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Dehydropyrrolizidine Alkaloids, Including Monoesters with an Unusual Esterifying Acid, from Cultivated *Crotalaria juncea* (Sunn Hemp cv.'Tropic Sun')

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Supporting Information

ABSTRACT: Cultivation of *Crotalaria juncea* L. (Sunn Hemp cv. 'Tropic Sun') is recommended as a green manure crop in a rotation cycle to improve soil condition, help control erosion, suppress weeds, and reduce soil nematodes. Because *C. juncea* belongs to a genus that is known for the production of toxic dehydropyrrolizidine alkaloids, extracts of the roots, stems, leaves, and seeds of 'Tropic Sun' were analyzed for their presence using HPLC-ESI/MS. Qualitative analysis identified previously unknown alkaloids as major components along with the expected macrocyclic dehydropyrrolizidine alkaloid diesters, junceine and trichodesmine. The dehydropyrrolizidine alkaloids occurred mainly as the *N*-oxides in the roots, stems, and, to a lesser extent, leaves, but mainly as the free bases in the seeds. Comprehensive spectrometric and spectroscopic analysis enabled elucidation of the unknown alkaloids as diastereoisomers of isohemijunceine, a monoester of retronecine with an unusual necic acid. The dehydropyrrolizidine alkaloid contents of the roots, stems, and leaves of immature plants were estimated to be 0.05, 0.12, and 0.01% w/w, respectively, whereas seeds were estimated to contain 0.15% w/w.

KEYWORDS: *Crotalaria juncea, 'Tropic Sun', pyrrolizidine alkaloids, N-oxides, HPLC-ESI/MS, junceine, trichodesmine, isohemijunceine, hemijunceine*

INTRODUCTION

Crotalaria juncea L. (sunn hemp) is an extensively cultivated, leguminous plant with a multitude of potential uses/benefits, including nonwood fiber and soil improvement, considered to have originated in India.[1] Agronomic development of the plant in the United States resulted in the 1983 release of the 'Tropic Sun' cultivar, which is recommended as a green manure crop cultivated in a rotation cycle to improve soil condition, help control erosion, suppress weeds, and reduce soil nematodes.[2,3]

The dehydropyrrolizidine alkaloid macrocyclic diesters junceine (**1**) (Figure 1), trichodesmine (**2**), senecionine, riddelliine, and seneciphylline have been reported to occur in *C. juncea* seeds.[4] However, another batch of seeds, extracted by the same authors under the same conditions, yielded only **1**. In a study of the total pyrrolizidine alkaloid content and relative toxicities of seeds of various *Crotalaria* species to chicks, Williams and Molyneux determined the total pyrrolizidine alkaloid concentration to be 0.02% based on dry weight of the seeds of *C. juncea* sourced from India.[5] The low levels and the variation in dehydropyrrolizidine alkaloid content of these earlier studies were later confirmed during the analysis of seeds from several accessions of *C. juncea* collected from various international locations.[6] This latter paper included an analysis of seeds from the 'Tropic Sun' cultivar that revealed the presence of only junceine using HPLC coupled to an evaporative light scattering detector (ca. 1.6 μmol/g seed or 0.06% w/w).

The 'Tropic Sun' cultivar has undergone 28 years of development since its release in 1983. For this reason, along with increasing attention being given to the potential for long-term or intermittent low-level exposure to dietary dehydropyrrolizidine alkaloids to cause chronic diseases in humans, including cirrhosis, genotoxicity, cancers, and pulmonary hypertension,[7] the 'Tropic Sun' cultivar was re-examined for the presence, type, and quantity of potentially toxic dehydropyrrolizidine alkaloids. In contrast to the previous works on the alkaloid content in seeds, this study examined the roots, stems, and leaves in addition to the seeds using an HPLC−electrospray ionization mass spectrometry (ESI/MS) approach.

MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile was an HPLC certified solvent (Honeywell Burdick and Jackson, Muskegon, MI, USA). Water for the HPLC mobile phase was Milli-Q-purified (18.2 MΩ/cm) (Millipore, Bedford, MA, USA). Methanol was of reagent ACS/USP/

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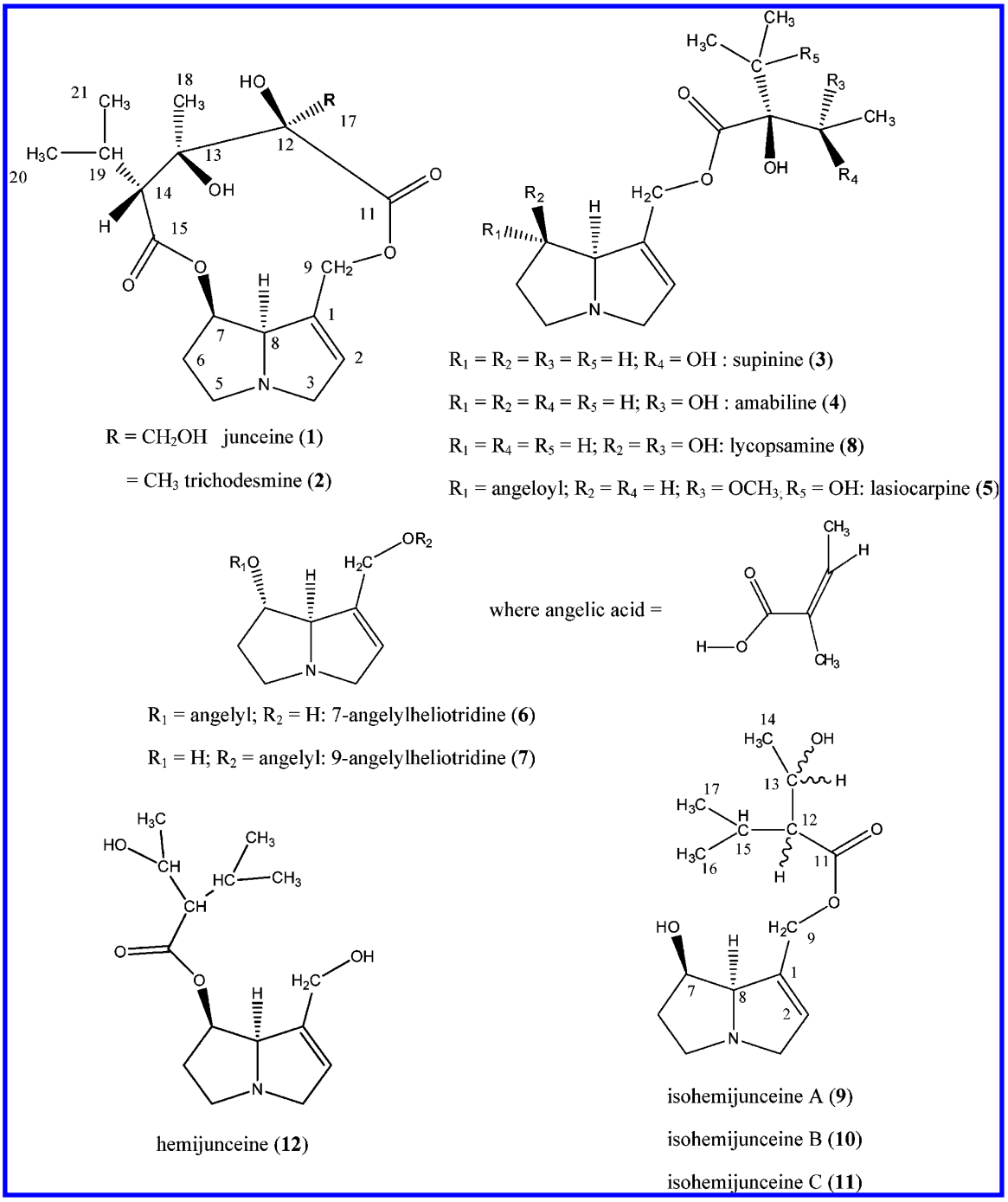

Figure 1. Structures of some dehydropyrrolizidine alkaloids observed in *Crotalaria juncea* or used in this study.

NF grade (Pharmaco Products, Brookfield, CT, USA). The mobile phase additives ammonium acetate, formic acid, acetic acid, and trifluoroacetic acid were each of the highest grade available. Junceine (**1**), trichodesmine (**2**), supinine (**3**), amabiline (**4**), lasiocarpine (**5**), 7-angelylheliotridine (**6**), and 9-angelylheliotridine (**7**) (Figure 1) were sourced from the stocks of extracted and purified (ca. 80−95% pure based on HPLC-ESI/MS analysis) pyrrolizidine alkaloids kept by the USDA/ARS Poisonous Plant Research Laboratory. Lycopsamine (**8**) was purchased from Planta Analytica (Danbury, CT, USA). The archived seed extract of *C. juncea*, sourced from India and previously analyzed using HPLC,[5] was supplied by Dr. R. J. Molyneux (University of Hawaii at Hilo). Unless otherwise stated, all solid phase extraction cartridges and HPLC columns and guard cartridges were sourced from Phenomenex (Torrance, CA, USA).

Extraction of Plant Samples. The plant samples were supplied by the Natural Resources Conservation Service, Plant Materials Center, U.S. Department of Agriculture, from cultivated, seed production fields in Hawaii in April 2011. Composite samples of roots, stems, and leaves were each derived from about 10 immature (90 days from planting) plants. The seeds, which are harvested approximately 150 days after planting, were derived from a general seed bank. The air-dried samples were milled to a fine powder.

For the quantitative comparison, an aliquot (ca. 400−500 mg) of each composite sample was extracted five times with methanol (10 mL) at room temperature and with gentle inversion mixing for 16 h. Each successive extract was monitored for dehydropyrrolizidine alkaloid content using HPLC-ESI/MS and then combined to yield the four total extracts, that is, roots, stems, leaves, and seeds, which were each concentrated to almost dryness in vacuo. The resultant

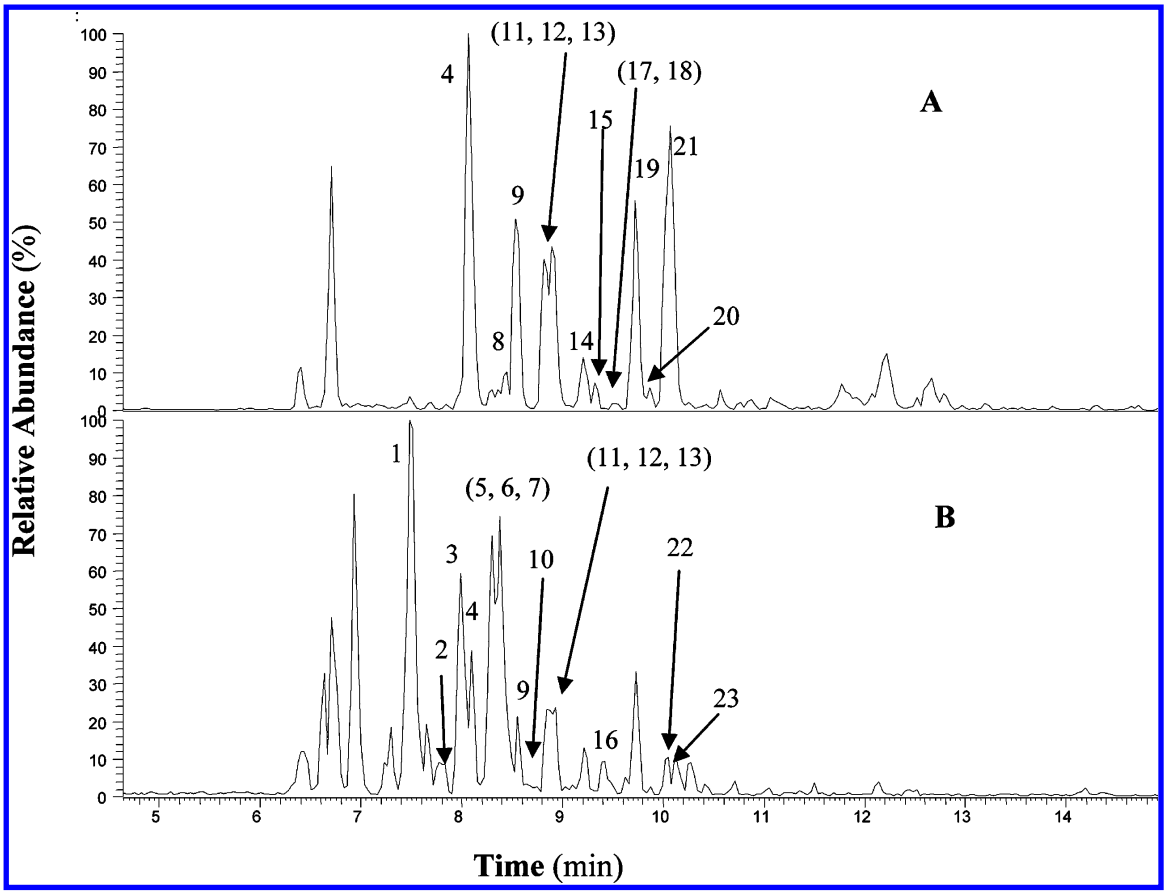

Figure 2. HPLC-ESI/MS base ion (m/z 200−800) chromatograms of the aqueous acid-soluble fraction of the methanolic extract of the stems (A) and seeds (B) of *Crotalaria juncea* (cv. 'Tropic Sun'). Peak numbers identify dehydropyrrolizidine alkaloids and are referred to in the text or in Table 1.

residues were extracted with 0.05 M sulfuric acid (ca. 10 mL) to provide the alkaloid (*N*-oxides as well as free base alkaloids) solution for further cation exchange enrichment.

For the structural elucidation studies, milled seed (160 g) was Soxhlet-extracted with methanol (1.2 L) for three periods of 16 h each. Fresh methanol was used for each extraction period to lessen or avoid any degradation of dehydropyrrolizidine alkaloids following extended periods in the boiling methanol. Again, the yield of dehydropyrrolizidine alkaloids after each extraction was monitored using HPLC-ESI/MS to ensure complete extraction. The combined methanol extracts were evaporated to almost dryness and re-extracted with 0.05 M sulfuric acid (3 × 50 mL) to yield the aqueous acid solution of the alkaloids for further purification.

Strong Cation Exchange Solid Phase Extraction (SCX SPE). The filtered 0.05 M sulfuric acid-soluble fraction from each of the concentrated methanolic extracts was applied to a separate stack of two conditioned (according to the manufacturer's guidelines) 500 mg/3 mL, Strata SCX 55 μm 70 Å SCX SPE columns. After loading, the stacked columns were separated, and each was washed with water, methanol and, finally, ammoniated methanol (a 10% solution of saturated ammoniated methanol in methanol) to elute the pyrrolizidine alkaloids and their *N*-oxides. The ammoniated methanol fractions were immediately evaporated to dryness under a stream of nitrogen, and the residues were reconstituted in methanol (1 mL). The efficiency of alkaloid capture and elution was monitored using HPLC-ESI/MS. The preparative extract of seeds was processed in a similar way except on a more appropriate, larger, scale of 25 mm ×100 mm cation exchange column using Sepra SCX, 50 μm, 65 Å bulk media.

HPLC-ESI/MS. Chromatographic separation of the dehydropyrrolizidine alkaloids and their *N*-oxides (Figure 2) was achieved using a Finnigan Surveyor HPLC system, comprising an Autosampler Plus and a MS Pump Plus (Thermo-Finnigan, San Jose, CA, USA), equipped with a 150 mm × 2 mm i.d., 4 μm, Synergi Hydro Reversed phase column, with a 4 mm × 2 mm i.d. AQ C18 guard column. Sample injections (2 μL) were eluted with a gradient flow (400 μL/min) in which the initial mobile phase composition of 5% acetonitrile in aqueous 0.05% trifluoroacetic acid/0.5% acetic acid was held for 2 min and then linearly increased to 50% acetonitrile over 13 min. This was held for a further 10 min before it was returned to the initial mobile phase composition and re-equilibration of the column. The column effluent was monitored using an LCQ Advantage ion trap mass spectrometer (Thermo-Finnigan) in the ESI positive ion mode. The mass spectrometer response was tuned to a solution of heliotrine (acquired from stocks held by the USDA/ARS Poisonous Plant Research Laboratory) in methanol. The capillary temperature was 275 °C with a capillary voltage of 46 V. The source voltage was 4.5 kV at a source current of 80 μA. The sheath and auxiliary gas flows were set at a ratio of 60:10. Data-dependent MS/MS spectra were acquired in the second scan of a two scan sequence in which ions identified in the first total ion scan were isolated and fragmented using 32% applied dissociation energy.

Qualitative Analysis. An even-numbered m/z for the MH^+ ion followed by observation of MS/MS fragment ions characteristic of dehydropyrrolizidine alkaloids, for example, m/z 94, 120, 122, 136, 137, 138, 156, and 180, highlighted HPLC-ESI/MS peaks as probable dehydropyrrolizidine alkaloids.[8,9]

Observation of a significant dimer ion $[2M + H]^+$ indicated potential dehydropyrrolizidine-*N*-oxide character under these ESI/MS conditions.[8] In situ treatment of an analytical sample indicative of *N*-oxides with indigocarmine-based redox resin reduced the *N*-oxides to their parent dehydropyrrolizidine alkaloids, thereby confirming the *N*-oxide character.[8]

Table 1. Positive Ion Electrospray Ionization Ion Trap MS/MS Data for the Dehydropyrrolizidine Alkaloids Detected in Extracts of *Crotalaria juncea* (cv. 'Tropic Sun')

peak[a]	identity	$MH^+/[2M + H]^+$ (m/z)	MS/MS[b] (% abundance)
1	junceine (**1**)	370	370 (20), 352 (15), 340 (7), 324 (100), 282 (8), 267 (9), 266 (58), 265 (7), 244 (14), 226 (53), 223 (20), 222 (36), 190 (5), 180 (23), 162 (8), 122 (18), 121 (7), 120 (28)
2	hemijunceine (**12**)	284	284 (42), 240 (100), 214 (2), 156 (45), 138 (36), 120 (10), 108 (5), 94 (2)
3	isohemijunceine A (**9**)	284	284 (<1), 240 (2), 222 (1), 204 (1), 156 (13), 138 (100), 120 (18), 94 (38)
4	junceine-*N*-oxide	386 (30)/ 771 (100)[c]	386 (14), 368 (96), 358 (6), 340 (100), 298 (20), 282 (12), 281 (8), 264 (22), 260 (10), 256 (6), 242 (6), 239 (10), 238 (21), 225 (7), 224 (4), 137 (10), 120 (10)
5	isohemijunceine B (**10**)	284	284 (1), 266 (1), 240 (2), 222 (1), 156 (9), 138 (100), 120 (17), 94 (28)
6	trichodesmine (**2**)	354	354 (21), 336 (3), 308 (100), 266 (14), 265 (9), 228 (8), 222 (30), 210 (21), 192 (5), 164 (14), 148 (3), 138 (1), 136 (3), 122 (4), 121 (5), 120 (7)
7	isohemijunceine C (**11**)	284	284 (<1), 240 (1), 222 (1), 156 (9), 138 (100), 120 (14), 94 (33)
8	dehydroisohemijunceine-*N*-oxide I	298 (70)/ 595 (100)	298 (51), 282 (9), 281 (100), 264 (9), 238 (8), 220 (29), 172 (4), 155 (17), 154 (27), 137 (18), 136 (26), 124 (7), 120 (3), 118 (6), 111 (5), 109 (9), 106 (44)
9	isohemijunceine A-*N*-oxide	300 (50)/ 599 (100)	300 (57), 283 (4), 282 (13), 256 (100), 172 (45), 155 (22), 154 (22), 139 (13), 138 (18), 137 (28), 136 (40), 118 (3), 112 (6), 108 (5), 94 (5)
10	dehydroisohemijunceine I	282	282 (14), 254 (1), 238 (2), 164 (2), 156 (1), 138 (100), 120 (33), 110 (2), 94 (73)
11	isohemijunceine B-*N*-oxide	300 (42)/ 599 (100)	300 (51), 283 (4), 282 (16), 256 (100), 172 (40), 155 (22), 154 (19), 150 (2), 138 (9), 137 (29), 136 (37), 112 (4), 111 (6), 109 (2), 108 (3), 94 (4)
12	trichodesmine-*N*-oxide	370/739 (20)	370 (1), 352 (5), 324 (100), 298 (7), 282 (4), 281 (6), 264 (11), 244 (6), 239 (14), 238 (23), 226 (6), 209 (3), 180 (2), 176 (3), 164 (2), 138 (4), 137 (9), 120 (6)
13	isohemijunceine C-*N*-oxide	300 (45)/ 599 (100)	300 (53), 282 (11), 256 (100), 172 (40), 155 (20), 154 (23), 139 (3), 138 (11), 137 (34), 136 (33), 121 (2), 120 (2), 109 (3), 108 (4), 94 (4)
14	dehydroisohemijunceine-*N*-oxide II	298 (89)/ 595 (100)	298 (63), 281 (2), 280 (4), 238 (1), 237 (3), 172 (100), 155 (16), 154 (37), 138 (11), 137 (33), 136 (44), 126 (3), 112 (3), 111 (3), 109 (1), 108 (2), 94 (4)
15	unidentified macrocyclic diester *N*-oxide	354 (40)/ 707 (100)	354 (100), 337 (4), 336 (3), 326 (8), 308 (7), 292 (6), 267 (3), 222 (2), 156 (2), 155 (11), 154 (10), 138 (39), 137 (40), 119 (3), 118 (19), 109 (2), 108 (6)
16	senecionine	336	336 (86), 318 (4), 308 (94), 290 (14), 199 (7), 153 (9), 138 (71), 120 (100)
17	acetylisohemijunceine I	326	198 (14), 180 (83), 162 (3), 120 (100), 118 (2)
18	unknown *N*-oxide	368 (85)/ 735 (100)	368 (100), 340 (55), 306 (15), 264 (31), 247 (14), 237 (12), 236 (66), 222 (13), 219 (14), 218 (42), 206 (13), 154 (26), 136 (18), 122 (11), 121 (22), 120 (30), 119 (57), 118 (50)
19	acetylisohemijunceine - *N*-oxide I	342 (40)/ 683 (100)	342 (51), 324 (28), 298 (100), 282 (6), 214 (72), 197 (14), 180 (14), 179 (8), 178 (28), 154 (9), 137 (4), 136 (8), 121 (5), 120 (9)
20	acetylisohemijunceine II	326	198 (17), 180 (61), 162 (4), 120 (100), 118 (1)
21	acetylisohemijunceine-*N*-oxide II	342 (44)/ 683 (100)	342 (38), 324 (23), 298 (100), 282 (4), 214 (55), 197 (15), 180 (7), 179 (6), 178 (23), 154 (5), 137 (4), 136 (8), 120 (7)
22	unidentified macrocyclic diester	338	338 (50), 310 (100), 292 (3), 222 (1), 138 (11), 120 (11),
23	unidentified dihydro macrocyclic diester	340	340 (100), 312 (40), 278 (2), 140 (3), 122 (8)

[a]Peak numbers refer to Figure 2. [b]Using a dissociation energy of 32% for the MH^+ ion. [c]Numbers in parentheses are percent relative abundances.

Quantitative Analysis. Standard solutions containing lycopsamine (**8**) (69, 34.5, 17.25, 8.63, 4.31, and 2.15 μg/mL) and junceine (**1**) (200, 100, 50, 25, 12.5, and 6.25 μg/mL) were made by adding a solution of **8** (200 μL of 138 μg/mL methanol) to a solution of **1** (200 μL of 400 μg/mL methanol) and serially diluting (1:1) an aliquot (200 μL) in methanol. Calibration standard samples were then prepared by adding lasiocarpine (**5**) solution (10 μL ca. 50 μg/mL methanol), to provide a normalizing injection standard (IS), to an aliquot (100 μL) of each of the standard solutions.

The analytical samples were similarly prepared by adding IS (10 μL) to an aliquot (100 μL) of the redox resin-reduced extract solution. Peak areas, generated from reconstructed ion chromatograms displaying the ions of interest, were adjusted within each run by division by the area of the IS for that run.

Preparative Flash Chromatography. The 10% ammoniated methanol eluate from the SCX SPE of seed extract was evaporated to dryness to afford a red gum (5 g) that was re-extracted with chloroform (3 × 50 mL). The pooled, orange chloroform extract was filtered, reduced in volume (to about 15 mL), and applied to a Biotage KP silica Samplet (10 g) cartridge of silica gel (Biotage, North America). A narrow orange band was retained on the Samplet cartridge. The loaded Samplet cartridge was fitted to a Biotage Snap KP-silica gel column (50 g) that had been equilibrated with ethyl acetate using the Isolera 1 automated flash chromatography system (Biotage, North America). Elution of retentates from the column assembly was achieved at a flow rate of 50 mL/min, collecting 20 mL fractions, using a linear gradient of 10% ammoniated methanol (0–40% over 5 min) into ethyl acetate, held for 6 min to elute nondehydropyrrolizidine alkaloid material, and then increased from 40 to 100% ammoniated methanol over 7 min to elute the dehydropyrrolizidine alkaloids, for a total run time of 18 min. The eluate was simultaneously monitored at 254 and 280 nm, which provided an indication, albeit poor due to low UV absorbance, of the alkaloid elution times. The collected fractions were then analyzed for dehydropyrrolizidine alkaloid content using HPLC-ESI/MS.

Preparative and Semipreparative HPLC. Alkaloid-enriched samples derived using the Biotage flash chromatography system were evaporated to dryness and reconstituted in 0.1% trifluoroacetic acid in water. Aliquots (up to 400 μL) of this solution were applied to a 100 mm × 21.2 mm i.d., 4 μm, 80 Å, AXIA-packed Synergi Hydro reversed phase column with a 15 mm × 21.2 mm i.d. C18 guard column. Using a Waters Prep LC 2000 System (Waters Corp., Milford, MA, USA), the alkaloids were eluted with an isocratic (0.1% trifluoroacetic acid/acetonitrile 93:7) flow (20 mL/min) and collected into bulk fractions determined by monitoring the absorbance at 220 nm. The dehydropyrrolizidine alkaloid content of the fractions was then determined using HPLC-ESI/MS.

Using a Finnigan Surveyor HPLC system, comprising a LC Pump Plus and a UV–vis Plus (Thermo-Finnigan), two isocratic, semi-preparative HPLC modes were used to achieve final separation of

Table 2. ^{1}H, ^{13}C, $^{1}H-^{1}H$ (COSY), and $^{1}H-^{13}C$ (HMBC) NMR Spectroscopic Data for Isohemijunceine A (9) Extracted from *Crotalaria juncea* (cv. 'Tropic Sun')

carbon[a]	^{1}H δ[b]	^{13}C δ[c]	COSY[d] (proton number)	HMBC[d,e] (proton number)
1	na[f]	133.38	na	9d (m), 9u (w), 3u (w)
2	5.85 (s)	129.83	3u[g] (w), 3d (w), 8 (w), 9u (w), 9d (w)	9d (m), 9u (w), 3u (w)
3	3.92 (d, $J_{3d,3u}$ = 15.4)	63.1	2 (w), 3u, 8 (w), 9u (w)	2 (w), 5u (m)
	3.40 (dd, $J_{3u,3d}$ = 15.5, $J_{3u,2}$ = 7.4)		2 (w), 3d, 8 (m), 9u (w)	
5	3.26 (m)	54.01	5u, 6 (w-m)	3u (w), 6 (w), 7 (m)
	2.73 (ddd, $J_{5u,5d}$ = 11.6, $J_{5u,6}$ = 9.1 and 6)		5d, 6 (m-s)	
6	ca. 1.86–2.01 (m, 2H)	35.97	5u (m-s), 5d (w-m), 7 (w)	5d (m), 5u (m)
7	4.29 (t, J = 2.7)	71.27	6 (w), 8 (m)	5d, 6,
8	4.16 (bs)	78.94	2 (w), 3u (m), 3d (w), 7 (m)	2 (w), 9d, 9u (w), 7 (w-m), 5d, 6d, 6u
9	4.92 (d, J = 13.0)	61.26	2 (w), 9u	5u (w),
	4.51 (d, J = 13.0)		2 (w), 3d (w), 3u (w), 9d	
11	na	173.43	na	9d (m), 12, 13, 15 (w-m)
12	2.36 (dd, J = 8.2, 6.2)	59.16	13 (m-s), 15 (w)	13, 15, 14, 16, 17
13	4.09 (p, J = 6.3)	66.91	12 (m-s), 14	12, 14, 15 (w), 16 (w), 17 (w)
14	1.21 (d, J = 6.3)	19.7	13	12, 13
15	ca. 2.0–2.13 (m)	27.49	12 (w), 16/17	12, 13, 14 (w), 16, 17
16	0.96 (d, J = 5)	20.63	15	12, 15, 17
17	0.94 (d, J = 4.8)	20.81	15	12, 15, 16

[a]Carbon numbering shown in Figure 1. [b]bs, broad singlet; m, multiplet; d, doublet; t, triplet; q, quartet; J in hertz. [c]Assignments of ^{13}C resonances were unambiguously established via a gradient enhanced HSQC experiment. [d]Strong interactions unless otherwise annotated (m) medium or (w) weak. [e]Data shown as proton to carbon correlations. [f]na, not applicable. [g]u, upfield; d, downfield partner of a geminal proton pair.

target analytes in the dehydropyrrolizidine alkaloid concentrates (up to 200 μL injections) derived from the preparative HPLC. The column effluent was monitored at 220 nm. Basic separation conditions were achieved using a 250 mm × 10 mm i.d., 5 μm, 110 Å Gemini NX C18 column with a 10 mm × 10 mm i.d. guard column of the same material with acetonitrile (7%) into 20 mM ammonium acetate at pH 9.5 (NH_4OH) (93%) at 5 mL/min. Acidic separation conditions were achieved using a 250 mm × 10 mm i.d., 4 μm, 80 Å Synergi Hydro reversed phase column with a 10 mm × 10 mm i.d. Synergi AQ C18 guard column with acetonitrile (7%) into 0.1% trifluoroacetic acid in water (93%) at 5 mL/min.

Structure Elucidation. Low-resolution MS and MS/MS data were acquired in the HPLC-ESI/MS mode described in a previous section. The high-resolution mass spectrometry (HRMS) measurement was achieved using an Agilent 6220 TOF mass spectrometer in the dual ESI positive ion mode. One-dimensional and two-dimensional (COSY, HSQC, and HMBC) ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR data were acquired using a JEOL Eclipse NMR spectrometer using solutions in $CDCl_3$ and the residual proton in the chloroform as the lock signal (except for junceine, for which d_6-DMSO was used as the solvent, and the crude seed extract, which was dissolved in d_4-methanol).

Junceine (**1**) (peak 1, Figure 2) and *trichodesmine* (**2**) (peak 6, Figure 2): HPLC-ESI/MS data (Table 1) were the same as for the authenticated standards, and the ^{1}H and ^{13}C NMR data were consistent with previous studies.[12−14]

Isohemijunceine A (**9**) (peak 3, Figure 2): obtained as a colorless oil estimated at >95% pure using HPLC-ESI/MS and ^{1}H NMR; ESI(+)/MS and MS/MS (Table 1); HRMS (found, 283.17842; calcd for $C_{15}H_{25}NO_4$ 283.1784; Δ 0.07 ppm); ^{1}H, ^{13}C, COSY, and HMBC NMR (Table 2).

Isohemijunceine B (**10**) (peak 5, Figure 2): obtained as a colorless oil estimated at 85–90% pure using HPLC-ESI/MS and ^{1}H NMR; ESI(+)/MS and MS/MS (Table 1); HRMS (found, 283.17892; calcd for $C_{15}H_{25}NO_4$ 283.1784; Δ 1.8 ppm); ^{1}H, ^{13}C, and COSY NMR (Table 3).

Isohemijunceine C (**11**) (peak 7, Figure 2): obtained as a colorless oil estimated at 85–90% pure using HPLC-ESI/MS and ^{1}H NMR; ESI(+)/MS and MS/MS (Table 1); HRMS (found, 283.17912; calcd for $C_{15}H_{25}NO_4$ 283.1784; Δ 2.5 ppm); ^{1}H, ^{13}C, COSY, and HMBC NMR (Table 4).

Combined NMR and HPLC-ESI/MS Analysis of Seed. Seeds (50 g) were milled to a fine powder. Three subsamples were dried in an oven at 40 °C for 5 days to determine the moisture content of about 5%. A larger sample (40.99 g) was Soxhlet-extracted with methanol (400 mL) for 20 h. The extraction solvent was removed and

Table 3. ^{1}H, ^{13}C, and $^{1}H-^{1}H$ (COSY) NMR Spectroscopic Data for Isohemijunceine B (10) Extracted from *Crotalaria juncea* (cv. 'Tropic Sun')

carbon[a]	^{1}H δ[b]	^{13}C δ[c]	COSY[d] (proton number)
1	na[e]	132.98	na
2	5.87 (s)	129.14	3u[f] (m), 3d (m), 7 or 8 (m), 9u (m), 9d (m)
3	4.05 (d, $J_{3d,3u}$ = 15)	62.6	2 (m), 3u, 7 or 8 (w), 9d (w-m), 9u (w-m)
	3.47 (bd, $J_{3d,3u}$ = 15)		2 (m), 3d, 7 or 8 (m), 9d (w), 9u (w)
5	3.41 (bm)	54.04	5u, 6 (m)
	2.82 (bm)		5d, 6
6	ca. 2.03 (m, 2H)	35.98	5u, 5d (m), 7 (w)
7	4.35 (bs, 2H)	71.04	6 (m), 2 (m), 3u (m), 3d (w)
8		78.89	
9	4.83 (d, J = 13.2)	61.16	2 (m), 3d (w), 3u (w), 9u
	4.67 (dd, J = 13.1, 1.5)		2 (m), 3d (w), 3u (w), 9d
11	na	174.44	na
12	2.13 (m)	59.75	13 (m-s)
13	4.05 (m)	66.25	12 (m), 14
14	1.22 (d, J = 6.3)	22.28	13
15	ca. 2.09 (m)	27.79	16, 17
16	1.01 (d, J = 6.4)	21.03	15
17	0.93 (d, J = 6.5)	20.06	15

[a]Carbon numbering shown in Figure 1. [b]bs, broad singlet; m, multiplet; d, doublet; t, triplet; q, quartet; J in hertz. [c]Assignments of ^{13}C resonances were unambiguously established via a gradient enhanced HSQC experiment. [d]Strong (s) interactions unless otherwise annotated (m) medium or (w) weak. [e]na, not applicable. [f]u, upfield; d, downfield partner of a geminal proton pair.

Table 4. ^{1}H, ^{13}C, $^{1}H-^{1}H$ (COSY), and $^{1}H-^{13}C$ (HMBC) NMR Spectroscopic Data for Isohemijunceine C (11) Extracted from *Crotalaria juncea* (cv. 'Tropic Sun')

carbon[a]	^{1}H δ[b]	^{13}C δ	COSY[c] (proton number)	HMBC[d,e] (proton number)
1	na[f]	132.99	na	2 (w-m), 3d, 3u, 9d, 9u
2	5.87 (bs)	130.43	3d[g], 3u (m), 8 (w-m), 9d (m), 9u	3d, 3u, 9d, 9u
3	4.03 (bd)	63.06	2 (m), 3u, 8 (w)	2, 5u,
	3.46 (bd)		2 (m), 3d, 8	
5	3.40 (m)	54.01	5u, 6d, 6u	3d (w), 3u, 7
	2.82 (m)		5d, 6d, 6u	
6	ca. 1.9−2.03 (2H, m)	36.08	5d, 5u, 7	5u
7	4.35 (bs)	71.16	6d, 6u,	3d (m-s), 5d, 6,
8	4.31 (bs)	78.89	2 (w-m), 3d (m), 3u, 5u	2, 3d (m), 5d, 6, 7 (m), 9d, 9u (w),
9	4.87 (bd, J = 13.2)	61.36	2, 9u, 3d (m), 3u (m)	2
	4.63 (bd, J = 13.2)		2, 9d, 3d (m-s), 3u (m)	
11	na	174.84	na	9d, 9u (w), 12, 13, 15
12	ca. 2.10−2.19 (m)	59.52	13	13, 14, 15, 16, 17
13	4.05 (m)	66.41	12, 14	12, 14, 15 (w)
14	1.20 (d, $J_{14,13}$= 6.3)	22.29	13	12 (w), 13
15	ca. 2.02 − 2.1 (m)	27.85	16, 17	12, 13, 16, 17
16	1.01 (d, $J_{16,15}$= 6.6)	21.0	15	12, 15, 17
17	0.93 (d, $J_{17,15}$= 6.6)	20.02	15	12, 15, 16

[a]Carbon numbering shown in Figure 1. [b]m, multiplet; s, singlet; d, doublet; t, triplet; q, quartet; b, broad; J in hertz. [c]Assignments of ^{13}C resonances were unambiguously established via a gradient enhanced HSQC experiment. [d]Strong interactions unless otherwise annotated (m) medium or (w) weak. [e]Data shown as proton to carbon correlations. [f]na, not applicable. [g]u, upfield; d, downfield partner of a geminal proton pair.

the extraction continued twice more with fresh solvent (20 and 48 h) to lessen any degradation that might result from prolonged boiling in methanol. The combined extracts were evaporated to dryness in vacuo to afford a yellow solid (5.7 g, 14%) that was subsequently extracted with 0.05 M H_2SO_4. The aqueous acid solubles were applied to a conditioned 5 g/20 mL Strata SCX 55 μm 70 Å SCX SPE column that discharged, under gravity, onto a second conditioned 5 g/20 mL Strata SCX 55 μm 70 Å SCX cartridge. After complete addition of the acid extracts, the two cartridges were separately washed with methanol (causing elution of an orange color) and then with 10% saturated ammoniated methanol to elute the alkaloids.

The combined ammoniated methanol eluates were evaporated to dryness to yield a dark red oil (0.127 g) that was reconstituted in d_4-methanol (700 μL) and the ^{1}H NMR spectrum recorded (see the Supporting Information). Then an aliquot of *p*-dinitrobenzene (25 μL of 23 mg/mL d_4-methanol) was added and the spectrum reacquired. A further aliquot (50 μL) of the *p*-dinitrobenzene was added and the spectrum again reacquired to provide an additional check on quantitation. The C2 proton signals in both NMR spectra were integrated relative to the *p*-dinitrobenzene singlet, as a total envelope (ca. 6.2−5.8 ppm) or as two separate envelopes, about 6.2 ppm for the macrocyclic diesters or about 5.8 ppm for the monoesters, and the amount of dehydropyrrolizidine alkaloids was estimated according to the method of Molyneux et al.[10]

The same sample, suitably diluted with methanol, was then assessed using the HPLC-ESI/MS method using trichodesmine (**2**) (isolated from *C. juncea* in this study; 38.1, 19.05, 9.53, 4.77, 2.38, 1.19, and 0.59 μg/mL) and lycopsamine (**8**) (34.5, 17.25, 8.63, 4.32, 2.16, 1.08, and 0.54 μg/mL) as the calibration standards for the macrocyclic diesters and monoesters, respectively.

RESULTS AND DISCUSSION

Qualitative Analysis of Roots, Stems, Leaves, and Seeds. HPLC-ESI/MS analysis (Figure 2) and the MS/MS data (Table 1) showed a differential distribution of free base dehydropyrrolizidine alkaloids and their *N*-oxides within the plant tissues. The seeds contained the free bases as the major pyrrolizidine alkaloid components (>95%), whereas the roots and stems contained the *N*-oxides with only traces of the free base dehydropyrrolizidine alkaloids. About equal, but relatively low, levels of the free base alkaloids and their *N*-oxides were extracted from the leaves. These observations are consistent with previous findings that the dehydropyrrolizidine alkaloids are biosynthesized in roots as their water-soluble *N*-oxides and then transported to the reproductive tissues, where they accumulate as the more lipophilic free bases.[11]

Detection of Previously Identified Alkaloids. Consistent with previous results[4,6] the presence of the macrocyclic diester alkaloids **1** and **2** was indicated by the peaks in reconstructed ion chromatograms showing a protonated molecule (MH^+) at m/z 370 (peak 1, Figure 2) and m/z 354 (peak 6, Figure 2), respectively, or by their *N*-oxides showing MH^+ and $[2M + H]^+$ ions at m/z 386/771 (peak 4) and m/z 370/739 (peak 12), respectively. Following isolation of the putative junceine and trichodesmine from seed extract, using preparative and semipreparative reversed phase HPLC, their identities were confirmed by comparison of their ^{1}H and ^{13}C NMR data with literature results.[12−14] On the basis of HPLC-ESI/MS/MS, only a minor amount of the previously reported[4] senecionine (MH^+ m/z 336, peak 16, Figure 2) was observed in these analyses. Contrary to this previous study[4] there were no HPLC-ESI/MS indications of the related, macrocyclic diester dehydropyrrolizidine alkaloids riddelliine or seneciphylline.

Detection and Identification of Previously Undescribed Alkaloids. At the commencement of this study, the analytical HPLC mobile phase conditions were the same as previously reported[8] using 0.1% formic acid in a gradient with acetonitrile. The MS/MS fragmentation ions at m/z 156, 138, 120, and 94 observed under these conditions for two unidentified major peaks in the seed extracts, each displaying a MH^+ at m/z 284, were indicative of dehydropyrrolizidine alkaloid character. However, the MS/MS data were inconsistent with the isobaric (MH^+ m/z 284) diastereoisomers of the known dehydropyrrolizidine alkaloids that lack oxygenation at C7 such as supinine (**3**) and amabiline (**4**), which show an abundant fragmentation ion at m/z 122 under the same conditions. The *N*-oxides of these putative dehydropyrrolizidine alkaloids, with $MH^+/[2M + H]^+$ ions at m/z 300/599 and confirmed by in situ reduction back to the parent free base alkaloids, were also observed, especially in the stems and roots.

However, preliminary NMR spectroscopy observations for the second of these peaks, in particular the unusual multiplicity of the C9 proton resonances and the doubling up of most signals in the proton-decoupled ^{13}C NMR spectrum, indicated that it was a mixture of very closely structurally related compounds. Subsequent HPLC-ESI/MS using trifluoroacetic acid as the acidic modifier rather than formic acid showed the partial resolution of the two free base components of this

second peak in the seed extract (peaks 5 and 7, Figure 2) and their respective *N*-oxides in the stem extract (peaks 11 and 13).

Preparative reversed phase HPLC under trifluoroacetic acid-based mobile phase conditions provided (≥95% pure) peak 3 (Figure 2), the first, and best separated, of the three observed *m*/*z* 284 peaks, and a fraction containing peaks 5, 6, and 7 (Figure 2). Subsequent semipreparative reversed phase HPLC using the basic mobile phase conditions with the Gemini NX column separated trichodesmine (peak 6, Figure 2) from peaks 5 and 7 that coelute from this column under these basic conditions. Finally, semipreparative HPLC using the Synergi Hydro reversed phase column and trifluoroacetic acid-based mobile phase conditions allowed isolation (ca. 85−90% pure) of peaks 5 and 7.

Structure Elucidation of Peak 3, 5, and 7 Alkaloids. These alkaloids had identical MS (MH^+ *m*/*z* 284) and very similar MS/MS fragmentation profiles (Table 1). High-resolution mass measurements supported a molecular formula $C_{15}H_{25}NO_4$ for each. The MS/MS data (Table 1) showed moderately abundant ions at *m*/*z* 120 and 156 and a base ion peak at *m*/*z* 138, which together support a necine C9/C7 monoester alcohol structure.[15] The fragmentation of *m*/*z* 284 to 138 therefore indicates a $C_7H_{14}O_3$ necic acid group that is unusual in that it is at least one oxygen atom less than C7 necic acids previously reported for pyrrolizidine alkaloid monoesters.[9,16] The loss of 44 Da from the protonated molecule (*m*/*z* 284 → 240) under LC-MS conditions is characteristic of a terminal hydroxy(methyl)methine (CH_3CHOH) group on the esterifying acid,[8,17] analogous to the loss of 45 Da due to the same terminal group under electron ionization MS.[18] Therefore, to be consistent with the putative $C_7H_{14}O_3$ necic acid group, the MS/MS data indicate a monoester with 3-hydroxy-2-isopropylbutanoic acid rather than the 2,3-dihydroxy-2-isopropylbutanoic acid more usually associated with the lycopsamine (**8**) type of dehydropyrrolizidine alkaloids.[19]

Analysis of the 1D and 2D NMR spectroscopic data for the peak 3 alkaloid (Table 2), the peak 5 alkaloid (Table 3), and the peak 7 alkaloid (Table 4) confirmed their common, two-dimensional structure as the C9 ester of 1,2-dehydro-7-hydroxypyrrolizidine with 3-hydroxy-2-isopropylbutanoic acid. Because this ester forms half of the junceine (**1**) macrocycle and because it is on the C9 rather than the C7 as in **1**, the peak 3, 5, and 7 alkaloids are given the trivial names isohemijunceine A (**9**), isohemijunceine B (**10**), and isohemijunceine C (**11**), respectively.

The determination of C9 esterification and the configuration around C7 and C8 were in accord with a review of proton NMR data by Logie et al.,[20] and a ^{13}C NMR study by Jones et al.[21] Thus, the relatively lower field resonance of the two C9 protons, that is, between δ 4.83−4.92 and 4.51−4.67, indicated C9 esterification in contrast to expected chemical shifts of approximately δ 3.6−4.3 for C7 esterification. This was supported by the HMBCs (Tables 2 and 4) between the carbonyl C11 and the protons on C9. The hydroxylation of C7, indicated by the MS/MS data (fragment ion at *m*/*z* 138 requires either C7 or C9 to be hydroxylated), was confirmed by the downfield chemical shifts of H7 at δ 4.29−4.31 and C7 at δ 71.04−71.27. The retronecine configuration of the necine base, that is, H8 and H7, both α relative to the plane of the ring, was indicated by the chemical shifts of C1 (δ 132.98−133.38), C2 (δ 129.14−130.43), C6 (δ 35.97−36.08), and C7 (δ 71.04−71.27) that are upfield, downfield, downfield, and upfield, respectively, from those expected of a heliotridine-based alkaloid.[21]

The C12 protonation postulated on the basis of the MS/MS data, in contrast to the more usual C12 hydroxylation,[19] was confirmed by NMR experiments, that is, HSQC; HMBCs to C11, C13, C14, C15, C16, and C17; and the COSY interactions with H13 and H15.

Whereas the chromatographic properties and NMR data for isohemijunceines B (**10**) and C (**11**) are very similar, indicating minor diastereoisomeric changes, the better chromatographic separation and the significant differences in the NMR spectra for isohemijunceine A (**9**) indicate a more substantial diastereoisomeric change. Previous papers describing saturated[22] and 1,2-unsaturated[18] pyrrolizidine alkaloid C9 monoesters of the lycopsamine (**8**) type, that is, esterified with viridifloric or trachelanthic acids that are 2,3-dihydroxy-2-isopropylbutanoic acids diastereoisomeric about C3, have differentiated the stereochemistry of the necic acid on the basis of the difference in chemical shifts ($\Delta\delta$) of the C9 protons, the $\Delta\delta$ of the methyl carbons (C16 and C17) of the isopropyl group, and the chemical shifts of C13 and H13 of the terminal methyl(hydroxy)methine group of the acid. However, because the isohemijunceines lack the C12 hydroxylation, it is doubtful these empirical observations can be applied as a general rule to the analogous data for the isohemijunceines (Table 5). Nonetheless, the most obvious difference in the ^1H NMR spectrum of **9** (peak 3, Figure 2) compared to **10** and **11** (peaks 5 and 7) is the large increase in the $\Delta\delta$ of the C9 protons and the downfield shift of the H12 (Table 5). Attempting to assign the relative configurations about C12 and C13 based upon the observations by Wiedenfeld and Röder[18] leads to contradictions in this case and is therefore not possible. However, it is postulated that the significant changes in the ^1H chemical shifts for C9 and C12 of **9** relative to those for **10** and **11** do reflect a diastereoisomerism about C12. Furthermore, because of the very minor NMR spectra changes, the diastereoisomerism leading to isohemijunceines B (**10**) and C (**11**) is therefore expected to be about C13, analogous to supinine (**3**) and amabiline (**4**). There was no evidence for C13-based diastereoisomerism of isohemijunceine A (**9**).

Table 5. Some Significant Differences in the NMR Spectra for the Isohemijunceines A (9), B (10), and C (11) of Potential Stereochemical Diagnostic Value[18]

isohemijunceine	$\Delta\delta$ C9 proton	δ H12	δ C13 ^1H / ^{13}C	^{13}C $\Delta\delta$ C16/C17
A (**9**)	0.41	2.36	4.09/66.91	0.18
B (**10**)	0.16	2.13	4.05/66.25	0.97
C (**11**)	0.24	2.10−2.19	4.05/66.41	0.98

Acetylated Derivatives of the Isohemijunceines. The *N*-oxides of the acetylated derivative of the *m*/*z* 284 alkaloids, that is, $MH^+/[2M + H]^+$ *m*/*z* 342/683 (peaks 19 and 21, Figure 2), and the much lower abundance free bases, that is, MH^+ *m*/*z* 326 (peaks 17 and 20, Figure 2), were observed in the stem and root extracts only. Examination of the MS/MS data for the *N*-oxides and the corresponding free bases (Table 1) supported a 7-acetoxy derivative by virtue of the abundant fragment ion at *m*/*z* 180.[8] Without NMR data, the stereochemistry and hence the relationship of the acetylated derivatives to the three isohemijunceines remain undefined. However, analogous to isohemijunceines A, B, and C (**9**−**11**),

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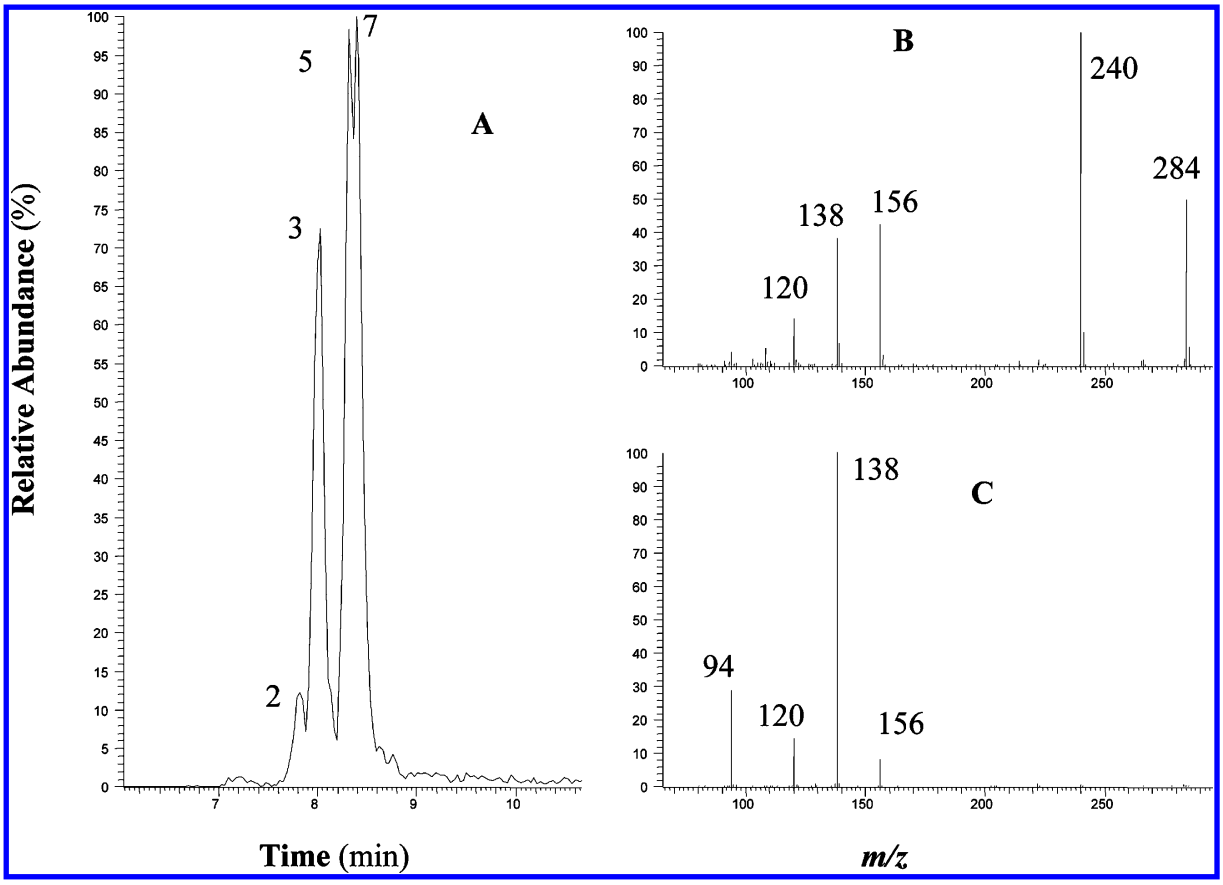

Figure 3. HPLC-ESI/MS, reconstructed ion chromatogram (A) displaying m/z 284 for the extract of seeds of *Crotalaria juncea* (cv. 'Tropic Sun'). The peak numbers in chromatogram A correspond to Figure 2. The subtle structural difference is highlighted by the MS/MS fragmentation profile of (B) hemijunceine (**12**) (peak 2) and (C) the isohemijunceines A (**9**), B (**10**), and C (**11**) (peaks 3, 5, and 7, respectively).

the MS/MS data for the two *N*-oxide peaks and the two free base peaks are very similar to each other in both cases. Therefore, it can be speculated that peaks 17 and 19 (Figure 2) may be 7-acetylisohemijunceine A and its *N*-oxide, respectively, whereas peaks 20 and 21 represent coeluting 7-acetylisohemijunceines B and C and their *N*-oxides, respectively.

Minor and Trace Levels of Additional Dehydropyrrolizidine Alkaloids. From an assessment of the MS and MS/MS data it is apparent that there are many dehydropyrrolizidine alkaloids, and their *N*-oxides, present at minor to trace levels. Because of their insignificance, at least to overall levels of dehydropyrrolizidine alkaloids in *C. juncea*, only some of these have been included in Figure 2 and Table 1 but, mostly, there are insufficient data to allow confident speculation on structures.

Another alkaloid with MH^+ at m/z 284 was observed in the seed extract (peak 2, Figure 2). The MS/MS data (Table 1) were very similar to those for the isohemijunceines except for the relative abundances (Figure 3). The increased stability of the protonated molecule, the $[MH^+ - 44]$ ion at m/z 240 (base ion), and the m/z 156 ion were all in contrast to the facile elimination of the necic acid from the isohemijunceines that gives rise to the base ion at m/z 138 in their mass spectra. A possible explanation for this is that this minor alkaloid is the C7 esterified analogue of the isohemijunceines, that is, hemijunceine (**12**). Because of resonance stabilization of the resultant carbocation, the C9 allylic esters would be expected to be more fragile, resulting in loss of the entire necic acid to yield the base ion at m/z 138.[23] The more stable C7 ester will have more opportunity for intranecic acid fragmentation such as the loss of 44 Da to yield the base ion at m/z 240. This effect of C7 esterification versus C9 esterification has been previously recognized in the electron ionization MS of 7- and 9-angelylplatynecine[24] and in the reported more facile cleavage of a C9 allylic ester compared to a C7 ester.[25] Indeed, under the HPLC-ESI/MS conditions used in this study, 9-angelylheliotridine (**7**; m/z (% abundance) 238 (5, MH^+), 220 (1), 156 (4), 138 (100), 120 (14), 96 (5), 94 (3)) also showed the facile cleavage of the entire necic acid to yield a base ion at m/z 138, whereas the C7 ester (**6**; m/z (% abundance) 238 (4, MH^+), 220 (7), 156 (7), 138 (31), 120 (100), 108 (6), 94 (19)) showed an increase in abundance of the $[MH^+ - H_2O]$ fragment (m/z 220), the intranecic acid fragmentation ion (m/z 156), and a base ion at m/z 120.

The stem extract included minor amounts of alkaloid *N*-oxides for which the MH^+ and $[2M + H]^+$ ions were observed at m/z 298/595 (peaks 8 and 14, Figure 2). One of the naturally occurring free base analogues (MH^+ m/z 282) was barely observable in the seed extract (peak 10, Figure 2) but was readily detected in the redox resin-reduced stem extract. The MS/MS data acquired for this free base (peak 10, Figure 2) showed a loss of 44 Da (m/z 282 → 238) followed by 100 Da (m/z 238 → 138) and therefore supported the presence of a methyl(hydroxy)methine derivative of senecioic acid, giving rise to the 12,15-dehydro analogue (or coeluting analogues) of one or more of the isohemijunceines. The MS/MS data for the *N*-oxides (peaks 8 and 14, Table 1), however, were quite different from each other, more so than might be expected for

simple C7 versus C9 esterification. Because of the facile loss of a hydroxyl group (m/z 298 (50% abundance) → 281 (100%)) it is possible that the minor peak 8 may be the *N*-oxide of the macrocyclic diester dicrotaline.[9] MS/MS data for the reduction product from peak 8 were not acquired due to coelution with more abundant alkaloids.

Quantitative Analysis of Roots, Stems, Leaves, and Seeds. Because the calibration standards are free base dehydropyrrolizidine alkaloids and it has been shown that the ESI/MS responses of an *N*-oxide and the corresponding free base can be quite different,[26] the *N*-oxides in the samples were reduced prior to analysis.[8] HPLC-ESI/MS analysis, using a 0.1% formic acid-based mobile phase with acetonitrile with the Synergi Hydro column, confirmed complete capture of the alkaloids by the top column of the SCX-SPE stack and their subsequent efficient elution by the ammoniated methanol. The peak areas in reconstructed ion chromatograms of the major resultant free base dehydropyrrolizidine alkaloids, that is, junceine (**1**), trichodesmine (**2**), isohemijunceines A (**9**), B (**10**), and C (**11**), and the acetylisohemijunceines I and II, were quantitated against six-point calibration curves generated using **1** (for the macrocyclic diesters, $y = 0.23x^2 + 10.71x$, $R^2 = 0.998$) or lycopsamine (**8**) (for the monoesters, $y = 2.95x^2 + 12.3x$, $R^2 = 0.999$) and expressed as a percentage of the dry weight of the plant (Table 6). The total amount of dehydropyrrolizidine alkaloids in seed was shown to be about 0.15%. The total dehydropyrrolizidine alkaloid contents of roots, stems, and leaves were determined to be about 0.05, 0.12, and 0.01%, respectively. Despite these estimates, which would be expected to fluctuate with the growth stage of the plant, the main observation is that the previously unrecognized monoester dehydropyrrolizidine alkaloids, that is, isohemijunceines A, B, and C (**9**−**11**) constitute the greater proportion of the total.

Table 6. Total Concentrations of Major Dehydropyrrolizidine Alkaloids in Roots, Stems, Leaves, and Seeds of *Crotalaria juncea* (cv. 'Tropic Sun') Following Reduction of *N*-Oxides to Free Bases

	alkaloid concentration (%, w/w)			
dehydropyrrolizidine alkaloid	roots	stems	leaves	seeds
junceine (**1**)[a]	0.021	0.026	0.003	0.03
trichodesmine (**2**)[a]	0.007	0.013	0.001	0.02
isohemijunceine A (**9**)[b]	0.007	0.009	0.001	0.03
isohemijunceines B (**10**) and C (**11**)[b]	0.014	0.023	0.002	0.07
acetylisohemijunceines I and II[b]	0.004	0.044	0.001	0.00
total (%, w/w)	0.053	0.115	0.008	0.15

[a]Quantitated from the junceine calibration curve. [b]Quantitated from the lycopsamine calibration curve.

As a more direct comparison with the brief report by Ji et al.,[6] an additional seed sample of the 'Tropic Sun' cultivar was extracted and analyzed using the quantitative NMR spectroscopy approach used by Ji et al.,[6] but originally described by Molyneux et al.[10] Thus, by quantitating the combined area of the C2 vinylic proton signals at 5.6−6.2 ppm against a known amount of *p*-dinitrobenzene, the total dehydropyrrolizidine alkaloid content was estimated to be 0.09% w/w (DW) of seed. A HPLC-ESI/MS analysis of this same sample extract, using seven-point calibration curves generated using trichodesmine (**2**), isolated from 'Tropic Sun' seed in this study, for the macrocyclic diesters ($y = 0.47x^2 + 2.43x - 0.16$, $R^2 = 0.999$) or **8** for the monoesters ($y = 1.63x^2 + 5.86x - 0.42$, $R^2 = 0.999$), returned an estimate of 0.1% dehydropyrrolizidine alkaloids (w/w, DW). This latter HPLC-ESI/MS estimate comprised 0.007% **1**, 0.009% **2**, and 0.08% of the combined isohemijunceines (**9**−**11**). It should be noted that **1** has a poor solubility in chloroform[13] and may not be totally accounted for in the chloroform-based analysis used in earlier studies.[5,6]

Implications of the Presence of Dehydropyrrolizidine Alkaloids in 'Tropic Sun'. The major use of 'Tropic Sun' is as a green manure crop that, among other beneficial attributes, helps control plant-parasitic nematodes.[2,3] Related to this, Thoden and Boppré[27] have highlighted the potential for at least some of the nematode management properties of plant extracts or mulches to be attributed to the presence of dehydropyrrolizidine alkaloids. They further hypothesize that accumulation (or primary biosynthesis) in the roots as the water-soluble *N*-oxides may be a defense against root-pathogenic nematodes. Thus, the presence of the dehydropyrrolizidine alkaloids in the 'Tropic Sun' cultivar may be responsible, at least in part, for the antinematode properties of the plant.

The archived seed extract of *C. juncea*, sourced from India, previously analyzed by Williams and Molyneux[5] was reconstituted in methanol and reanalyzed under the current HPLC-ESI/MS conditions. Not only were the expected macrocyclic diesters **1** and **2** observed but also the new isohemijunceines (**9**−**11**) were readily detected, demonstrating that these metabolites are not unique to the 'Tropic Sun' cultivar. In their paper Williams and Molyneux estimated that toxicity to chicks required levels of at least 1% w/w of pyrrolizidine alkaloids in the seed. They further speculated that levels of 0.1−0.3% of pyrrolizidine alkaloids in seed, if constituting >1% of the diet for several weeks, might present a health hazard to animals and fowl.[5]

A major assumption in this study is that the positive ion ESI/MS responses of **1**, **2**, and the isohemijunceines (**9**−**11**) are similar to those of the calibration standards trichodesmine, junceine, and lycopsamine, respectively, when used. With this caveat, the levels of dehydropyrrolizidine alkaloids determined in this study of the 'Tropic Sun' cultivar could be a cause for concern if simply compared to levels in *Senecio jacobaea* and *Senecio vulgaris*, for example, that are known problems to rangeland cattle in the United States with levels of between 0.09 and 0.18% total dehydropyrrolizidine macrocyclic diester alkaloids.[10] However, with over half of the dehydropyrrolizidine alkaloids in 'Tropic Sun' being the isohemijunceines that, as monoesters, may be less toxic,[28] such simple comparisons may not be valid. Additionally, *Crotalaria* species that have been associated with livestock intoxication have been reported to produce higher levels of alkaloids and include *C. spectabilis* (0.01−1.52% in leaves and 2.41−5.35% in seeds)[29] and *C. novae-hollandiae* subsp. *novae-hollandiae* (chemotype 2), *C. ramosissima*, *C. retusa* var. *retusa*, and *C. crispata*, each producing a mean alkaloid content of 0.6, 2.23, 1.22, and 0.77% w/w, respectively (expressed as retrorsine equivalents).[30] However, from the aspect of the potential for chronically developing diseases in humans[7] resulting from exposure to the *C. juncea* dehydropyrrolizidine alkaloids, it would be of value to determine and assess any such routes of potential exposure such as exposed animal-derived products (meat and milk), bee products (honey and pollen), and seed contamination of grain products.

■ ASSOCIATED CONTENT

Supporting Information

jpg copies of all NMR spectra described herein. This material is available free of charge via the Internet at http://pubs.acs.org.

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