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Crotalaria medicaginea Associated with Horse Deaths in Northern Australia: New Pyrrolizidine Alkaloids

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Supporting Information

ABSTRACT: *Crotalaria medicaginea* has been implicated in horse poisoning in grazing regions of central-west Queensland, which resulted in the deaths of more than 35 horses from hepatotoxicosis in 2010. Liver pathology was suggestive of pyrrolizidine alkaloidosis, and we report here the isolation of two previously uncharacterized pyrrolizidine alkaloids from *C. medicaginea* plant specimens collected from pastures where the horses died. The first alkaloid was shown by mass spectometric and NMR analyses to be 1β,2β-epoxy-7β-hydroxy-1α-methoxymethyl-8α-pyrrolizidine, which, like other alkaloids previously isolated from *C. medicaginea*, lacks the requisite functionality for hepatotoxcity. The second alkaloid isolated in this investigation was a new macrocyclic diester of otonecine, which we have named cromedine. The ^{1}H and ^{13}C NMR spectra of cromedine were fully assigned by 2D NMR techniques and allowed the constitution of the macrocyclic diester to be assigned unambiguously. *C. medicaginea* specimens implicated in this investigation do not belong to any of the three recognized Australian varieties (*C. medicaginea* var. *neglecta*, *C. medicaginea* var. *medicaginea*, and C. *medicaginea* var. *linearis*) and appear to be a local variant or form, referred to here as *C. medicaginea* (chemotype cromedine).

KEYWORDS: pyrrolizidine alkaloids, otonecine, cromedine, pumiline A, horse, hepatotoxic, *Crotalaria medicaginea*

INTRODUCTION

Trefoil rattlepod (*Crotalaria medicaginea*) is a native herb widespread in grazing lands of northern Australia, with three recognized varieties, *Crotalaria medicaginea* var. *neglecta* (Wight & Arn.) Baker, *C. medicaginea* Lam. var. *medicaginea*, and C. *medicaginea* var. *linearis* A.E. Holland.[1] Historically, there have been several reclassifications within this taxa,[2] and at least eight morphologically distinguishable forms of *C. medicaginea* have previously been recognized in Queensland, although not fully defined taxonomically.[3]

C. medicaginea is highly palatable to horses[4,5] and has been connected to a number of chronic diseases in horses. *C. medicaginea* (then called *Crotalaria trifoliastrum*) has been linked with hepatic lesions and photosensitization in the Northern Territory and with lesions of liver, lung, and urinary bladder in northwestern Australia.[3] Hepatic megalocytosis was observed in two horses that had readily consumed almost 720 kg of *C. trifoliastrum* over two successive seasons in a Northern Territory feeding trial.[5] *C. trifoliastrum* was also reported as an abundant pasture plant in a 1963 disease outbreak that caused the death of up to 200 horses in the eastern Kimberley region of Western Australia with bladder, liver, and lung lesions, although this species was not believed to be responsible for the observed bladder cystitis.[6]

In Queensland, *C. medicaginea* species have not previously been associated with hepatotoxicosis of grazing animals but have been associated with esophageal ulceration of horses. Original cases of this syndrome (then known as Chillagoe horse disease) were linked with *Crotalaria aridicola* subsp. *aridicola*, with further cases associated with *C. medicaginea* (then known as *C. triafoliastrum*).[7,8] Although the toxin responsible for this syndrome has not been identified, alkaloids of similar structure have been isolated from both *C. aridicola* subsp. *aridicola* and *C. medicaginea* var. *neglecta*.[3,9,10] Our previous analysis of *C. medicaginea* var. *neglecta* showed three distinct chemical profiles, and each chemotype contained mixtures of related simple pyrrolizidine derivatives **1**−**7** (Figure 1).[9] Methylethers **1** and **2** were identified in *C. medicaginea* var. *neglecta* chemotype 1 along with the chlorocarbene artifact **3** (previously incorrectly drawn as the hydroxymethylene analogue[9]), whereas chemotype 2 contained epoxyalcohols **4** and **5**. The profile of chemotype 3 consisted of epoxymethylethers **6** and the tentatively identified **7** along with methylether **1** and trace levels of epoxyalcohol **4**. Methylether **1** and the β-hydroxy epimer of methylether **2** have previously been identified as major components in *C. aridicola* subsp. *aridicola*.[3,10]

In the present study, abundant pasture plants identified as *C. medicaginea* were suspected of involvement in horse poisonings in pastures in central-west Queensland in 2010, which led to the deaths of more than 35 horses.[11] In these cases hepatic necrosis was accompanied by a predominantly neutrophilic inflammatory response, hepatic megalocytosis, and nodular regeneration in some areas of the liver. Megalocytosis (the presence of enlarged hepatocytes containing large, hyperchromatic nuclei)

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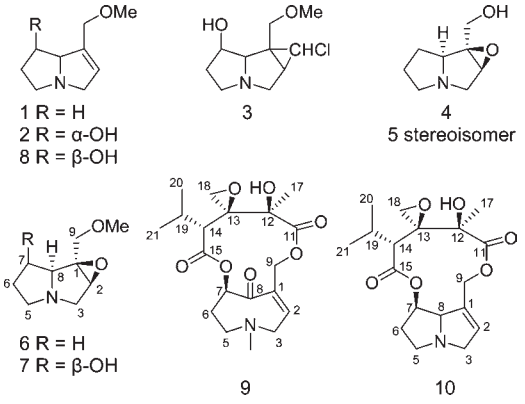

Figure 1. Structures of pyrrolizidine alkaloids identified in *Crotalaria medicaginea* var. *neglecta* (Wight & Arn.) Baker in chemotype 1 (**1**−**3**), chemotype 2 (**4** and **5**), and chemotype 3 (**6**, **7**, and **1**) and in *C. medicaginea* (chemotype cromedine) (**7**−**9**), with a revised structure for the related retonecine alkaloid, pumiline A, **10**.

is regarded as a characteristic feature of pyrrolizidine alkaloid-induced chronic hepatotoxicity,[12] which is inconsistent with alkaloids **1**−**7** as these structures lack the requisite unsaturation and/or esterification necessary for hepatotoxicity.[13]

We report here our investigation of the alkaloids present in *C. medicaginea* specimens implicated in these horse poisonings near Longreach in central-west Queensland, Australia.

MATERIALS AND METHODS

General Methods. Optical rotations were measured at 24 °C with a Jasco P-2000 polarimeter using a 1 mL cell with a 10 cm path length. NMR spectra were recorded on Bruker AV500 or AV750 MHz spectrometers. ^{1}H NMR spectra were recorded at 500 or 750 MHz with the residual protonated signal in the $CDCl_3$ (δ_H 7.24) or CD_3OD (δ_H 3.31) solvent as internal standards. ^{13}C NMR spectra were recorded at 125 or 188 MHz with the central peak of the $CDCl_3$ triplet (δ_C 77.0) or the central peak of the CD_3OD septet (δ_C 49.0) as internal standard. *J* values are reported in hertz. High-resolution mass spectra (HRESIMS) were recorded on a Bruker MicrOTOf-Q spectrometer equipped with a DIONEX UltiMate 3000 micro LC-MS system (ESI mode).

GC-MS analysis was carried out on a Shimadzu GC-17A and QP5050 MS instrument equipped with a 30 m × 0.25 mm i.d. DB-5 ms column (J&W Agilent Technologies, Melbourne, VIC, Australia). Analysis conditions were as follows: injector, 300 °C; temperature program from 50 °C (2 min), raised at 20 °C/min to 300 °C (held for 15 min); injection volume, 2 μL; injection mode, splitless; carrier gas, He; column flow, 1.5 mL/min; interface, 300 °C; and electron impact ionization at 70 eV.

Plant Material for Analysis. *C. medicaginea* samples were collected from a grazing paddock where horses had died approximately 60 km north of Longreach in central-west Queensland. Field-collected plant material (approximately 40 g) was frozen and transported to the laboratory, where the material was freeze-dried, milled, and stored frozen at −20 °C prior to analysis. A separate pressed mature plant sample was submitted for species identification by the Queensland Herbarium and confirmed as *C. medicaginea* (AQ863040).

Alkaloid GC-MS Analysis. Dried and milled plant material (2 g) was extracted as previously described to provide both a free pyrrolizidine alkaloid extract and a total alkaloid extract (after Zn reduction of *N*-oxides).[9] Alkaloid extracts were analyzed by GC-MS, with retrorsine obtained from Sigma (Sydney, NSW, Australia) as a co-injected internal standard for relative quantitation (Figure 2). Retention indices[14] were calculated with respect to a set of co-injected standard hydrocarbons (C_{10}−C_{28}).

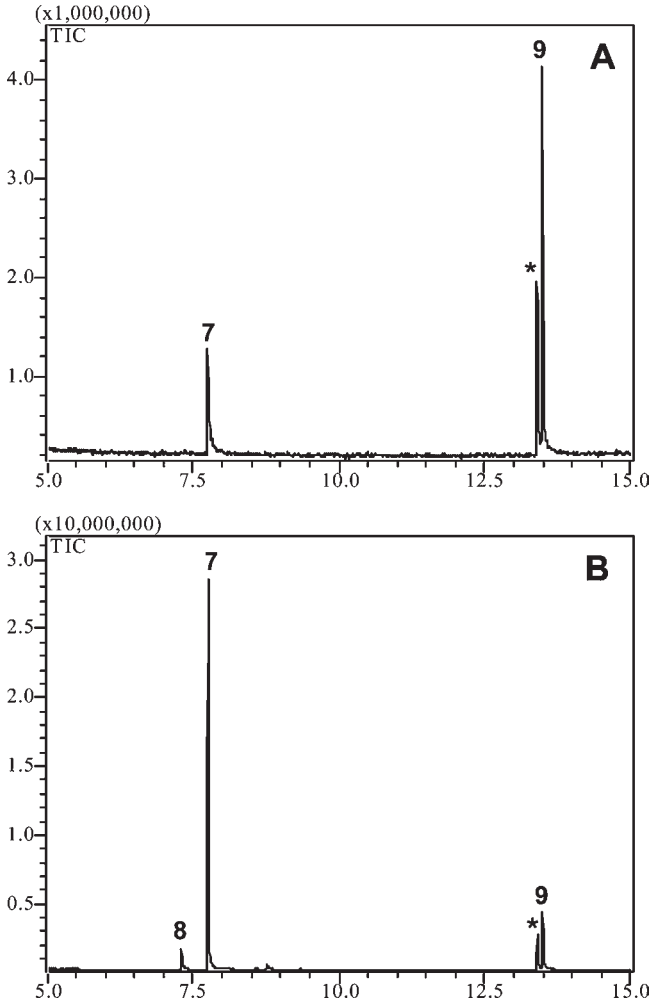

Figure 2. GC chromatograms of (A) a free pyrrolizidine alkaloid extract and (B) a total alkaloid extract (after zinc reduction of *N*-oxides), both with retrosine (∗) added as internal standard.

Purification of Alkaloids. The Zn-reduced alkaloid extract of *C. medicaginea* (AQ863040) (as employed in GC-MS analysis above) was purified by chromatography on a 12 cm × 0.5 cm i.d., 0.040−0.063 mm particle size, silica gel 60 column (Merck, Kilsyth, VIC, Australia) eluted with $CHCl_3$/MeOH (85:15 v/v). Eluted fractions (1 mL) were monitored by TLC (ninhydrin visualization) to provide pure 1*β*,2*β*-epoxy-7*β*-hydroxy-1*α*-methoxymethyl-8*α*-pyrrolizidine, **7** (1.9 mg from 1 g of dried plant material), together with cromedine, **9** (0.8 mg). Purity was determined by GC-MS and NMR analyses. The minor alkaloid, **8**, could not be obtained pure, and the structure of this compound was deduced by comparison of its GC-MS fragmentation pattern with those previously reported.[9]

Additional dried and milled *C. medicaginea* (AQ863040) (6 g) was similarly extracted as described,[9] to provide a free pyrrolizidine alkaloid extract (24 mg), which was purified by column chromatography (silica, $CHCl_3$/MeOH (85:15 v/v)) to provide pure cromedine, **9** (4 mg), which was recrystallized from pentane.

1β,2β-Epoxy-7β-hydroxy-1α-methoxymethyl-8α-pyrrolizidine, 7: RI 1462; HRESIMS, *m/z* 186.1125 (calcd for $C_9H_{16}NO_3$, 186.1130 [M + H$^+$]); $[\alpha]_D$ = −6.17 (*c*, 0.1, $CHCl_3$), 24 °C; GC-EIMS data in Table 1; NMR data in Table 2 (see also the Supporting Information).

7β-Hydroxy-1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine, 8: RI 1392; GC-EIMS data in Table 1.

Table 1. Mass Spectrometric Data for Pyrrolizidine Alkaloids in *Crotalaria medicaginea* (chemotype cromedine) with Retention Index (RI)

alkaloid	RI	mass spectrometric data (GC-EIMS)
1β,2β-epoxy-7β-hydroxy-1α-methoxymethyl-8α-pyrrolizidine (**7**)	1462	185 (32, [M$^+$]), 154 (61), 140 (28), 112 (24), 111 (100), 110 (68), 96 (47), 86 (98), 82 (36), 80 (32), 68 (35), 57 (17), 56 (19), 55 (92), 54 (24), 45 (81), 43 (27)
7β-hydroxy-1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**8**)	1392	169 (9, [M$^+$]), 139 (7), 138 (14), 137 (9), 136 (7), 125 (28), 95 (15), 94 (41), 93 (37), 82 (8), 81 (8), 80 (100), 67 (13), 53 (16), 45 (29)
cromedine (**9**)	2653	381 (1, [M$^+$]), 353 (1), 320 (2), 308 (3), 294 (18), 268 (3), 252 (3), 168 (59), 150 (11), 127 (16), 125 (17), 122 (24), 110 (34), 96 (18), 94 (22), 82 (24), 81 (25), 70 (29), 69 (22), 58 (27), 55 (24), 53 (43), 44 (57), 43 (100)

Table 2. ^{13}C and ^{1}H NMR Spectroscopic Data for Alkaloid 7, in $CDCl_3$ and CD_3OD

	$CDCl_3$		CD_3OD	
position	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)
1	68.4		70.2	
2	62.7	3.55 br s	63.8	3.63 br s
3	52.9	3.10 d (14.0)	55.4	3.08 br s
		3.11 br d (14.0)		3.09 br s
5	53.3	2.81 m	55.7	2.89 ddd (7.0, 8.9, 11.1)
		2.96 m		2.98 ddd (4.3, 7.0, 9.0)
6	35.9	1.92 m	36.4	1.95 m
7	71.5	4.50 m	73.4	4.56 ddd (4.4, 6.4, 6.4)
8	69.3	3.71 d (5.9)	70.5	3.66 d (6.4)
9	71.4	3.59 d (11.0)	72.7	3.80 d (11.0)
		3.91 d (11.0)		3.85 d (11.0)
11	59.5	3.42 s	60.7	3.43 s

Cromedine, **9**: mp 80−82 °C; RI 2653; HRESIMS, m/z 382.1866 (calcd for $C_{19}H_{28}O_7$, 382.1860 [M + H$^+$]); $[\alpha]_D$ = +61.1 (c, 0.34, $CHCl_3$), 24 °C; GC-EIMS data in Table 1; NMR data in Table 3 (see also the Supporting Information).

RESULTS AND DISCUSSION

GC-MS analysis of the zinc-reduced total alkaloid extract from *C. medicaginea* (AQ863040) demonstrated the presence of two nonesterified retronecine alkaloids, **7** and **8**, and a previously undescribed 11-membered macrocylic alkaloid, cromedine, **9**, with GC-EIMS mass spectrometric data presented in Table 1. Comparison of pyrrolizidine alkaloidal extracts with and without zinc reduction demonstrated that retronecine alkaloids **7** and **8** were present in the plant material primarily as their *N*-oxides (Figure 2). The major alkaloid, **7**, was isolated from this zinc reduced extract, and the minor alkaloid, **8**, detected by GC-MS (but not fully purified), was deduced by comparison with previously reported mass spectra[9] to be the 1,2-dehydro analogue of alkaloid **7**. Cromedine, **9**, a cyclic diester of the *N*-methylated necine base otonecine, was more readily isolated from the free alkaloid extract as it represented a greater proportion of this extract, which contained a lesser proportion of alkaloid **7** and only trace amounts of **8** (Figure 2).

Alkaloid Identification. Alkaloid **7** was isolated as an oil from the zinc reduced alkaloid extract and exhibited an ion (HRESIMS) at 186.1125 ([M + H$^+$]) corresponding to the molecular formula $C_9H_{16}NO_3$. This compound had an MS fragmentation pattern

Table 3. ^{13}C and ^{1}H NMR Spectroscopic Data for Cromedine, 9, in $CDCl_3$

position	δ_C	δ_H multiplicity (J in Hz)	HMBC crosspeaks
1	134.5		3, 7, 9
2	137.2	6.11 t (2.2)	3, 9
3	57.7	3.16 dt (2.2, 18.8)	N-Me, 2, 5
		3.47 br d (18.8)	N-Me, 2, 5
5	52.9	2.75 ddd (4.2, 10.4, 12.8)	N-Me, 3, 6, 7
		2.83 ddd (4.2, 4.2, 12.8)	N-Me, 3, 6, 7
6	35.5	1.97 dddd (4.2, 4.2, 4.2, 14.5)	5, 7
		2.35 dddd (4.2, 4.2, 10.4, 14.5)	5
7	76.8	5.20 dd (4.2, 4.2)	5, 6a, 14
8	191.1 (HMBC)		6, 7, 9b
9	66.4	4.43 dm (11.5)	2
		5.10 d (11.5)	2
11	175.7		9,17
12	76.9		17, 18b
13	60.1		14, 17, 18, 19
14	50.7	2.58 d (10.7)	17, 18, 19, 20, 21
15	172.9		7, 14, 19, 20
17	21.4	1.21 s	14
18	48.4	2.76 d (4.4) (H-18b)	14
		3.15 d (4.4) (H-18a)	14
19	27.3	1.65 m	14, 20, 21
20	22.0	1.04 d (6.6)	14, 19, 21
21	20.8	0.86 d (6.6)	14, 19, 20
N-Me	40.9	2.14 s	3, 5
OH		3.33 br s	

and a GC retention index (Table 1) identical to that reported for the previously tentatively identified epoxyalcohol **7**, for which the structure had been deduced from mass spectrometric evidence alone and the stereochemistry at C-7 was not previously defined.[9] NMR data reported here for isolated alkaloid **7** (Table 2) support this structure and allow full assignment of its stereochemistry by comparison with data reported for related epoxypyrrolizidines.[15] The stereochemistry of the C1/C2 epoxide relative to the C8 proton was established by comparison with NMR data reported for 1β,2β-epoxy-1α-hydroxymethyl-8α-pyrrolizidine (subulacine) **4** and its isomer, 1α,2α-epoxy-1β-hydroxymethyl-8α-pyrrolizidine.[15] In particular, C-1 (δ_C 70.2), C-2 (δ_C 63.8), and C-3 (δ_C 55.4) in the ^{13}C NMR (CD_3OD) spectrum of **7** were in close agreement with data for subulacine **4** (C-1, δ_C 70.9; C-2, δ_C 63.4; C-3, δ_C 54.0) and differed

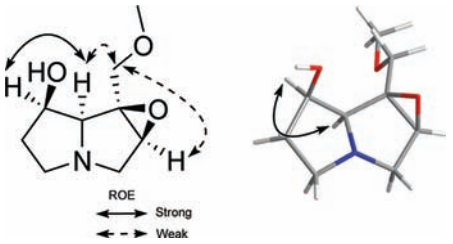

Figure 3. ROE correlations for alkaloid **7**.

substantially from its 1α,2α-epoxy isomer, where these same carbons were deshielded to 83.7, 65.2, and 62.3 ppm due to changes in electron density and repulsive interaction between nitrogen and oxygen lone pairs.[15] This evidence suggested the epoxy linkage of **7** was in the β-position, and this was confirmed by ROE interactions (weak) between H-8/H-9 and H-9/H-2 in 2D ROESY spectra (Figure 3). A strong ROE interaction between H-7 and H-8 combined with the observed coupling constant between H-7 and H-8 of 6.4 Hz (CD_3OD), in accord with Karplus equation predictions,[16] further established that H-7 and H-8 were on the same face of the molecule and placed the hydroxyl at C-7 in the β-position. The structure was thus confirmed as 1β,2β-epoxy-7β-hydroxy-1α-methoxymethyl-8α-pyrrolizidine, **7**, and GC-MS co-injection confirmed that this same compound is present in *C. medicaginea* var. *neglecta* (chemotype 3) (AQ751147).[9]

The minor retronecine alkaloid, 7β-hydroxy-1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine, **8**, was detected by GC-MS (Table 1), but could not be isolated pure, and was identified by comparison with the virtually identical mass spectrum for the 7α-hydroxy-1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine, **2**.[9] The GC retention index for the 7β-hydroxy isomer **8** (RI 1392) was less than that measured for the 7α-hydroxy isomer **2** (RI 1422),[9] and this was confirmed by co-injection with extracts of *C. medicaginea* var. *neglecta* (chemotype 1) containing alcohol **2** (AQ775300).[9] This elution order is consistent with the order of elution previously reported for stereoisomers **2** and **8** on a nonpolar SE 30 column.[3]

Cromedine, **9**, was a white solid, and the positive HRESIMS provided an ion at 382.1866 ([M + H^+]), corresponding to the molecular formula $C_{19}H_{28}NO_7$. The MS fragmentation of this compound exhibited several ions characteristic of otonecine pyrrolizidine alkaloids,[17] including characteristic peaks in the mass range m/z 168−94 (Table 1). The ^{1}H and ^{13}C NMR shifts for the otonecine skeleton were readily assigned by comparison with reported shifts for other otonecine alkaloids, such as that reported for crosemperine and croaegytptine,[18] including a characteristic low-field carbonyl resonance at δ_C 191.1 (C-8) and N-Me resonances (δ_C 41.1; δ_H 2.14 s). The carbonyl resonance was only detectable in the HMBC spectra, and this is consistent with previous references that report the otonecine carbonyl as a broad signal due to equilibration with a zwitterionic form (Figure 2).[19] X-ray analyses of all nine otonecine-type alkaloids measured to date have demonstrated that the otonecine conformation is identical to alkaloids having the C8−N bond (such as retronecine-type alkaloids).[20] HMBC crosspeaks confirmed the connectivity around the otonecine ring system of **9** and also through the necic acid chain (Table 3). Diesterification of the otonecine base was confirmed by HMBC crosspeaks between the carbonyls (C-15/C-11) in the necic acid chain

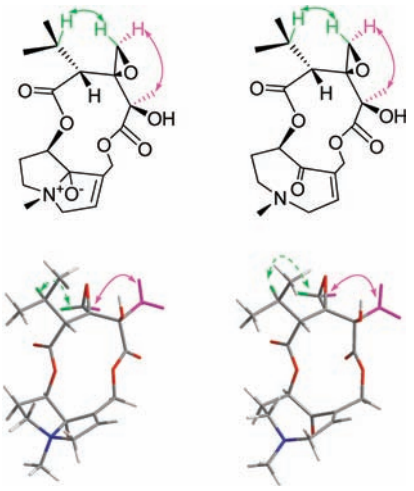

Figure 4. ROE correlations for cromedine, **9**.

and hydrogens of the otonecine core (H-7/H-9, respectively). An isopropyl group at C-14 was evidenced by two methyl doublets (δ_H 1.04 and 0.86) coupled to a common proton at δ_H 1.65 (H-19), and the adjacent spiroepoxide group at C-13 was characterized by a methylene resonance at δ_C 48.5 (C-18) and protons at δ_H 2.76 and 3.15 with a geminal coupling of 4.4 Hz. It was thus deduced that the necic acid in cromedine, **9**, has the same structure as that present in pumiline A, **10**,[21] in which the necine base is retronecine (rather than otonecine as present in cromedine, **9**). In support, ^{13}C and ^{1}H NMR resonances for the necic acid chain in cromedine very closely match those of pumiline A,[21] with differences of individual carbon resonances of only 0.2−0.4 ppm.

ROE correlations enabled the relative stereochemistry of cromedine, **9**, at C-12, C-13, and C-14 to be tentatively assigned (Figure 4). One of the epoxide protons (H-18b, 2.76 ppm) has a ROE with the methyl at C-12 (H-17, 1.21 ppm), whereas the other epoxide proton (H-18a, 3.15 ppm) has a ROE with the CH of the isopropyl moiety (H-19, 1.65 ppm). The assignment of the stereochemistry for pumiline A was similarly derived with reported NOESY crosspeaks between one epoxide proton and H-14 and the other epoxide proton and the protons of the C-12 methyl.[21] However, our examination of original unpublished spectra for pumiline A established that NOE correlations between H-14 (2.57 ppm) and the epoxide proton H-18a were very small and that the major NOESY crosspeak for this H-18a epoxide proton (3.25 ppm) was with the isopropyl methine H-19 (1.66 ppm). This evidence does not support the previously reported stereochemistry at C-14 for pumiline A,[21] and our conclusion to revise the stereochemistry at this position was supported by correspondence with the authors (Ludger Ernst, personal communication). It is thus concluded that cromedine, **9**, and pumiline A, **10**, have the same relative stereochemistry at C-12, C-13, and C-14 with C-17, C-18, and the isopropyl group on the same side of the molecule, as drawn in Figure 2. No ROE correlations between the necine base and the necic acid chain of cromedine, **9**, were observed, and the relative stereochemistry between these units could not be confirmed. Our tentative structural assignment for cromedine arbitrarily assumes the usual *R*-configuration at C-12. The usual β-configuration of the ester linkage exists at C-7 of the otonecine ring as evidenced by H-7α/H-6β and H-7α/H-6α couplings of 4.2 Hz, in line with

couplings predicted by the Karplus equation[16] from calculated dihedral angles of 51.5° and 120.0°, respectively.

Plant Identification. Suspect plants in this study were identified as a "local variety or form" of *C. medicaginea* (Ailsa Holland (Queensland Herbarium), personal communication, 2011), which does not correspond to any of the three previously described varieties (*neglecta*, *medicaginea*, or *linearis*) on the basis of differences in length of calyx, pod, and keel.[1] *C. medicaginea* of this "form" were found to be prevalent on seven properties where horse deaths had occurred in central-west Queensland, and all exhibited a comparable alkaloid profile (cromedine chemotype) comprising cromedine, **9**, along with the presumably nonhepatotoxic alkaloids **7** and **8**. Otonecine alkaloids (such as cromedine, **9**) are considered to be hepatotoxic, being metabolized by oxidative demethylation, ring closure, and dehydration to form the same reactive pyrrolic intermediates and consequent DNA and protein adducts arising from retronecine-based pyrrolizidine alkaloids.[13,20]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. 1D and 2D NMR spectra for cromedine, **9**, and 1β,2β-epoxy-7β-hydroxy-1α-methoxymethyl-8α-pyrrolizidine, **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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