Antibacterial Diterpenoid Acids from *Azorella compacta*

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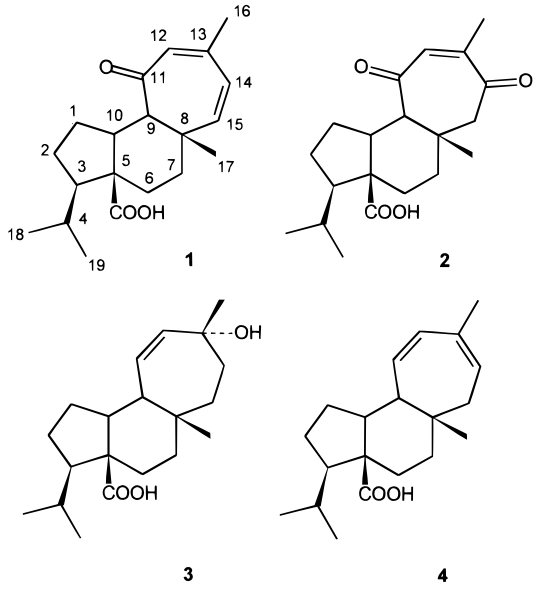
The two novel diterpenoid acids mulin-12,14-dien-11-on-20-oic acid (**1**) and mulin-12-ene-11,14-dion-20-oic acid (**2**) have been isolated from *Azorella compacta*. Their structures have been elucidated by 1D and 2D NMR methods. In contrast to the closely related known mulinolic acid (**3**) and its dehydration product (**4**) these new natural products have been shown to exhibit antimicrobial activity.

In Chilean folk medicine *Azorella compacta* Phil. (Apiaceae), which together with other *Azorella* and *Laretia* species is commonly known as "llareta", is used for the treatment of a variety of ailments.[1] So far, five mulinane diterpenoids have been isolated from *A. compacta*.[2−4] Recently, we reported the detection of 9,12-cyclomulin-13-ol in *A. compacta* by LC-MS. [5] The antimycobacterial activity of this compound,[5] which is also a major component of *Azorella madreporica*, prompted us to further evaluate the activity of mulinanes against other microorganisms. We report here the isolation and structure elucidation of two new mulinane diterpenoids **1** and **2** from *A. compacta* and the determination of their activity against *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, and *Candida albicans*.

HREIMS of **1** showed an ion consistent with a molecular formula of $C_{20}H_{28}O_3$. DEPT spectra revealed that of the 20 carbons four belonged to methyl, four to methylene, and seven to methine groups. Of the five quaternary carbons, two showed shifts of δ_C 179.0 (C-20) and 201.1 (C-11) typical for a carboxylic acid and a ketone, respectively. Four carbons at δ_C 128.5 (d, C-12), 146.1 (s, C-13), 127.4 (d, C-14), and 147.8 (d, C-15) together with the corresponding signals of three vinylic protons in the ^{1}H NMR indicated the presence of two double bonds. Two of these vinylic protons (H-14 and H-15) were located at a *cis*-configured double bond as shown by a coupling constant of 12.5 Hz. The proton shift of one methyl signal at δ_H 2.01 and its HMBC correlations to three of the olefinic carbons (δ_C 128.5, 146.1, and 127.4) suggested that these two double bonds were conjugated. No long-range coupling of the vinylic protons to the carbonyl carbon (δ_C 201.1) could be detected. However, a HMBC cross-peak for the proton at δ_H 2.56 (H-9) to this carbon was observed and carbon C-9 (δ_C 60.3), to which this proton corresponds in the HMQC, showed a cross-peak with the vinylic proton at δ_H 5.98 (H-12). HMBC cross-peaks of the methyl group at δ_H 1.05 (Me-17) with C-9 and the olefinic carbon C-15 of the conjugated dienone system suggested the presence of a seven membered ring. Based on these findings and other HMBC correlations found for this compound, we assigned **1** the structure of mulin-12,14-dien-11-on-20-oic acid.

HREIMS of **2** revealed a molecular formula of $C_{20}H_{28}O_4$. Of the 20 carbons appearing in the ^{13}C NMR four belonged to methyl, five to methylene, and five to methine groups. The ^{13}C NMR spectra indicated a carboxylic acid (δ_C 176.2), two keto groups (two signals overlapping at δ_C 203.8), and a double bond [δ_C 148.1 (s) and 133.5 (d)]. Comparison of the ^{13}C NMR data of **1** and **2** suggested that the five and six membered rings of both compounds were identical. Significant differences were only observed for the signals of carbons C-14 and C-15. On the basis of this comparison and on HMBC correlations, we assigned **2** the structure of mulin-12-ene-11,14-dion-20-oic acid.



Along with the new compounds **1** and **2** we isolated the known diterpenoid mulinolic acid **3** and its dehydration product **4**. We also prepared compound **4** by heating **3** in refluxing hexane. These known compounds were identified by comparison with published ^{1}H and ^{13}C NMR data.[2]

In an agar diffusion test, spots of 200 μg of compounds **1** and **2** in 10 μL of DMSO produced mostly hazy zones of inhibition of 8−12 mm in diameter against methicillin-susceptible and methicillin-resistant strains *of S. aureus*, a vancomycin-resistant strain of *E. faecium*, and a strain

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of *E. coli* with increased membrane permeability for compounds with high molecular weight. Interestingly, at the same concentration the known compounds **3** and **4** were completely inactive against all of these bacteria. Also, none of the compounds **1**−**4** showed any inhibition of *C. albicans* growth. Compounds **1** and **2** showed similar activities suggesting that the keto function in position 14 is not essential for their activity. It seems, however, that the keto function in position 11 is of importance for the observed weak antibacterial activities. Under the same experimental conditions clear zones of inhibition of 16−18 mm in diameter were found for antibiotic disks containing 30 μg of Vancomycin against methicillin-susceptible and methicillin-resistant *S. aureus*, for 10 μg of Bacitracin against vancomycin-resistant *E. faecium*, for 30 μg of Cefoxitin against *E. coli* with increased membrane permeability, and for 100 μg of Nystatin against *C. albicans*.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Jasco P1020 polarimeter. UV spectra were recorded with a Beckman DU 640 spectrophotometer, and IR spectra, with a Buck Scientifc 500 IR spectrometer. NMR spectra were recorded in $CDCl_3$ on a Varian Unity 300 spectrometer at 300 (^{1}H) and 75.4 MHz (^{13}C) with residual $CHCl_3$ (δ_H 7.24) and $CDCl_3$ (δ_C 77.0) as references. HMBC spectra were acquired with $^1/_{2J}$ = 0.05 s using standard software. Low-resolution EI spectra were obtained with a Hewlett-Packard 5988A spectrometer (70 eV). HREIMS were recorded on a JEOL HX 110 spectrometer with a resolution of 10 000.

Plant Material. Aerial parts of *A. compacta* Phil. were collected and identified in July 1995 by Gloria Montenegro in Chile at Farellones, Región Metropolitana. A voucher specimen (coll. no. 0587) has been deposited at the herbarium of the Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile. Intellectual Property Rights Agreements for plant collections and collaborative research have been fully executed between The University of Arizona and the Pontificia Universidad Católica de Chile.

Extraction and Isolation Procedure. The air-dried and ground plant material (1 kg) was extracted three times with CH_2Cl_2−MeOH (1:1). The resulting extract (64 g) was chromatographed on Si gel (700 g; 50−200 μm; Macherey und Nagel, Düren, Germany) with hexane−CH_2Cl_2 and CH_2Cl_2−acetone mixtures of increasing polarity. The fraction eluting with 10% of acetone in CH_2Cl_2 was rechromatographed on Si gel (300 g) with acetone−CH_2Cl_2 mixtures (2.5−10% acetone) containing 1% HCOOH. Final purification of **1** and **2** was carried out on a Büchi medium-pressure reversed phase column (460 × 15 mm) with 60% CH_3CN in water to yield 33 mg of **1** and 85 mg of **2**. Mulinolic acid (**3**, 285 mg) precipitated from fractions of the initial column which eluted with 40% of CH_2Cl_2 in hexane. Compound **4** (227 mg) was also isolated from the fraction eluting with 10% CH_2Cl_2 in hexane by chromatography on Si gel with EtOAc−hexane mixtures. Heating of the tertiary alcohol **3** in refluxing hexane for 1 h gave the dehydration product **4** which precipitated after cooling the solution.

Mulin-12,14-dien-11-on-20-oic acid (1): colorless solid; $[\alpha]_D^{25}$ −31.4° (*c* 2.0, MeOH); UV (MeOH) λ_{max} 300 nm (ϵ 3150); IR (KBr) ν_{max} 3430, 2959, 2923, 2870, 2855, 1714, 1644, 1616, 1456, 1436, 1376, 1344, 1298, 1224, 1182, 1156 cm $^{-1}$; ^{1}H and ^{13}C NMR see Table 1; EIMS *m/z* 316 ([M]$^+$, 26), 273 (18), 255 (20), 227 (58), 191 (46), 177 (25), 149 (56), 133 (51), 119 (52), 105 (70), 91 (86), 69 (57), 41 (100); HREIMS *m/z* [M]$^+$ 316.2039 (calcd for $C_{20}H_{28}O_3$, 316.2038).

Mulin-12-ene-11,14-dion-20-oic acid (2): colorless solid; $[\alpha]_D^{25}$ −2.6° (*c* 2.0, MeOH); UV (MeOH) λ_{max} 239 nm (ϵ 8060); IR (KBr) ν_{max} 3430, 2965, 2940, 2880, 1720, 1683, 1626, 1446, 1444, 1380, 1342, 1290, 1286, 1238, 1138, 1156 cm $^{-1}$; ^{1}H and ^{13}C NMR see Table 1; EIMS *m/z* 332 ([M]$^+$, 5), 304 (4), 286 (8), 271 (6), 243 (18), 215 (10), 177 (62), 165 (25), 133 (82), 111 (76), 91 (51), 69 (61), 40 (100); HREIMS *m/z* [M]$^+$ 332.1983 (calcd for $C_{20}H_{28}O_4$, 332.1987).

Table 1. ^{1}H and ^{13}C NMR Data of 1 and 2[a]

	1		2	
H/C	^{1}H	^{13}C	^{1}H	^{13}C
1a	1.87 m	24.1 t	1.84 m	24.9 t
1b	1.37 m		1.33 m	
2a	1.92 m	28.9 t	1.96 m	29.3 t
2b	1.57 m		1.52 m	
3	1.45 m	57.4 d	1.46 m	57.7 d
4	1.54 m	31.7 d	1.49 m	32.3 d
5		56.9 s		57.7 s
6a	2.58 m	33.7 t	2.55 m	33.1 t
6b	1.32 m		1.41 m	
7a	1.66 m	40.4 t	1.62 m	41.4 t
7b	1.56 m		1.55 m	
8		39.4 s		36.3 s
9	2.56 m	60.3 d	2.74 d (12.3)	63.4 d
10	2.32 m	46.8 d	2.17 m	48.8 d
11		201.1 s		203.8 s
12	5.98 s	128.5 d	6.28 t (1.2)	133.5 d
13		146.1 s		148.1 s
14	5.81 AB (12.5)	127.4 d		203.8 s
15a	5.78 AB (12.5)	147.8 d	3.40 d (13.2)	53.2 t
15b			2.40 d (13.2)	
16	2.01 d (1.2)	27.2 q	2.01 d (1.2)	20.4 q
17	1.05 s	24.5 q	1.02 s	28.9 q
18[b]	0.85 d (6.1)	22.7 q	0.86 d (5.7)	22.9 q
19[b]	1.02 d (6.1)	22.3 q	1.03 d (5.7)	22.5 q
20		179.0 s		176.2 s

[a] Shift values of overlapping proton signals from HMQC spectra; multiplicities of carbon signals from DEPT experiments; assignments based on HMQC and HMBC spectra. [b] Assigned by analogy to mulinolic acid.[2]

Determination of Antibacterial Activity. In vitro antimicrobial activities against methicillin-sensitive and methicillin-resistant *S. aureus*, Vancomycin-resistant *E. faecium*, *E. coli* imp (BAS849, a mutant strain with increased permeability to compounds with large molecular weight) and *C. albicans* were determined by the agar diffusion method. *E. coli* imp was obtained from S. Benson of Princeton University, NJ. All other test organisms were clinical isolates collected from various medical centers in the U.S.A. Media used were nutrient agar (pH 6.8) for *S. aureus*, LB (Luria-Bertani) agar for *E. faecium* and *E. coli*, and YM agar for *C. albicans*. All dehydrated media and antibiotic control disks (Dispens-O-Disc susceptibility test discs) were purchased from Difco Laboratories, Detroit, MI. Assay plates (12″ × 12″ Sumilon MS-12450 from Vanguard International Inc., Neptune, NJ) were prepared by pouring 125 mL of agar medium (tempered at 50 °C) inoculated with an overnight broth culture of the test organisms (adjusted to approximately 10^6 cells per mL). Test solutions of the compounds in DMSO were prepared (20 mg/mL) of which 10 μL were spotted directly onto the agar surface using a Multi Electrapette with Exp250 handle (a spreadable tip micropipetting device from Matrix Technologies Corporation, Lowell, MA). The plates were incubated at 37 °C for 18 h. Zones of growth inhibition were measured (in millimeters) using a hand-held Fowler Ultra-cal II digital caliper. A 10-μL amount of DMSO (solvent control) did not exhibit any growth inhibition.

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