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Phenolic aristolactams from leaves and stems of *Aristolochia chilensis*

[Aristolactamas fenólicas de hojas y tallos de *Aristolochia chilensis*]

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Abstract
Three phenolic aristolactams, aristolactam AII (3), velutinam (4) and piperolactam A (5), were identified from the leaves and stems of *Aristolochia chilensis* Bridges ex Lindl. The structures of these compounds were elucidated using a combination of HPLC-DAD, GC-MS and NMR experiments.

Keywords: *Aristolochia chilensis*; aristolactams; HPLC-DAD; GC-MS; NMR; aristolactam AL-AII; velutinam; piperolactam A

Resumen
Tres aristolactamas fenólicas aristolactama AII(3), velutinam(4) y piperolactama A(5), se identificaron en hojas y tallos de *Aristolochia chilensis* Bridges ex Lindl. Las estructuras de estos compuestos se determinaron por combinación de CLAE-DAD, CG-EM y experimentos de RMN.

Palabras Clave: *Aristolochia chilensis*; aristolactamas; CLAE-DAD; CG-EM; RMN; aristolactama AL-AII; velutinam; piperolactama A

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INTRODUCTION

Aristolactams (ALs) are a small group of aporphinoids containing a phenanthrene chromophore that are restricted to the families Annonaceae, Monimimaceae, Menispermaceae, Aristolochiaceae and Piperaceae (Kumar *et al*., 2003).

Forty-five aristolactams from 38 *Aristolochia* species have been identified and classified as di-, tri-, tetra- and penta-substituted derivatives according to their substitution patterns (Figure 1). With few exceptions, the substituents include hydroxy, methoxy and methylenedioxy groups. Glycosidic ALs have been isolated in which the glucoside substituent is attached to a nitrogen or hydroxyl group (Figure 1) (Mix *et al*., 1982; Kumar *et al*., 2003; Bentley, 2006).

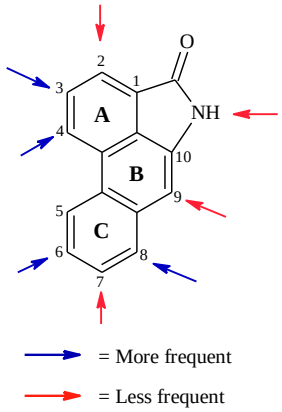


Figure 1
Representative aristolactam structures

The AL contents of a relatively small number of the *Aristolochia* species have been investigated; the studied species generally include those used in folk medicine. In addition, because ALs are neutral compounds, their isolation and purification are typically difficult. They are generally trace components in complex matrices of compounds from *Aristolochia* spp. and have intermediate polarities and similar chromatographic (TLC, CC) properties. An example of the difficulties encountered in isolating these compounds can be found in a report on the structural determination of 12 ALs from *Aristolochia argentina* (Priestap, 1985).

The modern approach for AL characterization involves the use of HPLC-DAD, LC-MS^n and other analytical techniques, but a lack of standards has significantly limited their analysis to only AL-I (1) and AL-II (2) because these compounds can be obtained commercially and are much more widely distributed within *Aristolochia*. (Zhang *et al*., 2006; Yuan *et al*., 2007; Yuan *et al*., 2008).

The biological properties of ALs have recently received additional attention since demonstrating potential medicinal properties including anti-inflammatory, antiplatelet, neuroprotective and anticancer activities (Choi et al., 2009).

In this communication, we report the isolation, analysis and identification of phenolic ALs from the leaves and stems of *Aristolochia chilensis* Bridges ex Lindl, a plant used in Central Chile as an anti-hemorrhagic agent and to expel the residual placenta after childbirth (Riedemann and Aldunate, 2001).

EXPERIMENTAL

Plant material

Representative samples of the leaves and stems of *Aristolochia chilensis* Bridges ex Lindl. were collected during the flowering season at Cuesta Lo Prado (15 km west of Santiago, 33º 28’ S, 70º 56’ W, 750 m above sea level) in October 2012. Voucher specimens (SGO-152461) were deposited in the Herbarium of the National Natural History Museum in Santiago, Chile.

AL Extractions

The oven-dried and powdered leaves and stems of *A. chilensis* (180 g) were extracted using light petroleum ether (35 - 65°) in a Soxhlet apparatus. The defatted plant material was then extracted with methanol (MeOH). The methanolic extracts were diluted with 200 mL of 3% $NaHCO_3$, warmed to 40° C, left to stand for 6 h and then extracted with $CHCl_3$ (5 × 150 mL). The basic solution was discarded, and the combined $CHCl_3$ extracts were dried and concentrated under reduced pressure. This extract was fractionated by CC over silica gel, using CH_2Cl_2 with gradually increasing concentrations of MeOH. The fractions were monitored via TLC on silica gel using CH_2Cl_2:MeOH (95:5), and the fractions containing ALs (UV irradiated at 364 nm) were evaporated *in vacuo* to yield the AL fraction. Preparative TLC (PTLC) on pre-coated silica gel plates (60 F_{254}, Merck, 1.0 mm thick, 20 × 20 cm) was used for the final purification with CH_2Cl_2:MeOH (95:5). The ALs were detected under UV irradiation at 364 nm as an intense fluorescent band (light green-yellow) and eluted from the plates using $CHCl_3$: MeOH (80:20). The extract obtained from the fluorescent band (3.2 mg) was analyzed via HPLC-DAD, GC-MS and NMR.

HPLC-DAD analysis of the ALs

The AL fraction in MeOH was directly injected (20 µL) into an analytical HPLC (Waters 600) with a reverse-phase Symmetry column (5 µm particle size; 25 × 0.46 cm). A gradient elution was performed with a mobile phase containing 0.1% acetic acid in water (solution A) and 0.1% acetic acid in acetonitrile (solution B) as follows: 0–5 min, isocratic elution with 70% A/30% B; 5–45 min, linear gradient from 70% A/30% B to 55% A/45% B. A Waters 2996 diode-array-detector (DAD) was used to record the spectra at wavelengths between 200 and 800 nm.

GC-MS analysis of the aristolactams

Qualitative analyses were conducted using a Thermo Scientific GC-MS (trace GC ultra, MS ISQ) apparatus with a DB-5 MS fused silica capillary column (60 m × 0.25 mm × 0.25 µm film thickness). The operating temperatures were as follows: injector, 270° C; detector, 300° C; and column oven, 100° C increasing to 300° C (5° C min^{-1}). Helium (1.3 mL min^{-1}) was used as the carrier gas.

NMR spectroscopy

NMR spectra were recorded on a Bruker 400 UltraShield spectrometer in CD_3OD.

RESULTS AND DISCUSSION

The HPLC analysis of the AL fraction (3.2 mg) from the *A. chilensis* leaves and stems yielded three peaks (ratio, 1:5:8), two showing the UV spectral characteristic of ALs with substitution at C-3 and C-4 (compounds 3 and 5) and one showing substitution at carbons C-3, C-4 and C-8 (compound 4) (retention times of 9.27, 11.67 and 14.20 min, respectively), Figure 2.

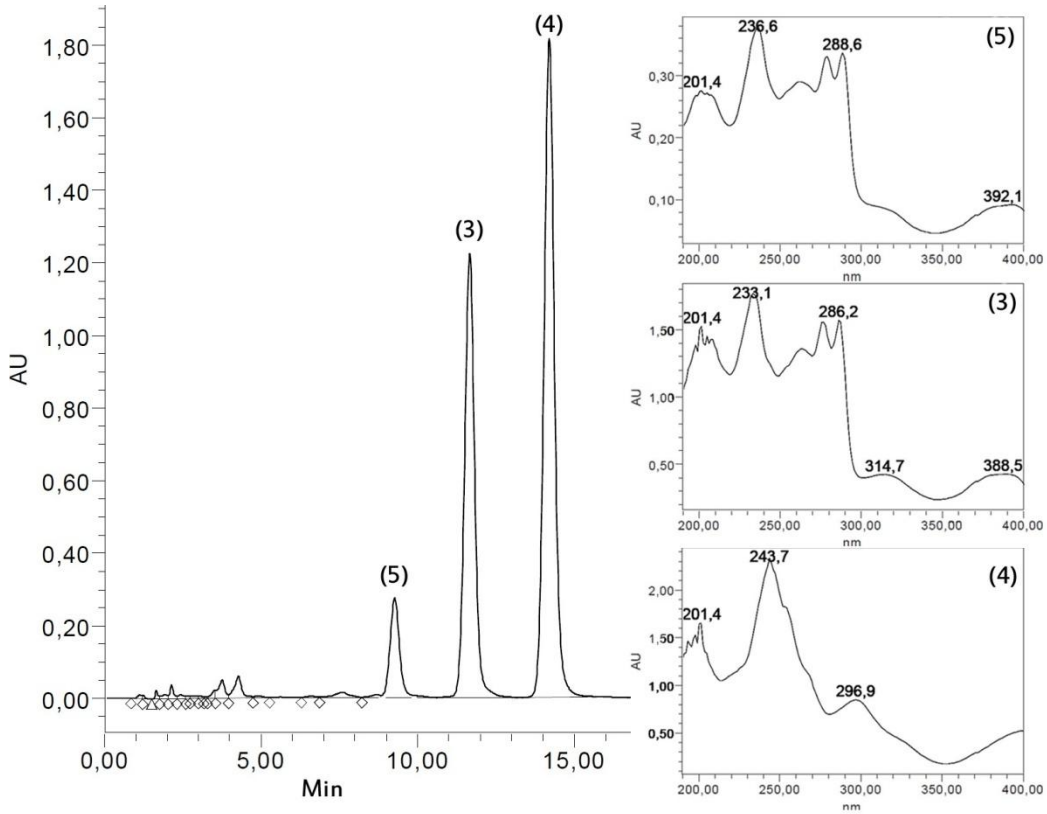


Figure 2
HPLC-DAD analysis and UV spectra of the AL fraction from *A. chilensis*

The GC-MS analysis of the AL sample (provided in Figure 3) indicated the presence of only two peaks in a 1:1 ratio (RI: 3078.2 , m/z $[M]^+$ 265.3 and RI: 3339.9 m/z $[M]^+$ 295.4). Peak $[M]^+$ 265.3, which is consistent with the molecular formula $C_{16}H_{11}NO_3$, was identified as a hydroxy-methoxy AL. Peak $[M]^+$ 295.3, which is consistent with the molecular formula $C_{17}H_{13}NO_4$, was identified as a hydroxy-dimethoxy AL. The fragmentation pattern of compound $[M]^+$ 265.3 was similar to that reported in the literature for AL-AII (3)

or piperolactam A (5), and the fragmentation pattern of compound $[M]^+$ 295.3 was similar to that reported in the literature for velutinam (4) or one of the known isomers (enterocarpam A or aristolactam AIII) (Lin *et al*., 2013; Kumar *et al*., 2003; Omar *et al*., 1992; Desai *et al*., 1988; Mahmood *et al*., 1986).

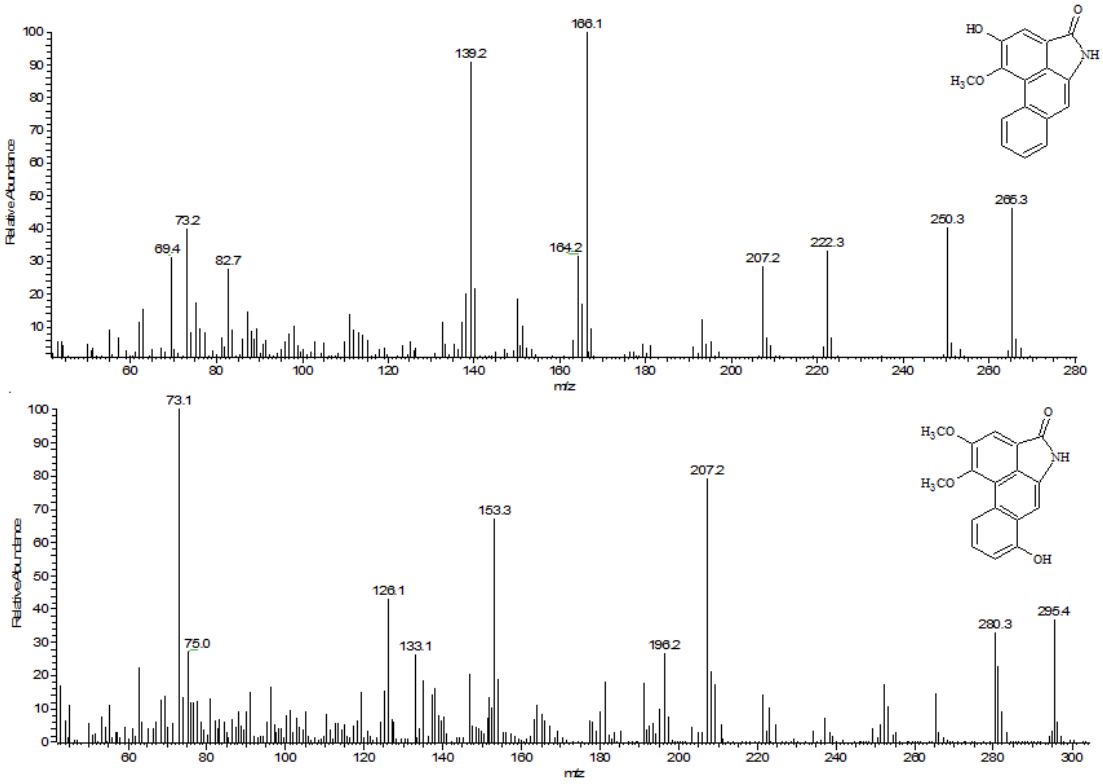
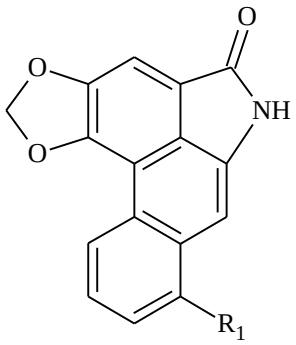


Figure 3
Mass spectra of ALs obtained from *A. chilensis*.

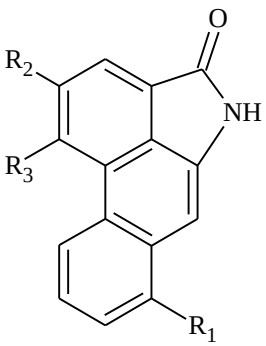
The structures of the ALs in the mixture were determined via NMR spectroscopy. An examination of the integral of the methoxy (OMe) groups in the 1H NMR spectrum confirmed the presence of a mixture containing three compounds in a ratio of 45:45:10. The aromatic protons were assigned via 1H-1H COSY correlations. Compound (4) exhibits an ABC pattern at δ: 8.79 (1H, d, J = 8.42 Hz), 7.48 (1H, br dd, J = 8.42; 8.05 Hz) and 7.14 (1H, d, J = 8.05 Hz), which can be attributed to H-5, H-6 and H-7, respectively. In the NOESY spectrum, the other signals, including δ: 7.67 (1H, s), 7.58 (1H, s), 4.06 (3H, s) and 4.04 (3H, s), were assigned to H-2, H-9, C-3-OMe and C-4-OMe, respectively, with correlations between H-5 and C-4-OMe; C-4-OMe and C-3-OMe; and C-3-OMe and H-2. Compound (4) was identified as velutinam (4).

The resonance of the aromatic protons in compound (3) exhibits the characteristic pattern of one AL with no substitution on ring C at δ: 9.17 (1H, m), 7.54 (2H, m) and 7.85 (1H, m), which are attributable to H-5, H-6, H-7 and H-8, respectively. In the NOESY spectrum, the signals at δ: 7.67 (1H, s), 7.10 (1H, s) and 4.02 (3H, s), were assigned to H-2, H-9 and C-4-OMe, respectively, and correlations between H-5 and C4-OMe were observed. Compound (3) was identified as AL-AII (3).



AL-I (1) R_1: -OCH_3

AL-II (2) R_1: -H



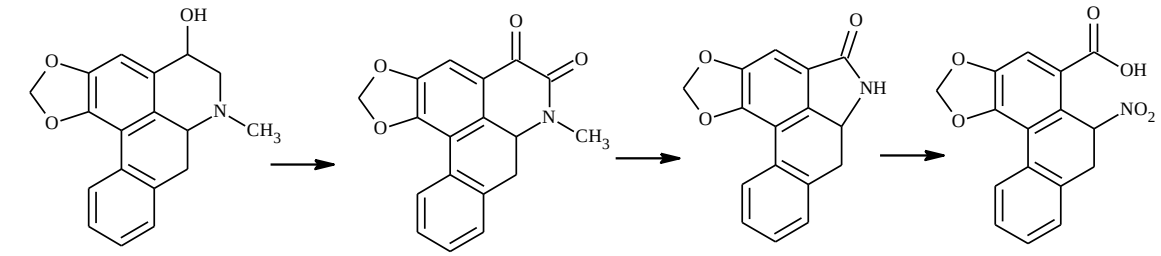
AL-AII (3) R_1: -H ; R_2: -OH ; R_3: -OCH_3

velutinam (4) R_1: -OH ; R_2: -OCH_3 ; R_3: -OCH_3

piperolactam (5) R_1: -H ; R_2: -OCH_3 ; R_3: -OH

Finally, the NMR spectrum of the minor component in the AL fraction showed a methoxy signal at δ 3.99 ppm. In addition, low-intensity signals of the aromatic protons were coincident with the AL-AII (3) proton resonances. According to the HPLC-DAD analysis, the compound is a phenolic AL with oxygenated substitution at C-3 and C-4, suggesting that compound may be piperolactam A (5), an isomer of AL-AII (3).

The structural relationship between the ALs and AAs suggests that the AAs are directly derived from the ALs via oxidation. A biogenetic route has been proposed (Figure 4) in which a 4-hydroxyaporphinic alkaloid is oxidized to a 4,5-dioxoaporphine, which generates lactam AL via CO extrusion and acid AA after oxidation (Priestap, 1985).



4-hidroxyaporphine 4,5-dioxoaporphine AL AA

Figure 4
Possible biogenetic route relating 4-hydroxyaporphines, 4, 5-dioxoaporphines, ALs, and AAs.

All aristolochic acids isolated from the *Aristolochia* species thus far have a methylenedioxy group on ring A. (Kumar *et al*., 2003). The ALs without a methylenedioxy group at C-3/C-4 are not directly oxidized to their respective AAs, and their biogenetic route should be more complicated, including additional enzymatic modification of the substituents to a methylenedioxy group.

Aristolactam AII (3) has been isolated from several species of *Aristolochia*, and piperolactam A (5) has been isolated from *Aristolochia cucurbitifolia*, *A. heterophylla* and *A. kaempferi.* Velutinam (4), isolated from *Fissistigma oldhamii* and a species of *Goniothalamus* (Kumar *et al*., 2003), is reported in the genus *Aristolochia* for the first time.

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