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Iridoids from the aerial parts of *Verbena littoralis* (Verbenaceae)

Ian Castro-Gamboa [a,*], Oscar Castro [b]

[a] *NuBBE-Núcleo de Bioensaio, Biossíntesse e Ecofisiologia de Produtos Naturais, Instituto de Química, Universidade Estadual Paulista, CP 355, Araraquara, CEP 14801-970, São Paulo, Brazil*
[b] *Departamento de Química, Universidad Nacional de Heredia (UNA), Heredia, Costa Rica*

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Abstract

The iridoids, 6*S*-hydroxy-8*S*-methyl-4-methylene-hexahydro-cyclopenta[*c*]pyran-3-one and 6*S*,9*S*-dihydroxy-8S-methyl-4-methylene-hexahydro-cyclopenta[*c*]pyran-3-one, were isolated from the aerial parts of *Verbena littoralis*. Their structures and stereochemistry were elucidated by means of NMR spectral data analysis. Both compounds showed moderate in vitro activity against gram positive and negative bacteria as well as moderate in vivo intestinal peristaltic action in mouse. The iridoids also showed moderate free radical scavenging activity against l,l-diphenyl-2-picrylhydrazyl (DPPH) as well as antioxidant activity, the latter being evidenced by redox properties measured using E1CD-HPLC.

Keywords: *Verbena littoralis*; Verbenaceae; Iridoids; Antibacterial activity; Antioxidant

1. Introduction

Verbena littoralis HBK (Verbenaceae) is currently used in traditional Central and South American folk medicine against diarrhea, fever, gastrointestinal disorders and some sexually transmitted diseases (Pérez-Arbeláez, 1978; Ocampo and Maffioli, 1987). Preliminary ethnobotanical and phytochemical information on *V. littoralis* has been published and various constituents were obtained (Umaña and Castro, 1990a,b; Li et al., 2001). Recently there has been interest in this plant because of its nerve growth factor action (Li et al., 2003a). A collection of the aerial parts of this plant from Guachipelin, Costa Rica, afforded an extract that revealed interesting preliminary antibacterial potential. Most of this activity was concentrated in the non-polar extract which triggered our curiosity since there are few reports on its chemical composition (Li et al., 2003b). In our studies on the chemosystematics and biological activity of this plant, we have isolated two new iridoids **1** and **2** that showed moderate antibacterial, intestinal peristaltic reflex and antioxidant activities.

2. Results and discussion

Sitosterol, stigmasterol, oleanoic acid and ursolic acid were identified by comparing their physical and spectral data with literature values (Breitmeier and Volter, 1987; Bruno et al., 1987; Srivastava and Jain, 1989).

Compound **1** was obtained as yellowish needles upon recrystallization from CH_2Cl_2–MeOH. Its molecular formula, $C_{10}H_{14}O_3$, was established by HRTOF-ESMS and the IR spectrum indicated the presence of hydroxyl (3450 cm^{-1} and ester (1743 cm^{-1}) groups. A conjugated terminal olefin (1653 and 887 cm^{-1}) was clearly confirmed in the ^1H NMR spectrum (Table 1) by a doublet at δ 6.42 (1H, $J =$ 2.3, Hz) and a broad singlet at δ 6.04

* Corresponding author. Tel.: +55 16 201 6664; fax: +55 16 222 7932.
E-mail address: castro@iq.unesp.br (I. Castro-Gamboa).

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Table 1
^{1}H NMR spectral data for iridoids **1** and **2** (500 MHz) in CD_3OD[a]

H	1	2
1-a	3.53 *d* (10.0)	3.50 *d* (9.5)
1-b	3.77 *dd* (10.0, 4.0)	3.61 *d* (9.5)
5	3.66 *ddd* (9.0, 7.0, 2.3)	3.48 *dd* (6.9, 2.0)
6	4.93 *m*	5.01 *ddd* (6.0,4.5,3.,8)
7-a	1.43 *m*[b]	2.01 *ddd* (9.8, 4.5, 3.0)
7-b	2.18 *m*[b]	2.30 *ddd* (9.8,5.0,3.8)
8	1.60 *m*	1.70 *ddq* (6.6,5.0,3.0)
9	1.83 *ddd* (9.0, 6.2, 4.0)	–
10	0.98 *d* (6.2)	0.94 *d* (6.6)
11-a	6.04 br *s*	6.00 br *s*
11-b	6.42 *d* (12.3)	6.35 *d* (12.0)

[a] Chemical shifts (relative to TMS) are in (δ) ppm, multiplicities and coupling constants in Hz in parentheses. Assignments were aided by ^{1}H–^{1}H COSY and HMQC

[b] May be interchanged.

(IH). The small constant (2.3 Hz) was the result of a long range interaction between this hydrogen and H-5 at δ 3.66 (IH, *ddd*, $J = 9.0$, 7.0, 2.3 Hz). In addition, H-9 appeared as a *ddd* at δ 1.80 (IH, $J = 9.0$, 6.3, 4.0 Hz). The smallest coupling constant for this signal was found in a *dd* at δ 3.77 (IH, $J = 10.0$, 4.0 Hz) attributed to H-δ H-1α only couples with H-1β showing a doublet at δ 3.53 (IH, $J = 10.0$ Hz). The methyl group at position 8 was attributed to a doublet at δ 0.98 (3H, $J = 6.2$ Hz). The configuration of **1** was determined by NOE observations. Irradiation of H-5 resulted in enhancement (5%) of H-9, H-6 and Me-10, showing that these were all *cis*. On the assumption that H-9 has the usual β-configuration, the methyl group at position 8, H-6 and H-5 could also be assigned with this configuration. Irradiation of H-9 led to enhancement of H-5, H-6 and H-10 signals. As a result, H-9 was deduced to be *cis* to H-5 and H-10 and *trans* to H-8. On the basis of the ^{1}H–^{1}H COSY and HMQC spectral data, the remaining proton signals of the cyclopentane ring in **1** were assigned. Based on comparision with analogues reported in the literature (Callant et al., 1983; Nagia and Prasuna, 1996; Pagnoni et al., 1976), we can define positions 5 and 9 with *S* and *R* absolute configuration, respectively. From this assumption, the other chiral centers in the molecule (C-6 and C-8) can be defined both as sinister.

The ^{13}C NMR procedure (Table 2) showed 10 absorptions: the carbonyl group at δ 170.9 (*s*), two signals at δ 134.9 (*s*) and 125.8 (*t*) typical for a terminal olefin methylene, a carbinolic carbon at δ 82.0 (*d*, C-6) and a carboxymethylene at δ 60.7 (*t*, C-l). HMBC defined the final connectivities between the pyran and the cyclopentane rings. Carbonyl at δ 170.9 (C-3) revealed correlations with δ 6.04 (H-11β) and δ 3.53 (H-1α), whereas H-5 showed strong correlations with C-1 (δ 60.72) and C-4 (δ 134.91). C-6 had correlations with H-5, and the multiplets attributed to H-7α and H-7β:δ 2.18 and δ 1.43, respectively. The methyl carbon at δ 17.37 correlated with H-7P and H-9. The structure of **1** was therefore 6*S*-hydroxy-8*S*-methyl-4-methylene-hexahydro-cyclopenta[*c*]pyran-3-one.

Table 2
^{13}C NMR spectral data for iridoids **1** and **2** (δ in ppm, CD_3OD solutions (δ_H, δ_C 125 MHz)

C	1	2
1	60.7	64.1
3	171.0	170.9
4	134.9	134.8
5	45.4	54.3
6	82.0	81.2
7	41.8	38.7
8	32.6	36.5
9	52.5	83.6
10	17.4	11.5
11	125.8	127.0

Compound **2** was also obtained as pale-yellow needles. Its molecular formula, $C_{10}H_{14}O_4$, was established by HRTOF-ESMS. The IR spectrum indicated the presence of hydroxyl (3451 cm^{-1}) and ester groups (1743 cm^{-1}). The terminal olefin functionality (1653 and 885 cm^{-1}) was also visible in the IR spectra.

The ^{1}H NMR spectrum of **2** was similar to that of **1** and showed the full multiplicities of hydrogens H-6, H-7α, H-7β and H-8. The absence of the signal at δ 1.83 present in **1**, was indicative a new hydroxyl at C-9. NOE measurements determined the relative configuration of **2** based on the assumption of a *cis* junction. Positive NOEs were observed for H-5, H-6, H-7β and Me-9 showing there were all on the same face of the molecule. Irradiation of the signal at δ 2.01 (H-7α) produced an increase at δ 1.70 (H-8) confirming the β-configuration for Me-10. On the basis of ^{1}H–^{1}H COSY and HMQC spectral data, the remaining proton signals of the cyclopentane and pyran ring in **2** were assigned. Due to the oxidation at C-9, the absolute configuration proposed for this center changes (sinister). The other chiral centers in the molecule (C-5, C-6 and C-8) remain sinister.

The ^{13}C NMR signals were almost identical to **1** with the appearance, as expected, of an additional quaternary carbinolic carbon at δ 83.6. HMBC measurements showed strong correlations between δ 83.6 (C-9) with H-5 and H-lβ. Thus, **2** was characterized as 6*S*,9*S*-dihydroxy-8*S*-methyl-4-methylene-hexahydro-cyclopenta[*c*]pyran-3-one.

The radical scavenging effects obtained for compounds **1** and **2** are shown in Table 3, using rutin as reference standard (IC_{50} 12.34 μg/mL). This result indicates that moderate free-radical scavenging activity of the iridoids is due to their hydrogen-donating ability, so increasing the number of hydroxyls, results in a more efficient radical scavenging effect (Son and Lewis, 2002). The electrochemical behavior displayed by these compounds, showed a good correlation with the radical scavenging effect measured in the DPPH assay. This observation clearly indicates that substances with oxida-

Table 3
Scavenging activity for DPPH radical and measured potentials (V) for electrochemically active compounds **1** and **2**

Compounds	IC_{50} (μmol)[a]	E_{ox} (+)	E_{red} (−)
1	34.70	0.35	0.40
2	18.50	0.40	0.35
Standard (rutin)	12.34	0.90	0.10

[a] Concentration in μmol required to scavenge 50% DPPH free radical.

tive peaks below +1.2V (Table 3) and a large oxidative capacity have more promising radical scavenging properties than those who are oxidized at higher potentials (Yamasaki et al., 1994).

On intraperitoneal administration of gradual doses of the decoctions from *V. littoralis's* aerial parts and also pure compounds **1** and **2** to rats the following effects were observed: reduction of motor activity and alarm reaction, ataxia, sedation, analgesia, anesthesia, ptosis, piloerection, anti-peristaltic movement and a significant reduction of body temperature of about 8.4 °C. These effects may confirm the popular usage of this plant as an antidiarrhetic and febrifuge. **1** and **2** were also detected in the non-polar extracts from the decoctions showing promising biological activities that must be evaluated by further specific bioassays.

The non polar extracts as well as **1** and **2** were evaluated against *Bacillus subtilis* and *Klebsiella pneumoniae* using direct autobiography (Rahalison et al., 1991). The crude extract showed moderate activity (11 mm inhibition halo at 770 μg/mL) whereas compound **1** (9 mm inhibition halo at 27 μg/mL) and compound **2** (8 mm inhibition halo at 2 μg/mL) showed slightly better activity when compared with the controls Streptomicine (16 mm inhibition halo at 10 μg/mL) and Nystatin (23 mm inhibition halo at 8 μg/mL).

3. Experimental

3.1. General

Optical rotations were measured in MeOH using a Perkin–Elmer polarimeter with a sodium lamp operating at 598 nm and 25 °C. UV spectra were recorded on Shimadzu UV-2401 PC spectrophotometer. IR spectra were run on a Perkin–Elmer FT-IR 600 spectrophotometer. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a VARIAN DRX-500 spectrometer, using TMS as an internal standard. ES-MS spectra were obtained on a VG Platform FISONS mass spectrometer. For HRRIMS Autospec-Micromass equipment was used. Column chromatography was carried out on Si gel 230-400 mesh (Merck), XAD-2 (Sigma–Aldrich) and Sephadex LH 20 (Pharmacia), respectively. TLC was performed using Merck silica gel 60 (>230 mesh) and precoated silica gel 60 PF_{254}. Spots on TLC were visualized under UV light and by spraying with anisaldehyde–H_2SO_4 reagent followed by heating at 120 °C. Preparative HPLC was performed on a Waters Prep LC 4000 system using a Phenomenex C-Phenyl (250 mm × 21.2 mm) preparative column. Radical scavenging activities were measured using a VARIAN STAR 9090 EICD composed of a glassy carbon-working electrode, an Ag/AgCl reference electrode.

3.2. Plant material

Authenticated *V. littoralis* plant material was collected in Guachipelín, Costa Rica, in January and August 2003. The botanical identification was made by Professor Oscar Castro and a voucher specimen was deposited at the Hebarium of the Museo Nacional de Costa Rica, San Jose (Voucher No. VL-2322).

3.3. Instestinal peristaltic reflex in mouse

The protocol adopted for this in vivo assay is described by Castro and co workers (Rodriguez and Castro, 1996).

3.4. Determination of radical scavenging activity

2,2-Diphenyl-picrylhydrazyl (DPPH) was used as a stable radical in methanol (200 μmol, 2 mL). The reagent was added to a 1 mL aliquot of the compounds, previously dissolved in MeOH, to yield final concentrations of 100, 80, 40, 20, 10 and 5 μmol. Each mixture was shaken and held for 30 min at room temperature, in the dark. Rutin was used as a reference compound. DPPH solution (2 mL) in MeOH (1 mL) served as control. The evaluation of the reduced form of DPPH generated in situ was performed measuring the decrease in the current (I) on the electrochemical cell of the E1CD (Castro-Gamboa et al., 2003). The areas obtained in each chromatogram were normalized and compared with the one obtained for the blank. The radical scavenging activity of the samples was expressed in terms of IC_{50} (concentration in μmol required for a 50% decrease in current (I) by the reduced form of DPPH). Rutin was used as reference compound.

3.5. Redox potential

Determination of the optimal potential (E_{ox}) were obtained by means of hydrodynamic voltammograms generated for each compound (Table 3).

3.6. Extraction and isolation

Fresh aerial parts of *V. littoralis* (1.5 kg) were extensively extracted using MeOH at room temperature. The

combined extracts were concentrated in vacua to give a dark solid (220 g). This residue was partitioned between *n*-BuOH and H_2O affording an *n*-BuOH (60 g) and an aqueous (105 g) soluble fraction. The *n*-BuOH was suspended in aqueous MeOH (1:4)% and successively partitioned with hexane, $CHCl_3$ and EtOAc. The hexane and CHC_3 fractions were recombined (25.8 g) and submitted to column chromatography using Sephadex LH 20 as stationary phase and MeOH as isocratic mobile phase, yielding 21 fractions (F1–F21).

F-5 (80.0 mg) was further purified by means of HPLC coupled to a phenyl-derived silica phase preparative column using MeOH:H_2O:AcOH (23:77:0.5) as mobile phase, UV detection at 254 nm and a flow rate of 10 mL min^{-1}, give pure **1** (12.3 mg), and **2** (10.6 mg). F-11 (105.20 mg) was purified with a similar chromatographic procedure, only varying the mobile phase MeOH/H_2O (27.5:72.5), giving ursolic (32.3 mg), and oleanoic acid (22.5 mg). Fraction F-6 (117.5 mg) was also subjected to HPLC purification using HEX/AcOEt (85:15) as mobile phase, flow rate set at 8 mL min^{-1}, using a preparative normal SiGel column and setting the UV detector at 210 nm. This afforded sitosterol (25.7 mg) and stigmasterol (22.8 mg).

3.6.1. 6-Hydroxy-8-methyl-4-methylene-hexahydro-cyclopenta[c]pyran- 3-one (*1*)

Amorphous powder; $[\alpha]_D^{25}$ − 36.0° (MeOH; *c* 1.4); UV λ_{max} (CH_3OH) nm (log ε): 246 (4.6); IR ν_{max} (KBr) cm^{-1}: 3450, 1743, 1653, 1635, 887. For ^1H and ^{13}C NMR see Tables 1 and 2; ES-MS: (70 eV) *m/z* (rel int.): $[M]^+$ 182 (35); HRTOF-ESMS: *m/z* 183.21630 $[M + H]^+$ Calcd. for $C_{10}H_{14}O_3$, 182.21636).

3.6.2. 6,9-Dihydroxy-8-methyl-4-methylene-hexahydro-cyclopenta[c]pyran-3-one (*2*)

Amorphous powder; $[\alpha]_D^{25}$ − 53.5° (MeOH; *c* 1.0); UV λ_{max} (CH_3OH) nm (log ε): 248 (4.4); IR ν_{max} (KBr) cm^{-1}: 3451, 1743, 1653, 1630, 885 cm^{-1}. For ^1H and ^{13}C NMR see Tables 1 and 2; ES-MS: (70 eV) *m/z* (rel. int.): $[M]^+$ 198 (40); HRTOF-ESMS: *m/z* 199.21569 $[M + H]^+$ Calcd. for $C_{10}H_{14}O_4$, 198.21576).

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