

Cytotoxic Clerodane Diterpenoids and Their Hydrolysis Products from *Casearia nigrescens* from the Rainforest of Madagascar¹

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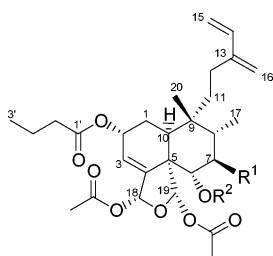
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Bioassay-guided fractionation of the cytotoxic leaf and flower extract of *Casearia nigrescens* led to the isolation of four new clerodane diterpenoids, designated caseanigrescens A–D (**1–4**). These compounds were subject to hydrolysis to dialdehydes when stored in CDCl₃. The structures of compounds **1–4** were determined using 1D and 2D NMR spectroscopy. All four compounds showed moderate cytotoxicity to the A2780 human ovarian cancer cell line, with an IC₅₀ range of 0.83–1.4 μM.

In our continuing search for bioactive molecules from the rainforests of Madagascar as part of an International Cooperative Biodiversity Group (ICBG) program,² an extract of the leaves and flowers of *Casearia nigrescens* Tul. (Flacourtiaceae) showed moderate cytotoxicity against the A2780 human ovarian cancer cell line. This extract was selected for fractionation on the basis of its cytotoxicity and the lack of phytochemical data for this species. The crude extract was purified by liquid–liquid partition, reversed-phase chromatography, and reversed-phase HPLC to yield the four new clerodane diterpenoids **1–4**, designated caseanigrescens A–D. These compounds were subject to hydrolysis to dialdehydes when stored in CDCl₃.

The genus *Casearia* is a member of the Saliciaceae. It consists of about 180 species and occurs throughout the tropics. Five species are found in Madagascar.^{3,4} The Flacourtiaceae family, and *Casearia* in particular, are well known for the production of clerodane diterpenoids.^{5–8} Such compounds have been reported to possess a wide range of biological activities including cytotoxic, antimalarial, antimycobacterial, DNA-damaging, trypanocidal, immunosuppressive, antifungal, and apoptosis-inducing activity.^{5–7,9–15} *C. nigrescens* is known locally as Hazondrano.



- 1 R¹ = OAc, R² = H
- 2 R¹ = OH, R² = H
- 3 R¹ = OH, R² = Ac
- 4 R¹ = H, R² = H
- 5 R¹ = αOAc, R² = H

Results and Discussion

Compound **1** was isolated as a colorless, amorphous solid, with a molecular formula of C₃₀H₄₂O₁₀ as determined by HRFABMS.

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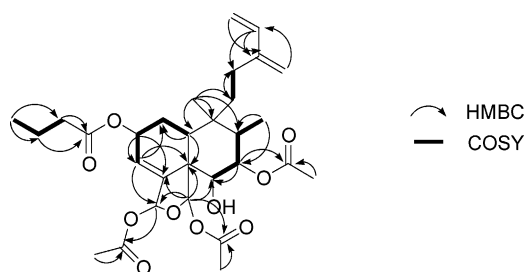


Figure 1. Selected HMBC correlations observed for **1**.

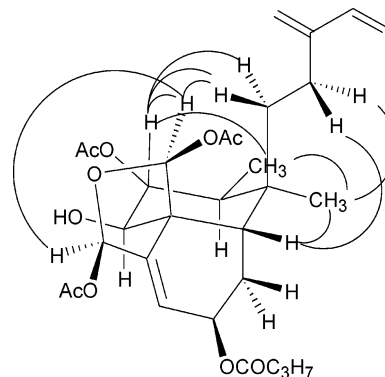


Figure 2. Selected NOE enhancements observed for **1**.

The ¹H and ¹³C NMR data (Table 1) indicated that it was a clerodane diterpenoid analogue. Correlations obtained from HMBC and COSY experiments were used to establish the 2D structure (Figure 1). The assignment of the 2D structure was straightforward with the exception of the butyrate group, which did not correlate to any of the oxymethine signals in the HMBC experiment. However, as all of the acetates had been accounted for via HMBC correlations, only two possibilities remained, the C-2 oxymethine that resonated at δ_C 66.4 (δ_H 5.47) and the C-6 oxymethine that resonated at δ_C 75.0 (δ_H 3.65). On the basis of the chemical shifts in the ¹H NMR spectrum it was clear that the butyrate group was located at the C-2 position.

The relative configuration of **1** was determined using coupling constants and NOE data (Figure 2) and was supported by comparison of the optical rotation to literature values for similar compounds. A NOE enhancement from the C-19 proton to the C-18 proton indicated that the acetate groups attached to the hemiacetal ring were in a *cis* configuration. Two 1,3-diaxial NOE enhance-

Table 1. ^1H and ^{13}C NMR Data for Caseanigrescens A (**1**), B (**2**), and C (**3**)

	caseanigrescen A (1) ^c		caseanigrescen B (2) ^c		caseanigrescen C (3) ^c	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1	1.62 ^a 1.77, m	27.1	1.84 ^a	27.2	1.72 ^a	27.1
2	5.47, m	66.4	5.53, brs	66.5	5.47, m	66.2
3	6.10, d (3.7)	121.9	6.15, d (2.8)	122.0	6.02, dd (1.4, 3.9)	123.6
4		145.7		145.4 ^b		144.0
5		54.3		53.5		53.1
6	3.65, d (10.7)	75.0	3.64, m	77.1	5.13, d (10.6)	77.0
7	5.35, dd (10.7, 11.0)	75.6	3.55, m	72.8	3.11, dd (10.6, 11.0)	71.2
8	1.64 ^a	41.9	1.70 ^a	43.3	1.46 ^a	43.2
9		39.1		38.8		38.6
10	2.46, dd (3.5, 13.8)	36.4	2.46, dd (5.6, 11.6)	36.5	2.38, dd (6.3, 10.9)	37.3
11	1.49 ^a 1.64 ^a	29.2	1.37 ^a 1.69 ^a	29.5	1.14, ddd (3.0, 12.8, 12.8) 1.48 ^a	29.3
12	2.04, m 2.17, m	24.2	2.08, m 2.19, m	24.3	2.01, m 2.12, m	24.2
13		144.8		145.3 ^b		145.3
14	6.34, dd (10.8, 17.7)	140.9	6.37, dd (11, 17.4)	140.9	6.34, dd (10.9, 17.7)	140.7
15	4.86, d (10.8) 5.07, d (17.7)	112.0	4.88, d (11) 5.10, d (17.4)	112.1	4.87, d (10.9) 5.07, d (17.7)	112.3
16	5.00, brs	116.6	5.09, s 5.23, s	116.2	5.02, s 5.05, s	115.9
17	0.83, d (6.7)	11.3	1.12, d (6.4)	11.3	0.85, d (6.7)	11.2
18	7.15 ^a	96.1	7.11, s	96.2	6.79, dd (1.6, 1.6)	95.1
19	6.98, s	98.7	6.76, s	99.0	6.69, s	98.6
20	0.67, s	25.6	0.76, s	25.7	0.64, s	25.7
1'		172.5		172.4		172.3
2'	1.89, m	36.2	1.88, m	36.2	1.85, m	36.1
3'	1.46, m	18.8	1.45, m	18.8	1.43, m	18.7
4'	0.73, t (7.4)	13.5	0.72, t (7.4)	13.5	0.70, t (7.3)	13.5
6-O ₂ CCH ₃						170.6
6-O ₂ CCH ₃					1.92, s	20.9
7-O ₂ CCH ₃		171.2				
7-O ₂ CCH ₃	1.93, s	20.8				
18-O ₂ CCH ₃		169.7		170.3		169.6
18-O ₂ CCH ₃	1.63, s	20.8	1.64, s	20.7	1.53, s	20.6
19-O ₂ CCH ₃		168.9		169.2		168.9
19-O ₂ CCH ₃	1.72, s	21.4	1.70, s	21.4	1.68, s	21.5

^a Signals were in overlapped regions of the spectrum. ^b Signals are interchangeable. ^c Spectra were collected in C₆D₆.

ments of the C-7 proton and one of the C-11 protons (δ_{H} 1.49) were also observed when the C-19 proton was irradiated. Thus, a chair conformation with the C-19 and C-11 groups in axial positions and the C-7 acetate in an equatorial position was indicated for the B-ring. A NOE enhancement of the C-17 protons when the C-7 proton was irradiated indicated that the C-17 methyl group was equatorial. No enhancement was observed for the C-6 proton, indicating that it was axial and *trans* from the C-7 proton and the C-19 group. This was supported by the observed coupling constants of the C-6 proton (δ_{H} 3.65, d, $J = 10.7$ Hz) and the C-7 proton (δ_{H} 5.35, dd, 10.7, 11.0 Hz), both of which indicated 1,2-diaxial couplings. Upon irradiation of the C-10 proton, an enhancement was observed for the protons of the C-12 and C-20 groups, which indicated that the C-10 proton was equatorial and that the rings were *cis* fused. The final position to be assigned was the C-2 position. The lack of any significant NOE enhancements when the C-2 proton was irradiated suggested that the C-2 proton was equatorial. This was confirmed by an examination of its chemical shift (δ_{C} 66.4); a chemical shift of approximately δ_{C} 66.0 indicates that the C-2 ester group is of the same relative configuration as C-11, C-17, and C-19, but a chemical shift of approximately δ_{C} 70.0 indicates that C-2 is of the opposite configuration.^{3,9} The specific rotation of **1** was +59.6, which was consistent with values for previously published compounds of similar structure. The relative configuration of **1** has been depicted in the same configuration as similar compounds whose absolute configuration has been determined.¹⁶ Compound **1** is a new clerodane diterpenoid and has been given the trivial name caseanigrescen A.

A recent paper reported the structure of caseamembrin N (**5**), which differed from **1** only in the configuration of C-7.¹⁷ The ^1H

and ^{13}C NMR data for **1** (CDCl₃) are very similar to those reported for caseamembrin N, but the two compounds differ significantly in their specific rotations, with that for caseamembrin N reported as +9.8.

Compound **2** was isolated as a colorless, amorphous solid, with a molecular formula of C₂₈H₄₀O₉ as determined by HRFABMS. The ^1H and ^{13}C NMR data (Table 1) for **2** were similar to those of **1** and indicated that it was the C-7 deacetate of **1**. This assignment was confirmed by analysis of the 2D NMR spectra of **2**. Compound **2** is a new clerodane diterpenoid and has been given the trivial name caseanigrescen B.

Compound **3** was isolated as a colorless, amorphous solid. Its HRFABMS indicated a molecular formula of C₃₀H₄₂O₁₀, which was the same as that of **1**. The ^1H and ^{13}C NMR data (Table 1) for **3** were virtually identical to those of **1**. The only significant differences were that in the ^1H NMR spectrum of **3** the oxymethine doublet ($J = 10.6$ Hz) resonated at δ_{H} 5.13 and the oxymethine doublet of doublets ($J = 10.6, 11.0$ Hz) resonated at δ_{H} 3.11. On the basis of HMBC and COSY experiments the doublet at δ_{H} 5.13 was assigned to the C-6 oxymethine and the doublet of doublets at δ_{H} 3.11 to the C-7 oxymethine. These values were reversed from the corresponding values for **1**, which were δ_{H} 3.65 and 5.35, respectively, and indicated that the acetate was located on the C-6 hydroxyl group in **3**. Compound **3** is a new clerodane diterpenoid and has been given the trivial name caseanigrescen C.

Compound **4** was isolated as a colorless, amorphous solid. Its HRFABMS indicated a molecular formula of C₂₈H₄₀O₈. Its ^1H and ^{13}C NMR spectra (Table 2) indicated that it had one less acetate than **1** and **3**, and its MS indicated that it also contained one less oxygen than **2**. Because the remaining oxymethine (δ_{H} 3.41, dd, J

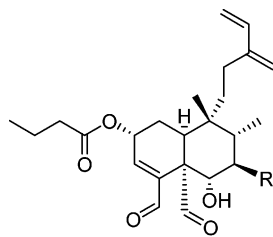
Table 2. ^1H and ^{13}C NMR Data for Caseanigrescen D (**4**) Caseanigrescen A-2 (**6**), and Caseanigrescen D-2 (**7**)

	caseanigrescen D (4) ^b		caseanigrescen A-2 (6) ^c		caseanigrescen D-2 (7) ^{c,d}	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1	1.50 ^a 1.73 ^a	27.1	1.65–2.00 ^e	25.8	1.92 ^a	25.8
2	5.45, m	66.5	5.55, m	64.5	5.52, m	64.8
3	6.04, d (4.1)	121.4	7.04, d (4.5)	147.3	7.05, d (5.1)	148.6
4		146.4		147.1		148.5
5		54.1		56.4		55.9
6	3.41, dd (3.7, 11.9)	72.8	3.71, d (9.8)	74.8	3.86, dd (6.6, 10.5)	71.8
7	1.10, m	36.8	5.36, dd (9.8, 11.5)	75.6	1.87 ^a	36.7
8	1.23	36.8		38.8	1.73 ^a	35.4
9		37.4		39.4		37.8
10	2.36, dd (3.4, 13.7)	37.1	2.12, dd (3.5, 13.5)	40.3	2.34 ^a	40.5
11	1.23 ^a	28.3	1.19, m	33.5	1.14, m	32.4
	1.35, ddd (4.5, 12.9, 12.9)		1.63, m		1.50, ddd (3.6, 12.8, 14.7)	
12	2.03, m 2.16, m	24.2	1.65–2.00 ^e	23.6	1.85 ^a 1.98, ddd (3.7, 13.4, 13.4)	23.4
13		145.6		145.7		146.3
14	6.36, dd (10.9, 17.5)	140.8	6.31, dd (11.0, 17.5)	138.8	6.32, dd (10.8, 17.5)	138.9
15	4.89, d (10.9) 5.11, d (17.5)	112.2	5.02, d (11.0) 5.15, d (17.5)	112.9	5.02, d (10.8) 5.17, d (17.5)	112.9
16	5.06, s 5.08, s	115.9	4.99, brs	116.5	4.97, s 4.99, s	116.2
17	0.60, d (6.7)	15.6	0.86, d (7.0)	10.7	0.89, d (6.7)	15.2
18	7.15 ^a	96.1	9.40, s	194.7	9.41, s	196.4
19	6.61, s	98.3	10.24, s	200.4	10.30, s	202.0
20	0.63, s	25.2	1.00, s	26.4	0.97, s	26.0
1'		172.4		172.8		172.9
2'	1.89, m	36.2	2.35, t (7.0)	36.1	2.35, t (7.5)	36.2
3'	1.46, m	18.8	1.67 ^a	18.5	1.68 ^a	18.5
4'	0.73, t (7.3)	13.5	0.97, t (7.5)	13.6	0.97, t (7.4)	13.7
7-O ₂ CCH ₃				171.2		
7-O ₂ CCH ₃			2.09, s	21.0		
18-O ₂ CCH ₃		169.6				
18-O ₂ CCH ₃	1.57, s	20.7				
19-O ₂ CCH ₃		169.1				
19-O ₂ CCH ₃	1.72, s	21.5				

^a Signals were in overlapped regions of the spectrum. ^b Spectra were collected in C₆D₆. ^c Spectra were collected in CDCl₃. ^d Assignments were based on chemical shift and comparison to other fully elucidated compounds. ^e Signals were not assignable due to the presence of multiple signals in the region.

= 3.7, 11.9 Hz) was a doublet of doublets, it must be adjacent to two protons, indicating that **4** is the C-7 deoxy analogue of compound **2**. This assignment was confirmed by HMBC and COSY experiments. Thus, **4** is a new compound and has been given the trivial name caseanigrescen D.

During the course of NMR analysis compounds **1–4** were stored in CDCl₃ for varying periods of time, and it was noted that all four compounds slowly hydrolyzed to their corresponding dialdehydes, as indicated by the disappearance of the hemiacetal resonances and the appearance of resonances for aldehydes.⁸ These dialdehydes were not stable, but partial NMR data could be obtained for two of the compounds. The hydrolysis was presumably due to the presence of traces of acid in the CDCl₃ used on this occasion, a conclusion supported by the fact that hydrolysis did not occur when the compounds were allowed to stand in a fresh sample of CDCl₃.



6 R = OAc
7 R = H

The hydrolysis product of **1** (**6**) was a colorless, amorphous solid. HRFABMS indicated a molecular formula of C₂₆H₃₆O₇ (*m/z* 461.2575 [M + H]⁺), which was consistent with the loss of the

two hemiacetal acetates and the formation of the dialdehyde from **1**. This conclusion was supported by both ^1H and ^{13}C NMR data (Table 2). This hydrolysis product has not previously been reported and has been given the trivial name caseanigrescen A-2 (**6**).

The hydrolysis product of **4** was a colorless, amorphous solid. No MS data were obtained prior to its decomposition. However, ^1H and ^{13}C NMR as well as HMBC and COSY experiments all confirmed that the hydrolysis product of **4** was the dialdehyde **7**. This hydrolysis product has not previously been reported and has been given the trivial name caseanigrescen D-2 (**7**).

The remaining two hydrolysis products were not stable enough to provide any spectroscopic data beyond ^1H NMR spectra, but they are presumably the dialdehydes corresponding to **2** and **3**.

Caseanigrescens A–D (**1–4**) and the two hydrolysis products (**6** and **7**) were found to exhibit good activity against the A2780 human ovarian cancer cell line, with IC₅₀ values of 1.4, 0.83, 1.0, and 1.0 μM , respectively, for the natural products and IC₅₀ values of 3.5 and 1.5 μM , respectively, for the hydrolysis products.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. NMR spectra were obtained on a JEOL Eclipse 500 or a Varian Inova 400 spectrometer in C₆D₆ or CDCl₃. Mass spectra were obtained on a JEOL JMS-HX-110 instrument. The chemical shifts are given in δ (ppm) with the residual C₆D₆ solvent peak referenced to δ_{H} 7.15 and δ_{C} 128.0 and the residual CDCl₃ solvent peak referenced to δ_{H} 7.24 and δ_{C} 77.0 as the internal reference; coupling constants are reported in Hz. HPLC was performed on a Shimadzu LC-10AT or an

LC-8A instrument with a C18 Varian Dynamax column (5 μm , 100 \AA , 250 \times 10 mm, or 8 μm , 100 \AA , 250 \times 21.4 mm, respectively).

Plant Material. The leaves and flowers of *Casearia nigrescens* were collected in the Zahamena region of Madagascar, in the province of Toamasina, 3 km northeast of Nosivola (17°41'01" S, 48°38'28" E) under the vernacular name Hazondrano, in November 2001. It was originally identified as an *Erythroxylum* sp. but was later reidentified as *C. nigrescens* Tul. (Salicaceae) by G. McPherson (MO), 2003, and C. Birkinshaw (MO), 2004. Duplicates of the voucher specimen (Ratovoson.F 589) were deposited at the Missouri Botanical Garden, the Muséum National d'Histoire Naturelle, Paris, the Département des Recherches Forestières et Piscicoles, Madagascar, and the Centre National d'Application des Recherches Pharmaceutique, Madagascar. The shrub had a height of 3 m and was collected in a secondary humid forest at an altitude of 900 m.

Extract Preparation. Dried leaves and flowers of *C. nigrescens* (305 g) were ground in a hammer mill, then extracted with EtOH by percolation for 24 h at rt at the Centre National d'Applications des Recherches Pharmaceutique, and evaporated to give the crude extract MG 1071 (19.6 g). A portion of the extract was shipped to VPISU for bioassay and isolation chemistry.

Bioassay-Guided Fractionation and Isolation of Compounds. The crude extract (820 mg) was partitioned between hexane and 90% aqueous MeOH. The MeOH fraction was adjusted to 50% aqueous MeOH and further extracted with CH_2Cl_2 . The CH_2Cl_2 fraction (170 mg) was the most active and was further fractionated using a reversed-phase C-18 SPE tube (1 g) eluted with 80% aqueous MeOH and 100% MeOH, to generate two fractions. The 80% aqueous MeOH fraction retained the activity in a preliminary fractionation, so it was further fractionated using reversed-phase C-18 HPLC and elution with 65% aqueous MeCN to yield HPLC fractions 1 and 6 along with compounds **2** (2.8 mg, t_R 15 min), **3** (6.5 mg, t_R 17.5 min), **1** (10.9 mg, t_R 22 min), and **4** (3.8 mg, t_R 26 min). Compounds **1–4** were responsible for the activity.

Cytotoxicity Bioassays. The A2780 human ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported.¹⁸ Actinomycin D was used as a positive control; it had IC_{50} values of $(8–24) \times 10^{-4} \mu\text{M}$ under the same conditions.

Caseanigrescen A (1): colorless, amorphous solid; $[\alpha]_D^{30} +59.6$ (c 0.77, MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (4.22) nm; IR ν_{max} 3480 br, 2968, 2930, 2880, 1729, 1597 cm^{-1} ; ^1H and ^{13}C NMR, see Table 1; HRFABMS m/z 585.2668 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{42}\text{O}_{10}\text{Na}$, 585.2676).

Caseanigrescen B (2): colorless, amorphous solid; $[\alpha]_D^{30} +42.9$ (c 0.21, MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (4.21) nm; IR ν_{max} 3396 br, 2966, 2923, 2878, 1754, 1734, 1597 cm^{-1} ; ^1H and ^{13}C NMR, see Table 1; HRFABMS m/z 543.2547 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{28}\text{H}_{40}\text{O}_9\text{Na}$, 543.2570).

Caseanigrescen C (3): colorless, amorphous solid; $[\alpha]_D^{30} +45.3$ (c 0.57, MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (4.28) nm; IR ν_{max} 3450 br, 2969, 2933, 2880, 1730, 1596 cm^{-1} ; ^1H and ^{13}C NMR, see Table 1; HRFABMS m/z 585.2728 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{42}\text{O}_{10}\text{Na}$, 585.2676).

Caseanigrescen D (4): colorless, amorphous solid; $[\alpha]_D^{30} +33.2$ (c 0.31, MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (4.16) nm; IR ν_{max} 3480 br, 2964, 2929, 2878, 1750, 1729, 1596 cm^{-1} ; ^1H and ^{13}C NMR, see Table 2; HRFABMS m/z 527.2595 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{28}\text{H}_{40}\text{O}_8\text{Na}$, 527.2621).

Caseanigrescen A-2 (6): colorless, amorphous solid; $[\alpha]_D^{30} +74.0$ (c 0.50, MeOH); IR ν_{max} 3430, 2967, 2930, 2883, 1726, 1596 cm^{-1} ; ^1H and ^{13}C NMR, see Table 2; HRFABMS m/z 461.2575 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{26}\text{H}_{37}\text{O}_7$, 461.2539).

Caseanigrescen D-2 (7): colorless, amorphous solid; ^1H and ^{13}C NMR, see Table 2.

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Foundation, the National Heart, Lung and Blood Institute, the National Institute of Mental Health, the Office of Dietary Supplements, and the Office of the Director of NIH, under Cooperative Agreement U01 TW000313 with the International Cooperative Biodiversity Groups, and this support is gratefully acknowledged. We thank Mr. B. Bebout for obtaining the mass spectra and Mr. T. Glass for assistance with the NMR spectra. Field work essential for this project was conducted under a collaborative agreement between the Missouri Botanical Garden and the Parc Botanique et Zoologique de Tsimbazaza and a multilateral agreement between the ICBG partners, including the Centre National d'Applications et des Recherches Pharmaceutiques. We gratefully acknowledge courtesies extended by the Government of Madagascar (Ministère des Eaux et Forêts).

Supporting Information Available: ^1H and ^{13}C NMR spectra for compounds **1–4** and **7**; ^1H NMR spectrum of compound **6**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

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