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Quinolizidine alkaloid status of *Acosmium* s.s., *Guianodendron* and *Leptolobium*, the segregate genera of *Acosmium* s.l.

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ABSTRACT

Extracts of leaves of five species of *Leptolobium*, the monospecific *Guianodendron* and all three species of *Acosmium* s.s. were examined for the presence of quinolizidine alkaloids by liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry. The species of *Leptolobium* all contained one or more of the quinolizidine alkaloids panacosmine, lupanacosmine or isomeric forms, while leaves of *Guianodendron praeclarum* accumulated guianodendrine, a previously unreported quinolizidine alkaloid with a novel skeleton. Quinolizidine alkaloids could not be detected in leaves of any of the three species of *Acosmium* s.s., which were found to accumulate stachydrine. The quinolizidine alkaloid status of *Acosmium* s.s, *Guianodendron* and *Leptolobium* agrees with their segregation from *Acosmium* s.l. and the exclusion of *Acosmium* s.s. from the genistoid clade of legumes, as suggested by morphological characters and molecular phylogenies.

1. Introduction

The ca 17 species of Neotropical trees assigned to *Acosmium* s.l. Schott (sensu Pennington et al., 2005) have recently been divided into three genera, *Leptolobium* Vogel, *Guianodendron* Sch.Rodr. & A.M.G.Azevedo and *Acosmium* s.s., on the basis of morphological analyses (Rodrigues and Tozzi, 2006, 2007, 2008). The new monospecific genus *Guianodendron* was created to accommodate *Acosmium praeclarum* (Sandwith) Yakovlev, a species displaying vegetative and floral character sets different from other species of *Acosmium* s.l. and other papilionoid genera (Rodrigues and Tozzi, 2006). The remainder of the species in *Acosmium* s.l. formed two unrelated groups in morphology-based analyses (Rodrigues and Tozzi, 2007), requiring the reinstatement of the genus *Leptolobium* to accommodate most species (Rodrigues and Tozzi, 2008) and leaving just three species in *Acosmium* s.s. (Rodrigues and Tozzi, 2007).

The phytochemical literature lists *Acosmium* s.l. as a genus producing quinolizidine alkaloids, and this chemical character is considered an important synapomorphy for the genistoid clade of papilionoid legumes (Kite et al., 2003; Van Wyk, 2003; Wink and Mohamed, 2003). In initial phylogenetic analyses of DNA sequence data, all sampled members of *Acosmium* s.l. were those now considered to be species of *Leptolobium*, and all were placed in the genistoid clade (Pennington et al., 2001; Wojciechowski et al., 2004). However, recent molecular analyses that have sampled all three segregate genera reveal that, while *Guianodendron* and *Leptolobium* remain within the genistoid clade, *Acosmium* s.s. is excluded from it and placed in the dalbergioid clade (Cardoso et al., 2012a, 2012b, 2012c). The NE African and Madagascan genistoid genus *Dicraeopetalum* Harms was also historically classified in the broader *Acosmium* circumscription (Yakovlev, 1969), but it is reported to accumulate quinolizidine alkaloids (Asres et al., 1997; Van Wyk et al., 1993) and to be phylogenetically closer to the core genistoids (Pennington et al., 2001; Cardoso et al., 2012c).

As the quinolizidine alkaloid status of *Acosmium* s.s. and *Guianodendron* cannot be determined from the literature, with all reports of quinolizidine alkaloids from *Acosmium* s.l. being from species now assigned to *Leptolobium* (Sousa et al., 2009), we have surveyed leaf material of the three segregate genera for the presence of quinolizidine alkaloids. Prior to the review of *Sweetia* and *Acosmium* by Yakovlev (1969), many species of *Acosmium* s.l. were included in *Sweetia* Spreng. s.l., giving rise to reports of quinolizidine alkaloids in this genus in the older phytochemical literature (Balandrin and Kinghorn, 1981). Thus we also examined *Sweetia* s.s., now reduced to just *Sweetia fruticosa* Spreng. (Yakovlev, 1969), to clarify its quinolizidine alkaloid status.

2. Materials and methods

2.1. Plant material and sample extraction

Details of the taxa analysed and the sources of materials are listed in Appendix 1. Dry leaf material (20–100 mg, weighed accurately) was powdered in a pestle and mortar with sand and transferred to an Eppendorf tube. Methanol was then added (1 μl/mg of plant material) and the sample was left overnight (ca 18 h) at room temperature (ca 22 °C). Following centrifugation, the supernatant was poured into

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an autosampler vial for analysis by liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS).

2.2. Preparation of alkaloidal fraction

Following analyses of the crude extract, the sample was dried under nitrogen and dissolved in 500 μl 2 M acetic acid. An equal volume of dichloromethane was added and the mixture was vortexed in an Eppendorf tube then centrifuged to separate the phases. The upper aqueous phase was transferred to a second Eppendorff tube and ammonia solution was added to pH 11 followed by 500 μl of dichloromethane. After vortexing and centrifugation, the lower dichloromethane phase was recovered into an autosampler tube. This alkaloidal fraction was analysed by GC-MS, while the upper aqueous phase was further purified to recover very polar nitrogen-containing compounds, if appropriate. After GC-MS analysis, the alkaloidal fraction was allowed to dry and dissolved in 500 μl methanol for LC-MS analysis. The alkaloidal fractions from the two specimens of *Guianodendron praeclarum* were combined and taken to dryness, yielding a pale yellow oil (0.9 mg) comprising **3** (ca 85%) and **3a** (ca 15%).

2.3. Preparation of polar nitrogen-compound fraction

The aqueous phase (from Section 2.2) was freeze dried and dissolved in 500 μl of water, and 50 mg of Dowex 50 (H^+) 100–200 mesh ion exchange resin was added, followed by gentle mixing. After allowing the resin to settle, the liquid was discarded and the resin was washed five times with 1 ml water. A fraction containing polar nitrogen-compounds was then eluted from the resin by adding 500 μl 2 M aq. ammonia and analysed by direct injection electrospray mass spectrometry. The fractions from all the specimens of *Acosmium* s.s. examined were combined and freeze dried, affording **4** as an off-white powder (0.4 mg).

2.4. Analysis by LC-MS

Samples were analysed using a Thermo Scientific LC-MS system comprising an 'Accela' 1290 pump and autosampler interfaced to an 'LTQ-Orbitrap XL' hybrid mass spectrometer via an 'Ion-Max' electrospray source. Chromatography of 2 μl injections was performed on a 150 mm × 2.1 mm, 1.9 μm Hypersil GOLD C18 column (Thermo Scientific) using a 400 μl/min mobile phase gradient of 95:0:5 (0 min), 95:0:5 (5 min), 50:45:5 (50 min), 0:95:5 (60 min), 0:95:5 (65 min) water/methanol/acetonitrile + 1% formic acid, following 3 min pre-injection equilibration in start conditions. Tuning of the electrospray source and calibration of the mass spectrometer followed the manufacturer's procedures and recommended settings. High resolution (30,000) first order mass spectra (MS^1) were acquired in positive mode over the range *m/z* 125 to 2000 by the orbitrap analyser while, simultaneously, the ion trap acquired low resolution MS^1 (*m/z* = 125–2000) and serial mass spectra (MS^2 and MS^3) in both positive and negative modes. For serial mass spectrometry, the most abundant three or four ions in the preceding lower order scan were selected successively and fragmented using an ion isolation window of ±2 *m/z* units and a relative collision energy of 35%. For some analyses, the positive ion MS^2 fragments generated by the ion trap were scanned at high resolution by the orbitrap to obtain accurate mass data on the product ions.

The same system was used for direct injection electrospray mass spectrometry simply by removing the column and injecting samples into a 400 μl/min flow of 50:45:5 water/methanol/acetonitrile + 1% formic acid.

2.5. Analysis by GC-MS

Samples were analysed using an Agilent Technologies GC-MS system consisting of a 7890A gas chromatograph and a 5975C single quadrupole mass spectrometer. Chromatography of 1 μl injections vaporised at 350 °C with a 10:1 split was performed on a 30 m × 0.25 mm (i.d.), 0.25 μm DB-5 capillary column (Agilent Technologies) using 1 ml/min helium carrier gas and an oven temperature programme of either 120–350 °C or 60–250 °C, both at 5 °C/min. The mass spectrometer recorded 70 eV electron ionisation mass spectra (EIMS) over the range *m/z* 38–650.

2.6. Analysis by NMR spectroscopy

NMR spectra were acquired in MeOH-d_4 at 30 °C on a Bruker 400 MHz (Avance) instrument. Standard pulse sequences and parameters were used to obtain one-dimensional ^{1}H, and two-dimensional gradient-enhanced COSY, HSQC, and HMBC spectra. Chemical shift referencing was carried out using the internal solvent resonances at δ_H 3.31 and δ_C 49.1 (calibrated to TMS at 0.00 ppm). Stachydrine (*4*). ^{1}H NMR (MeOH-d_4, 30 °C): δ 4.01 (1H, dd, J = 10.4, 8.7 Hz, H-2), 3.69 (1H, m, H-5a), 3.50 (1H, m, H-5b), 3.32 (3H, s, *N*-Me), 3.15 (3H, s, *N*-Me), 2.50 (1H, m, H-3a), 2.32 (1H, m, H-3b), 2.14 (2H, m, H-4a,b); ^{13}C NMR (MeOH-d_4, 30 °C): δ 170.9 (COO^-), 77.9 (C-2), 68.2 (C-5), 52.9 (*N*-Me), 46.5 (*N*-Me), 26.6 (C-3), 20.0 (C-4).

3. Results and discussion

3.1. Leptolobium

The positive mode base ion chromatogram from the LC-MS analysis of a crude methanol extract of leaves of *Leptolobium panamense* (Benth.) Sch.Rodr. & A.M.G.Azevedo showed two prominent peaks: *m/z* 342.2534 at t_R 28.8 min (**1**) and *m/z* 358.2847 at t_R 32.4 min (**2**) (Fig. 1). The compounds responsible for these peaks did not ionise in negative mode, indicating they were basic, and were present in the alkaloidal fraction following a classical alkaloid clean-up procedure (Section 2.2.) as determined by LC-MS analysis of the fraction—they were not sufficiently volatile to be observed by GC-MS analysis. Assigning the ions to $[M + H]^+$ suggested molecular formulae of $C_{21}H_{31}N_3O$ for **1** (calculated $[M + H]^+$ = *m/z* 342.2540, difference = 1.7 ppm) and $C_{22}H_{35}N_3O$ for **2** (calculated for $[M + H]^+$ = *m/z* 358.2853, difference = 1.6 ppm). These formulae agree with the quinolizidine alkaloids panacosmine (for **1**) and lupanacosmine (for **2**), reported from seeds of *L. panamense* [as *Acosmium panamense* (Benth.) Yakovlev] and root bark of *Leptolobium dasycarpum* Vogel [as *Acosmium dasycarpum* (Vogel) Yakovlev], respectively (Nuzillard et al., 1999; Trevisan et al., 2008) (Fig. 2).

Panacosmine is one of only five known natural products to contain the diaza-adamantane skeleton (Buckingham, 2013), the others being acosmine, acosmine acetate and dasycarpumine (all described in the aforementioned studies of *Leptolobium*) and bowdichine from the stem bark of *Bowdichia virgilioides* Kunth, which also contains acosmine (Barbosa-Filho et al., 2004). These diaza-adamantane alkaloids are considered to be related structurally to quinolizidine alkaloids and may be derived from them (Michael, 2001); panacosmine is clearly related structurally to lupanacosmine by bridging between N-12 and C-2 and elimination of the N-12 methyl group (Fig. 2). All five species of *Leptolobium* analysed contained **1** and/or **2**, and in some species isomeric or epimeric forms were also present. Neither **1**, **2** nor isomeric forms could be detected by displaying relevant single ion chromatograms from the LC-MS analysis of crude methanol leaf extracts of *G. praeclarum* or any of the three species of *Acosmium* s.s. (Fig. 1).

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