Cytotoxic Diterpenes from *Cassipourea madagascariensis* from the Madagascar Rainforest[1]

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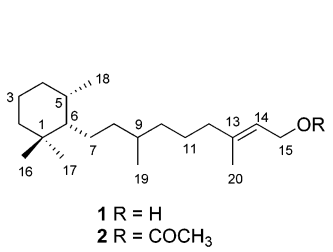
Bioassay-directed fractionation of ethanol extracts of the roots and leaves of the plant *Cassipourea madagascariensis* resulted in the isolation of the two new terpenoids cassipourol (**1**) and cassipouryl acetate (**2**) in addition to the three known compounds, 3*β*,30-dihydroxylup-20(29)-ene (**3**), 30-hydroxylup-20(29)-en-3-one (**4**), and combretol (**5**). The structures of the two new compounds were established on the basis of 1D and 2D NMR spectroscopic data and chemical conversion. All the isolated compounds were tested against the A2780 human ovarian cancer cell line; the two diterpenes (**1** and **2**) showed moderate cytotoxic activity, while the three known compounds (**3**−**5**) were weakly active.

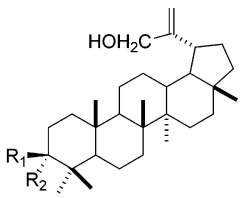
In our continuing research on the isolation of bioactive compounds from the Suriname and Madagascar rainforests as a part of the mission of the International Cooperative Biodiversity Group (ICBG),[2] we obtained a sample of *Cassipourea madagascariensis* DC. (Rhizophoraceae) from our collection work in Madagascar. *C. madagascariensis* is a shrub of 3 m height, with orange trilobed fruit, called "tavolopika" by the local population. The genus *Cassipourea* is a rich source of a variety of sulfur-containing amides,[3] but *C. madagascariensis* has not previously been investigated. Ethanol extracts of the roots and leaves of *C. madagascariensis*, designated MG1871 and MG1873, respectively, were selected for bioassay-guided fractionation on the basis of their cytotoxicity, with an IC_{50} of 18.2 μg/mL against the A2780 ovarian cancer cell line for the leaf extract, and also because of the absence of any phytochemistry on the species.

Initial liquid−liquid partition of MG1871 and MG1873 indicated that the activity was concentrated in the hexane- and $CHCl_3$-soluble fractions of the hexane/MeOH and $CHCl_3$/aqueous MeOH partitions. The combined hexane- and $CHCl_3$-soluble fractions were subjected to chromatography over MCI gel followed by reversed-phase preparative TLC and HPLC, and furnished the two new bioactive diterpenoids cassipourol (**1**) and cassipourin acetate (**2**), in addition to the three known compounds 3*β*,30-dihydroxylup-20(29)-ene (**3**),[4] 30-hydroxylup-20(29)-en-3-one (**4**),[5] and combretol (**5**).[6]

Compound **1** was isolated as a colorless optically active viscous liquid whose molecular formula was established as $C_{20}H_{38}O$ from its HRFABMS, ^{13}C NMR, and APT (attached proton test) spectroscopic data. These data indicated that the compound had two degrees of unsaturation, and an IR absorption band at 3380 cm^{-1} indicated the presence of a hydroxyl functional group. The ^1H NMR spectrum showed the presence of an olefinic proton as a quartet of triplets at δ 5.40 (J = 1.4, 7.0 Hz); an oxymethylene group as a doublet at δ 4.14 (J = 7.0 Hz); three methyl singlets at δ 1.66, 0.866, and 0.853; two methyl doublets at δ 0.846 (J = 7.1 Hz) and 0.838 (J = 6.6 Hz); and eight methylenes and three methines between δ 1.05 and 1.99. The ^{13}C NMR signals were assigned for all 20 carbons on the basis of APT, HMQC, and HMBC spectroscopic data, and these assignments are shown in Table 1. Since the ^{13}C NMR spectrum indicated the presence of only one double bond, **1** must be a monocyclic diterpene. A search of the literature indicated that the ^1H and ^{13}C NMR spectroscopic data of **1** were similar to those of the monocyclic diterpenoids viridiol A (**6**) and viridiol B (**7**) isolated from the red alga *Laurencia viridis*.[7]



1 R = H
2 R = $COCH_3$



3 R_1 = OH; R_2 = H
4 R_1 = R_2 = O

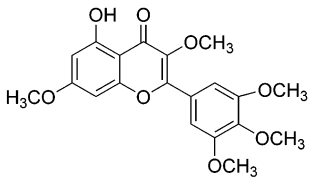


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Table 1. ^1H and ^{13}C NMR Data for Compounds **1** and **2** ($CDCl_3$)[a]

	1		**2**	
position	^1H	^{13}C	^1H	^{13}C
1		36.74		36.72
2	1.28 m, 1.05 m	39.45	1.28 m, 1.04 m	39.45
3	1.26 m	24.87	1.26 m	24.88
4	1.24 m	37.44	1.24 m	37.43
5	1.50 m	28.06	1.44 m	28.06
6	1.38 m	32.88	1.36 m	32.88
7	1.28 m	24.55	1.26 m	24.54
8	1.34 m	37.44	1.33 m	37.43
9	1.40 m	32.78	1.38 m	32.75
10	1.38 m, 1.28 m	37.51	1.36 m, 1.26 m	37.51
11	1.53 m	25.21	1.52 m	25.11
12	1.99 m, 1.13 m	39.95	2.01 m, 1.08 m	39.94
13		140.42		142.89
14	5.40 (qt, 1.4, 7.0)	123.16	5.32 (qt, 1.2, 7.0)	118.05
15	4.14 (d, 7.0)	59.52	4.57 (d, 7.0)	61.52
16	0.866 s	22.80[b]	0.866 s	22.80[d]
17	0.853 s	22.70[b]	0.853 s	22.70[d]
18	0.846 (d, 7.1)	19.83[c]	0.845 (d, 6.9)	19.83[e]
19	0.838 (d, 6.6)	19.80[c]	0.838 (d, 6.4)	19.80[e]
20	1.66 br s	16.26	1.68 br s	16.45
$OCOCH_3$				171.23
$OCOCH_3$			2.04 s	21.16

[a] Assignments made on the basis of HMQC and HMBC spectroscopic data and in comparison with the literature values.[7] [b−e] Values having the same superscript in the respective columns may be interchanged.

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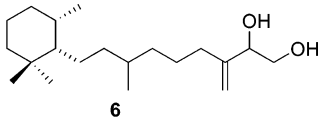

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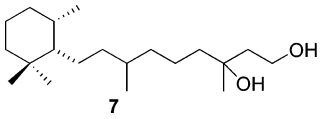

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A comparison of the ^{1}H and ^{13}C NMR data of **1** with those of **6** and **7** indicated that they had very similar diterpenoid skeletons, differing only at carbons C-12 to C-15 and C-20. The primary hydroxyl group and the trisubstituted double bond in **1** were assigned to C-15 and C-13/C-14, respectively, based on COSY (H-3/H-2, H-4; H-5/H-4, H-6, H-18; H-7/H-6, H-8; H-9/H-8, H-10, H-19; H-11/H-10, H-12; H-14/H-15) and HMBC (H-2/C-1, C-3, C-16, C-17; H-5/C-1, C-3, C-4, C-6, C-7, C-18; H-7/C-1, C-5, C-6, C-8, C-9; H-9/C-8, C-10, C-11, C-19; H-14/C-12, C-13, C-15, C-20) correlations; selected HMBC correlations are shown in Figure 1. The configurations of the three methyl groups at C-16, C-17, and C-18 and of the chiral centers at C-5 and C-6 were assigned to be the same as those of **6** and **7** on the basis of their almost identical coupling constants and ^{13}C NMR values. This was supported by the ROESY spectrum of **1**, in which the methyl group at C-16 showed a correlation to the methine proton at C-6, and the methyl groups at C-17 and C-18 showed correlation to the methylene group at C-7. A comparison of the ^{13}C NMR values of C-12−C-15 and C-20 in **1** with the values of the corresponding carbons of crotonadiol isolated from *Croton zambesicus*[8] suggested the *E* stereochemistry of the double bond between C-13 and C-14. This was supported by the ROESY spectrum of **1**, in which the methyl group at C-20 did not show any correlation to the olefinic proton at C-14 but did show a correlation to the oxymethylene group at C-15. The stereochemistry of the secondary methyl group at the C-9 position has not been established. On the basis of the above spectroscopic data, the structure of cassipourol was assigned as **1**.

The molecular formula of **2** was established as $C_{22}H_{40}O_2$ from HRFABMS (*m*/*z* 337.3120 $[M + H]^+$, calcd for $C_{22}H_{41}O_2$, 337.3107) and ^{13}C NMR spectroscopic data. The IR spectrum of **2** showed the absence of the absorption band corresponding to that of the hydroxyl group and the appearance of an absorption band corresponding to an ester carbonyl at 1742 cm^{-1}. A comparison of the ^{1}H and ^{13}C NMR spectra of **2** with those of **1** (Table 1) showed that they were identical except for the presence of signals for an acetate group [δ_H 2.04 (s, 3H) and δ_C 171.23 and 21.16] in the spectra of **2**. The acetate group was thus assigned to C-15, and this was supported by the key HMBC correlations H-14/C-13, C-15, O$\underline{C}$OCH$_3$; H-15/C-13, C-14, O$\underline{C}$OCH$_3$; and OCO$\underline{CH_3}$/C-15, O$\underline{C}$OCH$_3$. Finally, acetylation of **1** with Ac_2O/pyr furnished a product that was identified as **2** on the basis of co-TLC and ^{1}H NMR, confirming the structure of **2** as cassipouryl acetate.

All the isolated compounds were tested against the A2780 human ovarian cancer cell line. The two new diterpenoids **1** and **2** were modestly active, with IC_{50} values of 2.4 and 2.8 μg/mL, respectively, while the three known compounds **3**−**5** had weaker activity, with IC_{50} values of 12.2, 16.4, and 8.7 μg/mL, respectively.

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 spectrometer. The chemical shifts are given in δ (ppm) with TMS (tetramethylsilane) as internal reference, and coupling constants are reported in Hz. MCI gel (CHP20P) was used for column chromatography. Mass spectra were obtained on a JEOL HX-110 instrument. Reversed-phase preparative TLC was performed on Baker Si-C_{18}F plates. HPLC was performed on a Shimadzu LC-10AT instrument with an ODS A323 column (250 × 10 mm).

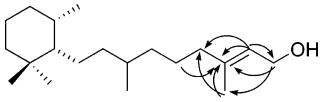

Figure 1. Selected HMBC correlations of compound **1**.

Cytotoxicity Bioassays. The A2780 ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported.[1,9] The concentration of each compound that inhibited 50% of the growth of the A2780 mammalian cell line was determined; actinomycin D (IC_{50} 1−3 ng/mL) was used as the positive control.

Plant Material. Samples of *Cassipourea madagascariensis* DC. (Rhizophoraceae) were collected in May 2003 by Fidy Ratovoson et al. in a forest adjacent to the national park of Zahamena, 3 km west of Ambatoharanana (17°33′34″ S and 48°53′42″ E, elevation between 750 and 900 m), in the province of Toamasina, on May 3, 2003. Duplicate voucher specimens (Ratovoson.F 694, TROPICOS specimen id 01902919[10]) were deposited at Centre National d'Application des Recherches Pharmaceutiques (CNARP) and Direction des Recherches Forestières et Piscicoles Herbarium in Antananarivo, Madagascar (TEF), Missouri Botanical Garden, St. Louis, Missouri (MO), and Museum National d'Histoire Naturelle in Paris, France (P).

Extraction and Isolation. Dried roots of *C. madagascariensis* (470 g) were extracted with ethanol to give extract MG1871 (15.6 g), and dried leaves (295 g) were extracted with ethanol to give extract MG1873 (11.2 g). Extract MG1871 (0.48 g) was suspended in aqueous MeOH (9:1 MeOH/H_2O, 100 mL) and extracted with three 100 mL portions of hexane. The aqueous layer was then diluted to 70% MeOH (v/v) with H_2O and extracted with three 100 mL portions of $CHCl_3$. The aqueous layer was concentrated, and the residue obtained was suspended in H_2O (25 mL) and extracted with two 25 mL portions of *n*-BuOH. The hexane- and $CHCl_3$-soluble fractions were found to be active. The leaf extract MG1873 (0.90 g) was treated similarly to give active hexane- and $CHCl_3$-soluble portions. Both the hexane and the $CHCl_3$ fractions of the roots and leaves were similarly cytotoxic and had similar TLC and ^{1}H NMR patterns, so all four fractions were combined. The combined residue (0.50 g) was fractionated over MCI gel using MeOH/H_2O (1:1 to 100:0) and MeOH/$CHCl_3$ (100:0 to 70:30) to furnish 20 fractions (A−T), of which fraction O was found to be the most active. Fraction O upon further chromatography over MCI gel using MeOH/H_2O (1:1 to 100:0) and MeOH/$CHCl_3$ (100:0 to 80:20) furnished nine subfractions (O-1 to O-9), of which fractions O-4 to O-7 were the most cytotoxic. Fractions O-4 and O-5 were combined and separated by preparative reversed-phase TLC (MeOH/H_2O, 80:20) to yield the known flavanoid combretol (**5**, 1.4 mg) and the known triterpene 30-hydroxylup-20(29)-en-3-one (**4**, 2.6 mg). Similarly, fractions O-6 and O-7 were combined and subjected to reversed-phase HPLC with the mobile phase MeOH/H_2O (75:25), thereby furnishing the two new diterpenes **1** (2.1 mg) and **2** (1.6 mg) in addition to the known triterpene 3β,30-dihydroxylup-20(29)-ene (**3**, 1.8 mg). The structures of the known compounds (**3**−**5**) were identified by comparison of their spectroscopic data with literature values.[4−6]

Cassipourol (1): colorless liquid; $[\alpha]_D$ +10.9 (*c* 0.042, $CHCl_3$); UV (MeOH) λ_{max} 208.2 nm (ϵ 11 400); IR ν_{max} 3380, 2926, 1461, 1377, 1366, 1215, 1004, 757 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 295.2983 $[M + H]^+$ (calcd for $C_{20}H_{39}O$, 295.3001).

Acetylation of 1. Compound **1** (0.8 mg) was dissolved in pyridine (0.2 mL) and acetic anhydride (0.2 mL), and the mixture was stirred for 16 h at room temperature. The product was dried under vacuum, and the residue obtained was purified over reversed-phase preparative HPLC with the mobile phase MeOH−H_2O (80:20), furnishing a product (0.7 mg) that was identified as **2** by co-TLC and ^{1}H NMR spectroscopic data.

Cassipouryl acetate (2): colorless liquid; $[\alpha]_D$ +10.6 (*c* 0.034, $CHCl_3$); UV (MeOH) λ_{max} 204.6 nm (ϵ 13 300); IR ν_{max} 2926, 1742, 1462, 1453, 1378, 1365, 1229, 1215, 1021, 747 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 337.3120 $[M + H]^+$ (calcd for $C_{22}H_{41}O_2$, 337.3107).

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References and Notes

(1) Biodiversity Conservation and Drug Discovery in Madagascar, Part 17. For Part 16, see: Yoder, B. J.; Cao, S.; Norris, A.; Miller, J. S.; Ratovoson, F.; Andriantsiferana, A.; Rasamison, V. E.; Kingston, D. G. I. *Nat. Prod. Res.* **2006**, *20*, in press.

(2) Adou, E.; Williams, R.; Schilling, J. K.; Malone, S.; Meyer, J.; Wisse, J. H.; Frederik, D.; Koese, D.; Werkhoven, M. C. M.; Snipes, C.; Werk, T. L.; Kingston, D. G. I. *Bioorg. Med. Chem.* **2005**, *13*, 6009−6014. (b) Murphy, B. T.; Cao, S.; Norris, A.; Ratovoson, F.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I. *J. Nat. Prod.* **2005**, *68*, 417−419. (c) Cao, S.; Guza, R. C.; Wisse, J. H.; Evans, R.; van der Werff, H.; Miller, J. S.; Kingston, D. G. I. *J. Nat. Prod.* **2005**, *68*, 487−492. (d) Chaturvedula, V. S. P.; Farooq, A.; Schilling, J. K.; Malone, S.; Derveld, I.; Werkhoven, M. C. M.; Wisse, J. H.; Ratsimbason, M.; Kingston, D. G. I. *J. Nat. Prod.* **2004**, *67*, 2053−2057. (e) Chaturvedula, V. S. P.; Schilling, J. K.; Miller, J. S.; Andriantsiferana, R.; Rasamison, V. E.; Kingston. D. G. I. *Planta Med*. **2003**, *69*, 440−444.

(3) Ichimaru, M.; Kato, A.; Hashimoto, Y. *J. Nat. Prod.* **2000**, *63*, 1675−1676. (b) Kato, A.; Okado, M.; Hashimoto, Y. *J. Nat. Prod*. **1985**, *48*, 289−292. (c) Kato, A.; Okado, M.; Hashimoto, Y. *J. Nat. Prod*. **1984**, *47*, 706−707.

(4) Abdel-Mogib, M. *Phytochemistry* **1999**, *51*, 445−448.

(5) Tinto, W. F.; Blair, L. C.; Alli, A.; Reynolds, W. F.; McLean, S. *J. Nat. Prod*. **1992**, *55*, 395−398.

(6) Auzi, A. A.; Hartley, T. G.; Waterman, P. G. *Biochem. Syst. Ecol.* **1997**, *25*, 611−617.

(7) Norte, M.; Souto, M. L.; Fernandez, J. J. *Nat. Prod. Lett.* **1996**, *8*, 263−269.

(8) Ngadjui, B. T.; Folefoc, G. G.; Keumedijo, F. K.; Dongo, E.; Sondengam, B. L.; Connolly, J. D. *Phytochemistry* **1999**, *51*, 171−174.

(9) Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F. *Cancer Res.* **1985**, *45*, 2110−2115.

(10) http://mobot.mobot.org/W3T/Search/vast.html; accessed 12/8/05.

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