Cytotoxic Flavanones of *Schizolaena hystrix* from the Madagascar Rainforest[1]

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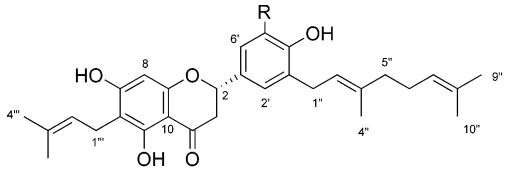
Bioassay-guided fractionation of an ethanol extract of a Madagascar collection of *Schizolaena hystrix* afforded three new flavanones, schizolaenone A (**1**), schizolaenone B (**2**), and 4′-*O*-methylbonannione A (**3**), as well as three known flavanones, nymphaeol A, bonannione A, and macarangaflavanone B, and the flavanol bonanniol A. The structures of compounds **1**−**3** were determined by various one- and two-dimensional NMR techniques. All of the isolates were tested for cytotoxicity against the A2780 human ovarian cancer cell line. Nymphaeol A (IC_{50} = 5.5 μg/mL) exhibited the greatest cytotoxicity, while the other flavanones were found to be only weakly active.

In our continuing search for biologically active natural products from tropical rainforests as part of an International Cooperative Biodiversity Group (ICBG) program, we obtained an extract from *Schizolaena hystrix* of the family Sarcolaenaceae. The fragmented remaining forests of Madagascar's eastern escarpment are particularly diverse and continue to yield new species at an astonishing rate. The ICBG project has focused on inventory and sample collection in the remaining patches of forest that surround the Zahamena National Park.

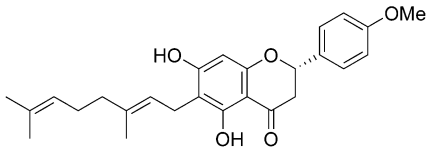
Indigenous to the humid forests of Anjanaharibe-Sud (Antsiranana) to Fort Carnot (Fianarantsoa) in Madagascar, *S. hystrix* is a 14−35 m tall tree brandishing large, leathery leaves.[2] Lowry et al. reported 18 species belonging to the genus *Schizolaena*, eight of which were reported for the first time.[2] The family Sarcolaenaceae is the largest of the nine vascular plant families endemic to Madagascar and comprises nine genera with about 50 species. The only past chemical investigation of *S. hystrix* was a fatty acid analysis, which revealed the presence of fatty acids predominantly of the 16:0, 18:1Δ^9, and 18:2Δ^{9,12}-types.[3] There are no other published reports on the chemical constituents of *S. hystrix*, and since many of the species of *Schizolaena* have only recently been discovered, there have been few attempts to characterize the chemistry of this genus.

An ethanol extract of the fruit of *S. hystrix* was found to be active in the A2780 ovarian cancer cytotoxicity assay, with an IC_{50} value of 10 μg/mL. Bioassay-guided fractionation of this extract led to the isolation of three new prenylated and geranyl substituted flavanones, schizolaenone A (**1**), schizolaenone B (**2**), and 4′-*O*-methylbonannione A (**3**), in addition to four known compounds. Three of the known natural products were the prenylated- and geranyl-substituted flavanones bonannione A,[4,5] nymphaeol A,[6] and macarangaflavanone B,[7] and one was a flavanol, bonanniol A.[4]

Compound **1** was obtained as a light yellow amorphous solid. Positive-ion HRFABMS analysis gave a pseudomolecular ion at *m/z* 477.2592, which suggested a formula of $C_{30}H_{37}O_5$ ([M + 1]$^+$). The ^{1}H NMR spectrum of **1** showed



1 R = H
2 R = OH



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the presence of one chelated hydroxyl group (δ_H 12.4, s, 5-OH), one methylene α to the carbonyl (δ_H 3.09, dd, J = 13.0, 17.0 Hz, H_{ax}-3 and δ_H 2.77, dd, J = 3.0, 17.0 Hz, H_{eq}-3), and one oxymethine (δ_H 5.30, dd, J = 3.0, 13.0 Hz, H-2) (Table 1). These data suggested that **1** possesses a flavanone skeleton. The observation of one doublet (δ_H 6.84, d, J = 8.0 Hz, H-5′), one doublet of doublets (δ_H 7.19, dd, J = 2.0, 8.0 Hz, H-6′), and one broad singlet (δ_H 7.18, br s, H-2′) implied the B ring substitution pattern as shown for **1**. In addition, the coupling constants of the B ring protons illustrated an ABX-type spin system. Prenyl and geranyl substituents gave rise to three olefinic (δ_H 5.03, m; δ_H 5.26, m; and δ_H 5.32, m), four methylene (δ_H 2.07, m; δ_H 2.09, m; δ_H 3.37, d, J = 4.0 Hz; δ_H 3.39, d, J = 3.6 Hz), and four methyl signals, representing a total of five methyl groups (δ_H 1.81, 1.78, 1.78, 1.67, and 1.59, all s). The HMBC spectrum of **1** revealed correlations of H-2′ (δ_H 7.18, s) with C-4′ (δ_C 155.2) and C-1″ (δ_C 130.3), and thus indicated the presence of the geranyl substituent on the B ring. Geranyl methyl protons H-9″ (δ_H 1.67, s) and H-10″ (δ_H 1.59, s) both showed correlations to olefinic carbons C-7″ (δ_C 124.0) and C-8″ (δ_C 132.5), and H-4″ (δ_H 1.81, s) showed a correlation with C-3″ (δ_C 140.1). Furthermore, the olefinic prenyl proton H-2‴ (δ_H 5.32, m) displayed correlations to C-6 (δ_C 107.0), while H-4‴ and H-5‴ (δ_H 1.78, s) correlated with C-2‴ (δ_C 121.6) and C-3‴ (δ_C 135.8). The chelated OH-5 proton (δ_H 12.4, s) correlated with three quaternary carbons (C-5, C-6, and C-10 at δ_C 161.5, 107.0, and 103.3). The lone A ring proton H-8 (δ_H 5.99, s) showed correlations with four of the six aromatic A ring carbons (C-7, C-9, C-6, and C-10

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Table 1. NMR Spectral Data of Flavanones **1**−**3** in $CDCl_3$[a]

	1		**2**		**3**	
position	^{13}C[b]	^{1}H[b,c] (*J*, Hz)	^{13}C[b]	^{1}H[b,c] (*J*, Hz)	^{13}C[b]	^{1}H[b,c] (*J*, Hz)
2	79.4	5.30 dd (3.0, 13.0)	79.2	5.27 dd (2.5, 10.0)	79.0	5.34 dd (2.8, 13.0)
3ax	43.7	3.09 dd (13.0, 17.0)	43.5	3.04 dd (10.0, 13.0)	43.4	3.09 dd (13.0, 17.0)
3eq		2.77 dd (3.0, 17.0)		2.75 dd (2.5, 13.0)		2.78 dd (2.8, 17.0)
4	196.6		196.5		196.3	
5	161.5		161.3		161.3	
6	107.0		107.2		106.9	
7	164.4		164.3		164.2	
8	96.0	5.99 s	95.9	5.98 s	95.8	5.99 s
9	161.5		161.4		161.3	
10	103.3		103.1		103.1	
1′	130.9		130.8		130.7	
2′	128.6	7.18[e] br s	119.8	6.73 d (2.0)	127.9	7.38 d (8.8)
3′	127.6		127.8		114.4	6.95 d (8.8)
4′	155.2		142.8		160.2	
5′	116.4	6.84 d (8.0)	144.3		114.4	6.95 d (8.8)
6′	126.1	7.19 dd (2.0, 8.0)	111.5	6.86 d (2.0)	127.9	7.38 d (8.8)
1″	30.3	3.39 d (3.6)	29.9	3.37 d (6.5)	29.8	3.37 d (7.2)
2″	121.6[d]	5.26[d] m	121.6[d]	5.25[d] m	121.4	5.25 t
3″	140.1		139.6		139.8	
4″	16.6	1.81 s	16.4	1.80 s	16.5	1.80 s
5″	40.1	2.07 m	39.9	2.07 m	39.9	2.04 m
6″	26.7	2.09 m	26.6	2.10 m	26.5	2.07 m
7″	124.0	5.03 m	123.9	5.05 m	123.8	5.05 m
8″	132.5		132.3		132.3	
9″	26.0	1.67 s	25.9	1.67 s	25.8	1.67 s
10″	18.1	1.59 s	17.9	1.59 s	18.0	1.59 s
1‴	21.4	3.37 d (4.0)	21.3	3.35 d (7.0)		
2‴	121.6[d]	5.32[d] m	121.5[d]	5.32[d] m		
3‴	135.8		135.6			
4‴	26.2	1.78 s	26.0	1.78 s		
5‴	18.3	1.78 s	18.1	1.78 s		
MeO-4′					55.5	3.83 s
HO-5		12.4 s		12.4 s		12.4 s

[a] Assignments based on COSY, HMBC, and HSQC. [b] Chemical shifts (δ) in ppm. [c] br s: broad singlet; d: doublet; m: multiplet. [d] Values are interchangeable. [e] Signal overlapped with H-6′.

at δ_C 164.4, 161.5, 107.0, and 103.3). These correlations confirmed the proposed structure of **1**. The absolute configuration of schizolaenone A was determined to be 2*S* by analysis of its circular dichroism (CD) spectrum and comparison with literature values.[8]

Compound **2** was obtained as a yellow amorphous solid. Positive-ion HRFABMS analysis gave a pseudomolecular ion at *m/z* 493.2569, which suggested a formula of $C_{30}H_{37}O_6$ ($[M + 1]^+$). Compound **2** had NMR and mass spectral data very similar to those of **1**, suggesting their structural similarity. A 16 mass unit difference in the HRFABMS as well as the absence of proton H-5′ in the ^1H NMR spectrum of **2** indicated the presence of an extra B ring hydroxyl group to be the sole difference between **1** and **2**. The ^1H NMR spectrum of **2** showed the presence of an aromatic proton in the A ring, a chelated hydroxyl proton, a methylene α to a carbonyl, an oxymethine, and the same signals for both prenyl and geranyl substituents as those of **1**. The B ring aromatic signals of H-2′ (δ_H 6.73, d, J = 2.0 Hz) and H-6′ (δ_H 6.86, d, J = 2.0 Hz) supported the proposed substitution pattern. The two B ring protons both showed HMBC correlations with C-2 (δ_C 79.2) and C-4′ (δ_C 142.8), while an additional HMBC correlation indicated the proximity of H-2′ (δ_H 6.73, d, J = 2.0 Hz) with C-1″ (δ_C 29.9). The absolute configuration of schizolaenone B was determined to be 2*S*, as deduced upon analysis of its CD spectrum and comparison with literature values.[8]

Compound **3** was obtained as a pale yellow solid. Positive-ion HRFABMS analysis gave a pseudomolecular ion at *m/z* 423.2139, which suggested a formula of $C_{26}H_{31}O_5$ ($[M + 1]^+$). The basic flavanone skeleton was present in **3** as indicated in the ^1H NMR spectrum by a downfield singlet (H-8, δ_H 5.99) and three characteristic doublets of doublets (δ_H 5.34, J = 2.8, 13.0 Hz, H-2; δ_H 3.09, J = 13.0, 17.0 Hz, H_{ax}-3; and δ_H 2.78, J = 2.8, 17.0 Hz, H_{eq}-3). In addition, a pair of downfield doublets characteristic of *para*-substitution (δ_H 6.95, J = 8.8 Hz, H-3′, 5′ and δ_H 7.38, J = 8.8 Hz, H-2′, 6′), a 3H singlet (δ_H 3.83, H-4′-OMe), a singlet for a chelated hydroxy proton (δ_H 12.4, 5-OH), and signals for a geranyl group were observed. The ^1H NMR spectrum also shared features with those of bonannione A,[4] indicating nearly identical structures. HMBC data placed the geranyl substituent on the A ring, as H-1″ (δ_H 3.37, d, J = 7.2 Hz) correlated with three nearby quaternary carbons (δ_C 161.3, C-5; δ_C 106.9, C-6; δ_C 164.2, C-7). Confirmation of **3** was finalized with correlations of aromatic protons H-2′, 6′ with C-2 (δ_C 79.0) and C-4′ (δ_C 160.2), and H-3′, 5′ with C-1′ (δ_C 130.7). The absolute configuration of 4′-*O*-methylbonannione A was determined to be 2*S*, as deduced upon analysis of its CD spectrum and comparison with literature values.[8]

All of the isolates were tested against the A2780 human ovarian cancer cell line, and the results are shown in Table 2. Of the seven compounds, all displayed weak cytotoxicity, with IC_{50} values ranging from 5.5 to 12 μg/mL. Interestingly, nymphaeol A contains an extra B ring hydroxyl substituent compared with its counterpart, bonannione A, and was found to be about twice as active, with an IC_{50} value of 5.5 μg/mL as compared to 12 μg/mL for the latter compound.

Experimental Section

General Experimental Procedures. CD analysis was performed on a JASCO J-720 spectropolarimeter. IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. The melting

Table 2. Cytotoxicity Data of Compounds **1**−**7**[a]

compound	IC_{50} (μg/mL)
schizolaenone A (**1**)	10
schizolaenone B (**2**)	11
4′-*O*-methylbonannione A (**3**)	17
nymphaeol A (**4**)	5.5
bonannione A (**5**)	12
macarangaflavanone B (**6**)	16
bonanniol A (**7**)	25

[a] Concentration of each compound that inhibited 50% of the growth of the A2780 human ovarian cell line according to the procedure described,[8] with actinomycin D (IC_{50} 1−3 ng/mL) as the positive control.

point was taken on a Buchi B-540 melting point apparatus. NMR spectra were obtained on JEOL Eclipse 500, Varion Inova 400, and Varion Unity 400 spectrometers. Mass spectra were obtained on a JEOL JMS-HX-110 instrument. Chemical shifts are given in δ (ppm), and coupling constants (J) are reported in Hz. HPLC was performed using either Shimadzu LC-8A pumps coupled with a Varian Dynamax preparative C_{18} column (250 × 21.4 mm) or Shimadzu LC-10AT pumps coupled with a Varian Dynamax semipreparative C_{18} column (250 × 10 mm). Both HPLC systems employed a Shimadzu SPD-M10A diode array detector. Preparative TLC was performed on Merck HPTLC cyano (CN) plates (10 × 10 cm), 200 μm thickness.

Cytotoxicity Bioassays. The A2780 ovarian cancer cell line cytotoxicity assay was performed at Virginia Polytechnic Institute and State University as previously reported.[9]

Plant Material. The plant sample used was a collection of fruits of *Schizolaena hystrix* (Sarcolaenaceae), and duplicates of the voucher specimen (Rakotondrafara 225) are deposited at the Missouri Botanical Garden (MO), the Muséum National d'Histoire Naturelle, Paris (P), the Département des Recherches Forestières et Pisicoles, Madagascar (TEF), and the Centre National d'Applications des Recherches Pharmaceutiques (CNARP), Madagascar. The collection was made in the province of Toamasina, 15−20 km SE of the village of Ambarifotsy, in forest adjacent to the Zahamena Protected Areas at 560 m in elevation on May 30, 2003, by A. Rakotondrafara, S. Randrianasolo, N. M. Andrianjafy, L. J. Razafitsalama, L. M. Randrianjanaka, A. Belalahy, Randrianjafisoa, and R. Mananjara.

Extraction and Isolation. The dried plant sample described above (336 g) was extracted with EtOH to give 27.3 g of extract designated MG 1938. Extract MG 1938 (0.93 g) was suspended in aqueous MeOH (MeOH−H_2O, 9:1, 350 mL) and extracted with hexanes (2 × 150 mL). The aqueous MeOH fraction displayed cytotoxicity (IC_{50} = 10 μg/mL) and was further chromatographed by preparative RP-C_{18} HPLC using MeOH−H_2O (87:13) to yield 10 fractions (A−J). Fraction J was identified as **1** (t_R 27.9 min, 122 mg), while fractions E and F were identified as nymphaeol A (t_R 12.1 min, 42.6 mg) and bonannione A (t_R 14.5 min, 50.4 mg), respectively. Fraction D (20.6 mg, IC_{50} = 9.0 μg/mL) was further separated via preparative TLC on CN plates (95:5, $CHCl_3$−hexane) to afford bonanniol A (R_f 0.31, 2.7 mg) and macarangaflavanone B (R_f 0.56, 1.79 mg). Fraction I (70.4 mg, IC_{50} = 10 μg/mL) was separated using CN preparative TLC ($CHCl_3$−hexane, 95:5) to afford compound **2** (R_f 0.60, 13.5 mg) and fraction K (R_f 0.85, 6.66 mg). Using a MeOH−H_2O system (85:15), fraction K required chromatographic purification over semipreparative RP-C_{18} HPLC to afford **3** (t_R 12.5 min, 1.99 mg). The structures of the known compounds were identified by comparison of their spectral data with literature values.[4−7]

Schizolaenone A (1): yellow amorphous solid; $[\alpha]_D$ +2.7° (*c* 0.44, MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (2.25) nm, 293 (2.10) nm; IR ν_{max} 2966, 2920, 2853, 1633, 1597, 1447, 1149, 1085, 1063, 819 cm^{-1}; CD (MeOH, *c* 0.039) $[\theta]_{293}$ −24; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 477.2592 $[M + 1]^+$ (calcd for $C_{30}H_{37}O_5$, 477.2641).

Schizolaenone B (2): yellow amorphous solid; $[\alpha]_D$ −3.9° (*c* 0.53, MeOH); UV (MeOH) λ_{max} (log ϵ) 231 (2.18), 292 (2.15) nm; IR ν_{max} 2966, 2914, 2855, 1633, 1597, 1446, 1297, 1152, 1085, 1065 cm^{-1}; CD (MeOH, *c* 0.076) $[\theta]_{295}$ −2.2; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 493.2569 $[M + 1]^+$ (calcd for $C_{30}H_{37}O_6$, 493.2590).

4′-*O*-Methylbonannione A (3): pale yellow solid, mp 133−136 °C; $[\alpha]_D$ −3.2° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 296 (1.97) nm; IR ν_{max} 2922, 2851, 1627, 1582, 1458, 1312, 1295, 1252, 1173, 1089, 830 cm^{-1}; CD (MeOH, *c* 0.012) $[\theta]_{290}$ −3.6; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 423.2139 $[M - 1]^+$ (calcd for $C_{26}H_{31}O_5$, 423.2172).

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Supporting Information Available: ^1H NMR spectra for compounds **1**−**3** and structures of all isolated compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Biodiversity Conservation and Drug Discovery in Madagascar, Part 13. For Part 12, see: Cao, S.; Guza, R. C.; Miller, J. S.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I. *J. Nat. Prod.* **2004**, *67*, 986−989.
(2) Lowry, P. P.; Schatz, G. E.; Leroy, J.; Wolf, A. *Adansonia, Sér. 3* **1999**, *21*, 183−212.
(3) Gaydou, E. M.; Ramanoelina, A. R. P. *Phytochemistry* **1983**, *22*, 1725−1728.
(4) Bruno, M.; Savona, G.; Lamartina, L.; Lentini, F. *Heterocycles* **1985**, *23*, 1147−1153.
(5) Wang, Y.; Tan, W.; Li, W. Z.; Li, Y. *J. Nat. Prod.* **2001**, *64*, 196−199.
(6) Phillips, W. R.; Baj, N. J.; Gunatilaka, A. A. L.; Kingston, D. G. I. *J. Nat. Prod.* **1996**, *59*, 495−497.
(7) Schütz, B. A.; Wright, A. D.; Rali, T.; Sticher, O. *Phytochemistry* **1995**, *40*, 1273−1277.
(8) Cao, S.; Schilling, J. K.; Miller, J. S.; Adriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I. *J. Nat. Prod.* **2004**, *67*, 454−456.
(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.

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