTMS. The elemental formula of compound **1** was thus deduced to be $C_{22}H_{28}O_5$. As evident from the ^1H and ^{13}C NMR spectra as well as HSQC correlations, the structure of **1** includes three methyl groups at δ_H 1.23/δ_C 20.3, δ_H 1.94/δ_C 20.9, and δ_H 1.82/δ_C 23.7, respectively, two methylene groups at δ_H 4.86, δ_H 5.00/δ_C 115.6 and δ_H 5.30, δ_H 5.91/δ_C 117.3, respectively, an α,β-unsaturated ketone at δ_C 195.1, and two ester groups at δ_C 169.7 and 170.5. Using these readily assignable groups, fragments were identified by the concerted interpretation of the 1D NMR, COSY, HSQC, and HMBC spectra. These units were then assembled on the basis of key HMBC correlations. Thus, the observation of a long-range coupling between the methyl at δ_H 1.94 and the carbonyl function at δ_C 170.5 and the observation of a correlation between the signal at δ_H 5.62 and the same carbonyl group indicated the presence of an acetoxy group attached to a methine. Starting from the signal at δ_H 5.62, the proton sequence H-14/H_2-13/H-12/H_2-11 was identified unambiguously by scalar H−H connectivities obtained by COSY experiments, and HMBC correlations were applied to confirm the assignments for this fragment (Table 1). Starting from the geminal methylene protons at δ_H 5.30 and 5.91 attached to the C-17 carbon (δ_C 117.3), their HMBC correlations with C-16 (δ_C 145.8), C-15 (δ_C 195.1), and C-12 (δ_C 35.5) indicated a α-methylene carbonyl group. An α-methylene cyclohexanone fragment was suggested on the basis of the observation of HMBC correlations between the H-12 proton (δ_H 3.06) and C-9 (δ_C 89.3) and C-15 and the observation of correlations between H-11a (δ_H 2.53) and C-16 and C-8 (δ_C 55.8). The two fragments were assembled as an α-methylenebicyclo-[2.2.2]octanone unit by fully connected HMBC experiments. At this point, key HMBC correlations included connectivities of H-14 (δ_H 5.62) with C-8 and C-12 and a connectivity of H-13a (δ_H 2.37) with C-16.

Another entry point for **1** was provided by the methyl group H-20 (δ_H 1.23), correlated to the oxygenated C-9 carbon, the quaternary C-10 carbon (δ_C 39.7), and the tertiary C-5 carbon (δ_C 44.2) as well as the C-1 methylene (δ_C 28.6). Then, the H-1 protons (δ_H 1.70 and 1.89) could be correlated to the same tertiary and quaternary carbons and to the C-2 methylene (δ_C 26.6) and the C-3 ester (δ_C 169.7). On the basis of these correlations, a δ-lactone fragment was suggested. This δ-lactone fragment was confirmed by the long-range correlations of the H-2 methylene protons (δ_H 2.49) with C-1, C-3, and C-10.

Correlations of the methylene protons at δ_H 4.86 and 5.00 (H-19b and H19a) to the C-4 (δ_C 146.7), C-5, C-10, and C-18 carbon (δ_C 23.7) were used to establish an isopropenyl group attached to the C-5 methine carbon. This was confirmed by correlations of the

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Table 1. NMR Spectroscopic Data for Compounds **1** (acetone-d_6, 600 MHz) and **2** ($CDCl_3$, 500 MHz and ^{13}C, 75.45 MHz)

	crotobarin **1**			crotogoudin **2**		
position	δ_C, mult.	δ_H (J in Hz)	HMBC	δ_C, mult	δ_H (J in Hz)	HMBC
1	28.6, CH_2	Ha: 1.89, m	2, 3, 5, 9, 10	28.0, CH_2	Ha: 1.74, m	3, 9
		Hb: 1.70, m	2, 3, 5, 9, 10		Hb: 1.69, m	
2	26.6, CH_2	Ha, Hb: 2.49, m	1, 3, 10	26.2, CH_2	Ha: 2.56, ddd, 19.4, 10.8, 9.2	1, 3, 10
					Hb: 2.41, ddd, 19.4, 8.4, 1.4	
3	169.7, qC			170.3, qC		
4	146.7, qC			144.5, qC		
5	44.2, CH	2.44, m	4, 7,19, 20	43.4, CH	2.38, dd, 12.7, 3.1	10, 18, 20
6	21.6, CH_2	Ha: 1.95, m	5, 8	22.9, CH_2	Ha: 1.83, m	8
		Hb: 1.57, m			Hb: 1.59, m	7, 8
7	23.5, CH_2	Ha: 1.89, m	5, 8, 9, 14	25.3, CH_2	Ha: 2.20, m	5
		Hb: 1.50, m	5, 8, 9, 14		Hb: 1.43, m	8
8	55.8, qC			50.1, qC		
9	89.3, qC			89.9, qC		
10	39.7, qC			38.7, qC		
11	39.6, CH_2	Ha: 2.53, m	8, 13, 16	39.5, CH_2	Ha: 2.33, dd, 14.3, 4.3	10, 12
		Hb: 1.86, m			Hb: 1.81, m	
12	35.5, CH	3.06, t, 2.2	9, 15	35.6, CH	2.90, m	9
13	36.0, CH_2	Ha: 2.37, dd, 14.8, 2.3	9, 16	25.2, CH_2	Ha: 1.79, m	
		Hb: 1.64, m			Hb: 1.65, m	8
14	67.2, CH	5.62, dd, 0.9, 10.1	8, 12, 15, 21	24.4, CH_2	Ha: 2.21, ddd, 15.1, 11.3, 3.4	8, 12
					Hb: 1.33, ddd, 15.1, 11.7, 6.6	8
15	195.1, qC			198.8, qC		
16	145.8, qC			145.4, qC		
17	117.3, CH_2	Ha: 5.91, d, 1.7	12, 15, 16	118.0, CH_2	Ha: 6.02, d, $J = 1.1$	11, 12, 15, 16
		Hb: 5.30, d, 1.7	12, 15		Hb: 5.22, d, $J = 1.1$	12, 15
18	23.7, CH_3	1.82, s	4, 5, 19	23.3, CH_3	1.77, m	4, 5, 19,
19	115.6, CH_2	Ha: 5.00, br s	4, 5, 18	115.1, CH_2	Ha: 4.95, m	4, 5, 18
		Hb: 4.86, br s	4, 5, 10, 18		Hb: 4.875, m	4, 5, 18
20	20.3, CH_3	1.23, s	1, 5, 9, 10	19.9, CH_3	1.82, s	9
21	170.5, qC					
22	20.9, CH_3	1.94, s	21			

H-18 proton to C-4, C-5, and C-19 carbons (δ_C 115.6). The H-5 protons showed correlations to the C-7 carbon, the H-7 methylene protons correlated to the C-5, C-8, and C-9 carbons, and the H-6 methylene protons correlated to the C-5 and C-8 carbons, extending the fragment to an isopropenylcyclohexane. This fragment could be linked to the δ-lactone unit via the C-9, C-10 bond to give a hexahydrochromanone fragment. Finally, the latter fragment was fused to the α-methylenebicyclo[2.2.2]octanone unit through the C-8, C-9 bond, for which the carbons showed HMBC correlations to key protons of both units (Table 1). Furthermore, the observation of HMBC correlations of the H-7 methylene protons with C-14 supported the hypothesized structure of **1** as a 3,4-*seco*-atisane derivative, as shown.

The molecular weight of compound **2** (crotogoudin) was determined as *m*/*z* 314 as a result of observation of a deprotonated molecular ion at *m*/*z* 313.1845 in the negative-ion electrospray TOFMS and a sodium-cationized molecular ion at *m*/*z* 337.2 in the positive-ion electrospray TOFMS, corresponding to the elemental formula $C_{20}H_{26}O_3$. Analysis of the ^1H NMR and ^{13}C NMR spectra suggested that it was a closely related diterpenoid to compound **1**. Thus, when the ^1H NMR spectrum was compared to that of compound **1**, there was the disappearance of the signal at δ_H 5.62 and the singlet at δ_H 1.94. Furthermore, in the ^{13}C NMR spectrum of compound **2**, the carbonyl signal at δ_C 170.5 and the signals at δ_C 67.2 and 20.9 were no longer present, but a new signal appeared at δ_C 24.4. These preliminary observations suggested that the acetoxy group was missing in compound **2**. Unambiguous assignments of ^1H NMR and ^{13}C NMR were obtained by the concerted interpretation of 1D and 2D NMR spectra (Table 1), confirming the structural hypothesis for **2** as shown.

Regarding the relative configuration of **1** and **2**, the bicyclic system imposes an equatorial orientation for H-12 and the C-7, C-8 bond. The observation of a NOE cross-peak between the protons of the C-20 methyl group at δ_H 1.23 and H-14 was pivotal for the determination of the relative stereochemistry at C-10, C-14 and C-9 (Figure 1). The spatial proximity of the C-20 methyl group to H-14 clearly indicated that the C-20 methyl group, the C-8−C-14 bond, and the C-9−O bond are oriented axially and impose the relative configuration 14*S** for **1**. Finally, in both structures, the observation of cross-peaks from the C-18 methyl group to the C-20 methyl group and the C-2 methylene group to H-5 supported the relative configuration, 5*S**, representing an equatorial orientation of the isopropenyl group. The relative configuration of each compound was assigned as *rel*-(5*S*,8*S*,9*S*,10*S*,12*R*,14*S*) for **1** and *rel*-(5*S*,8*R*,9*S*,10*S*,12*R*) for **2**.

Crotobarin (**1**) and crotogoudin (**2**) showed marked cytotoxic activities, with IC_{50} values of 0.14 ± 0.05 and 0.13 ± 0.01 μg/mL, respectively, against the murine P388 cell line used for bioassay-guided fractionation. These activities were confirmed on human cell lines as shown in Table 2.

The effect of compounds **1** and **2** on the cell cycle of the K562 human leukemia cell line was then investigated by flow cytometry. As shown in Figure 2, after 24 h of treatment, a net progression in the number of K562 cells arrested at the G2/M growth stage was observed with 4 μM **1** or **2**. It should be noted that in all cells treated with **1** or **2** for 24 h, a subdiploid DNA content was observed (data not shown), indicating that cells have undergone apoptosis, probably as a result of the cell cycle being arrested in the G2/M phase.

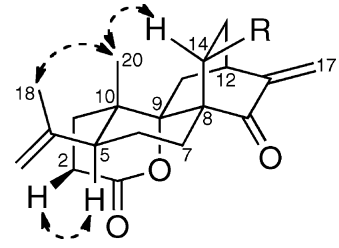

Figure 1. Key NOESY correlations for compounds **1** (R = acetoxy) and **2** (R = H).

Table 2. Cytotoxic Activities of Crotobarin (**1**) and Crotogoudotin (**2**) for Human Tumor Cell Lines[a,b]

	KB	HT29	A549	HL60
1	2.5 ± 0.10	2.1 ± 0.60	0.79 ± 0.15	0.56 ± 0.02
2	1.5 ± 0.03	1.9 ± 0.25	0.54 ± 0.02	0.49 ± 0.01
Taxotere[c]	0.29 ± 0.03	0.92 ± 0.02	0.37 ± 0.01	0.52 ± 0.02

[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 duplicate experiments. [b] For the cell lines used, see Experimental Section. [c] Positive control substance.

The cytotoxic activities of **1** and **2** may be due to the presence in each case of the α-methylene ketone group, which has been found to react preferably with nucleophiles by a Michael-type addition.[8] It was reported that the anti-inflammatory activity of sesquiterpene lactones possessing a α,β-unsaturated carbonyl moiety is due to the inhibition of the transcription factor NF-kappaB, probably by alkylating cysteine-38 in the DNA binding domain of the p65 subunit.[9] It was also recently reported that this structural element is necessary for the inhibition of the tubulin carboxypeptidase (TCP) activity of the sesquiterpene lactone parthenolide, since the dihydro derivative did not show any inhibition.[10] However, this structural unit alone was found to be insufficient for TCP inhibition, and additional molecular requirements are needed for activity. Crotobarin (**1**) and crotogoudin (**2**) are therefore good candidates for further biological investigation including TCP inhibition. Inhibitors of TCP, by reversing abnormal detyrosinated tubulin accumulation in tumor cells, could impair tumor progression.

3,4-*seco*-Atisane diterpenes are rare natural products. To the best of our knowledge, they have been reported hitherto in *Euphorbia acaulis, Euphorbia fidjiana*, and the mangrove plant *Excoecaria agallocha*.[11−13]

Experimental Section

General Experimental Procedures. A JASCO-P1010 polarimeter was used for measurement of optical rotations. IR spectra were recorded on a Nicolet FT-IR 205 spectrophotometer. One- and two-dimensional NMR spectra were recorded in acetone-d_6 for compound **1** and $CDCl_3$ for compound **2** at 300 K using a Bruker 600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) with TMS as an internal standard. ESIMS and FT-MS were recorded on an Orbitrap Thermo-Finnigan mass spectrometer (Thermo-Finnigan, Bremen, Germany). Column chromatography was carried out on silica gel 60 F 254 and silica gel 100 (Merck, Darmstadt, Germany). Precoated plates of silica gel 60 F254 (Merck) were used for TLC, and the zones were detected with a UV lamp at 254 and 366 nm and by spraying with phosphomolybdic acid in H_2SO_4 followed by heating at 100 °C for 1−2 min.

Plant Material. *Croton barorum* was collected at Ampanihy in the south region of Madagascar in October 2006, and *Croton goudotii* at Ankililoaka in the southwestern region of Madagascar, in June 2007. They were identified by Armand Rakotozafy by comparison with voucher specimens held at the Department of Botany, Parc Botanique et Zoologique de Tsimbazaza, Antananarivo. Voucher specimens with assigned sample numbers MAD-0393 for C. *barorum* and MAD-0578 for C. *goudotii* have been deposited at the herbarium of IMRA.

Extraction and Isolation. The dried aerial parts of *C. barorum* (200 g) were extracted (2 × 3 h) with ethyl acetate (2 × 1 L) at room temperature on a shaker. The pooled organic solvents were dried over Na_2SO_4 and evaporated to dryness at 40 °C under reduced pressure to yield 6.38 g of crude extract. The crude extract was dissolved in methanol (0.5 L) and passed through a thin layer of CXVO charcoal to remove chlorophylls to yield 6.17 g of depigmented extract. Then, 6 g of the depigmented extract was submitted to silica gel chromatography using successively hexane, dichloromethane, ethyl acetate, and acetone as eluents. The ethyl acetate fraction (2.15 g) was found to be the most cytotoxic and was fractionated over silica gel chromatography using a gradient of dichloromethane and ethyl acetate as eluents. Elution with dichloromethane/ethyl acetate (98:2) furnished eight fractions, of which the seventh gave pure crotobarin (**1**, 50.3 mg).

Crotobarin (1): amorphous solid; $[\alpha]^{20}_D$ −29.9 (*c* 0.05, $CHCl_3$); IR ν_{max} 2942, 1730, 1636, 1450, 1372, 1239, 1192, 1129, 1107, 1118, 970 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); HRFT-MS *m/z* 395.1849 $[M + Na]^+$ (calcd for 395.1834).

Following the same protocol, 200 g of dried aerial parts of *C. goudotii* yielded 5.44 g of a depigmented crude extract. In initial silica gel column chromatography, the cytotoxic activity was found in the dichloromethane fraction (1.25 g), which was subsequently submitted to silica gel column chromatography using a gradient of hexane and ethyl acetate as eluents. Elution with hexane/ethyl acetate (60:40) gave six fractions, which were combined on the basis of TLC similarities (306 mg). Further silica gel column chromatography using a gradient of hexane and ethyl acetate (9:1 to 7.5:2.5), then pure dichloromethane, gave pure crotogoudin (**2**, 64.7 mg) in the dichloromethane fraction.

Crotogoudin (2): amorphous solid; $[\alpha]^{20}_D$ +7 (*c* 0.40, $CHCl_3$); IR ν_{max} 2941, 2872, 1728, 1634, 1468, 1359, 1276, 1187, 1166, 1021, 982, 950 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); HRESIMS *m/z* 313.1845 $[M - H]^-$ (calcd for 313.1809).

Cytotoxicity Bioassay. A standard method was used to assess cytotoxicity.[14] Murine P388 leukemia cells were grown in RPMI 1640 medium containing 0.01 nM β-mercaptoethanol, 10 mM L-glutamine, 100 IU/mol penicillin G, 100 μg/mL streptomycin, 50 μg/mL gentamycin, and 50 μg/mL nystatin, supplemented with 10% fetal calf serum. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The inoculum was seeded at 104 cells/mL at an optimal volume of 0.1 mL per well and introduced into flat-bottomed 96-well plates containing a single concentration of extract at 10 μg/mL in triplicate, alongside untreated controls. Camptothecin was used as a positive control. Cultures were then incubated at 37 °C for 72 h in the required atmosphere. Thereafter, cells were incubated at 37 °C with 0.02% neutral red dissolved in 1:9 methanol/water (0.1 mL per well) for 1 h and then washed with 1 N PBS and finally lysed with 1% SDS. After a brief agitation on a microculture plate shaker, the plates were transferred to a Titertek Twinreader equipped with a 540 nm filter to measure absorbance of the extracted dye. Cell viability was expressed as the percentage of cells incorporating dye relative to the untreated controls. For the determination of the median inhibitory concentration

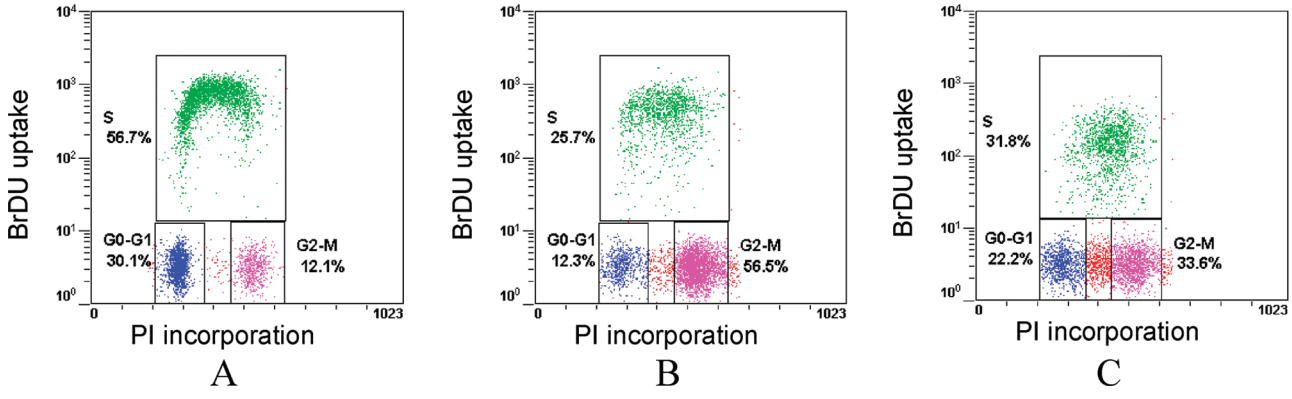

Figure 2. Cell cycle distribution, measured by bromodeoxyuridine (BrdU) uptake and propidium iodide (PI) staining, of K562 cells (control, A) and K562 cells treated with **1** at 4 × 10^{-6} M (B) and **2** at 4 × 10^{-6} M (C) for 24 h.

(IC_{50}), serial concentrations of extracts were used, and IC_{50} was calculated by linear regression method. Three separate experiments were carried out.

The KB (human oral epidermoid carcinoma), HT29 (human colon adenocarcinoma), A549 (human lung adenocarcinoma), and HL60 (human promyelocytic leukemia) cell lines were obtained originally from ATCC (Manassas, VA). The cytotoxicity assays were performed according to a published procedure.[15] Taxotere was used as the reference compound.

Cell Cycle Analysis. Exponentially growing K562 human leukemia cells were incubated with test compounds or DMSO for 24 h. Cell-cycle profiles were determined by flow cytometry on a FC500 flow cytometer (Beckman-Coulter, Villepinte, France) as described previously.[13]

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Supporting Information Available: NMR spectra of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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