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ORIGINAL ARTICLE

Alkaloids from *Crinum erubescens* Aiton

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Abstract Eight alkaloids have been identified from fresh leaves of *Crinum erubescens* (Amaryllidaceae) collected in Costa Rica. The alkaloid 1-epidemethylbowdensine, detected by means of GC–MS as part of a global Amaryllidaceae Phytochemical Program, is reported for the first time and completely characterized by physical and spectroscopic methods. The absolute configuration of this compound is also reported.

1. Introduction

Plants belonging to the Amaryllidaceae family are well known for containing an exclusive group of alkaloids, which have been considered a distinctive taxonomic characteristic of this family (Bastida et al., 2006). *Crinum* is a pantropical genus that has extensive traditional use in Africa in a wide range of therapeutic applications, including antitumoral and antimalarial, and treatment of rheumatism and kidney and bladder infections, among others (Nair and van Staden, 2013). Alkaloids purified from the *Crinum* species have been confirmed as responsible for some of these properties, particularly the antiproliferative action (Berkov et al., 2011a). The alkaloid-rich extracts from *Crinum angustum* have shown significant antibacterial and antifungal activities (Ianello et al., 2014a). To date, approximately 130 species found throughout Africa, America, southern Asia and Australia have been classified within the *Crinum* genus (Refaat et al., 2012).

GC–MS has proven to be a useful tool in the identification and quantification of Amaryllidaceae alkaloids. This technique has been successfully used to assist with the isolation of new structures from alkaloid-rich extracts by comparing their component electron impact-mass fragmentation spectra (EI-MS) with those of known standards. Such a guided approach has been extensively applied as part of a global Amaryllidaceae

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Phytochemical Program, in which our research group is closely involved, and whose main focus is to characterize the alkaloid profiles of these species. Within this framework, the anti-*Trichomonas vaginalis* agent candimine and the acetylcholinesterase (AChE) inhibitory 11β-hydroxygalanthamine have been recently isolated from Brazilian Amaryllidaceae species (de Andrade et al., 2011; Giordani et al., 2010), and the anti-Alzheimer's disease (AD) drug, galanthamine, has been quantified in several natural sources (Berkov et al., 2008a, 2011b; Torras-Claveria et al., 2013). The global program has also led to the characterization of several new compounds from *Leucojum aestivum* (Berkov et al., 2008b), *Pancratium illyricum* (Iannello et al., 2014b), and others. All together, the obtained results represent a valuable contribution to the understanding of chemical and biological aspects of Amaryllidaceae alkaloids.

In the present work, the phytochemical study of the indigenous Costa Rican *Crinum erubescens* led to the identification of eight alkaloids using an in-home GC–MS database. Some of them have been previously isolated from the same species (Wildman et al., 1967; Fales et al., 1959; Wildman and Bailey, 1968). In particular, the new compound 1-epidemethylbowdensine (**1**) was completely characterized by NMR, while its absolute configuration was established by CD spectroscopy.

2. Experimental

2.1. General procedures

NMR spectra were collected in a Varian 500 MHz instrument using $CDCl_3$ as the solvent and TMS as the internal standard. Chemical shifts were reported in δ (ppm), and coupling constants (J) in Hz. GC–MS data were obtained on an Agilent 6890 N GC 5975 inert MSD operating in EI mode at 70 eV (Agilent Technologies, Santa Clara, CA, USA). A DB5 MS column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies) was used. The temperature program was: 100–180 °C at 15 °C min^{-1}, 1 min hold at 180 °C and 180–300 °C at 5 °C min^{-1}, and 10 min hold at 300 °C. The injector temperature was 280 °C. The flow rate of the carrier gas (helium) was 0.8 ml min^{-1} and the split ratio was 1:20. The EI-MS spectra were recorded on an Agilent MSD 5973 detector with a temperature of 40–300 °C at 10 °C min^{-1} at the ion source. The HRESIMS spectra were obtained on a LC/MSD-TOF (2006) mass spectrometer (Agilent Technologies) by direct injection of the compounds dissolved in H_2O:CH_3CN (1:1). Optical rotations were measured on a Perkin–Elmer 241 polarimeter. A Jasco-J-810 spectrophotometer was used to obtain the CD spectra, all recorded in MeOH. The UV spectra were obtained on a DINKO UV2310 instrument and the IR spectra were recorded on a Nicolet Avatar 320 FT-IR spectrophotometer. Silica gel (Kieselgel – mesh 0.15/0.30) was used for all of the vacuum liquid chromatography procedures (VLC). For thin layer chromatography (TLC), silica gel F_{254} was used as the stationary phase. A plate with dimensions of 20 cm × 20 cm × 0.20 mm was used for analytical TLC and a plate with dimensions of 20 cm × 20 cm × 0.25 mm was used for semi-preparative TLC (SPTLC). Exclusion chromatography was carried out using a Sephadex LH 20 column.

2.2. Plant material

Crinum erubescens Aiton was collected in October 2012 during the flowering period from a population located in Guácimo (Limón, Costa Rica). The species was identified by the botanist Mr. Luis Poveda (National University of Costa Rica). The voucher specimen of *C. erubescens* was deposited in the Herbarium JVR at the National University of Costa Rica under reference number JVR 14283.

2.3. Samples for GC–MS

Five mg of the alkaloid-rich extracts, including the *n*-Hex, EtOAc and EtOAc:MeOH extracts (Section 2.5), was diluted separately in 300 µl of MeOH, filtered and submitted for GC–MS analysis under the specific conditions presented in Section 2.1. A split ratio of 1:20 was used.

2.4. Identification of the alkaloids by GC–MS

The alkaloids were identified by comparing their GC–MS spectra and Kovats retention indices (RI) with our library database, which contains Amaryllidaceae alkaloids that have been isolated and identified using other spectroscopic techniques such as NMR, UV, CD and MS, as well as literature data, and is continually being updated and reviewed. Mass spectra were analyzed using AMDIS 2.64 software (NIST) (WA, USA). The compound RIs were recorded with a standard *n*-hydrocarbon calibration mixture (C9–C36).

The proportion of each individual compound in the alkaloid fractions analyzed by GC–MS (Table 1) is expressed as a percentage of the total alkaloids measured by the total ion current (TIC). The area under the GC–MS peaks depends on the concentration of the corresponding compound and the intensity of their mass spectral fragmentation. Although these data do not express a real quantification, they can be used for a relative comparison of the alkaloid content.

2.5. Extraction and isolation of alkaloids

Fresh leaves (2 kg) of *C. erubescens* were crushed and thrice extracted for 48 h with MeOH at room temperature and the combined macerate was filtered and evaporated to dryness under reduced pressure. The crude extract from the leaves (98.4 g) was acidified with sulfuric acid (2%) to pH 2 and extracted with Et_2O (3 × 200 ml) to remove the neutral material. The aqueous solution was basified with ammonia (25%) up to pH 9 and extracted with *n*-Hex (3 × 200 ml) to give the *n*-Hex extract (87.4 mg). Another extraction using EtOAc (10 × 200 ml) afforded the EtOAc extract (1.45 g) and a final extraction using EtOAc:MeOH (3:1, 3 × 200 ml) yielded the EtOAc:MeOH extract (3.37 g). The EtOAc extract was selected for alkaloid isolation and was subjected to VLC (2.5 × 4 cm) on silica gel (10 g), starting with *n*-Hex (100%), gradually enriching with EtOAc (0–100%), and finally with MeOH (0–20%). A total of 270 fractions (40 ml) were collected, monitored by analytical TLC (using Dragendorff's reagent and UV light λ 254 nm) and combined according to their profiles. From a total of 22 fractions, fraction 18 (120 mg) was subjected to a new VLC column (1.0 × 4 cm)

Table 1 GC–MS alkaloid profile of *Crinum erubescens*. Values are expressed as a relative percentage of TIC.

Compound	R.I.	$[M]^+$	MS data	% in *n*-Hex	% in EtOAc	% in EtOAc:MeO
Ismine (**7**)[a]	2280	257(35)	238(100), 225(5), 211(6), 196(8), 180(6), 139(5), 115(3), 77(3)		1.34	
Trisphaeridine (**8**)[a]	2282	223(100)	193(2), 164(14), 138(20), 111(13), 75(2), 50(1)		1.70	2.23
11,12-Dehydroanhydrolycorine (**6**)[b]	2606	249(61)	248(100), 191(13), 190(32), 163(11), 123(13), 95(29)			4.42
Crinamine (**4**)[a]	2648	301(< 1)	272(100), 242(10), 211(17), 199(8), 181(23), 153(14), 128(18), 115(16), 77(6), 56(11)	2.76	17.76	
1-Epidemethoxybowdensine (**3**)[a]	2764	373(100)	314(61), 254(48), 242(22), 225(22), 224(27), 201(34), 172(19), 115(14), 68(10)	6.54		
Macronine (**5**)[a]	2824	329(10)	299(91), 254(18), 245(68), 225(30), 201(37), 167(16), 139(36), 115(19), 82(28), 70(100)	77.20	51.54	23.20
Bowdensine (**2**)[a]	2955	403(100)	344(66), 314(18), 284(47) 272(22), 254(30) 232(29), 231(26), 202(24), 68(10), 43(38)	tr		
1-Epidemethylbowdensine (**1**)[a]	3038	389 (100)	330(65), 300(12), 270(38), 258(28), 241(25), 240(55), 218(30), 207(30), 188(19), 177(14), 68(11)		14.84	

Identification:
[a] Compounds identified using in-home MS database.
[b] MS data from the literature (Berkov et al., 2011b). Values less than 1.0 are described as "traces" (tr).

on silica gel and the alkaloids were eluted using the same gradient outline previously detailed. A total of 238 10-ml aliquots were collected, monitored by TLC (using Dragendorff's reagent and UV λ 254 nm) and combined according to their profiles, affording 13 major fractions. Fractions 5, 6 and 7 were combined (55 mg) and submitted to semi-preparative TLC using EtOAc:MeOH (4:1) to yield 4.8 mg of the alkaloid **1** after purification by Sephadex LH20.

3. Results and discussion

The known alkaloids bowdensine (**2**), 1-epidemethoxybowdensine (**3**), crinamine (**4**), macronine (**5**), 11,12-dehydroanhydrolycorine (**6**), ismine (**7**) and trisphaeridine (**8**) were identified by means of GC–MS (Table 1). Macronine (**5**) was the major component present in *C. erubescens*, and a significant relative percentage of crinamine (**4**) was also found. The GC–MS technique cannot provide the absolute stereochemistry of the Amaryllidaceae alkaloids, although the previous isolation of various bowdensine variants and crinamine in *C. erubescens* was taken into account for the identification of compounds **2**, **3** and **4** (Fales et al., 1959; Wildman et al., 1967; Wildman and Bailey, 1968). The structures of the alkaloids identified in *C. erubescens* in the present work are shown in Fig. 1. The GC–MS chromatogram of the alkaloid-rich fractions is shown in Fig. 2.

The GC–MS data for **1** were similar to those observed for the 5,10b-ethanophenanthridine derivatives (Bastida et al.,

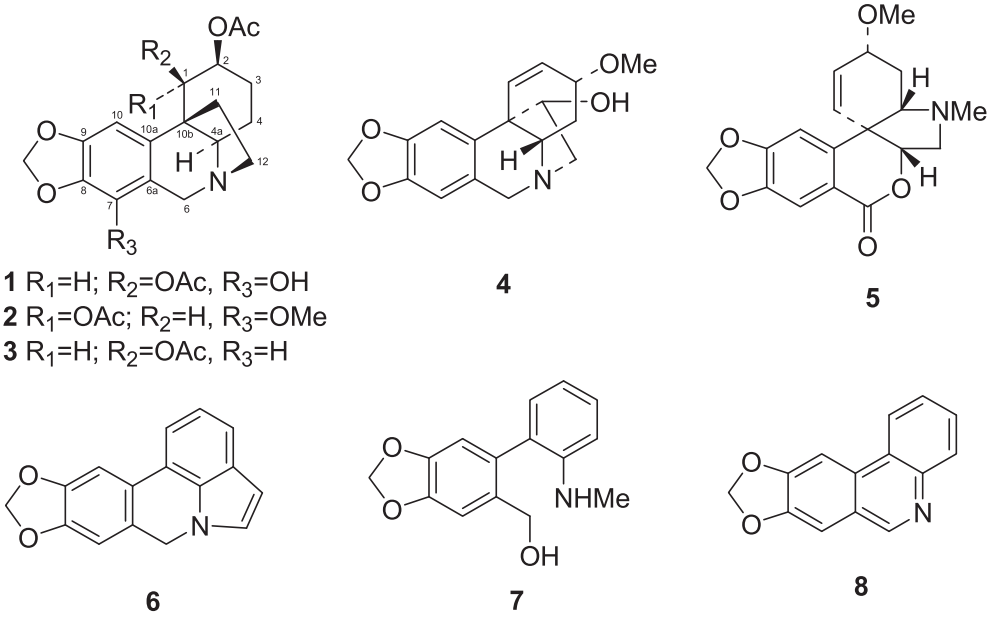

Figure 1 Alkaloids identified from *Crinum erubescens*.

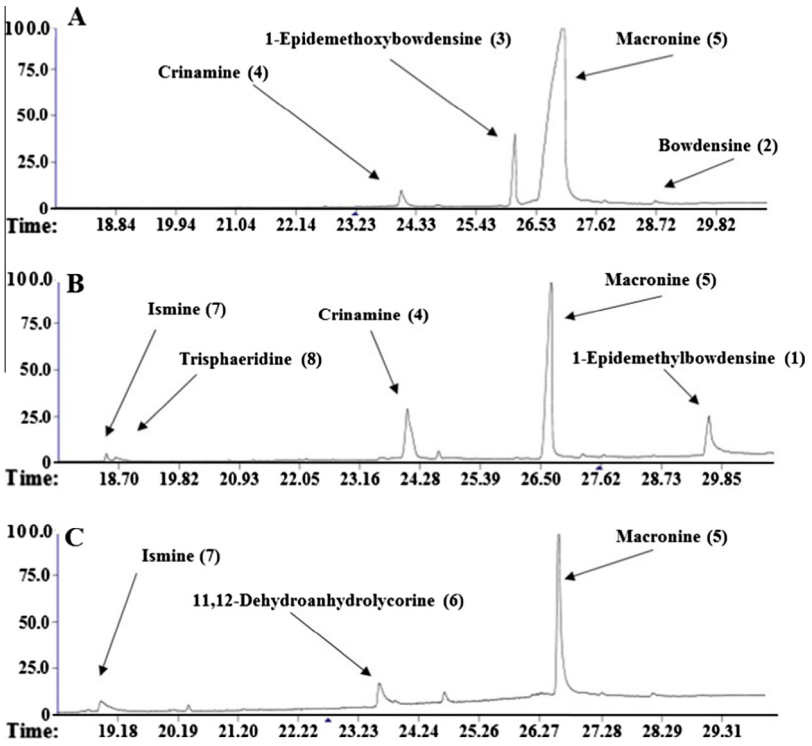

Figure 2 GC–MS chromatogram of *n*-Hex (**A**), EtOAc (**B**) and EtOAc:MeOH (**C**) extracts from *Crinum erubescens* with the identified alkaloids.

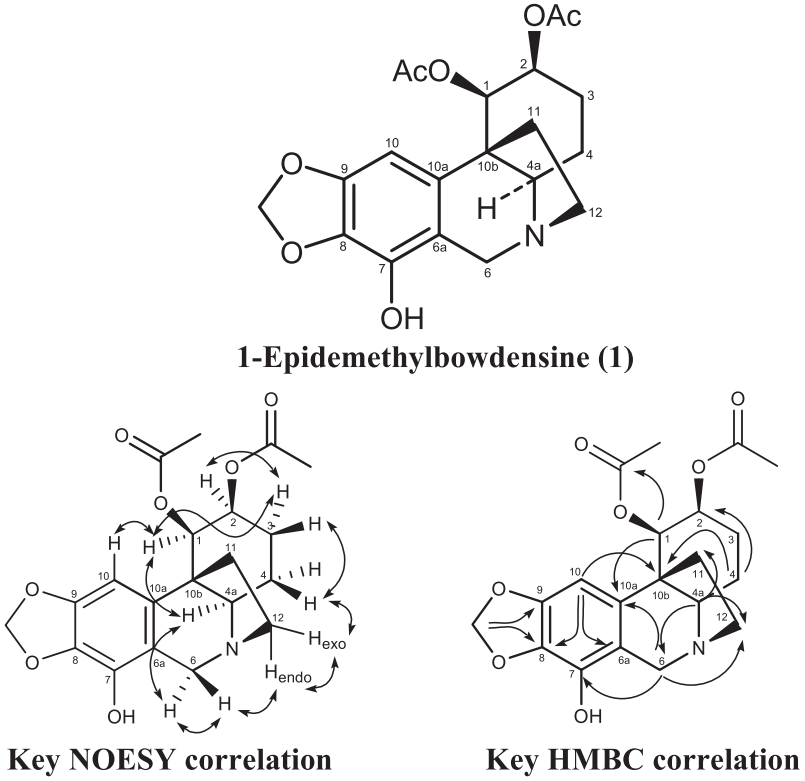

Figure 3 Key NOESY (↔) and HMBC (→) correlation of compound **1**.

Table 2 ^{1}H NMR, COSY, NOESY, HSQC and HMBC data for 1-epidemethylbowdensine (**1**) (500 MHz, $CDCl_3$).

Position	^{1}H δ (J in Hz)	COSY	NOESY	HSQC – ^{13}C	HMBC
1	5.34 *d* (4.4)	H2	H2, H3α, H4a, H10, OCOMe	74.1 *d*	C2, C4a, C10a, C10b, C11, CO
2	5.58 *dt* (5.5, 4.0)	H1, H3α, H3*β*	H1, H3*β*, H3α, OCOMe	68.4 *d*	
3α	1.60 *m*	H2, H3*β*, H4α, H4*β*, H4a	H1, H2, H3*β*, H4a	26.5 *d*	C4
3*β*	1.95 *dq* (14.6, 3.0)	H2, H3α, H4α, H4*β*	H2, H3α, H4*β*		C4
4α	1.67–1.73 *m*	H3α, H3*β*, H4a	H4a	21.1 *t*	C2, C4a, C10b
4*β*	1.67–1.73 *m*	H3α, H3*β*, H4a	H3*β*, H4a, H11*exo*, H12*exo*		C2, C4a, C10b,
4a	3.09 *m*	H3α, H4α, H4*β*	H1, H3α, H4α, H4*β*, H6α,	68.3 *d*	C4, C6, C10a, C11, C12
6α	4.29 *d* (17.0)	H6*β*	H4a, H6*β*	57.8 *t*	C6a, C7, C10a, C11, C12
6*β*	3.86 *d* (17.0)	H6α	H6α, H12*endo*		C4a, C6a, C7, C10a, C12
6a				114.4 *s*	
7				137.3 *s*	
8				132.5 *s*	
9				147.3 *s*	
10	6.13 *s*		H1, OCOMe	96.3 *d*	C6a, C8, C9, C10b
10a				141.0 *s*	
10b				47.5	
11exo	2.79 *ddd* (12.5, 10.5, 6.0)	H11*endo*, H12*endo*, H12*exo*	H4*β*, H11*endo*, H12*exo*	37.8 *t*	C10a, C10b, C12,
11endo	2.02–2.09 *m*	H11*exo*, H12*endo*, H12*exo*	H11*exo*, H12*endo*		
12exo	3.48 *ddd* (13.0, 10.5, 4.0)	H11*endo*, H11*exo*, H12*endo*	H4*β*, H12*endo*, H11*exo*	52.3 *t*	C6
12endo	2.86 *ddd* (13.0, 9.0, 6.0)	H11*endo*, H11*exo*, H12*exo*	H6*β*, H11*endo*, H12*exo*		C4a, C6, C11
OCH_2O	5.87–5.86 2*d* (1.5)			101.2 *t*	C8, C9
1,2-OCOMe	2.11 *s*		H1, H2, H10	21.3–21.4 *q*	CO
1,2-OCOMe				170.5–170.2 s	

2006; Duffield et al., 1965). Its HRESIMS suggested the molecular formula $C_{20}H_{24}NO_7$ for the parent ion $[M + H]^+$ at m/z 390.1553 (calcd 390.1547). The EI-MS spectra showed the molecular ion peak $[M^+]$ at m/z 389 (100%) and two major fragments at m/z 330 $[M\text{-}59]^+$ and m/z 270 $[M\text{-}119]^+$, which are typical for the loss of two vicinal acetoxy groups (Viladomat et al., 1996). Characteristic ^{1}H NMR signals included the following: (1) one aryl singlet at δ 6.13, confirmed as H-10 due to the NOESY correlation with H-1; (2) two doublets (J = 1.5) at δ 5.87–5.86, typical of the methylenedioxy group; (3) a *d* at δ 5.34 (J = 4.4) and a *dt* at δ 5.58 (J = 5.5, 4.0) assigned to H-1 and H-2, respectively, which were shifted to a lower field due to the presence of 1- and 2-acetoxy substituents. The magnitude of the coupling constant in H-1 and H-2 resonances confirmed that both 1- and 2-acetoxy substituents were *β*-oriented, as in 1-epibowdensine (Viladomat et al., 1996); (4) two AB doublets at δ 4.29 and 3.86, corresponding to the benzylic protons at C-6, with H-6α being assigned to a lower field due to its *cis* relationship with the nitrogen lone pair. All remaining NMR data, including NOESY and HMBC experiments (Fig. 3), were in agreement with the structure of a new bowdensine derivative 1-epidemethylbowdensine (**1**).

Although the basic crinane structure of **1** was well established by 1D and 2D NMR experiments, the absolute stereochemistry of the 5,10b-ethanophenanthridine alkaloids should be confirmed by CD spectroscopy. Indeed, the shape, amplitude and sign of the CD spectra are crucial for the correct orientation of the 5,10b ethano bridge in crinane variants (De Angelis and Wildman, 1969; Wagner et al., 1996). The CD spectrum of **1** displayed a positive and negative Cotton effect at ca. 250 and 290 nm, respectively, which is characteristic of a crinine-type compound (Wagner et al., 1996). Therefore, by means of NMR and CD spectroscopy, the compound 1-epidemethylbowdensine (**1**) was completely characterized and its NMR data are shown in Table 2.

1-Epidemethylbowdensine (**1**): amorphous solid; $[\alpha]_D^{24}$ + 18.7 (c 0.23, $CHCl_3$); CD $[\Theta]_\lambda^{20}$: $[\Theta]_{248}$ + 703.95, $[\Theta]_{300}$ – 58.90; UV (MeOH) λ_{max}(log ε) 240 (3.39), 276 (2.99) nm; IR ($CHCl_3$) ν_{max} 2924, 2854, 1743, 1625, 1480, 1376, 1247, 1036, 946, 757 cm^{-1}; EIMS data are shown in Table 1; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) are detailed in Table 2; HRESIMS of $[M + H]^+$ m/z 390.1553 (calcd for $C_{20}H_{24}NO_7$, 390.1547).

4. Conclusions

The phytochemical investigation of *C. erubescens*, native to Costa Rica, led to the identification of eight alkaloids by GC–MS. A complete spectroscopic characterization, including absolute configuration determined by CD, of the new compound 1-epidemethylbowdensine has been performed, leading to its confirmation as a new crinine-type derivative.

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