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# Mulinane-type diterpenoids from *Azorella compacta* display antiplasmodial activity

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**Abstract**

Two mulinane-type diterpenoids were isolated from *Azorella compacta*; namely 20-hydroxymulin-11,13-dienyl acetate and 13,14-dihydroxymulin-11-en-20-oic acid. The structures were elucidated by analysis of their spectroscopic data. These compounds, as well as three previously isolated diterpenes, were evaluated as potential in vivo growth inhibitors of *Plasmodium berghei* NK 65 on infected mice at an intraperitoneal dose of 10 mg/kg/day. Sixty percent and forty-two percent growth inhibition were obtained with 17-acetoxymulin-11,13-dien-20-oic acid and 13, 14-dihydroxymulin-11-en-20-oic acid, respectively.


*Keywords:* *Azorella compacta*; Umbelliferae; Diterpenoids; *Plasmodium berghei*

## 1. Introduction

Previous chemical work on *Azorella* (Apiaceae) has shown that this genus accumulates diterpenes with mulinane and azorellane skeleta (Loyola et al., 1997a,b, 1998a,b). *Azorella compacta* is a compact resinous cushion shrub that grows in the Andes of Peru, Bolivia, Argentina and Chile and has been used in folk medicine. The common name "llareta" is used for several species of the genus *Azorella* (Wickens, 1995). We have studied a recently collected sample of *A. compacta* in continuing our investigation on the biological effects of secondary metabolites from Apiaceae and to obtain larger amounts of bioactive diterpenes. In this communication, we report the isolation and spectroscopic characterization of two new diterpenes and provide evidence for in vivo biological activities of mulinane-type diterpenes from *A. compacta* against *Plasmodium berghei*.

## 2. Results and discussion

The petroleum ether extract of the aerial parts of *A. compacta* was subjected to open column chromatography on silica gel, using increasing proportions of ethyl acetate in petrol ether to afford new compounds ***1*** and ***2***.

The $^{13}C$ NMR data for ***1*** (multiplicities of the carbon signals shown in Table 1 were determined from the DEPT (45°, 90° and 135°) spectra) revealed the presence of one carbonyl, two $sp^3$ quaternary carbons, two olefinic linkage, four $sp^3$ methines, six methylenes (one of them substituted by an heteroatom) and five methyl groups. The total of 22 carbons suggested the presence of acetylated diterpenes. This, together with accurate mass spectral measurements (HREIMS: requires 330.2557, found 330.2559) indicated that the molecular formula $C_{22}H_{34}O_2$ had six sites of unsaturation. The $^1H$

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Table 1
$^{13}C$ (125 MHz) and $^{1}H$ (500 MHz) NMR spectroscopic data for compound **1**

| Number of C/H | $\delta_C$[a] | $\delta_H$[b] | NOESY | HMBC |
|---|---|---|---|---|
| 1 | 23.1 ($CH_2$) | β 0.98 *m* | | |
| | | α 1.56 *m* | | |
| 2 | 28.0 ($CH_2$) | β 1.66 *m* | | |
| | | α 1.20 *m* | | |
| 3 | 57.6 (CH) | 0.85 *m* | | |
| 4 | 31.3 (CH) | 1.46 *m* | | |
| 5 | 46.5 (C) | | | |
| 6 | 30.4 ($CH_2$) | β 2.07 *m* | | 8, 7, 5, 10, 20 |
| | | α 1.14 *m* | | |
| 7 | 38.7 ($CH_2$) | β 1.59 *m* | | |
| | | α 1.17 *m* | | |
| 8 | 34.6 (C) | | | |
| 9 | 48.8 (CH) | 1.92 *dd* (6.3, 11.9) | | 17, 8, 15, 5, 10, 12, 11 |
| 10 | 53.6 (CH) | 1.33 *dq* (7.4, 11.9) | | |
| 11 | 132.5 (CH) | 5.36 *dd* (6.3, 12.5) | | 8, 9, 13 |
| 12 | 127.4 (CH) | 5.65 *d* (12.5) | | 16, 9, 14, 13, 11 |
| 13 | 135.5 (C) | | | |
| 14 | 125.3 (CH) | 5.45 *d* (8.4) | | 16, 8, 12 |
| 15 | 36.3 ($CH_2$) | β 1.50 *m* | | 17, 8, 14, 13 |
| | | α 2.66 *d* (17.0) | 10, 7α | |
| 16 | 25.5 ($CH_3$) | 1.78 *s* | | 14, 12, 13 |
| 17 | 26.9 ($CH_3$) | 0.94 *s* | | 8, 7, 9, 15 |
| 18 | 22.6 ($CH_3$)* | 0.81 *d* (6.4) | 6β | 19, 4, 3 |
| 19 | 23.5 ($CH_3$)* | 1.02 *d* (6.4) | | 18, 4, 3 |
| 20 | 61.5 ($CH_2$) | 4.27 and 3.89 *d* (11.9) | 17, 9, 7β, 1β | 6, 5, 10, 3, C=O |
| 21 | 169.7 (C) | | | |
| 22 | 20.1 ($CH_3$) | 1.70 s | | |

Spectra taken Bz-$d_6$.
[a] By DEPT sequence.
[b] Coupling constants ($J$ in Hz) in parentheses.
* Interchangeable.

NMR spectrum of ***1*** (Table 1) showed signals corresponding to olefinic protons at $\delta$ 5.36 (1H, *dd*, $J = 6.3$, 12.5), 5.65 (1H, *d*, $J = 12.5$), a doublet at $\delta$ 5.45 (1H, $J = 8.4$) and a singlet at $\delta$ 1.78 (3H). The $^{13}C$ NMR spectra data also confirmed the existence of two double bonds (one being *cis* disubstituted and the other trisubstituted) in a homoannular system: $\delta$ 132.5 (CH), 127.4 (CH), 135.5 (C) and 125.3 (CH). Two doublets at $\delta$ 4.27 and 3.89 (each 1H with $J = 11.9$) were attributed to geminal protons on a carbon atom carrying an acetate. This was confirmed by analysis of the IR data (1.688 $cm^{-1}$) and from the presence of corresponding $^{13}C$ NMR signals at 61.5 (CH), 169.7 (C) and 20.1 ($CH_3$), [$\delta_H$ 1.70 (3H, *s*)]. The $^{1}H$ and $^{13}C$ NMR spectra of ***1***, together with analysis of $^{1}H$ COSY, HMQC and HMBC experiments, revealed the presence of an isopropyl group [$\delta_C$ 31.3 (CH), 22.6 ($CH_3$) and 23.5 ($CH_3$); $\delta_H$ 1.46 overlapped signal, 0.81 *d* and 1.02 *d* ($J = 6.4$ Hz in both signals)]. The similarities observed in the $^{13}C$ NMR and $^{1}H$ NMR spectra of ***1*** and mulin-11,12-dien-20-oic acid (Loyola et al., 1997b), were indicative of the presence of a mulinane diterpenoid moiety in ***1***. We assumed, by analogy with other mulinane diterpenoids (Loyola et al., 1990, 1997a,b, 1998a) that the substituents at C-3, C-5 and C-8 were β orientated as well as the proton at C-9. Additional proof of the relative stereochemistry of ***1*** was obtained from ROESY NMR spectroscopy experiments. Thus, there was a correlation between the signal at $\delta_H$ 2.66 (H-15α) and the signals at $\delta$ 1.33 (C-10) and 1.17 (C-7α). The methyl group which appeared at $\delta$ 0.81 (C-18) was correlated with H-6β ($\delta$ 1.59). Finally, the doublets at $\delta$ 4.27 and 3.89 (C-20) showed correlations with H-17 ($\delta$ 0.94), H-9 ($\delta$ 1.92), H-7β($\delta$ 1.59) and H-1β($\delta$ 0.98). Also, no ROESY cross-peak was found between H-10 and $CH_2$-20, which is in agreement with a *trans* configuration at this ring junction. Hence structure ***1*** was assigned as 20-hydroxymulin-11,13-dienyl acetate.

The IR spectrum of ***2*** exhibited absorptions due to hydroxyl and carboxylic functionalities (3325 $cm^{-1}$, broad and 1710 $cm^{-1}$, strong). The $^{1}H$ and $^{13}C$ NMR spectra of ***2*** suggested the presence of an isopropyl group, two primary methyl, one disubstituted olefine, five methylenes, four methines, one oxygen-bearing methine, two quaternary carbons, one oxygen-bearing quaternary carbon and one carboxylic acid (see Table 2). These spectroscopic data are similar to those of mulinolic acid ***3*** (Loyola et al., 1996). In fact, the main difference is the presence of an additional hydroxy group in ***2***. This suggested that ***1*** and ***2*** were structurally related to each

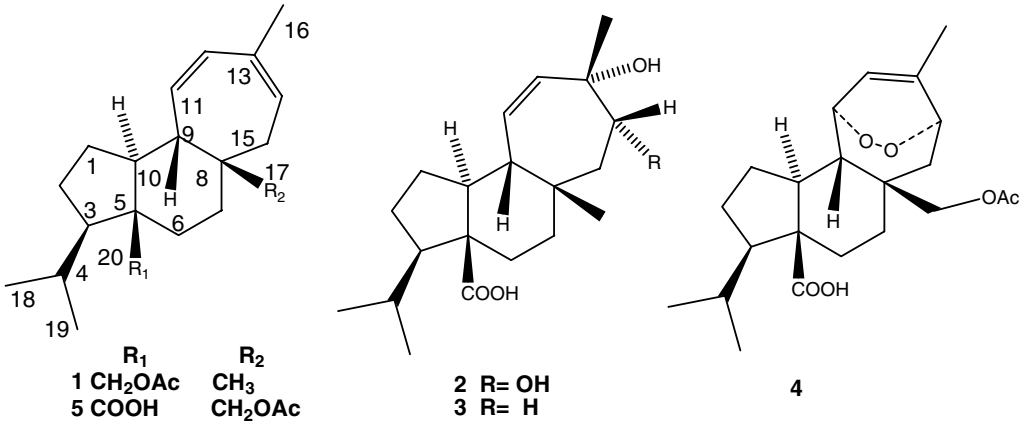
Table 2
$^{13}C$ (125 MHz) and $^{1}H$ (500 MHz) NMR spectroscopic data for compound **2**

| Number of C/H | $\delta_C$[a] | $\delta_H$[b] | NOESY | HMBC |
|---|---|---|---|---|
| 1 | 29.7 ($CH_2$) | β 1.75 *m* | | |
| | | α 1.58 *m* | | |
| 2 | 25.6 ($CH_2$) | β 2.29 *dq* (7.0, 11.6) | 15β | 1 |
| | | α 1.86 *m* | | |
| 3 | 57.5 (CH) | 1.21 *dd* (10.0, 15.5) | | 1, 5, C=O |
| 4 | 32.4 (CH) | 1.62 *m* | | |
| 5 | 58.4 (C) | | | |
| 6 | 33.3 ($CH_2$) | β 2.61 *dt* (3.2, 12.7) | 18, 7β | 8 |
| | | α 1.54 *m* | | |
| 7 | 43.2 ($CH_2$) | β 1.78 *m* | | 5 |
| | | α 1.46 *m* | | |
| 8 | 36.6 (C) | | | |
| 9 | 47.6 (CH) | 2.41 *dd* (8.4, 11.6) | 17, 7β | 8, 15, 10, 11, 12 |
| 10 | 51.1 (CH) | 2.71 *dq* (8.4, 11.9) | 3, 6α, 2α, 15α | 2, 9, C=O |
| 11 | 134.2 (CH) | 5.77 *dd* (8.3, 12.6) | | 8, 3, 12 |
| 12 | 135.3 (CH) | 5.69 *d* (12.6) | | 16, 9, 13 |
| 13 | 72.8 (C) | | | |
| 14 | 72.4 (CH) | 3.93 *dd* (2.8, 11.3) | 17, 16, 15β | |
| 15 | 41.6 ($CH_2$) | β 1.56 *m* | | 17, 14 |
| | | α 3.12 (*dd* 11.3, 13.5) | 10 | |
| 16 | 30.6 ($CH_3$) | 1.65 *s* | | 14, 12 |
| 17 | 28.3 ($CH_3$) | 1.01 *s* | | 8, 7, 15, 9 |
| 18 | 23.1 ($CH_3$)* | 1.05 *d* (6.4) | | 4, 3 |
| 19 | 22.8 ($CH_3$)* | 0.71 *d* (6.4) | | 4, 3 |
| 20 | 177.1 (C) | | | |

Spectra taken Py-$d_5$.
[a] By DEPT sequence.
[b] Coupling constants ($J$ in Hz) in parentheses.
* Interchangeable.

other and to other diterpenoids isolated from the same source. The HREIMS of ***2*** had an ion corresponding to $C_{20}H_{30}O_3$ (requires $m/z$ 318.2195, found 318.2194) indicating the loss of water from $C_{20}H_{32}O_4$ with the $^{1}H$ NMR and $^{13}C$ NMR spectra also in agreement. Compared with the $^{13}C$ NMR spectrum of ***3***, the most significative differences were in the chemical shifts of the cycloheptene ring with upfield shifts for C-16 and C-12 and downfields shifts for C-13 and C-15, attributable to a C-14 hydroxyl group. Additional proof of the location of the secondary hydroxy group of ***2*** was obtained from HMBC experiments. A long range correlation of the proton of H-15β at $\delta$ 1.56 with C-17 and C-14 was observed. Analogously, H-16 protons at $\delta$ 1.65 correlated with C-14 and C-12 and H-17 protons at $\delta$ 1.01 correlations with C-8, C-7, C-9 and C-15. The relative stereochemistry of ***2*** was deduced from analysis of ROESY NMR experiment which showed a correlation between the signal at $\delta$ 2.41 (H-9) and H-17 and H-7β ($\delta$ 1.01 and 1.78, respectively). In addition, H-10 gave a correlation with the signals at 1.21 (H-3), 1.54 (H-6α), 1.86 (H-2α) and 3.12 (H-15α). The α orientation of the hydroxyl group at C-14 was also deduced by the correlation between H-14 ($\delta$ 3.93) and H-16 ($\delta$ 1.65); H-15β ($\delta$ 1.56) and H-17 ($\delta$ 1.01). Hence, the relative stereochemistry is that shown in ***2*** and corresponds to 13,14-dihydroxymulin-11-en-20-oic acid. The acetyl derivative of ***2*** has been recently published (Chiaramello et al., 2003).

As a worldwide public health problem, malaria requires the urgent search for, and identification of, new drugs. Bioactive compounds of vegetal origin and some derivatives are presently most efficient in coping with this serious crisis (Bustos et al., 1994; Hoffman, 1996; Price et al., 1998). The secondary metabolism of *Azorella compacta* has provided us with various mulinane-type diterpenes (Loyola et al., 1997a,b, 1998b) and their effect on parasitemia were investigated. Five mulinane-type diterpenes were evaluated as potential in vivo growth inhibitors of *Plasmodium berghei* NK 65 on infected mice at an intraperitoneal dose of 10 mg/kg/day. The effect of compounds ***2***, ***4***, ***5***, ***3*** and ***1*** on the parasite erythrocytic life cycle, was 42%, 26%, 60%, 25% and 29% growth inhibition, respectively. Chloroquine, a more intensively studied antiplasmodial drug, has been reported to have an $IC_{50}$ of 2.5 mg/kg/day (Baelmans et al., 2000). Based on these encouraging results, studies on 50% inhibitory concentration ($IC_{50}$), cytotoxicity and other side effects on the animal model are under consideration for the most promising bioactive compounds. This report, nevertheless provides the first evidence for antiplasmodial activity of mulinane-type diterpenoids isolated from natural sources.




## 3. Experimental

### 3.1. General

Mp. uncorr. Optical rotations were measured on a Perkin–Elmer model 241 polarimeter using a Na lamp at 25 °C. IR spectra were obtained with a Perkin–Elmer 1650/FTIR spectrometer in KBr and $CHCl_3$ solution. EIMS and HREIMS spectra were taken on a Micromass Autospect spectrometer. $^1H$ NMR, $^{13}C$ NMR, HMQC, HMBC, 2D ROESY and COSY spectra were measured employing a Bruker AMX 500 instrument operating at 500 MHz for $^1H$ NMR and at 125 MHz for $^{13}C$ NMR, using TMS as internal standard. Two-dimensional spectra were obtained with the standard Bruker software.

### 3.2. Plant material

Plant material was collected in January, 2001 at "Quebrada de las llaretas", Vallenar, Chile. It was identified by Professor C. Marticorena and voucher specimens were deposited at the Herbarium of Universidad de Concepción, Concepción, Chile.

### 3.3. Extraction and isolation of diterpenoids

Dried and finely powdered whole plant of *A. compacta* (3.0 kg) were extracted with petroleum ether at room temperature. After filtration, the solvent was evaporated in vacuo (and low temperature) yielding a gum (220 g). The concd. petrol ext. was adsorbed on silica gel (300 g) and slurried onto the top of a column containing silica gel (2.0 kg) in petrol, and eluted with a P–EtOAc gradient with increasing amounts of EtOAc to produce six fractions. Fraction 2 (100 g) eluted with P/EtOAc (18:2) was further separated and purified by silica gel column chromatography (P/EtOAc, 19:1) to give: ***1*** (0.5 g), ***2*** (0.6g).

#### 3.3.1. 20-Hydroxymulin-11, 13-dienyl acetate (*1*)

Colorless oil. $[\alpha]_D^{25} + 89.28$ ($CHCl_3$, *c* 0.168); IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3450, 2880, 1688, 1386, 1371, 1142. For $^1H$ and $^{13}C$ NMR spectra, see Table 1. HREIMS *m/z* $[M]^+$ 330.2559 ($C_{22}H_{34}O_2$ 330.2557), $[M-CH_3]^+$ 315.2398 ($C_{21}H_{31}O_2$ requires 315.2324), EIMS (70 eV) *m/z* rel. Int.): 330 (30), 315 (26), 270 (47), 255 (54), 227 (94), 145 (47), 134 (83), 119 (97), 105 (100), 91 (82), 81 (57), 55 (65).

#### 3.3.2. 14-Dihydroxymulin-11-en-20-oic acid (*2*)

Amorphous solid. $[\alpha]_D^{25}$ – 60 (MeOH, *c* 0.10); IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3325, 1710, 1520, 1250, 1020. For $^1H$ and $^{13}C$ NMR spectra, see Table 2. HREIMS *m/z* 318.2194 $[M-H_2O]^+$ ($C_{20}H_{30}O_3$ requires *m/z* 318.2195); EIMS (70 eV) *m/z* rel. Int.: 318 (19), 300 (12), 272 (21), 255 (18), 229 (32), 221 (36), 175 (73), 147 (28), 119 (37), 105 (68), 91 (100), 79 (60), 55 (62).

### 3.4. In vivo antimalarial activity

The activities against *Plasmodium berghei* NK 65 of diterpenoids ***1–5*** isolated from *A. compacta* were determined by the four days suppressive test as previously described (Peters and Robinson, 1992). Swiss male mice (Charles River, France; $20 \pm 2$ g) were infected intraperitoneally with $10^8$ parasitized erythrocytes in 0.9% saline on day 0. A daily treatment dose of the pure compound (10 mg/kg/day) was given intraperitoneally to groups of five mice from day 0 to day 3. Each test included a positive control with 5 mg chloroquine/kg (Sigma, USA) with control animals administered the appropriate solvent (DMSO or Tween). On day 4, the malaria-suppressive effect of each compound under evaluation was estimated by examination of Giemsa-

stained thin blood smears made from each mouse tail blood. At least 9000 red blood cells were examined under 1000-fold magnification and percentage of parasitized cells was determined for each compound. Percentage of parasitemia inhibition was calculated as

$$\frac{(\text{parasitemia in control} - \text{parasitemia with drug})}{\text{parasitemia in control}} \times 100 = \% \text{ of inhibition.}$$

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