

Antiproliferative Xanthenes of *Terminalia calcicola* from the Madagascar Rain Forest¹Shugeng Cao,[†] Peggy J. Brodie,[†] James S. Miller,[‡] Richard Randrianaivo,[§] Fidy Ratovoson,[§] Chris Birkinshaw,[§] Rabodo Andriantsiferana,[⊥] Vincent E. Rasamison,[⊥] and David G. I. Kingston^{*†}

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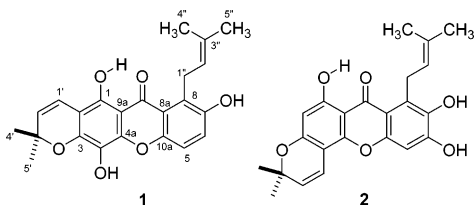
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A bioassay-guided fractionation of the EtOH extract of the Madagascar plant *Terminalia calcicola* led to the isolation of two new cytotoxic xanthenes, termicalcicolanone A (**1**) and termicalcicolanone B (**2**). The structures of the new compounds were established on the basis of one- and two-dimensional NMR spectroscopic data. Both compounds showed modest antiproliferative activity toward the A2780 human ovarian cancer cell line.

In our continuing search for bioactive molecules from the Madagascar rainforests as part of an International Cooperative Biodiversity Group (ICBG) program,¹ we obtained extracts of various parts of the tree *Terminalia calcicola* H. Perrier (Combretaceae) collected in Madagascar. The EtOH extract of the immature fruit proved to have moderate antiproliferative activity, with an IC₅₀ value of 14 $\mu\text{g/mL}$ against the A2780 human ovarian cancer cell line. This extract was thus selected for bioassay-guided fractionation on the basis of its cytotoxicity and also the absence of any previous detailed phytochemical studies on this species.

Previous phytochemical studies have revealed the genus *Terminalia* to be a rich source of secondary metabolites, such as lignans,² flavonoids,³ terpenoids,⁴ and tannins.⁵ Some of these metabolites have shown a wide range of biological activities, including anti-HIV-1,² antimalarial,² antifungal,^{2,4} antibacterial,^{4,6} and cytotoxic⁵ activities. The anthelmintic and hemolytic properties of the terpene esters from *T. macroptera* have been studied.⁴ The antioxidant effects of the aqueous extract of *T. chebula* have also been investigated.⁷

Activity-guided fractionation of the dichloromethane extract (IC₅₀, 10 $\mu\text{g/mL}$) by passage over a C18 open column, followed by purification of active fractions using C18 HPLC, led to the isolation of the two new compounds **1** and **2**.



Termicalcicolanone A (**1**) was obtained as a yellow powder. Its positive HRFABMS revealed a pseudomolecular ion $[(M + H)^+]$ consistent with the molecular formula $\text{C}_{23}\text{H}_{22}\text{O}_6$. The IR spectrum for **1** revealed the existence of hydroxy (3435 cm^{-1}) and conjugated ketone (1652 cm^{-1}) functionalities. Its UV absorptions at 237, 262, 298, and 401 nm suggested a xanthone skeleton as its base structure.⁸ The ¹H NMR spectrum (pyridine-*d*₅) of **1** revealed the

presence of a hydrogen-bonded hydroxy group [δ_{H} 13.83 (1H, s)], two *ortho*-coupled protons [δ_{H} 7.51 (1H, d, $J = 9.0$ Hz) and 7.33 (1H, d, $J = 9.0$ Hz)], a dimethylchromene system [δ_{H} 7.07, 5.63 (1H each, d, $J = 10.1$ Hz) and 1.39 (6H, s)], and a 3-methylbut-2-enyl group [δ_{H} 5.83 (1H, br t, $J = 7.1$ Hz), 4.68 (2H, d, $J = 7.1$ Hz), 2.04 (3H, s), and 1.75 (3H, s)] (Table 1). The ¹³C NMR spectrum (DMSO-*d*₆) of **1** exhibited the 23 carbon signals reported in Table 1, including one carbonyl group, two aromatic rings with six oxygenated carbons, and two prenyl groups. The H-1'' resonance appeared at δ_{H} 4.68, which was a more deshielded value than that usually found for this functionality, due to the effect of the C-9 carbonyl group.⁹ Clearly, both the C-1 hydroxy and the C-8 3-methylbut-2-enyl groups were *peri* to the carbonyl group. The *ortho*-coupled aromatic H-5 and H-6 showed ³J HMBC correlations (Figure 1) to C-7 and C-8a, and C-8 and C-10a, respectively, while H-1'' correlated to C-7, C-8, C-8a, C-2'', and C-3'', which provided further evidence for the position of the 3-methylbut-2-enyl group at C-8. The HMBC correlations between the hydrogen-bonded proton (1-OH) and C-1, C-2, and C-9a, H-1' and C-1, and H-2' and C-2 were also observed, indicating that the 3',3'-dimethylpyrano ring was fused at C-2 and C-3, which was confirmed by a ROESY correlation between the hydrogen-bonded proton and H-1'. On the basis of the molecular formula of **1**, the remaining two hydroxy groups must be located at C-4 and C-7, respectively. Thus, the structure of **1** was determined to be 5,8,12-trihydroxy-2,2-dimethyl-7-(3-methylbut-2-enyl)pyrano[3,2-*b*]xanthen-6(2H)-one.

Termicalcicolanone B (**2**) was also obtained as a yellow powder. It was deduced to have a molecular formula of $\text{C}_{23}\text{H}_{22}\text{O}_6$ by HRFABMS and ¹³C NMR spectroscopy. A spectroscopic comparison of **2** and **1** indicated that both compounds had a hydrogen-bonded hydroxy group, a 3-methylbut-2-enyl group, a 3',3'-dimethylpyrano ring, and two aromatic protons. The hydrogen-bonded hydroxy and the 3-methylbut-2-enyl groups of **2** were located at C-1 and C-8, respectively, by the HMBC correlations (Figure 1) between the hydrogen-bonded proton (1-OH) and C-1, C-2, and C-9a and between H-1'' and C-7, C-8, and C-8a. The 3',3'-dimethylpyrano ring of **2** was fused at C-3 and C-4, rather than at C-2 and C-3, as indicated by the HMBC correlations (Figure 1) between H-2 and C-3 and C-4, between H-1' and C-3, C-4, and C-4a, and between H-2' and C-4. In the HMBC spectrum, proton H-2 correlated to C-1, C-3, C-4, and C-9a, while H-5 correlated to C-6, C-7, C-8a, C-9, and C-10a. A ROESY correlation between the hydrogen-bonded proton and H-2 confirmed its location. Therefore, the structure of **2** was determined to be 6,9,10-trihydroxy-3,3-dimethyl-8-(3-methylbut-2-enyl)pyrano[2,3-*c*]xanthen-7(3H)-one.

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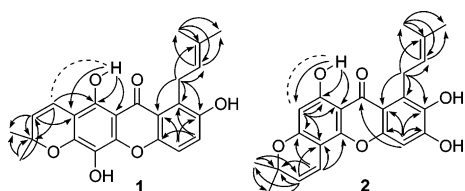
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Table 1. ^1H and ^{13}C NMR Data for Compounds **1** and **2**^a

no.	1			2		
	$^1\text{H}^b$	$^1\text{H}^c$	$^{13}\text{C}^b$	$^1\text{H}^b$	$^1\text{H}^c$	$^{13}\text{C}^b$
1			149.1			162.5
2			103.0	6.03 s	6.51 s	97.9
3			149.1			158.6
4			144.6			99.7
4a			148.1			150.4
5	7.35 br s	7.33 d (9.0)	115.1	6.59 s	7.22 s	99.9
6	7.35 br s	7.51 d (9.0)	123.4			155.3
7			151.5			142.0
8			128.3			126.0
8a			117.8			108.6
9			183.2			181.3
9a			103.3			102.9
10a			150.4			152.7
1'	6.64 d (10.1)	7.07 d (10.1)	116.1	6.74 d (9.8)	6.77 d (12.0)	114.7
2'	5.76 d (10.1)	5.63 d (10.1)	127.0	5.67 d (9.8)	5.52 d (12.0)	127.2
3'			77.8			77.9
4'	1.45 s	1.39 s	27.7	1.41 s	1.44 s	27.8
5'	1.45 s	1.39 s	27.7	1.41 s	1.44 s	27.8
1''	4.03 d (6.4)	4.68 d (7.1)	25.6	3.93 d (6.1)	4.77 d (6.9)	25.4
2''	5.18 br t (6.4)	5.83 br t (7.1)	124.7	5.25 br t (6.1)	5.92 br t (6.9)	123.8
3''			130.3			130.0
4''	1.78 s	2.04 s	18.0	1.75 s	2.08 s	18.1
5''	1.61 s	1.75 s	25.1	1.60 s	1.76 s	25.7
1-OH	13.12 s	13.83 s		14.55 s		

^a δ (ppm), 500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR; multiplicities; J values (Hz). ^b DMSO- d_6 . ^c Pyridine- d_5 .

**Figure 1.** Key HMBC (arrows) and ROESY (dashed) correlations of **1** and **2**.

Both compounds **1** and **2** were evaluated for antiproliferative activity in the A2780 assay and had IC_{50} values of 40.6 and 8.1 μM respectively.

Experimental Section

General Experimental Procedures. IR and UV spectra were measured on a Spectrum One FT-IR spectrometer (Perkin-Elmer Instruments) and a Shimadzu UV-1201 spectrophotometer, respectively. NMR spectra were obtained on a JEOL Eclipse 500 and a Varian INOVA 400 spectrometer in DMSO- d_6 or pyridine- d_5 . The chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. Mass spectra were obtained on a JEOL JMS-HX-110 instrument, in the positive ion mode. HPLC was performed on a Shimadzu LC-10AT instrument with a semipreparative C18 Varian Dynamax column (5 μm , 250 \times 10 mm) and a preparative C18 Varian Dynamax column (8 μm , 250 \times 21.4 mm).

Antiproliferative Bioassays. Antiproliferative activities were obtained at Virginia Polytechnic Institute and State University against the drug-sensitive A2780 human ovarian cancer cell line.¹⁰ In brief, human A2780 ovarian cancer cells grown to 95% confluency were harvested and resuspended in growth medium (RPMI1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine). Cells were counted using a hemacytometer, and a solution containing 2.7×10^5 cells per mL was prepared in growth media. Eleven columns of a 96-well microtiter plate were seeded with 180 μL of cell suspension per well, and the remaining column contained media only (100% inhibition control). The plate was incubated for 3 h at 37 $^\circ\text{C}/5\%\text{CO}_2$ to allow the cells to adhere to the wells. Following this incubation, potential antiproliferative agents, prepared in 50:50 H_2O –DMSO, were added to the wells in an appropriate series of concentrations, 20 μL per well. One column of wells was left with no inhibitor (0% inhibition control), and four dilutions of a known compound (paclitaxel or actinomycin D) were included as a positive control. The plate was incubated for 2

days at 37 $^\circ\text{C}/5\%\text{CO}_2$, then the medium gently shaken from the wells and replaced with reaction medium (supplemented growth medium containing 1% AlamarBlue) and incubated for another 3 h. The level of AlamarBlue converted to a fluorescent compound by living cells was then analyzed using a Cytofluor series 4000 plate reader (Perseptive Biosystems) with an excitation wavelength of 530 nm, an emission wavelength of 590 nm, and gain of 45. The percent inhibition of cell growth was calculated using the 0% and 100% controls present on the plate, and an IC_{50} value (concentration of agent that produces 50% cell growth inhibition) was calculated using a linear extrapolation of the data that lay on either side of the 50% inhibition level. Samples were analyzed in triplicate on at least two separate occasions to produce a reliable IC_{50} value.

Plant Material. Samples of roots, bark, wood, leaves, and immature fruit of *Terminalia calcicola* H. Perrier (Combretaceae) were collected by Randrianaivo et al. (collection #1086) on September 8, 2004, in the forest of Andranonakomba, Andavakoera, in the Montagne des Français, Province of Antsiranana Madagascar (12.21.00 S/49.21.34 E, elevation 112 m). The plant was a tree 12 m in height, diameter 40 cm, with green fruits. Duplicate voucher specimens were deposited at the Centre National d'Application des Recherches Pharmaceutiques (CNARP) and the Parc Botanique et Zoologique de Tsimbazaza (PBZT) herbarium (TAN) in Antananarivo Madagascar, at the Missouri Botanical Garden, St. Louis, Missouri (MO), and at the Muséum National d'Histoires Naturelles (MNHN), Paris, France (P). The specimens were collected from a tree 12 m in height, with a diameter of 40 cm.

Extraction and Isolation. Dried leaves of *T. calcicola* (420 g) were ground in a hammer mill, then extracted with EtOH by percolation for 24 h at rt to give the crude extract MG 2581 (36.6 g), of which 7.8 g was shipped to Virginia Polytechnic Institute and State University (VPISU) for fractionation. Extract MG 2581 (IC_{50} : 14 $\mu\text{g}/\text{mL}$, 96.2 mg) was suspended in aqueous MeOH (MeOH– H_2O , 9:1, 10 mL) and extracted with hexane (3 \times 10 mL portions). The aqueous layer was then diluted to 70% MeOH (v/v) with H_2O and extracted with CH_2Cl_2 (3 \times 10 mL portions). Both the hexane and the CH_2Cl_2 extracts were evaporated *in vacuo* to leave 43.9 and 35.9 mg of residues (IC_{50} : 11 and 10 $\mu\text{g}/\text{mL}$, respectively). The aqueous MeOH extract (16.4 mg) was inactive. The CH_2Cl_2 extract was first fractionated using a SPE cartridge over C18, and five fractions were collected. Fractions I, II, III, IV, and V (19.1, 3.5, 6.1, 1.2, and 0.3 mg) had IC_{50} values of 14, 11, 6, 18, and 17 $\mu\text{g}/\text{mL}$, respectively. Fraction III was separated by C-18 HPLC (80% MeOH– H_2O), and compounds **1** (0.8 mg, t_R 29 min) and **2** (0.8 mg, t_R 41 min) were isolated.

Termicalcicolanone A (1): yellow powder; IR (film) ν_{max} 3435, 2975, 2955, 1652, 1614, 1580, 1485, 1380, 1258, 1139, 1061, 1026,

897, 819 cm^{-1} ; UV (MeOH) λ_{max} (log ϵ) 237 (4.21), 262 (4.22), 298 (4.48), 330 (sh), 401 (3.45) nm; ^1H NMR (500 MHz, $\text{DMSO-}d_6$ and pyridine- d_5) and ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$), see Table 1; HRFABMS m/z 395.1504 (calcd for $\text{C}_{23}\text{H}_{23}\text{O}_6$, 395.1495).

Termicalcicolanone B (2): yellow powder; IR (film) ν_{max} 3401, 3293, 2973, 2927, 1647, 1618, 1575, 1556, 1484, 1460, 1375, 1275, 1177, 1156, 1113, 833 cm^{-1} ; UV (MeOH) λ_{max} (log ϵ) 262 (4.48), 287 (4.38), 321 (sh), 377 (3.83) nm; ^1H NMR (500 MHz, $\text{DMSO-}d_6$ and pyridine- d_5) and ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$), see Table 1; HRFABMS m/z 395.1488 (calcd for $\text{C}_{23}\text{H}_{23}\text{O}_6$, 395.1495).

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