A new mulinane diterpenoid from the cushion shrub 
Azorella compacta growing in Perú

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INTRODUCTION

Terrestrial flora has formed the basis of our traditional medicine systems and even today invokes tremendous interest in the scientific world. There is an optimism placed on natural products in the search of new drugs because the world of the plants represents an untapped reservoir of natural compounds waiting for us. The Azorella genus is well known for a wide range of secondary metabolites such as diterpenoids, triterpenoids and flavonoids present in several species of the genus.¹¹⁻¹⁷ In particular Andean species of the genus Azorella, Bolax, Mulinum and Laretia are recognized for the production of diterpenoids having unique mulinane and azorellane skeletons.¹¹ These diterpenoids have displayed a wide variety of interesting biological activities, including trypanosomicidal,³ trichomonacidal,³ toxoplasmocidal,⁴ antiplasmodial,⁵ antibacterial,⁶,⁷ antiviral,⁸ spermicidal,⁹ cytoxic,¹⁰,¹¹ antihyperglycemic,¹² antitubercular,¹³,¹⁴ antiinflammatory and analgesic activities.¹⁸,¹⁹ Azorella compacta known as “llareta” is distributed in the Andes mountains of southern Peru, northeastern Chile, Bolivia, southern Ecuador and northwestern Argentina among boulders in the High-Andean belt at altitudes between 3800-4000 and 4800-5200 m. Llareta is a compact cushion shrub of extremely slow growth (only 1 cm in 20 years).²⁰ The Andean people utilized this plant since precolombian times for the treatment of colds, pains, diabetes, asthma, bronchitis, womb complaints, gastric disorders, backache,
wounds, and altitude sickness.\textsuperscript{[21]} As a continuation of our current chemical studies on the \textit{Azorella} genus, we report in this work the isolation of five known diterpenoids along with a new mulinane diterpenoid from a sample of \textit{A. compacta} growing in Perú. Furthermore, the gastroprotective activity of the new isolated diterpenoid against the HCl/ethanol-induced gastric ulcer test in mice and comparison with the known compounds is reported.

**MATERIAL AND METHODS**

**General procedures**

Aluminum-coated silica gel thin layer chromatography (TLC, Kieselgel F254) was developed using $n$-hexane/EtOAc mixtures (1:0; 9:1; 7:3 and 1:1 v/v) as solvent systems while the spots were revealed by spraying the plates with H$_2$SO$_4$-MeOH (5:95, v/v) and heating at 120$^\circ$C. Silica gel (Kieselgel 60, Merck 0.063-0.200 mm) and Sephadex (LH-20) were used for open column chromatography (CC). Technical solvents used in bulk plant extraction were previously distilled and dried according to standard procedures.

**Instrumentation**

A Bruker Avance AM-400 spectrometer equipped with 5 mm probes was used for all NMR experiments. Compounds were dissolved in CDCl$_3$ with tetramethylsilane (TMS) as internal standard. $^1$H-NMR spectrum was obtained at 400.13 MHz;\textsuperscript{[13]} C-NMR spectrum at 100.61 MHz. IR spectra was recorded on a Vector 22 FT-IR spectrometer. Mass spectra were recorded on a MAT 95XP Thermo Finnigan model spectrometer with an accelerating voltage of 3kV and ionization energy of 70 eV. The temperature of the ion source was maintained at 250$^\circ$C. Optical rotations were obtained in CHCl$_3$ on a Polax-2L ATAGO, polarimeter.

**Plant material**

Specimens were collected in 2012 at “Patapampa” (4980 m Arequipa, Perú) and identified as \textit{Azorella compacta} \cite{1} by the expert botanist Prof. Fatima Cáceres. A voucher specimen (Nº AC031112) is kept at the Herbarium of the Departamento de Biología, Universidad Nacional de San Agustín (Arequipa, Perú).

**Extraction and isolation**

The aerial parts of \textit{A. compacta} (400 g) were extracted with DCM (2 l) three times for 7 days. The concentrated DCM extract (10.5 g) was submitted to an open silica gel column (10 × 70 cm) using $n$-hexane/EtOAc mixtures (100:0 to 0:100 v/v) to afford five fractions. Fraction 1 (100% $n$-hexane; 950 mg) was subjected on a Sephadex LH-20 column (7 × 70 cm) using as mobile phase only $n$-hexane to afford \textit{1} (20 mg).

Fraction 2 ($n$-hexane: EtOAc; 8:2; 2.2 g) was permeated through Sephadex LH-20 (7 × 70 cm, $n$-hexane: DCM: MeOH; 3:1:1 v/v/v) to afford two fraction 2A and 2B. Fraction 2A (200 mg) was submitted to a silica gel column (4 × 30 cm) using $n$-hexane/EtOAc mixtures (1:0 to 9:1 v/v) to yield 1 (10 mg) and \textit{2}\textsuperscript{[22]} (mulin-11,13-dien-20-oic acid; 50 mg). Fraction 2B (1.8 g) was rechromatographed on silica gel (4 × 60 cm) using mixtures of $n$-hexane/EtOAc of increasing polarity as eluents (1:0 to 8:2 v/v) to give \textit{2} (1.1 g) and \textit{3}\textsuperscript{[1]} (13α-hydroxyazorellane; 350 mg). Fraction 3 ($n$-hexane: EtOAc; 6:4; 3.5 g) was permeated on Sephadex LH-20 (7 × 70 cm; $n$-hexane: DCM: MeOH; 3:2:1 v/v/v) and then was purified over silica gel using open column chromatography (4 × 60 cm) and $n$-hexane/EtOAc as eluents (0:1 to 0:10 v/v) to yield \textit{3} (100 mg), \textit{4}\textsuperscript{[1]} (mulinic acid; 300 mg), \textit{5}\textsuperscript{[1]} (mulinolic acid; 1g) and \textit{6}\textsuperscript{[1]} (azorellanol; 1.1g). Fraction 4 ($n$-hexane: EtOAc; 3:7; 1.5 g) and fraction 5 (100% EtOAc; 1.0 g) were permeated on Sephadex LH-20 (7 × 70 cm; MeOH). No presence of diterpenoids or other metabolites were observed in this last polar fraction. The chemical structures of the diterpenoids are shown in Figure 2.

**Computational procedures**

Conformational analysis of \textit{1a} and \textit{1b} was carried out using a protocol described in previous publications\textsuperscript{[26,27]} First, a Monte Carlo search was done using the MMFF94 molecular mechanics force field through the SPARTAN 02 program.\textsuperscript{[28]} Next, lower-energy conformers were obtained and then reoptimized using DFT at the B3LYP/6-31G (d) level of theory (GAUSSIAN 09 program).\textsuperscript{[29]} Finally, NOE distances were calculated via GAUSSIAN 09.

**Gastroprotective activity**

**Animals**

Swiss albino mice (30 ± 3 g) were purchased at the Instituto de Salud Pública de Chile in Santiago-Chile. Mice were fed...
RESULTS

The dichloromethane extract of the leaves of *A. compacta* afforded the new muline diterpenoid 1 and the known compounds mulin-11,13-dien-20-oic acid 2, 13α-hydroxyazorellane 3, mulinic acid 4, mulinolic acid 5, and azorellanol 6, by Sephadex LH-20 permeation and open column silica gel chromatography. The structures of the known diterpenoids 2-6 and the new compound 1 were elucidated by comparison of their NMR data with those found in previous works.[3,5,7,22,24]

DISCUSSION

Structural elucidation of the new compound 1

The structure of 1 was mainly elucidated by NMR spectroscopy as follows: The $^1$H-NMR spectrum of 1 indicated resonances for a diene moiety at δ $^1H$ 5.64 (1H, d, J = 12.7, H-12), 5.52 (1H, dd, J = 12.7; 6.4, H-11), 5.48 (1H, d, J = 8.1, H-14) along with four methyl groups at δ $^1H$ 1.80 s, 0.93 d (6.6), 0.87 s, 0.84 d (6.6) and 0.74 s. These data were similar to the resonances of the muline diterpenoids particularly mulin-11,13-dien-20-oic acid, which Loyola et al. earlier reported.[22] The major difference was the presence of an additional methyl group in 1 in place of a carboxylic group in 2. DEPT 135 and $^{13}$C-NMR data confirmed the presence of that methyl group (δ$c$ 11.8) instead of carboxylic acid (δ$c$ 182.1). $^{13}$C-NMR data and low-resolution mass spectrometry indicated the molecular formula $C_{13}H_{24}$ for 1. The structure of 1 was determined as mulin-11,13-diene. 1D and 2D NMR allowed the correct assignments of all 1H NMR signals for compound 1. In the HMBC spectrum, the proton signal at δ $^1H$ 1.33 (H-10) showed long-range correlations with the carbon signals at δ $^1C$ 11.8 (C-20), 24.1 (C-1) and 44.3 (C-5), and the proton signal at δ $^1H$ 0.72 (H-20) had cross peaks with δ $^1C$ 57.8 (C-3), 44.3 (C-5), 35.9 (C-6) and 54.6 (C-10) indicating that δ$c$ 11.8 to be the C-20 signal. The other known signals were deduced by comparison with the spectra of 2.[22] The relative stereochemistry of 1 was assigned on the basis of 2D NOESY experiment. In the NOESY spectrum, correlations between δ $^1H$ 0.87 (H-17) and δ $^1H$ 2.02 (H-9), and δ $^1H$ 0.73 (H-20) and δ $^1H$ 2.02 (H-9) were observed, indicating that they are on the same molecular face of 1. No NOESY correlation was detected between δ $^1H$ 1.33 (H-10) and δ $^1H$ 0.87 (H-17) and δ $^1H$ 0.73 (H-20). Surprisingly, a strong NOE interaction was observed between δ $^1H$ 2.02 (H-9) and δ $^1H$ 1.33 (H-10). The last one data is inconsistent with the relative stereochemistry for muline diterpenoids. To clarify this data, molecular modeling was used to examine the two possible structures of 1, corresponding to the α (1a) and β (1b) orientations of H-10. Conformational distribution using a Monte Carlo search was carried out to

on certified Champion diet with free access to water under standard conditions of 12-h dark-light cycle and at 20°C room temperature. The protocols were followed according to the recommendations of the Canadian Council on Animal Care with the ethical guidelines for investigations in conscious animals and were approved by the Universidad de Chile Animal Use and Care Committee (certificate approved on July 2, 2010 by Dr. Nicolas Giuliani).[30]

The gastroprotective activity of the diterpene 1 was evaluated in the HCl/EtOH-induced lesion model as described previously.[31,32] Mice were randomly distributed into groups of seven animals each and fasted for 12 h with free access to water prior to the experiment. Diterpenoid 1 and lansoprazole were suspended in Tween 80 (1% solution), free access to water prior to the experiment. Diterpenoid 1 and lansoprazole were suspended in Tween 80 (1% solution), and administered intragastrically to mice at a dose of 20 mg/kg in a 10 ml/kg vol. Fifty minutes after administration of 1, lansoprazole or 1% Tween 80 (10 ml/kg), all groups were orally treated with 0.2 ml of a solution containing 0.3 M HCl/60% ethanol solution (HCl/EtOH) for gastric lesion induction. Animals were sacrificed 1 h after the administration of HCl/EtOH; the stomachs were excised and inflated by saline injection (1 ml). The ulcerated stomachs were fixed in 5% formalin for 30 min and opened along the greater curvature. Gastric damage visible to the naked eye was observed in the gastric mucosa as elongated black-red lines, parallel to the long axis of the stomach similar to the HCl/EtOH-induced lesions in rats. The length (mm) of each lesion was measured, and the lesion index was expressed as the sum of the length of all lesions.

Statistical analysis

Results are presented as the mean ± sem. In all experiments, statistical differences between treatments and their respective control were determined by one-way analysis of variance (ANOVA) followed by Dunnet’s pair-wise test. The level of significance was set at $P < 0.01$. GraphPad Prism 4 for Windows was used for all statistical tests.
define stable conformations within a 10 kcal/mol window. Low-energy conformers were then submitted to further optimization using B3LYP functional with a 6-31G(d) basis set. Two optimized structures 1a and 1b were obtained [Figure 3], in which the distances between H-9 and H-10, H-17 and H-20 were calculated. In both structures the distances between H-9 and H-10 led to a cross peak in the NOESY spectrum [see Table 1]. However, the NOESY spectrum of 1 showed the expected cross peaks which are consistent with the configuration of the optimized structure 1a and the relative configuration reported for mulinane-type diterpenoids.[3,5,7,22-24] The spectroscopic and physical data for new compound 1 is presented below.

Mulin-11,13-diene (1): White oil; FT-IR νmax: 3090, 1440, 1260, 1125 cm⁻¹; HREIMS: Calcd. for C27H32 [M+] 272.2504, found: 272.2473; EI-MS: m/z (rel. int. %): C27H32 [M+] 272 [M⁺] (4), 257 (2), 247 (4), 245 (68), 242 (13), 227 (24), 191 (66), 175 (19), 163 (12), 161 (28), 149 (33), 147 (33), 136 (27), 135 (43), 133 (25), 123 (31), 121 (83), 119 (41), 117 (9), 109 (51), 107 (100), 105 (48), 95 (54), 91 (48), 85 (26), 82 (47), 79 (53), 77 (35), 69 (44), 67 (46), 55 (64), 53 (27), 43 (48), 41 (61).[10] H NMR (400.13 MHz, CDCl3); 1.62 m; 1.22 m (H-1); 1.82 m; 1.26 m (H-2); 1.00 m (H-3); 1.50 m (H-4); 1.66 m; 1.40 m (H-6); 2.74 brd (16.9); 1.56 m (H-7); 2.04 dd (6.4; 8.8) (H-9); 1.33 m (H-10); 5.52 dd (6.4; 12.7) (H-11); 5.63 d (12.7) (H-12); 5.48 d (8.1) (H-14); 1.63 m; 1.23 m (H-15); 1.8 s (H-16); 0.87 t (H-17); 0.84 d (6.6) (H-18); 0.92 d (6.6) (H-19); 0.73 s (H-20).[15] C NMR (100.61 MHz, CDCl3); 24.1 t(C-1); 82.5 t (C-2); 57.8 d (C-3); 31.1 d (C-4); 44.3 s (C-5); 35.9 t (C-6); 36.4 t (C-7); 34.9 s (C-8); 48.9 d (C-9); 54.6 d (C-10); 133.7 d (C-11); 127.6 d (C-12); 131.5 s (C-13); 125.5 d (C-14); 39.0 t (C-15); 25.8 q (C-16); 27.3 q (C-17); 23.1 q (C-18); 22.4 q (C-19); 11.8 q (C-20).

Gastroprotective activity
The effect of mulin-11,13-diene 1 on the model of gastric ulcers induced by HCl/EtOH in mice is shown in Table 2. A single oral administration of 1 and lansoprazole at 20 mg/kg inhibited the appearance of gastric lesions by 18% and 68%, respectively.

The imbalance between aggressive (chemical agents, gastric acid, pepsin, bile, lysolecithin, pancreatic enzymes, stress and drugs) and protective factors (mucus, PGs, bicarbonate, fast epithelial cell renewal, sulfhydryl compounds and gastric blood flow) in the gastric mucosa may lead to gastric damage.[33] On the other hand, various natural compounds from medicinal plants are a source of gastroprotective agents.[34-37] In a previous study, several mulinine and azorellane diterpenoids showed gastroprotective activity at 20 mg/kg on the HCl/EtOH-induced gastric lesion model in mice.[38] Among the compounds studied, azorellanol (6), 13β-hydroxyazorellane and mulin-11,13-dien-18-acetoxy-16,20-dioic acid showed the greater gastroprotective effect, being as active as lansoprazole and reducing the gastric lesions by at least 69%. The same study demonstrated that in the mulinine diterpenoid series, the best activity was at least 69%. The same study demonstrated that in the mulinine diterpenoid series, the best activity was at least 69%.

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lansoprazole reducing the gastric lesions by 18%. Clearly, the same trend was observed for the gastroprotective activity when compared to the natural compounds mulin-11,13-dien-20-ol (26%), mulin-11,13-dien-20-oic acid (39%) and mulin-11,13-dien-18-acetoxy-16, 20-dioic acid (73%). Therefore, according to the results, it is suggested that the gastroprotective effect increases with the degree of oxidation at both carbons C-16 and C-20 in mulinane diterpenoids.

**CONCLUSION**

To the best of our knowledge, no previous studies on the chemistry of *A. compacta* samples growing in Perú were published, thus this is the first report of secondary metabolites from the Peruvian plant and the presence of the new mulinane compound 1 in the leaves, which was fully elucidated using NMR and molecular modeling techniques. Furthermore, the gastroprotective activity against ulcers in mice was tested for this new compound which was low (18%) compared to the positive control lansoprazole (68%) and other known mulinanes isolated which indeed confirm the role of an oxygenated group (OH or carboxylic acid) at C‑16 and other known mulinanes isolated.

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