Phytochemistry Letters xxx (2012) xxx–xxx

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Phytotoxic halimanes isolated from *Baccharis salicifolia* (Ruiz & Pad.) Pers.

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ARTICLE INFO

Article history:
Received 18 November 2011
Received in revised form 21 December 2011
Accepted 6 February 2012
Available online xxx

Keywords:
Baccharis salicifolia
Halimane
Germination inhibition
Growth inhibition
Panicum miliaceum
Raphanus sativus

ABSTRACT

From the EtOH extract of the medicinal native plant, *Baccharis salicifolia*, two novel halimane-type diterpenoids, salicifolic acid (**1**) and 5-hydroxy-6-hydro-salicifolic acid (**2**) together with the known compounds sakuranetin (**3**), apigenin (**4**) and scopoletin (**5**) were bioguided isolated against *Panicum miliaceum* (monocotyledonous). The structures of **1** and **2** were established by extensive spectroscopic analyses. The effective concentration for 50% inhibition of germination (EC_{g50}) and the root (EC_{r50}) and shoot (EC_{s50}) elongations was determined for **1**–**5** against *P. miliaceum* and *Raphanus sativus* (dicotyledonous). Compound **2** was the most active in the inhibition of germination of *P. miliaceum* (EC_{g50} = 1 mM), followed by **1**, **5** and **3**, although **1** was the most effective in regulating the growth of *P. miliaceum* seedlings, with a EC_{r50} and EC_{s50} values of 1.8 and 6.6 mM, respectively. Compounds **1** and **3** were the only samples capable of inhibiting the germination of *R. sativus*, while seedling development was affected by **1**, **2**, and **3** with different effectiveness.

1. Introduction

In a search for plants with high allelopathic potential, 101 native plant species of Central Argentina (Palacios et al., 2007, 2010) were screened, and *Baccharis salicifolia* (Ruiz & Pad.) Pers. (Asteraceae) proved to be the most promising species, due to its highly effective inhibition of the germination (IG) and seedling growth of *Avena sativa* L. (monocotyledonous) and *Raphanus sativus* L. (dicotyledonous) (Palacios et al., 2010). *B. salicifolia* is a resinous medicinal plant that grows as a shrub up to 2 m. It is very resistant to extreme climate conditions and is widely distributed in North and Central Argentina (Barboza et al., 2006) as well as in other parts of Latin America (Loayza et al., 1995).

The phytochemistry of the *Baccharis* genus has been studied for many years by different authors. Particularly, in *B. salicifolia*, 15 flavones, 7 flavanones (Zdero et al., 1986), 17 labdanes (Gonzaga Verdi et al., 2005; Jakupovic et al., 1990), 3 clerodanes (Zdero et al., 1986), 2 triterpenes (Gonzaga Verdi et al., 2005), phytoesterols (Domínguez et al., 1972), mono- and sesquiterpenes (García et al., 2005), and cadinene derivates (Zdero et al., 1986) have been described.

We have found only one report (Céspedes et al., 2006) on the phytotoxicity of *B. salicifolia* where the germination inhibition against *Lactuca sativa* of some flavonoids isolated from this plant (quercetin, apigenin and naringenin) was described. The most effective compound was quercetin with activity in the range of 15–40 ppm (Céspedes et al., 2006).

B. salicifolia is used for the ethnopharmacological treatment of several chronic illnesses. The green leaves/leafy branches are used as anti-inflammatory agents, against diarrhea, dysentery, respiratory illnesses and gynecological disorders (Barboza et al., 2006; Ladio and Lozada, 2009).

In the present study, we identified phytotoxic compounds from the ethanol extract of *B. salicifolia*, through bioguided isolation of those chromatographic fractions which inhibited germination of *Panicum miliaceum*. Subsequently, we determined the EC_{50} for germination and seedling growth for each compound in a mono- as well as a dicotyledonous species.

2. Results and discussion

After liquid–liquid partition of the ethanol extract of *B. salicifolia* (yield 10.2%), the organic layer was submitted to flash silica gel column chromatography, and the major activity was separated into three hexane/EtOAc (35:65) eluted fractions (IG% = 94–100). Then, these fractions were further separated by silica gel column chromatography and the active fractions yielded

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doi:10.1016/j.phytol.2012.02.001

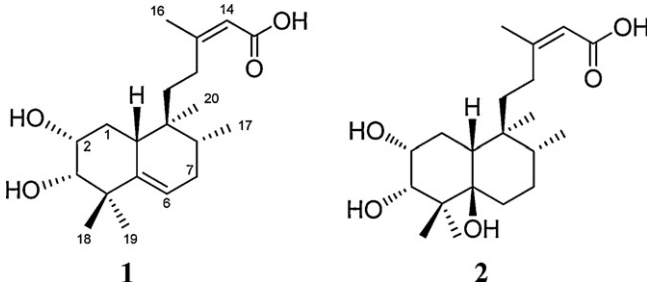

Fig. 1. Structures of salicifolic acid (**1**), 5-hydroxy-6-hydro-salicifolic acid (**2**).

two new halimanes (**1**, **2**) (Fig. 1) together with two known flavonoids (**3**, **4**) and a coumarin (**5**).

Compound **1** was isolated as white crystals and had the molecular formula $C_{20}H_{32}O_4$ as determined by HR-TOF-MS (ESI positive) at *m/z* 359.2179 $[M+Na]^+$. The ^1H NMR spectrum (Table 1) showed signals for a secondary methyl group at δ_H 0.85 (*d*, *J* = 6.8 Hz, H_3-17) and four tertiary resonances at δ_H 1.87 (*s*, H_3-16); 1.06 (*s*, H_3-18), 0.97 (*s*, H_3-19) and 0.60 (*s*, H_3-20). The ^{13}C NMR data (Table 1) for **1** showed 20 carbon signals and, based on DEPT and HSQC spectra, we assigned the signals as follows: two oxygen-bearing methines at δ_C 67.7 (C-2) and δ_C 78.7 (C-3); four olefinic carbons at δ_C 143.0 (C-5), 118.2 (C-6), 160.7 (C-13) and 116.6 (C-14), together with a carboxylic group at δ_C 167.6 (C-15). The high field data were assigned to four methylene, two methine and two quaternary carbons, resonating at 41.5 (C-4) and 37.3 (C-9). With the aid of 2D NMR experiments, such as COSY, HSQC, and HMBC, and comparisons with a previously reported halimane structure (Hara et al., 1995), these spectroscopic data culminated in the structure of **1** which was given the trivial name of salicifolic acid.

Crystallization of compound **1** via the slow evaporation of a methanol solution yielded white crystals suitable for X-ray crystallography. The structure obtained matched the structure deduced from the above NMR data and revealed the 2*R*, 3*S*, 8*R*, 9*S* and 10*R* configuration of the asymmetric carbons (Fig. 2A).

Table 1
^1H and ^{13}C NMR assignments[a] for **1** and **2** (DMSO-d_6).

Position	**1**		**2**	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1a[b]	1.38, m	28.9	1.37, m 1.45, m	26.2
1b[c]				
2	3.84, dt (3.6,10, 2,4)	67.7	3.80, m	68.6
3	3.22, bs	78.7	3.31, d (3.2)	78.3
4	–	41.5	–	38.8
5	–	143.0	–	76.3
6a	5.37, d (5.6)	118.2	1.16, d (13.2)	32.3
6b			1.61, dd (3.2, 13.2)	
7a	1.72, m	31.8	1.28, m	26.9
7b	1.81, t (3.6)			
8	1.45, m	33.6	1.39, m	36.2
9	–	37.3	–	40.6
10	2.37, m	37.7	1.76, d (11.2)	38.5
11a[b]	1.38, m	34.9	1.23, d (9.6)	37.1
11b[c]	1.54, m		1.45, m	
12a	2.33, td (5.6, 12.0)	26.7	2.14, dd (4.4, 12.0)	27.3
12b	2.68, td (4.4, 12.0)		2.66, td (3.6, 12.0)	
13	–	160.7	–	160.5
14	5.58, d (0.8)	116.6	5.56, d (1.2)	116.7
15	–	167.6	–	167.4
16	1.87, s	25.2	1.85, s	25.4
17	0.85, d (6.8)	15.3	0.77, d (6.0)	16.5
18	1.06, s	26.4	1.09, s	21.8
19	0.97, s	28.4	0.97, s	17.5
20	0.60, s	16.3	0.66, s	18.7

[a] Diastereotopic protons designated by a and b (unknown stereochemistry).
[b] Assignments may be interchanged in **1**.
[c] Assignments may be interchanged in **2**.

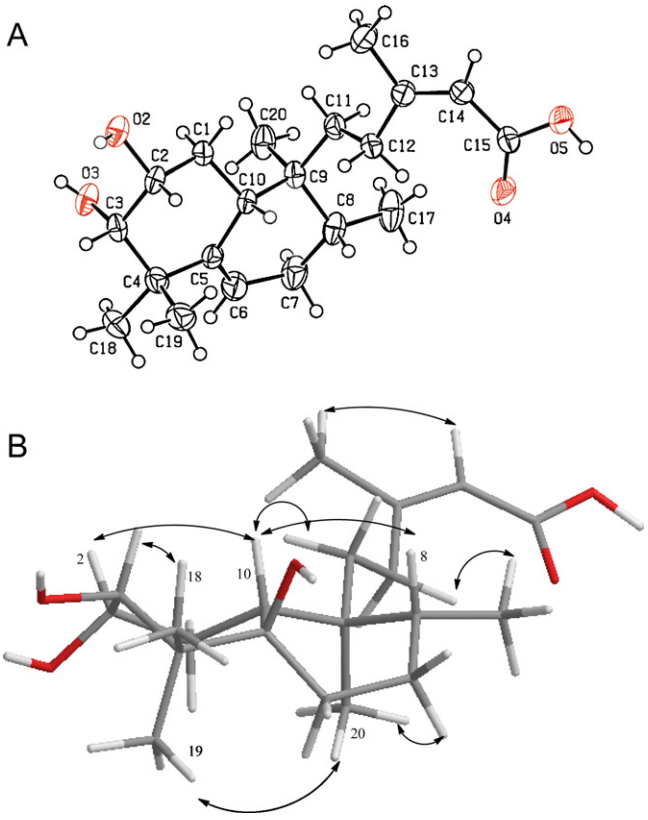

Fig. 2. (A) ORTEP view of the compound **1**. Ellipsoids are drawn at the 40% level of probability. (B) Relative configuration of compound **2**. Arrows indicate the observed ROESY correlations.

Compound **2** was isolated as white crystalline rosettes, and its molecular formula was established as $C_{20}H_{34}O_5$ by HR-TOF-MS (ESI positive), *m/z* 377.2291 $[M+Na]^+$. Its IR spectrum revealed the presence of a hydroxyl (3382 cm^{-1}) and a carbonyl (1683 cm^{-1}) group. The ^1H NMR spectrum (Table 1) was quite similar to that of compound **1**; ^{13}C NMR data (Table 1) for **2** also showed 20 carbon signals where the major differences were one oxygen-bearing quaternary carbon at δ_C 76.3 and a CH_2 at δ_C 32.3 assigned to C-5 and C-6, respectively, in place of the C-5–C-6 double bond of **1**. In the HMBC spectrum, the hydroxylated methine carbon at δ 78.3 (C-3) showed correlations with H-18, H-19, H-1, and H-2, whereas the quaternary hydroxylated carbon at δ 76.3 (C-5) revealed $^3J_{C-H}$ interactions with H-3, H-18, and H-19 and $^2J_{C-H}$ interactions with H-10. Thus, the quaternary carbon C-4, bearing two methyl groups, was linked to C-3 and C-5. On the basis of the HMBC correlations, the locations of three hydroxyl groups at C-2, C-3 and C-5 were also confirmed. Besides, the COSY correlation between H-2 and H-3, confirmed that assignment. The quaternary carbon at δ 40.6 (C-9) displayed $^3J_{C-H}$ interactions with H-1, H-7, and H-17. The analyses of other 2D NMR experiments, and comparisons with previously reported halimanes (Chen et al., 2000), suggested the structure of **2** (Fig. 1). The trivial name 5-hydroxy-6-hydro-salicifolic acid was assigned to this compound.

Contrary to the crystallization ability of **1**, crystals of **2** obtained from methanol were not suitable for RX diffraction studies. In consequence, the relative configuration of **2** was proposed according to ROESY experiment data (Fig. 2B). The ROESY correlations of H-10 to H-2, H-11, H-6b and H-8, together with the correlation of H-8 to H-12, suggest that H-10, H-2, H-6 and H-8 have the same spatial orientations, while the C-11-C-12 side chain

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Table 2
Inhibition of germination and seedling growth of the isolated compounds against *Panicum miliaceum* and *Raphanus sativus*.

Compound	ECg_{50} (mM)	ECr_{50} (mM)	ECs_{50} (mM)
Panicum miliaceum			
1	1.9 (1.5–2.4)	1.8 (1.4–2.2)	6.6 (5.0–8.7)
2	1.0 (0.7–1.3)	2.0 (1.6–2.5)	>14
3	6.3 (2.6–15.2)	>14	>14
4	>14	>14	>14
5	2.7 (2.3–3.2)	2.3 (1.7–3.3)	>14
Dicamba[a]	0.04 (0.03–0.06)	0.04 (0.03–0.05)	8.1×10^{-3} (6.3×10^{-3} to 0.01)
Raphanus sativus			
1	4.6 (3.7–5.9)	4.5 (3.7–5.5)	4.4 (3.7–5.2)
2	>14	1.5 (1.1–2.1)	3.9 (3.3–4.6)
3	11.5 (6.9–19.2)	>14	9.6 (6.2–14.9)
4	>14	>14	>14
5	>14	>14	>14
Dicamba[a]	2.9 (2.4–3.7)	0.04 (0.02–0.06)	1.5×10^{-4} (6.1×10^{-5} to 3.9×10^{-4})

[a] The commercial herbicide Banvel® (Novartis) was used.

is also on the same face of the molecule. These correlations, together with the correlation of H-20 to H-7 and the correlation between H-20 and H-19, which was reported for a *cis* A/B ring junction of the bicyclic diterpenoid nucleus (Chen et al., 2000), suggested the proposed structure. Other correlations, such as H-3 to H-18 and H-17 to H-12, support the configuration suggested in Fig. 2B. The chemical shift of H-14 (δ = 5.56) and of H-16 (δ = 1.85), and the ROESY correlation of H-16 to H-14 and H-11, suggested a *Z* configuration for the double bond.

Compounds **3** and **4** were flavonoids and according to their ^{1}H and ^{13}C NMR spectra and 2D NMR experiments, they were assigned as sakuranetin (Vasconcelos et al., 1998) and apigenin (Nakasugi and Komai, 1998), respectively. Compound **5** was assigned as scopoletin, according the ^{1}H and ^{13}C NMR spectra and HMBC experiments (Carpinella et al., 2005). Compounds **3** and **4** were previously isolated from *B. salicifolia* (Zdero et al., 1986).

The phytotoxic activities of compounds **1**–**5** were evaluated on *P. miliaceum* and *R. sativus* seeds. The effective concentrations for 50% inhibition of germination (ECg_{50}) and 50% inhibition of elongation of root (ECr_{50}) and shoot (ECs_{50}) tissues were determined for both test species. Compound **2** was the most active inhibitor of germination of *P. miliaceum*, followed by **1**, **5** and **3**, in descending order, although **1** was the most effective in regulating the growth of *P. miliaceum* seedlings (Table 2). A commercial herbicide containing dicamba as active ingredient, was simultaneously assayed showing an ECg_{50} and ECr_{50} of 0.04 and 0.04 mM, respectively, against *P. miliaceum*. This herbicide was 25 and 50 more effective than **2** for inhibition of the germination and root elongation, respectively, of the monocot.

Compounds **1** and **3** were the only compounds capable of inhibiting the germination of *R. sativus* (Table 2). Root growth was inhibited by **1** and **2**, while **1**, **2**, and **3** inhibited shoot growth (Table 2). Dicamba was 1.5 and 118 times more active than **1** in the inhibition of germination and root growth of *R. sativus*, respectively, while the former was much more effective than **1** for the inhibition of shoot growth. The isolated halimanes were rather germination than growth inhibitors, and probably without auxinic effect, in contrast to the action exerted by dicamba (Yajima et al., 2004). These results showed the halimanes **1** and **2** to be highly phytotoxic toward *P. miliaceum* and *R. sativus*, with **2** showing greater effectiveness in inhibiting the germination of *P. miliaceum* and the growth of *R. sativus* seedlings, while **1** inhibited the germination and growth of both test species. The combination of the activity of both compounds accounts for the high phytotoxicity shown by *B. salicifolia* plant extract of both mono- and dicotyledonous species. Our study demonstrates that the phytotoxity of *B. salicifolia* is more supported by the presence of the halimanes than the flavonoids, as was suggested previously (Céspedes et al., 2006).

3. Experimental

3.1. General experimental procedures

Melting points were determined using a Kofler Heizbank Reichert RB 9577 and are uncorrected. Optical rotations were recorded on a JASCO DIP 370360 digital polarimeter in MeOH. UV spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer in MeOH solution. IR spectra were obtained using KBr disks on a Shimadzu spectrophotometer. ^{1}H and ^{13}C NMR spectra and 2D experiments, were recorded in DMSO-d_6 with a Bruker AVANCE II 400 spectrometer operated at 400 MHz for ^{1}H and at 100 MHz for the ^{13}C nucleus. Chemical shifts (parts per million) are relative to internal tetramethylsilane (TMS) used as a reference (δ = 0.00). The high resolution mass spectra was determined in a Bruker Micro-QTOFII mass spectrometer (Bruker Daltonics, MA, USA), equipped with an ESI source operated in positive mode at 180 °C with a capillary voltage of 4500 V. Mass accuracy was verified by calibration before and after sample introduction, using sodium formate (1 mM). Both samples and calibrant were introduced using a syringe pump at 10 μl min^{-1}. HPLC Shimadzu was performed on a Phenomenex Prodigy 5 μ ODS (4.6 mm i.d. × 250 mm) column eluted with MeOH/H_2O/trifluoroacetic acid (TFA) (7:3:0.1) as mobile phase, flow rate 0.8 ml min^{-1} and UV detection at 210 nm for **1** and **2**. The compounds **3**, **4** and **5** were eluted MeOH/H_2O/TFA (7:3:0.1), 0.8 ml min^{-1} and detection at 280 nm. Silica gel grade 70–230 mesh and 220–440 mesh (Sigma Chemical Co., Inc.) and SPE cartridge (Sep-Pak Plus C18, Waters) was used.

3.2. Plant material

Aerial parts of *B. salicifolia* were collected in the hills of Córdoba Province, Argentina, in December 2008. A voucher specimen (UCCOR 175) has been deposited in the "Marcelino Sayago" Herbarium of the School of Agricultural Science, Catholic University of Córdoba and was authenticated by the botanist, Gustavo Ruiz.

3.3. Seeds

Proso millet, *P. miliaceum*, and radish, *R. sativus*, seeds were purchased from Semillería Florensa in Córdoba, Argentina.

3.4. Germination bioassay

The germination assay was performed as previously reported (Palacios et al., 2010) with the following changes. Fourteen seeds of *P. miliaceum* or six seeds of *R. sativus* were placed in a twelve

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multiwell plate lined with filter paper. The chromatographic fractions were assayed at 5, 2.5 and 1.25 mg ml^{-1}; pure compound at 14, 7, 3.5, 1.8, 0.9 and 0.45 mM (all in water–acetone 3%); the commercial herbicide Banvel® (Novartis) which contained 48% (p/v) of dicamba was equally assayed after dissolution in water at 5, 0.1, 0.01, 0.001, 0.0001 and 0.00001 mM of dicamba. After 2 and 7 days for *R. sativus* and *P. miliaceum*, respectively, the number of germinated seeds and the lengths of seedling roots and shoots were recorded and the corresponding inhibition index were calculated. These results were analyzed by *t*-test ($p < 0.05$). Effective dose 50 values for germination and for root and shoot growth inhibition were calculated by Probit analysis as ECg_{50}, ECr_{50} and ECs_{50}, respectively, based on the inhibition percent.

3.5. Plant extraction

The vegetable material (208.8 g) of *B. salicifolia* was air-dried at room temperature, crushed, and extracted by 48 h maceration with ethanol. An extract was obtained after solvent removal (21.3 g) by vacuum evaporation. Yields of the plant extract, or the isolated compounds, are expressed as a percentage of the weight of the air-dried plant material.

3.6. Bioguided isolation

The ethanol extract of *B. salicifolia* (21.3 g) was partitioned between MeOH/H_2O (50:50) (500 ml) and diethyl ether (3 × 250 ml), yielding an aqueous and an ether fraction, representing 19% and 81% of the original extract, respectively. The IG% against *P. miliaceum* for the aqueous and ether fractions was 60% and 100%, respectively. A portion of the ether fraction (6.2 g) was fractionated by silica gel column chromatography eluted with a gradient of hexane/EtOAc. The active fractions 9–11 (IG% = 94–100 at 5 mg ml^{-1}), eluted with hexane/EtOAc (35:65), were then re-chromatographed on silica gel with a gradient of hexane/diethyl ether. The fractions 6–13 (IG% = 95–100) were combined and their components were separated by a new column chromatography eluted with ether. Five compounds were isolated with purities better than 80%. Compound **1** was further purified by means of SPE, eluted with approximately 30 ml of MeOH/H_2O/TFA (40:60:0.1), affording 169 mg of **1** with 99% purity by HPLC analysis (0.5% yield). Compound **2** was equally purified by SPE, yielding 42 mg (HPLC: purity 98%; 0.12% yield). Compounds **3**, **4**, and **5** were purified by crystallization.

3.7. Compound characterization

2,3-Dihydroxy-haliman-5,13Z-diene-15-oic acid or salicifolic acid (**1**): white needles (diethyl ether); m.p. uncorr. 122–124 °C; $[\alpha]_D^{21}$ −5.7 (*c* 0.80, MeOH); UV (MeOH) λ_{max} (log ε) 206 (3.78) nm; IR (KBr) ν_{max} 3369, 2968, 1647 (CO–OH) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz) see Table 1; HR-TOF-MS (ESI positive) *m*/*z* 359.2179 $[M+Na]^+$ (calcd for $C_{20}H_{32}O_4Na$, 359.2193).

2,3,5-Trihydroxy-cis-haliman-13Z-ene-15-oic acid or *5-hydroxy-6-hydro-salicifolic acid* (**2**): white crystalline rosettes (diethyl ether); m.p. uncorr. 208–210 °C; $[\alpha]_D^{22}$ +12.8 (*c* 0.91, MeOH); UV (MeOH) λ_{max} (log ε) 216 (3.18) nm; IR (KBr) ν_{max} 3382, 2852, 1683 (CO–OH) cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz) see Table 1; HR-TOF-MS (ESI positive) *m*/*z* 377.2291 $[M+Na]^+$ (calcd for $C_{20}H_{34}O_5Na$, 377.2298).

3.8. X-ray crystallography

Crystals of **1** that were suitable for X-ray work were grown via the slow evaporation of a methanol solution. The intensity data were collected with an Enraf-Nonius CAD4 diffractometer equipped with graphite-monochromated Mo Kα radiation, at room temperature. Cell parameters were determined from 25 carefully centered reflections in the θ range 6.66–15.21°. 4608 intensities were collected using the $\omega - 2\theta$ scan technique. All data were corrected for Lorentz and polarization effects. The structure was solved by direct methods and refined by full-matrix least-squares methods using SIR97 and SHELXL97 programs, respectively. All non-hydrogen atoms were refined anisotropically. The H atoms bonded to carbon atoms were placed at idealized positions using standard geometric criteria. The H atoms of the hydroxyl groups, acid group and methanol solvate were found from a difference Fourier map and treated as free atoms. Crystallographic data in CIF format have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 838928). Crystal data of **1**: $C_{20}H_{32}O_4$, CH_4O, fw = 368.50, orthorhombic, $P2_12_12_1$ (no. 19), a = 7.2311(14) Å, b = 24.7167(17) Å, c = 11.5316(9) Å, V = 2061.0(5) $Å^3$, Z = 4, ρ_{calc} = 1.188 Mg/m^3, F(000) = 808, N_{tot} = 4608, N_{unique} = 2824 (R_{int} 0.0499), N_{obs} ($I > 2\sigma(I)$) = 1675, R_1 (obsd data) = 0.0467, wR_2 (all data) = 0.1158.

Acknowledgments

This work was supported by a Grant from FONCyT, PICT 2005 and PICTO-CRUP 2005. S.L. Cuffini thanks CAPES for the financial support of the PVE program and Fundación Sauberan. S.d.C. gratefully acknowledges receipt of a fellowship from FONCyT. The authors thank Joss Heywood for revising the English language.

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Please cite this article in press as: del Corral, S., et al., Phytotoxic halimanes isolated from *Baccharis salicifolia* (Ruiz & Pad.) Pers.. Phytochem. Lett. (2012), doi:10.1016/j.phytol.2012.02.001