Notes

Genipatriol, a New Cycloartane Triterpene from *Genipa spruceana*

Chowdhury Faiz Hossain,[†] Melissa R. Jacob,[†] Alice M. Clark,[†,‡] Larry A. Walker,[†,§] and Dale G. Nagle*[,†,‡]

National Center for Natural Products Research, Department of Pharmacognosy, Department of Pharmacology, and Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, Mississippi 38677-1848

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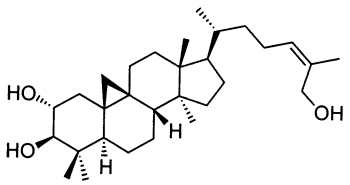
Genipatriol (**1**), a new 2α,3β-dihydroxylated cycloartane triterpene, was isolated from the aerial parts of *Genipa spruceana*. The structure of genipatriol was determined by a combination of spectroscopic methods.

As part of an ongoing project to discover potential new drugs to treat opportunistic fungal infections, a bioassay system was developed to identify natural products that reverse multidrug resistance (MDR) efflux pump-mediated azole resistance in *Candida glabrata*. The Peruvian plant *Genipa spruceana* Steyerm. (Rubiaceae) was selected for chemical investigation from evaluation of more than 2000 plants. Among the seven species of *Genipa*, only *G. americana* has been reported to have been chemically investigated.[1,2] Its elliptically shaped fruit, known as "genipap," is edible and the source of a popular beverage in Central and South America.[1] Bioassay-guided fractionation yielded an active fraction, from which ursolic acid and a new cycloartane triterpene, trivially named genipatriol (**1**), were isolated. However, neither ursolic acid nor genipatriol (**1**) significantly reversed azole resistance in *C. glabrata*. The isolation and structure elucidation of compound **1** is described herein.



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The crude CH_2Cl_2 extract of *G. spruceana* was fractionated by Si gel VLC (hexanes/EtOAc step gradient). The active fraction (eluting with 70% EtOAc in hexanes) was further separated by successive normal-phase and reversed-phase chromatography. Compounds **1** and **2** were isolated from the most active fraction by reversed-phase HPLC.

Genipatriol (**1**) was obtained as a white amorphous powder. Analysis by ^{13}C NMR and HRESIMS provided a molecular formula of $C_{30}H_{50}O_3Na$, indicating six degrees of unsaturation. The ^1H NMR spectrum of **1** displayed two characteristic cyclopropane methylene proton resonances at δ 0.38 (1H, d, J = 4.0 Hz) and 0.58 ppm (1H, d, J = 4.0 Hz), five tertiary methyl singlet resonances at δ 2.04, 1.24, 1.13, 0.97, 0.90, and one secondary methyl resonance at δ 0.91 (3H, d, J = 8.5 Hz). Additionally, signals for one olefinic methine proton at δ 5.40 (1H, t, J = 7.1 Hz), two oxymethine protons at δ 4.13 (1H, d, J = 16.0, 9.0 Hz) and 3.49 (1H, d, J = 9 Hz), and two oxymethylene protons at δ 4.51 (2H, s) were observed. The ^{13}C DEPT (135° and 90°) spectra of **1** revealed the presence of six primary, 11 secondary, and seven tertiary carbons among the 30 signals in the ^{13}C NMR spectrum. Considering the molecular formula and that only one degree of unsaturation (from a total of six) could be attributed to a single double bond, the structure of compound **1** appeared to be that of a tetracyclic triterpene with an additional cyclopropane ring. The $^1H-^1H$ COSY spectrum together with HMQC data revealed that **1** has five distinct $^1H-^1H$ spin systems: (a) [$-CH_2-CH(O)-CH(O)-$], (b) [$>CH-CH_2-CH_2-CH<$], (c) [$>CH_2-CH_2<$], (d) [$-CH_2-CH_2-CH<$], and (e) [$-CH(CH_3)-CH_2-CH_2-CH<$]. Long-range $^1H-^{13}C$ couplings observed in the $^1H-^{13}C$ HMBC spectrum (Table 1) supported the connectivity of these spin systems. Long-range $^1H-^{13}C$ couplings between δ_H 3.49 (H-3) and four ^{13}C resonances at δ_C 41.4 (C-4), 26.9 (C-29), 16.3 (C-30), and 71.6 (C-2) revealed the position of the oxymethine protons to be at H-2 and H-3 in the first spin system (a). The coupling constant between these proton resonances (9.0 Hz) indicated that they are both in an equatorial orientation. The δ value of C-3 (83.8 ppm) and C-30_{eq} (16.3 ppm) suggested the configuration of OH-3 to be β.[3,4] Long-range $^1H-^{13}C$ couplings ($^1H-^{13}C$ HMBC) between δ_H 0.38 (H-19_a) and the four carbon resonances at δ_C 19.6 (C-10), 27.1 (C-11), 41.5 (C-1), and 48.2 (C-8) indicated **1** to be a C9−C19 cyclopropane derivative. Analysis of the $^1H-^{13}C$ HMBC spectrum confirmed the position of unsaturation ($\Delta^{23,24}$) and oxymethylene as that of a terminal methylene at C-26. The rotating frame nuclear Overhauser spectrum (ROESY) indicated the stereochemistry of **1** (see Table 1) is as in the structure presented. Specifically, NOE interactions between H-2 and H-30, H-3, and H-29 unambiguously indicated that the hydroxy groups attached to C-2 and C-3 are axially oriented in the chair form of the A-ring. Therefore, the structure of **1** was assigned as (2α,3β,24*E*)-9,19-cyclolanost-24-ene-2,3,26-triol.

The structure of the other major component in the active fraction was determined to be ursolic acid by 1D and 2D

* To whom correspondence should be addressed. Tel: (662) 915-7026. Fax: (662) 915-6975. E-mail: dnagle@olemiss.edu.
[†] National Center for Natural Products Research.
[‡] Department of Pharmacognosy.
[§] Department of Pharmacology.

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Table 1. NMR Spectral Data for Genipitriol (**1**) at 500 MHz (^{1}H) and 125 MHz (^{13}C) in Pyridine-d_5[a]

position	δ_H (J in Hz)	δ_C	ROESY	HMBC
1 ax	1.85 (m)	41.5	H-2	C-3, C-2, C-5, C-9, C-19
1 eq	1.86 (m)		H-3	
2	4.13 (dd, J = 9.0, 16.0)	71.6	H_3-30	C-3
3	3.49 (d, J = 9.0)	83.8	H-1$_{eq}$, H-5, H_3-29	C-2, C-4, C-29, C-30
4		41.4		
5	1.45 (dd, J = 4.3, 12.5)	47.8	H-3	C-6, C-16, C-19, C-29
6 ax	1.57 (m)	21.7		C-7
6 eq	0.78 (dd, J = 1.9, 12.7)		H-5	
7 ax	1.08 (m)		H_3-18	
7 eq	1.31 (m)			
8	1.51 (dd, J = 4.8, 12.3)	48.2		
9		26.0		
10		19.6		
11 ax	2.00 (dd, J = 6.0, 6.5)	27.1		
11 eq	1.17 (m)		H_3-28	
12	1.56 (2H, m)	33.4		
13		45.8		
14		49.4		
15	1.29 (2H, m)	36.0		
16 ax	1.28 (m)	28.7	H-15	
16 eq	1.90 (m)			
17	1.58 (m)	52.8		
18	0.97 (3H, s)	18.5	H-8, H-19$_a$, H-19$_b$, H-20	C-12, C-13, C-14
19a	0.38 (d, J = 4.0)	30.2	H_3-30	C-1, C-8, C-10, C-11
19b	0.58 (d, J = 4.0)		H-6$_{eq}$, H-8, H-30	
20	1.44 (d, J = 4.0)	36.4		
21	0.91 (3H, d, J = 8.5)	18.7	H-22$_b$, H_3-27	C-17, C-22
22a	1.17	37.3		
22b	1.54			
23a	2.11	25.1		
23b	2.27			
24	5.40 (t, J = 7.4)	127.7		C-23, C-26, C-27
25		149.6		
26	4.51 (2H, s)	61.1	H_3-27	
27	2.04 (3H, s)	22.1	H_3-21	C-24, C-25, C-26
28	0.90 (3H, s)	19.8	H_3-12	
29	1.24 (3H, s)	26.9	H-3	
30	1.13 (3H, s)	16.3	H-2	C-3, C-4, C-5, C-30

[a] ^{13}C assignments by HMQC and DEPT experiments. Correlations from ambiguous overlapping resonances are not presented.

NMR spectral analysis and confirmed by direct comparison by TLC and ^{1}H and ^{13}C NMR with an authentic sample.[5]

To the best of our knowledge, the only previously reported 2α-hydroxylated cycloartane triterpene was isolated from the Asian medicinal/food plant *Mussaenda pubescens* (also Rubiaceae).[6] Since neither **1** nor ursolic acid reverses azole resistance in *C. glabrata*, it seemed possible either that these substances functioned in a direct synergistic manner or that ursolic acid served a role in solubilizing **1**. Simultaneous testing of **1** and ursolic acid and evaluation of various recombined mixtures of these two components in the *C. glabrata* assays failed to reestablish significant activity. The reason for this loss of bioactivity remains uncertain. However, the possibility that **1** is a degradation or isomerization product of a yet-undetermined active constituent cannot be ruled out.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a RUDLPH research autopol IV atomatic polarimeter. The IR spectrum was obtained using an AATI Mattson Genesis Series FTIR. The ^{1}H and ^{13}C NMR spectra as well as 2D spectra (COSY, ROESY, HMQC, and HMBC) of **1** were recorded on a Bruker DRX 500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. The NMR spectra of ursolic acid were recorded on a Bruker DRX 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. NMR spectra were recorded running gradients and using residual solvent peaks as internal references. The HRESIMS and ESIMS data were acquired on a Bruker BioAPEX 30es mass spectrometer. TLC was performed on Merck TLC plates precoated with Si_{60} F_{254} or Si_{60} RP18 F_{254} with visualization by spraying with 1:1 H_2SO_4 in EtOH and heating. HPLC was carried out on a Waters Millennium system with a 996 photodiode array detector.

Plant Material. The plant *Genipa spruceana* Steyerm. (Rubiaceae) was collected in the rainforest (elevation 85−165 m) of Peru (Departmento Loredo, Provincia Maynas, Distrito Punchana) in March 1993. The sample was identified by Manuel Rimachi Y., dried in a plant drier at 38 °C, and deposited in the National Center for Natural Products Research repository. A voucher specimen of the dried plant is on deposit at the Mississippi State University Herbarium-MISSA (voucher # IBE 10461).

Extraction and Isolation. Dried leaves of the plant (600 g) were exhaustively extracted with CH_2Cl_2 (2 L × 2 days, 3 times). The CH_2Cl_2 extract (14 g, 2.3% yield of dry plant weight) was chromatographed by VLC (Si gel, 32−63 μm, 100:0:0−0:0:100 hexanes/EtOAc/MeOH, step gradient) to give 13 fractions, A_1−M_1. Fraction G_1 (492 mg, eluted with 30:70 hexanes/EtOAc) showed activity and was further separated on an open column (Si gel, 100−200 μm, step gradient 1% to 50% MeOH in CH_2Cl_2) to afford fractions A_2−I_2. The major fraction E_2 (92 mg) was separated using a solid-phase extraction cartridge (Alltech) (C_{18}, 10 g, 70:30 to 100:0 (v/v), MeOH/H_2O, step gradient) to obtain fractions A_3−N_3. The most active fractions, H_3 (6.0 mg) and I_3 (4.4 mg), were combined and purified by RP-HPLC (Prodigy ODS-3, 5 μm, 21.2 × 250 mm column, 80:20 (v/v), CH_3CN/H_2O, 14 mL min^{-1}, photodiode-array detection monitored at 220 nm) to obtain **1** (2.8 mg, 0.02% yield) and ursolic acid (6.3 mg, 0.045% yield), $[\alpha]^{25}_D$ +31.2° (*c* 0.03, MeOH); ^{1}H and ^{13}C NMR consistent with literature values.[6]

(2α,3*β*,24*E*)-9,19-Cyclolanost-24-ene-2,3,26-triol (1): white amorphous powder; $[\alpha]^{25}_{D}$ +20.0° (*c* 0.05, MeOH); IR (film) ν_{max} 3303, 2955, 2928, 2863, 1570, 1257, 1087, 1038, 800 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz), see Table 1; ^{13}C NMR (pyridine-d_6, 125 MHz), see Table 1; ESIMS *m*/*z* $[M + Na]^+$ 481.4; HRESIMS *m*/*z* $[M + Na]^+$ 481.3628 (calcd for $C_{30}H_{50}O_3$ + Na, 481.3652).

Assay for Reversal of Azole Resistance in *Candida glabrata*. Temporary cultures (for immediate use in assays) of *Candida glabrata* (ATCC 32312, obtained from ATCC, Manassas, VA) were prepared from frozen stocks in SDB broth for 24 h at 37 °C prior to susceptibility testing. Long-term storage of *C. glabrata* 32312 is accomplished via freezing cells in 10% glycerol/SDB broth at −70 °C. Susceptibility testing was performed using a modified version of the NCCLS methods.[7] On the day of the assay, prepared samples (dissolved in DMSO) were serially diluted using 0.9% saline, and 25 μL was transferred in duplicate to flat-bottomed 96-well microtiter plates (non-tissue culture treated, Costar). Inocula were prepared after comparing the absorption (630 nm) of a 100 μL aliquot of the 24 h culture to a 100 μL aliquot of a 0.5 McFarland standard using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont). The *C. glabrata* 32312 culture was diluted in RPMI broth (2% glucose, without phenol red, buffered at 7.0 with MOPS, Cellgro, VA) to achieve a final target inoculum of 1.0×10^4 CFU/mL. Crude extracts and column fractions were evaluated at an initial test concentration of 200 μg/mL. Pure compounds were tested at 50 μg/mL. A subinhibitory concentration of fluconazole (Pfizer), dissolved in DMSO, was added to the inoculum prior to addition to the microplates (FLU +). An equivalent volume of DMSO only was added to the inoculum for a duplicate set of microplates (FLU −). Growth (saline only), solvent (DMSO), and blank (media only) controls were included on each test plate. Beauvericin (Sigma) was used as a positive control. The inocula were added to afford a final volume of 200 μL. The plates were read at 630 nm prior to and after incubation at 37 °C for 24−48 h. Percent growth was calculated and plotted versus test concentration to afford the IC_{50} (sample concentration that affords 50% growth of *C. glabrata* 32312).

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