New Antimycobacterial Saponin from *Colubrina retusa*

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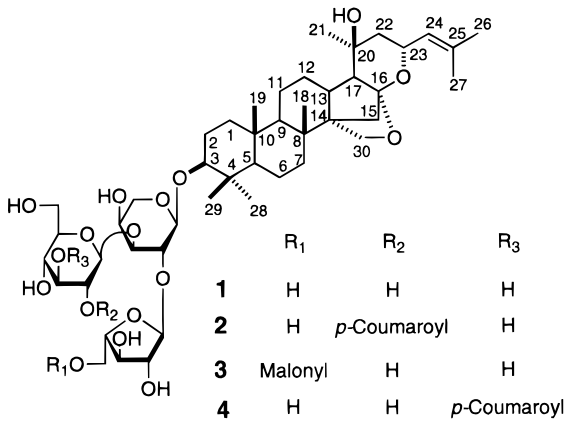
A new jujubogenin saponin was isolated from the stems of *Colubrina retusa* and identified as jujubogenin 3-*O*-α-L-arabinofuranosyl (1→2)-[3-*O*-(*trans*)-*p*-coumaroyl-β-D-glucopyranosyl (1→3)]-α-L-arabinopyranoside (**4**) on the basis of chemical and spectroscopic data. The antimycobacterial activity expressed as minimum inhibitory concentration (MIC) for compound **4** was 10 μg/mL.

Colubrina retusa (Ditter) Cowan is a dicotyledonaceous plant of the Rhamnaceae family, whose 55 genera and 900 species are widely distributed especially in the tropics. The only rhamnaceous plants of some economic importance are *Ziziphus* species and *Rhamnus purshiana*.[1] A previous study carried out on the stem of *C. retusa*[2] resulted in the isolation of a known saponin identified as jujubogenin [3-*O*-α-L-arabinofuranosyl (1→2)-β-D-glucopyranosyl (1→3)]-α-L-arabinopyranoside (**1**),[3] along with two new saponins: jujubogenin 3-*O*-α-L-arabinofuranosyl (1→2)-[2-*O*-(*trans*, *cis*)-*p*-coumaroyl-β-D-glucopyranosyl (1→3)]-α-L-arabinopyranoside (**2**) and jujubogenin 3-*O*-(5-malonyl)-α-L-arabinofuranosyl (1→2)-[β-D-glucopyranosyl (1→3)]-α-L-arabinopyranoside (**3**). To isolate new constituents from this plant, further investigation of a column fraction yielded a new saponin (**4**) whose structure was established by spectroscopic means. ESI-MS showed a molecular ion peak at *m*/*z* 1067 $[M + Na]^+$ suggesting the molecular formula $C_{55}H_{80}O_{19}$.



	R_1	R_2	R_3
1	H	H	H
2	H	*p*-Coumaroyl	H
3	Malonyl	H	H
4	H	H	*p*-Coumaroyl

Comparison of the ^1H and ^{13}C NMR spectra of **4** with the previously identified saponins **1** and **2** suggested the same type of jujubogenin aglycone. The ^{13}C NMR of **4** displayed three anomeric carbon signals at δ105.4, 104.8, and 110.4 as well as a typical carboxylic ester carbon signal at δ167.5 along with some aromatic and olefinic carbon signals. Aromatic and olefinic proton signals were also observed in the downfield region of the ^1H NMR spectrum. On alkaline hydrolysis, compound **4** afforded **1** and *p*-coumaric acid. The presence of *p*-coumaroyl group was indicated by the carbon signals at δ 115.9 (d), 116.9 (d), 126.5 (s), 130.5 (d), 145.1 (d), 161.3 (s), and 167.5 (s). The ^1H NMR spectrum showed the aromatic signals as two ortho-coupled doublets at δ 7.43 (2H, J = 8.6 Hz) and 7.10 (2H, J = 8.6 Hz) together with another two doublets at δ 6.49 (1H, J = 15.9 Hz) and 7.85 (1H, J = 15.9 Hz) integrating for one proton each; these were assigned to the olefinic protons, and the size of the coupling constant indicated a trans configuration. Complete assignments of the ^1H and ^{13}C NMR signals were accomplished by 2D NMR including COSY, HMQC, and HMBC (Table 1). The linkage position of the *p*-coumaroyl residue to the sugar moiety was determined by 2D NMR. $^1H-^1H$ COSY and HMQC established each sugar's spin−coupling network. The signal of H-3 of glucose in **4** was significantly shifted to a downfield value of δ 5.92 when compared with **1**, in which it resonated at δ 4.22.[2] This indicated that the *p*-coumaroyl group was linked at the 3 position of glucose. Key HMBC correlations are sum-

Table 1. ^{13}C and ^1H NMR Data for Compound **4** in Pyridine-d_5 (ppm)

	δ_C	δ_H (J, Hz)		δ_C	δ_H (J, Hz)
aglycone-1	39.2	1.54, 0.75	Ara (*p*)-1	105.4	4.75 (d, 7)
2	26.8	2.10, 1.79	2	76.9	4.45
3	88.9	3.21 (dd, 11.7, 4.3)	3	83.6	4.16
4	40.0	−	4	68.3	4.50
5	56.7	0.66	5	65.4	4.15, 3.65
6	18.4	1.45, 1.33	Glc(*p*)-1	104.8	5.14 (d, 7.8)
7	36.4	1.47	2	73.4	4.02
8	37.8	−	3	79.3	5.92
9	53.6	0.86	4	70.0	4.32
10	37.6	−	5	78.5	3.94
11	22.0	1.51	6	62.6	4.43, 4.22
12	28.7	1.91, 1.88	Ara (*f*)-1	110.4	6.03 (d, 2.8)
13	37.5	2.79	2	83.4	4.97
14	53.9	−	3	78.3	4.78
15	37.0	2.42/1.49 (ABq, 8)	4	85.4	4.71
16	110.6	−	5	62.6	4.30, 4.14
17	54.6	1.35			
18	19.0	1.04 (*s*)	*p*-Coum-1	126.5	−
19	16.4	0.68 (*s*)	2,6	130.5	7.43 (d, 8.6)
20	68.7	−	3,5	116.9	7.10 (d, 8.6)
21	30.1	1.33 (*s*)	4	161.3	−
22	45.6	1.74	α-	115.9	6.49 (d, 15.9)
23	68.8	5.12	β-	145.1	7.85 (d, 15.8)
24	127.2	5.48	O=C	167.6	−
25	134.2	−			
26	25.5	1.66 (*s*)			
27	18.6	1.71 (*s*)			
28	28.2	1.24 (*s*)			
29	16.7	1.02 (*s*)			
30	65.9	4.23			
20-OH		5.93 (*s*)			

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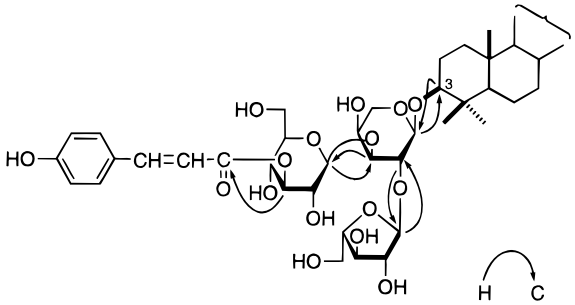

Figure 1. Key HMBC correlations of **4**.

marized in Figure 1. On the basis of the evidence above, compound **4** was established to be jujubogenin 3-*O*-α-L-arabinofuranosyl (1→2)-[3-*O*-(*trans*)-*p*-coumaroyl-*β*-D-glucopyranosyl (1→3)]-α-L-arabinopyranoside. Compounds **2** and **4** were active against *Mycobacterium intracellulare* (MIC of 50 and 10 μg/mL, respectively); while **1** was inactive.

Experimental Section

General Experimental Procedures. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. NMR were recorded in pyridine-d_5 with TMS as internal standard, using Brüker Avance DPX-300 (300 MHz) DRX-400 (400 MHz), and DRX-500 (500 MHz) instruments. ESI-FTMS spectra were measured on a Brüker-Magnex BioAPEX 30es ion cyclotron high-resolution HPLC-FT spectrometer. HPLC was performed on a Waters LC Module 1, using a Phenomenex Prodigy reversed-phase (150 × 4.6 mm, 5 μm) for screening and a Phenomenex Prodigy reversed-phase (250 × 10 mm, 10 μm) for preparative work; UV detection was achieved at 203 nm. Optical rotations were measured on a JASCO DIP 370 Digital Polarimeter.

Isolation. The stems from *C. retusa* L. (Rhamnaceae) were collected in Venezuela; a Voucher specimen (# V13021) was deposited at the National Center for Natural Products Research, University of Mississippi. Air-dried stems (500 g) were ground to a coarse powder and extracted at 37° with 95% EtOH (2.5 L × 4, 3 h, each). The EtOH extract was suspended in water (1.5 L) and extracted with $CHCl_3$ (3 × 1 L) and then with 1-BuOH (3 × 1 L). The combined BuOH fractions were evaporated to dryness in-vacuo at 45° to afford a yellow residue (5.9 g). Part of the residue (5.0 g) was subjected to column chromatography on silica gel using a mobile phase consisting of $CHCl_3$/MeOH/H_2O mixtures starting at 70:10:1 and ending at 20:10:1(6 L). Fractions of 25 mL each were collected, spotted on TLC, and combined according to TLC similarities. A total of 17 combined fractions were obtained. The new saponin (**4**) was obtained from fraction #5 (109 mg) by further purification on HPLC using an isocratic mixture of 37% acetonitrile in water at a flow rate of 3.5 mL/min (t_R 19 min). This material was dissolved in 1.5 mL of MeOH, and 24 injections of 40−65 μL each were made. The combined fractions were evaporated to dryness to yield compound **4**.

Jujubogenin 3-*O*-α-L-arabinofuranosyl (1→2)-[3-*O*-(*trans*)-*p*-coumaroyl-*β*-D-glucopyranosyl(1→3)]-α-L-arabinopyranoside (4). White powder (26.5 mg), mp 212 °C, $[\alpha]^{25}_D$ −79.7° (*c*, 0.025, MeOH); ^{1}H and ^{13}C NMR spectra are listed in Table 1; ESI-MS *m/z*, 1067.5199 $[M + Na]^+$ (calcd for $C_{55}H_{80}0_{19}Na$, 1067.5186).

Alkaline Hydrolysis. A solution of **4** (1 mg) in 1% KOH (0.2 mL) was kept at room temperature for 30 min. The reaction mixture was subjected to TLC analysis using $CHCl_3$/MeOH/H_2O (30:10:1) as a developing system and 10% H_2SO_4 as a chromogenic reagent. Saponin **1** was detected with an R_f value of 0.72. For detection of the acid group, the reaction mixture was acidified with 2 N HCl, evaporated to dryness under a stream of N_2 and the residue dissolved in a drop of MeOH. The methanolic solution was spotted on TLC along with reference *p*-coumaric acid and the plate developed in $CHCl_3$/MeOH (6:1). *p*-Coumaric acid with an R_f value of 0.47 was detected by UV.

Antimycobacterial Assay. *Mycobacterium intracellulare* (ATCC#23068) was maintained on Lowenstein−Jenson slants at 4 °C. OADC-supplemented Middlebrook broth (5 mL) was inoculated with bacteria and incubated at 37 °C for 72 h. Compounds **1**, **2**, and **4** were solubilized in DMSO, further diluted in sterile saline and a 1:30 diluted inoculum of the bacteria in Middlebrook broth was added to the diluted samples in the wells of round-bottomed microtiter plates. The plates were incubated at 37 °C for 48 h and then visually assessed for growth. The antimycobacterial activity expressed as minimum inhibitory concentration (MIC) for compound **4** was 10 μg/mL. Rifampicin was used as a positive control (MIC = 0.3 μg/mL).

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