A New Antitubercular Mulinane Diterpenoid from *Azorella madreporica* Clos

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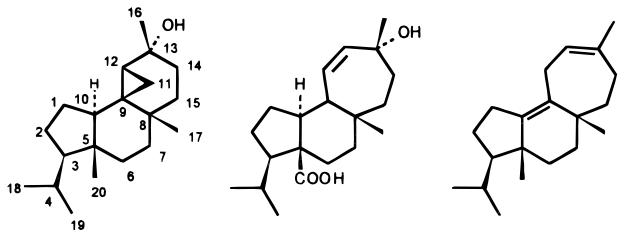
Bioactivity-guided fractionation of the petroleum ether extract of *Azorella madreporica* Clos has led to the isolation of the novel, antitubercular mulinane diterpenoid **1**. The structure has been elucidated on the basis of its 1D and 2D NMR spectra and by comparison with mulinolic acid **2** and a dehydration product **3** obtained from **1**. The MIC of **1** for growth inhibition of the $H_{37}Rv$ strain of *Mycobacterium tuberculosis* was determined as 20 μg/mL. LC-MS and NMR have suggested the presence of this new compound in four other species of *Azorella*.

Azorella (Apiaceae) is a genus of 70 species of Central America, west temperate South America, the Antarctic and Malvinas Islands, and the Southern Ocean.[1,2] *A. madreporica* Clos, a woody cushion plant that grows up to 5 cm on an underground rhizome, is a dominant species in the alpine communities of the Andes Mountains facing the mediterranean-arid region in Central Chile. This species is distributed from the northern part of the O'Higgins Province to the Mountains of Coquimbo, where it forms abundant patches and is commonly known as "llareta de Coquimbo".[3] The common name "llareta" is used for several species in the genera *Azorella* and *Laretia* (Apiaceae). In folk medicine, infusions made of llareta are employed as stomach stimulants and the extracts obtained with vegetable oils are used for the treatment of wounds.[4] During our ongoing investigation of medicinal plants from Latin America, we found that the petroleum ether extract from *A. madreporica* showed antitubercular activity with a complete growth inhibition of the $H_{37}Rv$ strain of *Mycobacterium tuberculosis* at a concentration of 100 μg/mL. Activity-guided fractionation of this extract led to the isolation of the novel, antitubercular diterpene alcohol **1** with a MIC of 20 μg/mL.

HREIMS of **1** showed an ion consistent with a molecular formula of $C_{20}H_{34}O$ (requires *m/z* 290.2610, found 290.2603). The base peak corresponded to a molecular formula of $C_{20}H_{32}$ (requires *m/z* 272.2504, found 272.2504), indicating the loss of water from **1**. DEPT spectra revealed that of the 20 carbons appearing in the ^{13}C NMR, five belonged to methyl, seven to methylene, and four to methine groups. One quaternary carbon showed a typical oxygen shift of δ_C 70.1, which together with a broad band at 3420 cm^{-1} in the IR spectrum and the loss of water observed in the MS indicated the presence of a tertiary diterpene alcohol. The ^1H NMR of **1** showed five methyl groups, two doublets (δ_H 0.80 and 0.90, J = 6.6 Hz, Me-18 and Me-19, respectively), and three singlets [δ_H 0.82 (Me-20), 0.95 (Me-17), and 1.24 (Me-16)]. A prominent signal at δ_H 2.27 (dd, J = 16.8, 7.2 Hz, H-10) and three signals at δ_H 0.04 (dd, J = 5.4, 5.4 Hz, H-11β), 0.68 (dd, J = 5.4, 9.8 Hz, H-11α), and 0.59 (ddd, J = 9.8, 5.4, 1.2 Hz, H-12) indicated the presence of a three-membered ring. HMBC cross-peaks of the proton at δ_H 0.04 (H-11β) with the hydroxy-substituted carbon at δ_C 70.1 (s, C-13) and the carbon at δ_C 25.3 (d, C-12) were also observed. For the latter carbon, a one bond correlation to the proton at δ_H 0.59 (H-12) in the three-membered ring was shown by HMQC. In addition, the methyl group at δ_H 1.24 (Me-16), a shift typical for a methyl group geminal to a hydroxy group, showed cross-peaks to carbons at δ_C 25.3 (C-12) and 70.1 (C-13). On the basis of these observations, we conclude that the three-membered ring had to be located next to the methyl- and hydroxy-substituted carbon C-13. Comparison with the NMR spectra of mulinolic acid **2**,[5,6] isolated from *A. compacta*,[6] suggested that **1** is a closely related mulinane-type diterpenoid lacking the carboxylic group of mulinolic acid **2**. The double bond of the seven-membered ring of **2** is replaced with a three-membered ring in **1**. The position of this cyclization was confirmed by HMBC cross-peaks of the methyl singlet at δ_H 0.95 (Me-17) with two secondary carbons at δ_C 35.3 (C-15) and 37.6 (C-7) and two quarternary carbons at δ_C 26.1 (C-9) and 30.5



1 **2** **3**

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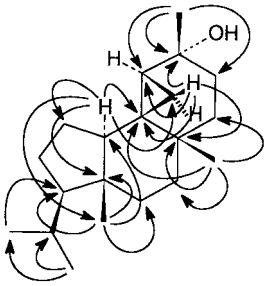


Figure 1. Significant HMBC correlations of **1**.

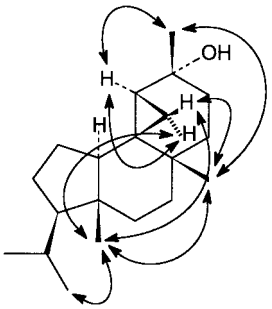
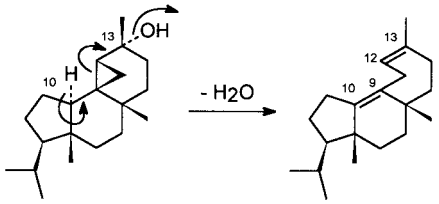


Figure 2. Significant NOESY correlations of **1**.

(C-8). Carbons C-8 and C-9 correlated with the proton at δ_H 0.04 (H-11β); therefore, we concluded that the cyclopropane moiety is located between the methyl groups Me-17 and Me-16. Because the carbons of the three-membered ring at δ_C 26.1 (s, C-9) and 10.5 (t, C-11) had HMBC cross-peaks to the prominent proton signal at δ_H 2.27 (H-10), which itself is correlated to the methyl group Me-20 at δ_C 17.8, the structure of 9,12-cyclomulin-13-ol was assigned to **1**. All other HMQC and HMBC correlations (Figure 1) of **1** were in agreement with this structure. The cross-peaks of a NOESY spectrum (Figure 2) suggested the positions on the same side of the mulinane skeleton for the methyl and isopropyl groups as well as the three-membered ring of **1**. We assumed, by analogy to mulinolic acid **2**, that these substituents are β-configurated. Proton 11β (δ_H 0.04), whose coupling constant of 5.4 Hz to H-12 is typical for a trans-configuration in three-membered rings,[7] exhibited NOESY cross-peaks to Me-17 and Me-20. The absence of a NOESY cross-peak of Me-16 with proton 11β can be explained by the equatorial position of this methyl group. Also, no NOESY cross-peak was found between H-10 and Me-20, which is in agreement with a trans-configuration at this ring junction.

The formation of the dehydration product **3**, which we isolated as a major component from a mixture of decomposition products of **1**, further confirmed the structure of **1**. ^{1}H and ^{13}C NMR of **3** showed a multiplet for one vinylic proton at δ_H 5.52 (H-12), a low-field shift of the proton signal of Me-16 to δ_H 1.65 (t, J = 1.5 Hz), and four olefinic carbons at δ_C 141.9 (s), 138.6 (s), 132.5 (s), and 122.8 (d). These findings are in agreement with the elimination of water from positions 10 and 13 in **1** with a simultaneous opening of its three-membered ring. Two isolated double bonds are formed in this reaction (Figure 3). The structure of **3** was assigned as mulin-9(10),12(13)-diene.

The MIC of **1** for growth inhibition of the R_{37}Hv strain of *M. tuberculosis* was determined in the BACTEC system as 20 μg/mL.[8,9] A mixture of decomposition products of **1**, with **3** as the major component, showed



1 **3**

Figure 3. Proposed formation of **3** by dehydration and simultaneous ring opening of **1**.

a MIC of 20 μg/mL. These results suggest that the activity of **1** could be partly or completely due to the formation of unsaturated decomposition products, such as **3**, during the bioassay. A high affinity to the lipophilic mycobacterial cell wall is likely for both structures and one might expect that this is where they exert their activity. The moderate toxicity of **1** against Vero cells[8] with an IC_{50} of 184 μg/mL indicated that the observed antitubercular activity is not due to unspecific toxic effects. The selectivity of **1** for inhibition of *M. tuberculosis* versus its toxicity against Vero cells is, however, lower than for drugs currently used in the treatment of tuberculosis (e.g., rifampin MIC = 0.125 μg/mL, IC_{50} (Vero cells) > 158 μg/mL).

We also observed antitubercular activity of extracts obtained from seven other *Azorella* species. To clarify whether these activities are due to the presence of **1**, we employed isocratic normal-phase LC-MS of hexane-soluble fractions and NMR spectra of crude extracts from each species. Only extracts of *A. madreporica* and *A. compacta* contained sufficient amounts of **1** calculated with a standard as equivalent to 0.5% and 0.3% in the dry biomass. The strong inhibition of mycobacterial growth by extracts from *A. monanthos* and *A. patagonica* (95% growth inhibition at 100 μg/mL) must be largely due to the presence of antitubercular compounds other than **1** of which only traces were detected. Also, the moderate antitubercular activities observed for *A. filamentosa*, *A. trifurcata*, and *A. crassipes* (<75%), in which **1** could not be detected, are necessarily due to the presence of other, still unidentified components. *A. cryptantha* contained **1** in low concentrations and was only weakly active (<25%), showing that no other potent antitubercular components are present in sufficiently high concentrations in this species.

These results show that of the eight investigated *Azorella* species, *A. patagonica* and *A. monanthos* are most likely to contain antitubercular compounds different from **1**, thus providing a rationale for further investigations of antitubercular extracts from *Azorella*. Additional LC-MS studies should reveal whether the presence of the rare mulinane diterpenoids, a class of compounds that previously had been reported only in *Mulinum* species and in *A. compacta*,[10−14] is a common feature in this genus.

Experimental Section

General Experimental Procedures. The melting point of **1** was determined on a Electrothermal capillary melting point apparatus and is uncorrected. ^{1}H and ^{13}C

NMR spectra were recorded on a Varian Unity 300 (300 and 75.4 MHz) in $CDCl_3$ using the proton resonance of residual undeuterated $CHCl_3$ (δ_H 7.24 ppm) and the carbon signal of $CDCl_3$ (δ_C 77.0 ppm) as reference. For the detection and quantification of **1** in crude extracts by ^1H NMR its signal at δ_H 0.04 was used. The mixing time for the NOESY experiment was 0.6 s, and the HMBC spectra were recorded with $^1/_2J = 0.05$ s. LC-MS experiments were carried out on a Finnegan TSQ 7000 in APCI positive mode with an Alltech adsorbosil (5 μm) 4.6 × 250 mm column, 10% EtOAc in hexane as isocratic eluent at a flow rate of 1 mL/min, and an ion trace at *m/z* 273 $[M - H_2O + 1]^+$ for detection. A retention time of 11.8 min was observed for **1** under these conditions. HREIMS was carried out on a JEOL HX 110 with a resolution of 10 000. Optical rotation was determined with a JASCO P1020 polarimeter. For normal-phase flash chromatography, silica gel 60 (40−63 μm, Lagand Chemical Co., Inc., Elmhurst, NY) was used. A Büchi medium-pressure chromatography system with Polygoprep RP18 (25−40 μm, Macherey & Nagel, Düren, Germany) was used for reversed-phase chromatography. The HPLC system employed for the isolation of **3** consisted of a Varian 9002 pump, a Varian Star 9040 refractive index detector, and an Alltech econosil RP18 (10 μm) 10 × 250 mm column.

Plant Material. *A. madreporica*, *A. cryptantha*, and *A. compacta* were collected and identified in July 1995 by Gloria Montenegro in Chile at Laguna Dolores, Departamento Colina, Region Metropolitana (*A. madreporica* and *A. cryptantha*), and Farellones, Region Metropolitana (*A. compacta*). Voucher specimens (coll. nos. 0663, 0667, and 0587, respectively) have been deposited at the herbarium of the Pontificia Universidad Católica de Chile, Santiago, Chile. *A. filamentosa*, *A. trifurcata*, *A. crassipes*, *A. monanthos*, and *A. patagonica* were collected and identified in January and February 1995 by Renée H. Fortunato in Argentina at the following locations: 9 km E of Puerto Haberton, Ushuaia, Tierra del Fuego (*A. filamentosa*); 63 km SE of San Julio, Departamento Rio Grande, Tierra del Fuego (*A. monanthos*); Route 40, 1.8 km SE of Route 19, Departamento Lago Argentino, Santa Cruz (*A. patagonica*); on the beach toward Puerto Moat, Ushuaia, Tierra del Fuego (*A. trifurcata*); and 20 km W of Lago Yehuin, Departamento Rio Grande, Tierra del Fuego (*A. crassipes*). Voucher specimens (coll. nos. RF 4838, RF 4906, RF 4943, RF 4837, and RF 4874, respectively) have been deposited at the herbarium of the Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, Buenos Aires, Argentina. Intellectual Property Rights Agreements for plant collections and collaborative research have been fully executed between The University of Arizona and each of the collaborating institutions in this study.

Extractions and Isolation. Air-dried and ground aerial parts of *A. madreporica* (750 g) were extracted five times with petroleum ether to give 15 g of extract, which was fractionated on a silica gel column with hexanes−EtOAc mixtures of increasing polarity. The growth inhibitory activity of the resulting fractions against *M. tuberculosis* was determined at 100 and 33 μg/mL. Compound **1** was isolated from the most active fraction by column chromatography on silica gel with 8% EtOAc in hexane and finally purified on a medium-pressure reversed-phase column with CH_3CN containing 3% water and by crystallization from CH_3CN.

Table 1. ^1H and ^{13}C NMR Data of **1**

H/C	HMQC[a]	^{13}C[b]
1a	1.20	20.5 t
1b	0.78	
2a	1.74	27.8 t
2b	1.08	
3	1.13	59.1 d
4	1.42	31.5 d
5		42.0 s
6a	1.82	33.9 t
6b	1.58	
7a	1.80	37.6 t
7b	1.24	
8		30.5 s
9		26.1 s
10	2.27 (dd, J = 16.8, 7.2 Hz)	48.3 d
11α	0.68 (dd, J = 5.4, 9.8 Hz)	10.5 t
11β	0.04 (dd, J = 5.4, 5.4 Hz)	
12	0.59 (ddd, J = 9.8, 5.4, 1.2 Hz)	25.3 d
13		70.1 s
14a	1.23	31.2 t
14b	1.18	
15a	1.35	35.3 t
15b	0.99	
16	1.24 (s)	30.1 q
17	0.95 (s)	25.1 q
18	0.80 (d, J = 6.6 Hz)	22.6 q
19	0.90 (d, J = 6.6 Hz)	22.5 q
20	0.82 (s)	17.8 q

[a] Multiplicities and coupling constants from 1D-^1H NMR. [b] Multiplicities from DEPT experiment.

Sample Preparation for LC-MS. Air-dried and ground samples of aerial parts (200 g) of all eight investigated species were extracted once with CH_2Cl_2−MeOH (1:1). Four milliliters of hexane was used to prepare hexane-soluble fractions from 100 mg of each of these dried CH_2Cl_2−MeOH extracts. One milliliter of each of these hexane solutions was passed through pasteur pipets half filled with silica gel 60 (63−200 μm, Macherey & Nagel, Düren, Germany). One milliliter of hexane, 1 mL of 10% EtOAc−hexane, and 1 mL of 20% EtOAc−hexane were successively passed through the pipets. Ten microliters of the last wash were used for the LC-MS investigation.

9,12-Cyclomulin-13-ol (1): colorless powder; mp 56−58 °C; $[\alpha]^{25}_D$ +26.1° (*c* 2.0, $CHCl_3$); IR (KBr) 3420, 3065, 2950, 2870, 1634, 1624, 1612, 1465, 1378, 1364, 1158, 1110, 1068, 1028, 922, 898 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) see Table 1; APCI-MS *m/z* (rel int) 273 (100), 217 (24), 203 (14); HREIMS *m/z* 290.2603 ($C_{20}H_{34}O$ requires 290.2610).

Mulin-9(10),12(13)-diene (3). For the preparation of the dehydration product **3**, a solution of 30 mg of **1** in $CDCl_3$ was kept at room temperature until the starting material was not detectable anymore by NMR (2−5 days). Compound **3** was isolated as a colorless oil using semipreparative reversed-phase HPLC with CH_3CN containing 3% water:^1H NMR ($CDCl_3$) δ 5.52 (1 H, m, H-12), 2.66 (1 H, br d, J = 15.3 Hz, H-11a), 2.49 (1 H, dd, J = 15.3, 7.8 Hz, H-11b), 1.65 (3 H, t, J = 1.5 Hz, Me-16), 1.10 and 0.95 (2 × 3 H, s, Me-17, Me-20), 0.88 and 0.85 (6 H, 2 d, J = 6.6 Hz, Me-18 and Me-19); ^{13}C NMR ($CDCl_3$) δ 141.9 (s), 138.6 (s), 132.5 (s), 122.8 (d), 58.6 (d), 43.7 (s), 41.0 (t), 37.7 (s), 37.5 (t), 35.2 (t), 30.5 (t), 30.0 (d), 28.1 (t), 28.1 (q), 27.6 (t), 26.1 (t), 26.0

(q), 23.0 (q), 22.8 (q), 18.5 (q); APCI-MS *m*/*z* (rel int) 273 (100), 217 (24), 203 (14).

Determination of Biological Activity. The crude extract, fractions, and purified compounds were tested for inhibition of *M. tuberculosis* H_{37}Rv ATCC 27294 using the BACTEC 460 system as previously described.[8,9] Percent inhibition was calculated as [1 − (growth index of test sample/growth index of control)] × 100. The minimum inhibitory concentration (MIC) is defined as the lowest concentration that inhibited 99% of the inoculum. Cytotoxicity was assessed against Vero cells (ATCC CCL-81) using the CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI). The IC_{50} is defined as the reciprocal dilution resulting in 50% inhibition of the Vero cells.[8]

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