

Hydroxylic Solvent-Induced Ring Opening of the Dehydropyrrolizidine Alkaloids Riddelliine and Seneciphylline: Implications for Toxicity and Analytical Studies

Steven M. Colegate^{1*}, Dale R. Gardner¹, William Resager¹, Nathan Bollar¹, Joseph M. Betz², and Kip E. Panter¹

¹USDA-ARS Poisonous Plant Research Laboratory, Logan, Utah, USA

²Office of Dietary Supplements, National Institutes of Health, Bethesda, Maryland, USA

*Corresponding author: Steven M. Colegate, steven.colegate@ars.usda.gov

Abstract

Dehydropyrrolizidine alkaloids are potentially hepato-, pneumo-, and geno-toxic plant secondary metabolites that can also cause various cancers in animal models. Riddelliine and seneciphylline are closely related, macrocyclic diester dehydropyrrolizidine alkaloids produced by various species in the Asteraceae. Despite the evidence of carcinogenicity in animal models and the increasing concerns with respect to human health, riddelliine and lasiocarpine are the only dehydropyrrolizidine alkaloids so far officially described as Group 2B carcinogens. This, combined with the ready accessibility of quite pure riddelliine from some plant sources, has led to riddelliine often being used in toxicology studies. A routine HPLC-esi(+)MS analysis of a stored, standard solution of riddelliine in methanol revealed the formation of two new isobaric (m/z 382) peaks at slightly longer retention time. A similar formation of two new isobaric (m/z 396) peaks was observed for riddelliine in ethanol. The methanol- and ethanol-related compositional changes were also observed with seneciphylline and the *N*-oxides of riddelliine and seneciphylline but not with retrorsine, senecionine, heliotrine, or the *N*-oxides of senecionine and heliotrine. There was no evidence for complete hydrolysis of the alkaloids to the retronecine base. Isolation of the major riddelliine–methanol adduct and subsequent 1D and 2D NMR spectroscopic analysis unambiguously determined that the methanol adds to the riddelliine in a ring-opening transesterification at the allylic C9 ester bond forming a new C7 monoester of retronecine with methylriddelliic acid. The spontaneous formation of riddelliine and seneciphylline adducts should be checked prior to using solutions of riddelliine and seneciphylline, or their *N*-oxides, as analytical standards or for toxicological studies.

Keywords: dehydropyrrolizidine alkaloids, heliotrine, HPLC-esiMS, NMR, *N*-oxides, retrorsine, riddelliine, seneciphylline, senecionine, transesterification

Introduction

The toxicity of the 1-hydroxymethyl-1,2-dehydro-7-hydroxypyrrolizidine alkaloid mono- and diesters (dehydroPAs), produced by numerous plant species in genera of the Boraginaceae, Asteraceae, and Fabaceae, to livestock and to humans is well

documented (Bull et al. 1968, Edgar et al. 2011, Molyneux et al. 2011). Since the liver is a primary source of bioactivation of the pro-toxic dehydroPAs to the toxic didehydroPAs, otherwise referred to as the “pyrrolic” form, then hepatotoxicity is a usual

observation in livestock and humans. In some circumstances, the dehydroPAs or their hepatic metabolites can escape the liver and circulate systemically to cause pneumotoxic sequelae (Pessoa et al. 2013). Some dehydroPAs are also documented genotoxins and can cause cancers in various animal models (Edgar et al. 2011). Humans have been poisoned, with consequent liver-related adverse effects, by dehydroPAs in contaminated grain and in some herbal products. However, it is the potential for low-level, long-term or intermittent dietary exposures that cause, contribute to, or exacerbate chronically developing disease in humans (e.g. various cancers, pulmonary arterial hypertension, and cirrhosis) (Edgar et al. 2015) that has led to international concern about the levels of exposure to these alkaloids in the human diet and dietary supplements.

Over the past 5 to 6 decades, there have been a myriad of international studies of the occurrence and toxicity of dehydroPAs. One of the more commonly studied dehydroPAs, probably because of its facile and abundant isolation from plants such as *Senecio riddellii* (Molyneux et al. 1991), has been the macrocyclic, retronecine-based diester riddelliine (figure 1). As a consequence, it is at present only one of two dehydroPAs, the other being the open chain diester lasiocarpine (figure 1), for which enough data have been accumulated to enable a risk assessment that concluded there were reasonable grounds to classify it as a Group 2B carcinogen, i.e., “possibly carcinogenic to humans” (IARC 2014).

Materials and Methods

Chemicals, Reagents, and Plant Material

Methanol and ethanol were reagent ACS/USP/NF grade (Pharmaco Products, USA). Acetonitrile was HPLC-certified solvent (Honeywell Burdick and Jackson, USA), and water was Milli-Q-purified (18.2 M Ω /cm) (Millipore, USA). The mobile phase additive, formic acid, was “For Analysis” grade (>99%; Acros Organics/Thermo Fisher Scientific, USA). The dehydroPAs heliotrine, riddelliine, retrorsine, seneciphylline, senecionine, and lasiocarpine, and the *N*-oxides of riddelliine, heliotrine, senecionine, and seneciphylline (figure 1), confirmed using NMR spectroscopy and/or HPLC-*esi*(+)MS, were all sourced from the stocks of extracted and purified or semi-purified pyrrolizidine alkaloids kept by the USDA-ARS Poisonous Plant Research Laboratory. The purity of the alkaloids,

determined using HPLC-*esi*(+)MS, varied from about 90% to >99%. Ammoniated methanol was prepared by bubbling dry ammonia gas through cooled (ice bath) methanol. The resultant saturated ammoniated methanol solution was diluted 1:9 with methanol for use in strong cation exchange, solid phase extraction (SCX SPE). The SCX SPE, HPLC, and guard columns were obtained from Phenomenex (USA). For normal phase chromatography, KP silica gel cartridges (10 g Samplets) and KP-silica gel-HS columns (120 g) were obtained from Biotage (USA).

One dimensional (1D) and 2D (COSY, HSQC, HMBC) ¹H (300 MHz) and ¹³C (75 MHz) NMR data were acquired using a JEOL Eclipse NMR spectrometer using solutions in deuteriochloroform (Sigma-Aldrich, USA) and the residual proton in the CHCl₃ (δ = 7.2) as the chemical shift reference.

HPLC-MS Analysis

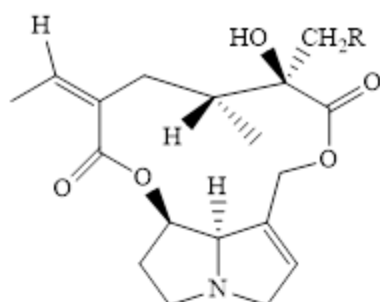
HPLC-*esi*(+)MS and MS/MS analysis. Using an Agilent 1260 Infinity HPLC System (Agilent Technologies, USA), analytical samples (2 μ L) were injected onto a Synergi Hydro RP column (150 x 2 mm, 4 μ) fitted with a guard column of similar adsorbent (AC C18, 4 mm diameter x 2 mm) (Security Guard Cartridge system). A gradient flow (400 μ L/min) of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) was used to elute sample components from the column. Mobile phase B was held at 3% for 2 min before linearly increasing to 70% by 10 min. After holding at 70% for another 5 min, data acquisition was stopped, and the column was re-equilibrated to 3% mobile phase B over 2 min and held for a further 8 min before the next injection.

Despite the widespread use of riddelliine in toxicology studies, there has been no reference to its potential for degradation or reactivity in solution. A routine high-pressure liquid chromatography-electrospray ionization-positive ion trap mass spectrometry (HPLC-*esi*(+)MS) analysis of a stored, standard solution of riddelliine in methanol revealed a significant change that was subsequently investigated in more detail.

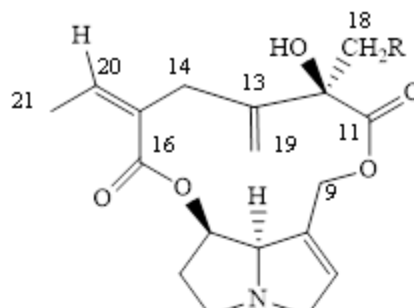
The eluate from the HPLC column was monitored using a Velos Pro LTQ mass spectrometer (Thermo Scientific, USA) in a 2 scan, positive ion mode, and equipped with a heated electrospray ionization (HESI) source. The first full scan (*m/z* 200-800) was followed by a data-dependent (most intense ion in a peak), collision-

induced dissociation (CID) scan using a generic CID energy of 35%, activation Q of 0.25, and an activation time of 10.0 ms. The capillary temperature was set at 275 °C, the ionization spray

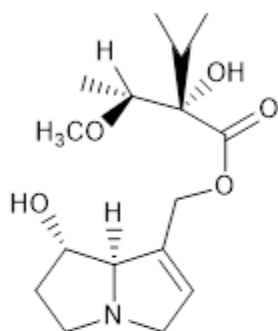
voltage at 3.45 kV, the HESI source heater temperature at 305 °C, and the sheath gas flow was 40 units with an auxiliary flow of 5 units.



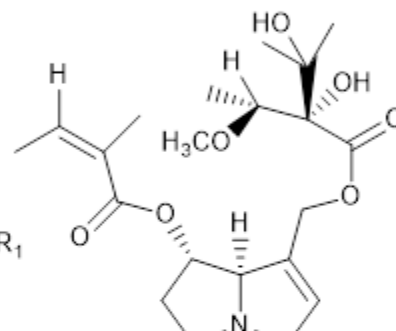
R = H: senecionine
R = OH: retrorsine



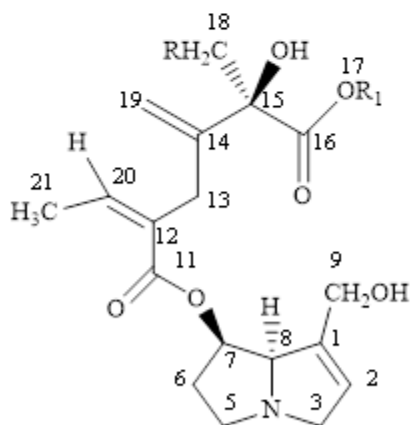
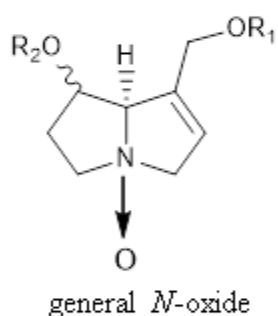
R = H: seneciphylline
R = OH: riddelline



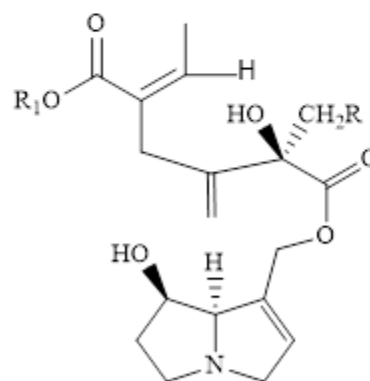
heliotrine



lasiocarpine



Ring-opening at C9



Ring-opening at C7

R = H, R₁ = CH₃: seneciphylline-methanol addition product
R = H, R₁ = CH₂CH₃: seneciphylline-ethanol addition product
R = OH, R₁ = CH₃: riddelline-methanol addition product
R = OH, R₁ = CH₂CH₃: riddelline-ethanol addition product

Figure 1. Structures of dehydropyrrolizidine alkaloids as discussed in the text.

HPLC-esi(+)HRMS. High-resolution m/z measurements of the dehydroPAs were made using an Ultimate 3000 HPLC (Thermo Scientific, USA) to inject a sample aliquot (2 μ L) onto the Synergi Hydro column and elute components using the same acetonitrile/0.1% formic acid gradient as described above. The column effluent was monitored using an Exactive Plus Orbitrap high-resolution mass spectrometer (Thermo Scientific, USA): scan range 100-1,000 Da; resolution 35,000; microscans 1; lock mass 445.12003; sheath gas flow 35; auxiliary gas flow 10; spray voltage 4 kV; capillary temperature 320 °C; S lens RF field 55; auxiliary gas temperature 300 °C; and maximum inject time 250 ms.

Reaction Conditions

Analytical scale. Samples (2-10 mg) of the dehydroPAs and their *N*-oxides were separately dissolved in methanol or ethanol, or in methanol or ethanol containing 0.5% (v/v) of hydrochloric acid (37%) or 0.5% (v/v) or 0.05% (v/v) of ammonium hydroxide (0.88). The solutions were left at room temperature or heated in a sand bath at solvent reflux temperatures. Aliquots (10 μ L) were regularly sampled, diluted with lasiocarpine-spiked mobile phase (100 μ L), and analysed using HPLC-esi(+)**MS** and MS/MS.

Preparative scale reaction of riddelliine and isolation of riddelliine-methanol adduct. Riddelliine (ca. 100 mg) was dissolved in methanol (50 mL) containing 0.5% of ammonium hydroxide (0.88). The solution was left at room temperature (ca. 22 °C), and the progress of the reaction was monitored using HPLC-esi(+)**MS**. After about 2 weeks, or whenever the HPLC-esi(+)**MS** monitoring indicated near completion of the reaction, the solvent was removed in vacuo by rotary evaporation. The residue was extracted with 0.05 M sulphuric acid (50 mL), filtered, and the consequent aqueous acid solution loaded onto an SCX SPE column (70g/150 mL; 55 μ m, 70 Å; Strata Giga Tubes) preconditioned by washing with methanol followed by 0.05 M sulphuric acid. The loaded column was washed with water (50 mL) and methanol (100 mL) before the captured alkaloids were eluted from the column using ammoniated methanol. All loadings and column washes were monitored for the dehydroPAs using HPLC-esi(+)**MS**. The ammoniated methanol fractions containing the alkaloids were pooled and evaporated to dryness in vacuo using a rotary evaporator. The residue was reconstituted in chloroform and, to maintain a narrow band, was

added batch wise to a KP-silica gel cartridge (10g Biotage Samplet) and allowed to air dry between additions. The loaded Samplet cartridge was fitted to a KP-silica-HS column (120 g) previously equilibrated with chloroform:10% saturated ammoniated methanol (9:1) using a programmable Isolera 1 flash chromatography system (Biotage, USA). The alkaloid components were eluted from the Samplet-column combination using an isocratic flow (12 mL/min) of chloroform:10% saturated ammoniated methanol (9:1). Collected fractions (5 mL) were analysed using HPLC-esi(+)**MS**. Fractions with a high (>95%) proportion of the major riddelliine-methanol addition product were pooled and evaporated to dryness (35 mg) for subsequent reconstitution in CDCl₃ for NMR analysis.

Results and Discussion

The instability of riddelliine was first observed during a routine HPLC-esi(+)**MS** analysis of a standard methanol solution of riddelliine (0.9 mg/mL) that had been stored at room temperature (ca. 22°C) for several months. Two additional peaks (one major and one minor) with protonated molecules (MH⁺) at m/z 382 were observed in the ion chromatogram (figure 2). The mass difference (Δ MH⁺ 32 Da) between the riddelliine and the new products implied an addition of methanol to riddelliine. Addition products were also observed when riddelliine was dissolved in ethanol, giving rise to a major and a minor new compound each equivalent to the addition of ethanol (MH⁺ m/z 396) (figure 3).

Various experiments were conducted to investigate the conditions and salient structural features that might be required for, or that might facilitate, the formation of the addition products. The intent was to accelerate the formation of addition products and allow isolation for NMR spectroscopy analysis. Initially, riddelliine and its *N*-oxide were each dissolved in refluxing methanol or ethanol for several days. Secondly, riddelliine and its *N*-oxide were dissolved in acidified methanol either at room temperature or at methanol-refluxing temperature. Thirdly, riddelliine was dissolved in basified methanol at room temperature. Although formation of the addition products was observed in all cases, it was much faster, going to completion within a week, in the basified methanol or ethanol at room temperature. Therefore, further investigations of

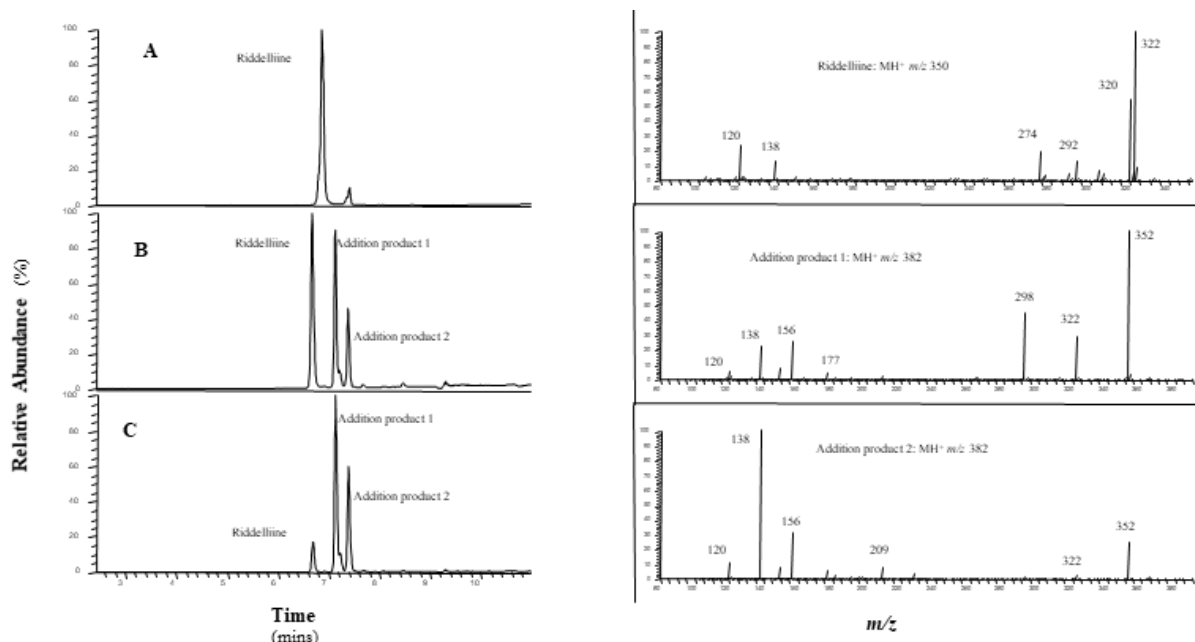


Figure 2. HPLC-esiMS base ion chromatograms (m/z 340-400) of A, fresh solution of riddelliine in methanol showing a minor contamination with retrorsine; and B and C, progressive loss of riddelliine and formation of the two addition products. Also shown are the MS/MS fragmentation profiles for riddelliine and the two addition products. m/z , mass to charge ratio.

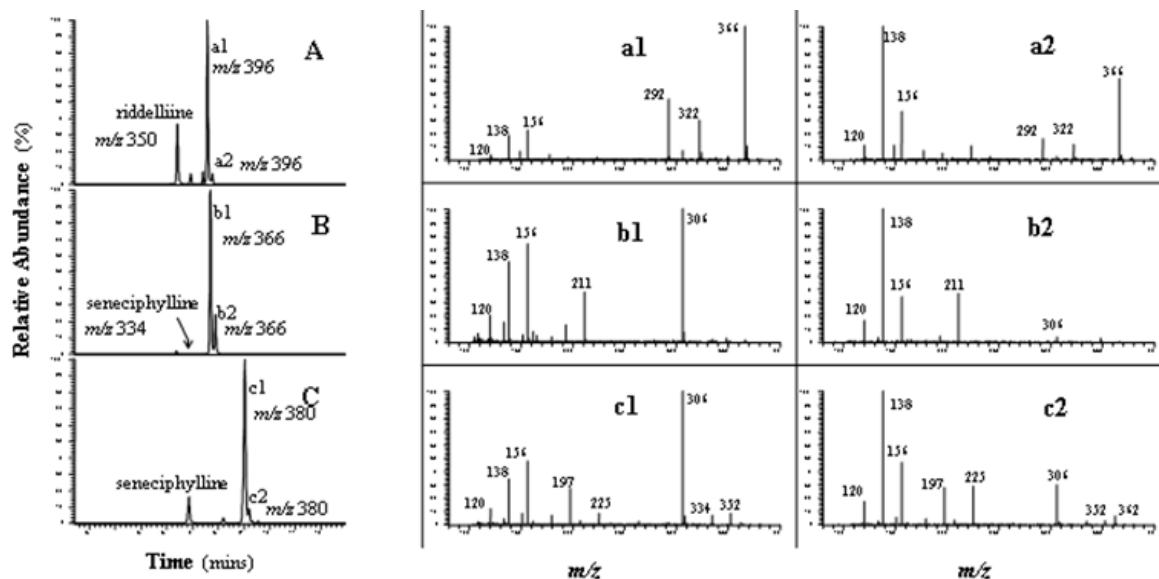


Figure 3. HPLC-esi(+)MS base ion (m/z 300-400) chromatograms and MS/MS fragmentation spectra for the ring-opening transesterifications of riddelliine with ethanol (A, a1, a2) and seneciophylline with methanol (B, b1, b2) and ethanol (C, c1, c2). m/z , mass to charge ratio.

seneciophylline, analogous to riddelliine with its C13-C19 exocyclic double bond in the macrocyclic diester ring (figure 1), and retrorsine and senecionine, the C13 double bond-reduced analogues of riddelliine and seneciophylline respectively, were conducted using the basic conditions. Also investigated were the reactions of

the monoester heliotrine and the *N*-oxides of riddelliine, seneciophylline, senecionine, and heliotrine, in methanol and ethanol, all basified with ammonium hydroxide. Only the macrocyclic diesters with an exocyclic double bond, i.e., riddelliine and seneciophylline, and their *N*-oxides, reacted with the methanol or ethanol (table 1, figures 2 - 4). Neither

the monoester heliotrine nor the macrocyclics without an exocyclic double bond, i.e., retrorsine and senecionine, nor *N*-oxidation per se, i.e., senecionine-*N*-oxide and heliotrine-*N*-oxide (table 1) resulted in any additive or hydrolytic transformation of the parent alkaloids under the experimental conditions examined. Thus, two addition products were observed in each of the reactions of the free

base and *N*-oxide forms of riddelliine and seneciophylline with methanol or ethanol. Despite the same molecular formula calculated from the accurately determined molecular masses within any pair of addition products, there were major MS/MS differences in ion formation and relative abundance between the two products indicating a significant difference in their structures (table 1, figures 2-3).

Table 1. HPLC-esi(+)MS, MS/MS, and high-resolution MS data for dehydropyrrolizidine alkaloids and transesterification products discussed in the text. Also shown are the calculated and expected, where applicable, molecular formulae

Dehydropyrrolizidine alkaloid	Expected protonated molecular formula	Retention time (relative abundance) (min)	HRMS (<i>m/z</i>)	Calculated protonated molecular formula ^a	Δ (ppm) ^a	MS/MS
riddelliine	C ₁₈ H ₂₄ NO ₆	6.35	350.16172	C ₁₈ H ₂₄ NO ₆	3.9	332(2), 323(8), 322(100), 320(57), 306(5), 304(7), 292(13), 288(4), 276(3), 274(21), 149(2), 138(13), 120(26), 118 (2)
riddelliine-methanol product 1		6.88 (100)	382.18658	C ₁₉ H ₂₈ NO ₇	0	364(1), 352(100), 322(28), 292(47), 209(2), 177(4), 156(25), 149(7), 138(22), 120(5)
riddelliine-methanol product 2		7.13 (15 - 50)	382.18646	C ₁₉ H ₂₈ NO ₇	0.3	364(1), 352(27), 338(0.4), 322(3), 292(3), 227(3), 209(7), 181(2), 177(6), 156(30), 149(8), 138(100), 120(11)
riddelliine-ethanol product 1		7.58 (100)	396.2035	C ₂₀ H ₃₀ NO ₇	3.2	368(10), 367(6), 366(100), 350(2), 338(1), 324(5), 323(2), 322(30), 306(7), 292(46), 223(2), 195(2), 177(4), 156(22), 149(6), 138(18), 120(4)
riddelliine-ethanol product 2		7.78 (8)	396.20324	C ₂₀ H ₃₀ NO ₇	2.4	378(1), 368(2), 367(1), 366(52), 350(1), 324(1), 322(9), 306(2), 292(11), 241(2), 223(10), 195(5), 177(7), 156(35), 149(10), 138(100), 120(11)
riddelliine-water product 1		6.15 (100)	368.16969	C ₁₈ H ₂₆ NO ₇	3.4	350(31), 338(2), 332(2), 324(100), 322(30), 306(95), 156(2), 138(1), 120(0.5)
riddelliine-water product 2		6.54 (15)	368.17026	C ₁₈ H ₂₆ NO ₇	1.8	350(9), 338(0.5), 332(0.5), 324(100), 322(3), 306(31), 156(2), 138(17), 120(2)
seneciophylline	C ₁₈ H ₂₄ NO ₅	7.24	334.16487	C ₁₈ H ₂₄ NO ₅	1.8	334(1), 316(1), 306(100), 290(4), 288(22), 274(2), 246(2), 151(4), 138(20), 122(3), 120(32), 118(3)
seneciophylline-methanol product 1		7.7 (100)	366.19168	C ₁₉ H ₂₈ NO ₆	0.05	348(3), 307(8), 306(100), 211(38), 193(13), 179(4), 165(5), 161(8), 157(3), 156(74), 151(5), 139(3), 138(61), 133(15), 120(20), 118(2), 110(3), 108(6)

seneciophylline-methanol product 2		7.9 (25)	366.19165	C ₁₉ H ₂₈ NO ₆	0.04	348(3), 306(4), 211(36), 193(4), 179(1), 165(2), 161(2), 156(34), 151(1), 138(100), 120(60)
seneciophylline-ethanol product 1		9.04 (100)	380.20591	C ₂₀ H ₃₀ NO ₆	3.7	362(1), 352(8), 334(7), 308(6), 306(100), 264(2), 225(8), 207(3), 197(27), 179(7), 161(2), 156(47), 151(8), 138(34), 133(4), 120(12), 118(1), 108(2)
seneciophylline-ethanol product 2		9.2 (10)	380.20613	C ₂₀ H ₃₀ NO ₆	3.1	362(6), 352(2), 334(2), 308(1), 306(21), 225(29), 207(3), 197(26), 179(4), 161(1), 156(43), 151(5), 138(100), 133(2), 120(17)
riddelliine- <i>N</i> -oxide	C ₁₈ H ₂₄ NO ₇	7.13	366.15420	C ₁₈ H ₂₄ NO ₇	2.9	348(16), 338(100), 336(69), 318(12), 308(10), 290(15), 274(18), 246(13), 244(6), 154(13), 140(8), 138(15), 137(8), 136(17), 122(6), 121(9), 120(42), 119(31), 118(48)
riddelliine- <i>N</i> -oxide-methanol product 1		7.74 (75)	398.18007	C ₁₉ H ₂₈ NO ₈	3.6	368(100), 338(21), 332(11), 308(17), 304(6), 298(4), 288(6), 286(4), 274(5), 272(4), 262(4), 244(5), 209(4), 177(6), 172(25), 154(13), 149(11), 137(8), 136(11), 118(3)
riddelliine- <i>N</i> -oxide-methanol product 2		8.08 (100)	398.18014	C ₁₉ H ₂₈ NO ₈	3.4	380(41), 368(100), 352(3), 350(3), 348(1), 339(1), 338(55), 308(1), 172(8), 156(2), 155(2), 154(4), 138(15), 137(3), 136(9), 120(4)
riddelliine- <i>N</i> -oxide-ethanol product 1		8.23 (50)	412.19565	C ₂₀ H ₃₀ NO ₈	3.6	384(10), 383(5), 382(100), 346(9), 338(24), 322(6), 308(24), 304(7), 302(5), 286(4), 274(6), 244(5), 223(4), 195(4), 177(7), 172(29), 154(13), 149(12), 137(5), 136(11)
riddelliine- <i>N</i> -oxide-ethanol product 2		8.50 (100)	412.19565	C ₂₀ H ₃₀ NO ₈	3.6	394(42), 383(5), 382(100), 376(2), 366(5), 364(3), 340(2), 339(3), 338(61), 322(3), 308(1), 172(8), 156(1), 155(1), 154(4), 138(11), 137(2), 136(10), 120(4)
seneciophylline- <i>N</i> -oxide	C ₁₈ H ₂₄ NO ₆	8.71	350.15870	C ₁₈ H ₂₄ NO ₆	4.7	332(16), 322(100), 288(12), 274(7), 246(27), 244(11), 218(8), 178(7), 167(10), 154(17), 151(7), 138(13), 136(16), 122(13), 121(13), 120(44), 119(28), 118(55), 117(8), 106(8)
seneciophylline- <i>N</i> -oxide-methanol product 1		8.8 (25)	382.18507	C ₁₉ H ₂₈ NO ₇	3.9	322(73), 302(11), 288(77), 270(28), 262(12), 260(13), 244(46), 226(11), 216(15), 211(63), 193(19), 172(100), 161(14), 154(49), 151(11), 137(27), 136(41), 133(24), 120(11), 118(13)
seneciophylline- <i>N</i> -oxide-methanol product 2		9.09 (100)	382.18483	C ₁₉ H ₂₈ NO ₇	4.6	364(95), 322(100), 288(1), 262(1), 252(1), 211(1),

						172(10), 156(1), 155(3), 154(7), 138(17), 137(4), 136(12), 133(1), 120(4), 118(1)
seneciphylline- <i>N</i> -oxide –ethanol product 1		9.31 (42)	396.20125	C ₂₀ H ₃₀ NO ₇	2.5	368(14), 350(10), 322(100), 316(10), 297(10), 288(80), 270(31), 260(12), 244(36), 226(10), 225(33), 216(11), 197(52), 179(19), 172(96), 154(42), 151(24), 137(22), 136(34), 133(11), 118(11)
seneciphylline- <i>N</i> -oxide –ethanol product 2		9.55 (100)	396.20119	C ₂₀ H ₃₀ NO ₇	2.6	378(100), 350(5), 322(100), 288(1), 262(1), 252(1), 197(1), 172(8), 156(1), 155(2), 154(5), 151(1), 138(15), 137(3), 136(10), 120(3), 118(1)
retrorsine	C ₁₈ H ₂₆ NO ₆	6.92	352.17560	C ₁₈ H ₂₆ NO ₆	1.2	352(2), 324(100), 322(28), 308(3), 306(7), 304(3), 294(2), 290(3), 276(17), 220(3), 169(2), 151(4), 138(17), 122(3), 121(2), 120(25), 118(2), 103(2)
senecionine	C ₁₈ H ₂₆ NO ₅	7.77	336.18094	C ₁₈ H ₂₆ NO ₅	0.5	336(2), 308(100), 292(4), 290(15), 274(3), 238(2), 220(3), 153(5), 140(1), 138(27), 120(37), 118(4), 103(2)
senecionine- <i>N</i> -oxide	C ₁₈ H ₂₆ NO ₆	9.47	352.17563	C ₁₈ H ₂₆ NO ₆	1.1	334(14), 324(85), 254(25), 248(25), 246(57), 220(100), 218(22), 202(9), 154(16), 152(10), 138(13), 137(9), 136(25), 122(10), 121(27), 120(44), 119(28), 118(69), 117(9), 106(11)
heliotrine	C ₁₆ H ₂₈ NO ₅	7.76	314.19577	C ₁₆ H ₂₈ NO ₅	3.1	296(2), 282(1), 156(4), 152(1), 138(100), 120(3), 96(4), 94(2)
heliotrine- <i>N</i> -oxide	C ₁₆ H ₂₈ NO ₆	8.05	330.19122	C ₁₆ H ₂₈ NO ₆	1.3	298(4), 172(100), 155(2), 154(4), 138(7), 137(3), 136(5), 124(1), 94(1)
lasiocarpine	C ₂₁ H ₃₄ NO ₇	9.49	412.233579	C ₂₁ H ₃₄ NO ₇	0.1	394(25), 336(100), 322(2), 312(1), 254(1), 238(5), 220(32), 120(8)

^aCalculated using ChemCalc (http://www.chemcalc.org/mf_finder/mfFinder_em_new).

MS, mass spectrometry; MS/MS, tandem mass spectrometry; HRMS, high resolution mass spectrometry

It has been previously noted that the MS/MS fragmentation spectra of macrocyclic dehydroPAs with a hydroxymethyl substituent at C12, i.e., α to the C9 ester carbonyl (e.g. riddelliine structure, figure 1) can show a significant loss of 30 Da in addition to the more usual loss of 28 Da from the protonated macrocyclic dehydroPA diester molecule (Boppré et al. 2008, Colegate and Gardner 2008). However, although retrorsine, riddelliine, and riddelliine-*N*-oxide showed this characteristic pair of fragment ions, the riddelliine and riddelliine-*N*-oxide addition products with methanol and ethanol did not, with the exception of the minor product of riddelliine-*N*-oxide with ethanol that showed a 10%

relative abundance of $MH^+ - 28$ at m/z 384 (table 1). Instead, they simply showed a loss of 30 Da from the MH^+ i.e., m/z 382 \rightarrow m/z 352 and m/z 396 \rightarrow m/z 366, for the methanol and ethanol addition products to riddelliine respectively, or m/z 398 \rightarrow m/z 368 and m/z 412 \rightarrow m/z 382, for the methanol and ethanol addition products to riddelliine-*N*-oxide, respectively. This observation implies a significant alteration of the structure involving the C9 and/or C7 ester linkages thereby facilitating alternative fragmentations. This change in MS/MS fragmentation was not observed with seneciphylline or its *N*-oxide and their addition products with methanol or ethanol since seneciphylline does not

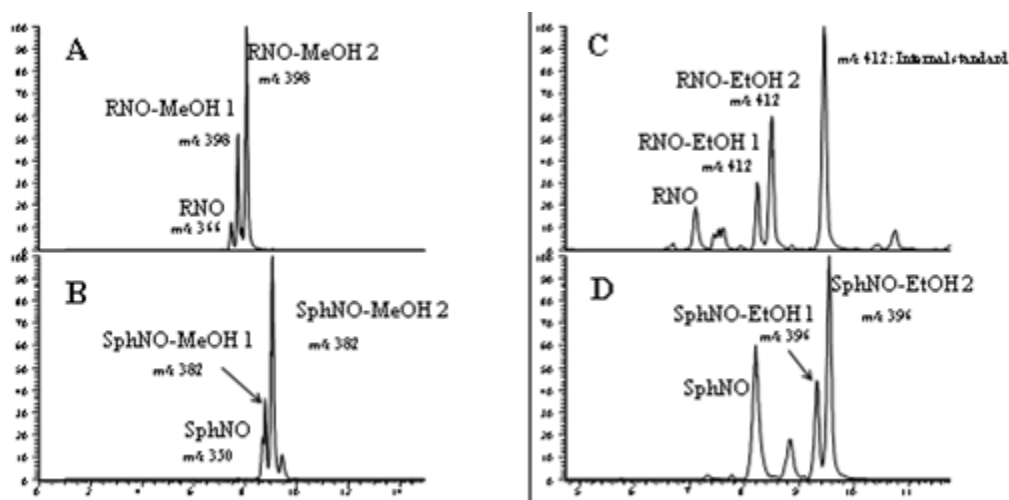


Figure 4. HPLC-esi(+)-MS base ion chromatograms of the reactions of riddelliine-*N*-oxide (RNO) with methanol (MeOH) (A) and ethanol (EtOH) (B); and of seneciophylline-*N*-oxide (SphNO) with methanol (MeOH) (C) and ethanol (EtOH) (D). Each of the alkaloids shows two addition products each with the same MH^+ (labeled on peaks as m/z) but with different MS/MS fragmentation spectra (table 1). m/z , mass to charge ratio.

possess the requisite hydroxymethyl substituent (figure 1, table 1).

While all the initial experiments were conducted on a small scale that allowed HPLC-esi(+)-MS and MS/MS monitoring, the reaction of riddelliine with methanol was also conducted on a larger scale, and the major riddelliine-methanol addition product was separated and purified in a two-stage process. The first stage involved SCX SPE to concentrate the alkaloidal material from the reaction mixture. The dried alkaloidal fraction, reconstituted in chloroform, was then chromatographed on silica gel to separate the addition product from unreacted riddelliine, the minor addition product, and minor contaminants. Unambiguous structure elucidation of the major riddelliine-methanol addition product was achieved using 1D (1H , ^{13}C) and 2D (COSY, HSQC, HMBC) NMR spectroscopy (tables 2 and 3, figures 5-7).

All direct carbon-hydrogen bonds were readily established using the heteronuclear single quantum coherence (HSQC) NMR experiment (figure 5, table 2), while 1H - 1H couplings were established via the 2D correlation (COSY) NMR spectrum (figure 6, table 3). It was evident that the exocyclic methylene group of the riddelliine (C13→C19, riddelliine in figure 1) had been retained in the methanol addition product (C14→C19, riddelliine-methanol addition product in figure 1). It was also determined that a methoxy group had been added (1H 17, δ 3.79; ^{13}C 17, δ 53.33) for which the heteronuclear multiple bond correlation (HMBC) NMR experiment (figure 7,

table 3) showed its protons (numbering shown on riddelliine-methanol addition product in figure 1) correlated only to the downfield carbonyl (C16, δ 173.92). Also correlated to this downfield carbonyl are the C18 protons and, weakly, H19d. There are no correlations between the H9 protons and either carbonyl carbon (C11 or C16). The upfield carbonyl (C11, δ 166.94) is correlated to H21 (vinyl methyl), H20 (alkene proton), H13d, and 13u (methylene carbon protons) and, very weakly, to H7, clearly establishing its sub-structure environment. These observations confirm a ring-opening transesterification between C9 and C11 of the riddelliine molecule to form the C7 monoester of retronecine with methylriddelliic acid (figure 1).

Although not unambiguously determined using NMR spectroscopy, based on the MS, MS/MS, and HRMS data, it is reasonable to expect that ethanol causes a ring-opening transesterification of riddelliine to yield the ethyl ester. Similarly, it is reasonable to deduce from the data that seneciophylline reacts in the same way as riddelliine, resulting in the major production of the C7 monoester of retronecine with methyl(or ethyl)senecinic acid (figure 1). Furthermore, HPLC-esi(+)-MS analysis ascertained that riddelliine is stable in water containing 0.5% (v/v) of HCl (37%) but hydrolyses rapidly, starting within a couple of hours and going to completion over a week at room temperature, in 0.5% (v/v) of ammonium hydroxide (0.88). Two products were formed, the major one eluting just before, and the minor product just after,

the riddelliine peak. Both displayed protonated molecules at m/z 368 and accurate masses corresponding to ring-opening hydrolysis of a single ester bond in riddelliine. Similar to the formation of the methanol and ethanol ring-opening addition

products, the MS/MS fragmentation spectra (table 1) indicated that the major product was the C7 monoester of retronecine with riddelliic acid while the minor product was the analogous C9 monoester.

Table 2. Carbon-13 (^{13}C) and proton (^1H) nuclear magnetic resonance spectroscopy chemical shifts for the major methanol transesterification product resulting from ring-opening at C9 ester of riddelliine (figure1)

Carbon ^a	Chemical shift (ppm)	Proton	Chemical shift (ppm)
1	139.18	-	-
2	123.41	2	5.64, bs
3	62.91	3d	3.92, m
		3u	3.38, m
5	53.64	5d	3.34, m
		5u	2.67, m
6	34.79	6d and u	2.05, m
7	74.46	7	5.38, bs
8	75.84	8	4.32, bs
9	59.94	9d and u	4.11, bs
11	166.94	-	-
12	129.63	-	-
13	36.19	13d and u	3.02, ABq, J=10.9, 16.2
14	145.14	-	-
15	80.25	-	-
16	173.92	-	-
17	53.33	17	3.79, 3H, s
18	66.46	18d	3.98, d, J=11.34
		18u	3.73, d, J=11.37
19	114.4	19d	5.23, s
		19u	4.94, s
20	140.5	20	6.04, q, J=7.45
21	15.95	21	1.96, d, J=7.4

^aNumbering according to figure 1. All assignments confirmed using gradient-enhanced HSQC NMR spectroscopy: s, singlet; bs, broad singlet; d, doublet; q, quartet; ABq, AB quartet; m, multiplet.

Table 3. Proton-proton correlations (COSY) and proton-carbon multiple bond correlations (HMBC) for the major methanol addition product (MH⁺ m/z 382) resulting from ring-opening transesterification at the C9 ester of riddelliine (figure1)^a

Proton	Correlated protons	Correlated carbons
2	3u(w), 3d(m), 9d and u(m), 8(w)	1(w), 3(m), 8(m), 9(w)
3d	3u(s), 2(w), 9(m)	1(w), 2(wm)
3u	3d(s), 2(w), 9(m), 8(s)	1(w), 2(wm)
5d	5u(s), 6d and u(s)	6(w), 3(w), 7(wm), 8(wm)
5u	5d(s), 6d and u(s)	6(ms), 3(ms)
6	5d(m), 5u(s), 7(m)	8(w), 7(w)
7	6d and u(m), 8(s)	5(wm), 2(w), 11(vw)
8	3u(s), 7(m), 2(w)	1(w)
9d and u	2(m), 3u(s), 3d(m)	1(s), 2(s), 8(m)
13d and u	13u and 13d(s), 21(m), 19u(m), 19d(w)	11(ms), 12(s), 14(s), 15(m), 19(s), 20(s)
18d	18u(s)	14(m), 15(w), 16(m), 18(w)
18u	18d(s)	14(m), 15(s), 16(s)
19d	13d and u(m)	13(s), 14(m), 15(s), 16(w)
19u	13d and u(m)	13(s), 14(wm), 15(s), 16(vw)
20	21(s), 13(w)	11(ms), 21(m), 13(ms)
21	13d and 13 u(m), 20(s)	11(m), 12(s), 13(w), 14(m), 20(s)
OMe	-	16(s)

^aProton and carbon numbers refer to figure 1.

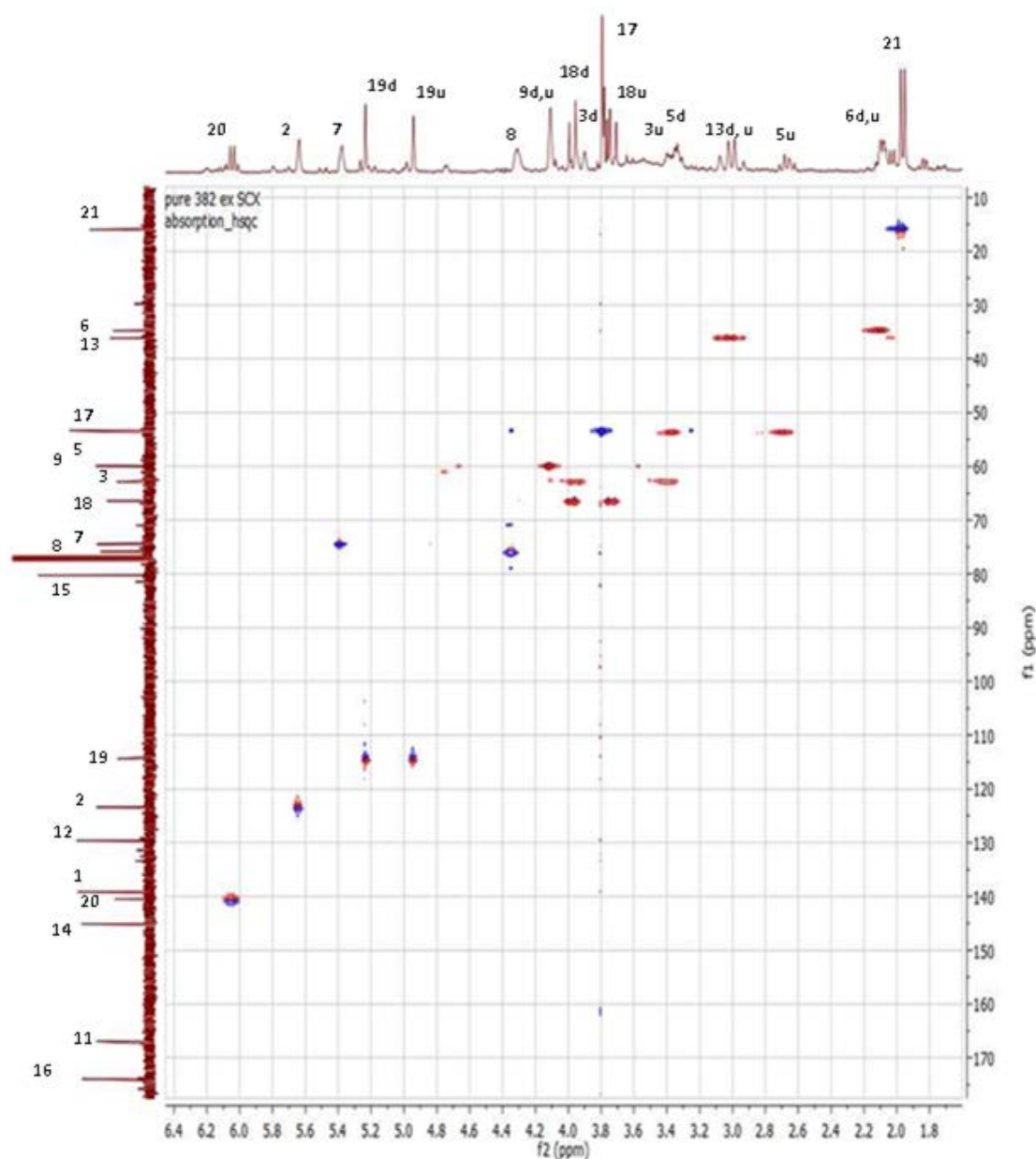


Figure 5. A gradient-enhanced ^1H - ^{13}C heteronuclear single quantum coherence (HSQC) NMR spectrum of the major riddelliine-methanol addition product resulting from a ring-opening transesterification at the C9 ester of riddelliine (figure 1).

Each of the riddelliine and riddelliine-*N*-oxide addition products with methanol or ethanol exhibits a facile MS fragmentation of 30 Da to yield a different base ion, i.e., m/z 352 and 356 for the methanol and ethanol adducts, respectively, with riddelliine and m/z 368 and 382 for the methanol and ethanol adducts, respectively, with riddelliine-*N*-oxide. This most likely represents a McLafferty-type elimination of formaldehyde from the hydroxymethyl-carbonyl entity (figure 9). Without this α -hydroxymethyl-carbonyl entity, each pair of seneciophylline and seneciophylline-*N*-oxide addition products fragments differently, i.e., $\text{MH}^+ - 60$ for

methanol adducts or $\text{MH}^+ - 74$ for ethanol adducts, to produce the same ion, m/z 306 and m/z 322 for seneciophylline and its *N*-oxide, respectively (table 1, figure 8). This most likely represents hydrogen transfer to the terminal carbonyl oxygen from the adjacent hydroxyl group thereby releasing the entire terminal ester function (figure 9).

It is noted that with riddelliine and seneciophylline, the major product with methanol or ethanol eluted before the minor product, whereas the reverse was observed with the *N*-oxides (table 1, figures 2-4). In every case, the minor product of the reaction of methanol or ethanol with riddelliine and

seneciophylline shows a base ion peak at m/z 138 that is consistent with a C9 monoester (Pedersen and Larsen 1970, Colegate et al. 2012) and as reported herein for heliotrine (figure 1, table 1). The increased abundance of fragments with higher m/z observed for the major addition products, indicating an increased tendency for intra-necic acid fragmentation, supports them all being C7 monoesters similar to the structure unambiguously described for the major riddelliine-methanol addition product (figure 1). In contrast to the observations of the riddelliine and seneciophylline adducts, a similar examination and interpretation of the MS/MS fragmentation of the addition products with the *N*-oxides (figure 8, table1) indicates that the minor products are C7 monoesters, eluting before the major C9 monoester products in each case. This interpretation is supported by the increased fragmentation, including the much higher relative abundance of m/z 172, in the MS/MS fragmentation spectra of the minor products (figure 8 A, C, E, and G), and by the presence of m/z 138 only in the

MS/MS fragmentation spectra of the major products (figure 8 B, D, F, and H). Thus, it seems that in both cases of methanol or ethanol addition to the free base or *N*-oxides of riddelliine and seneciophylline, the C7 monoester product elutes before the C9 isomer. However, the relative abundances of the two isomers formed from the free bases are reversed when formed from the *N*-oxides. There was no evidence for complete hydrolysis of the alkaloids to the retronecine base.

These transesterification addition products are previously undescribed dehydroPAs with unknown toxicities that may contribute to or mitigate against toxic effects during a toxicological evaluation of riddelliine, seneciophylline, and, perhaps, similar macrocyclic dehydroPAs that are susceptible to ring-opening transesterification. It also poses questions about the potential for such ring-opening *in vivo* and how it may contribute to or mitigate against toxicity by affecting absorption, distribution, metabolism, and excretion of the alkaloids.

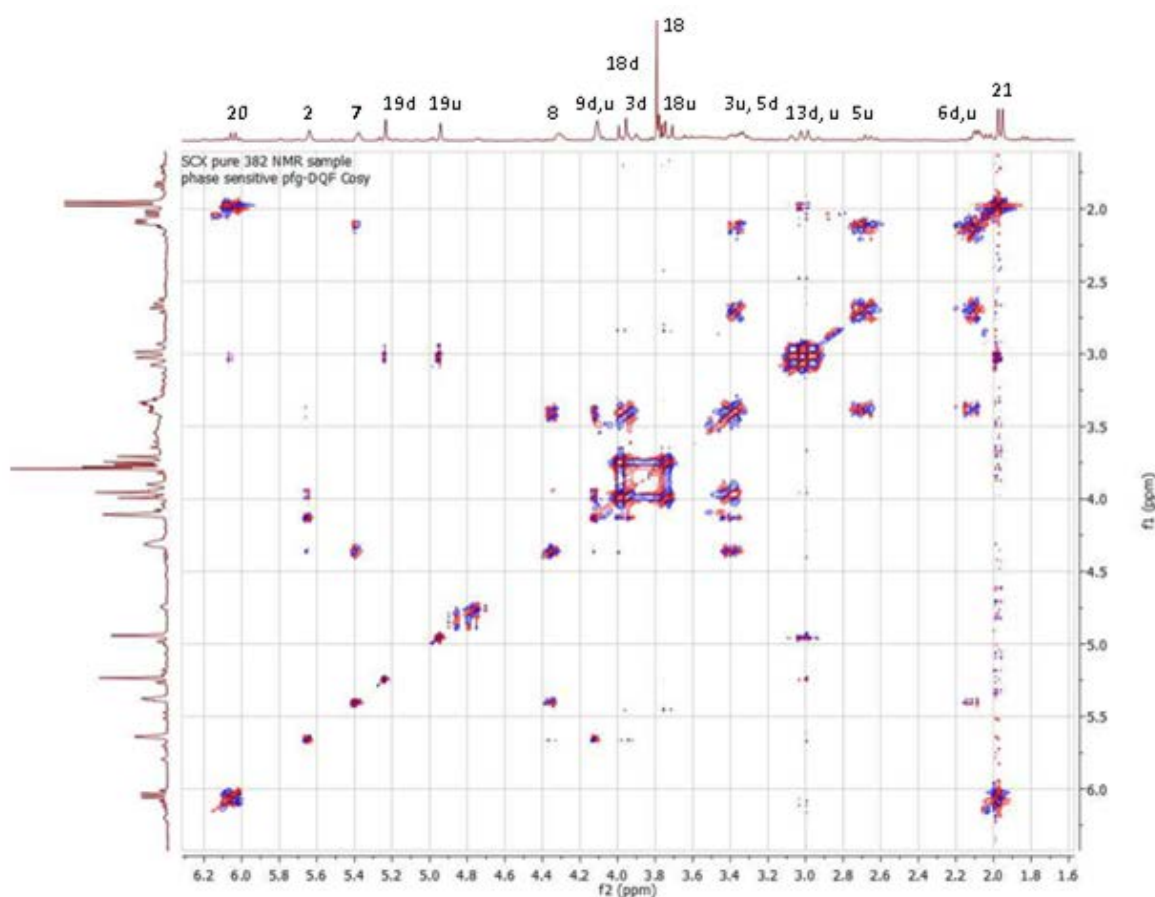


Figure 6. Phase-sensitive gradient enhanced $^1\text{H} - ^1\text{H}$ correlation NMR (COSY) of the major riddelliine – methanol addition product resulting from a ring-opening transesterification at the C9 ester of riddelliine (figure1).

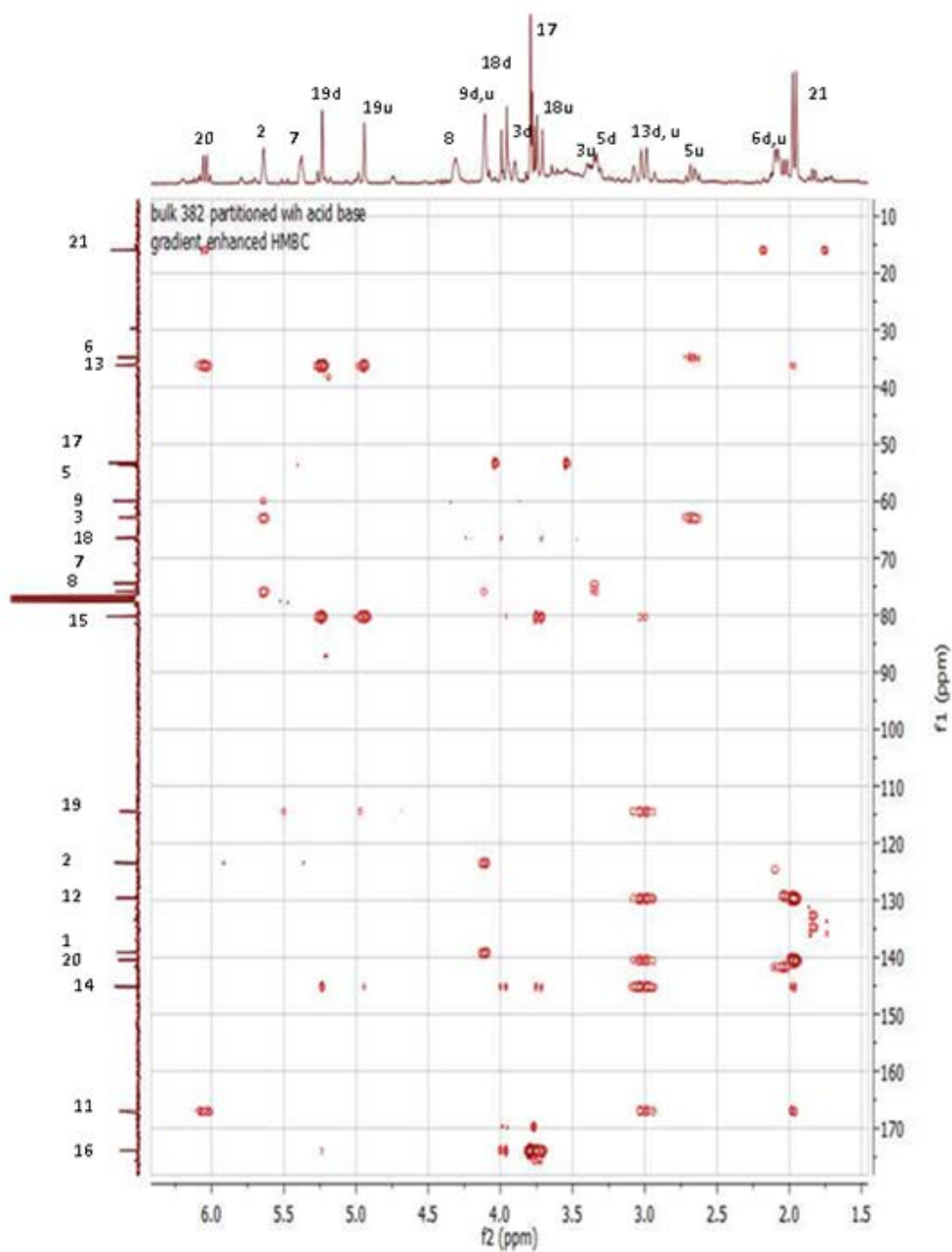


Figure 7. A gradient-enhanced ^1H - ^{13}C heteronuclear multiple bond correlation (HMBC) NMR spectrum of the major riddelliine-methanol addition product resulting from a ring-opening transesterification at the C9 ester of riddelliine (figure 1).

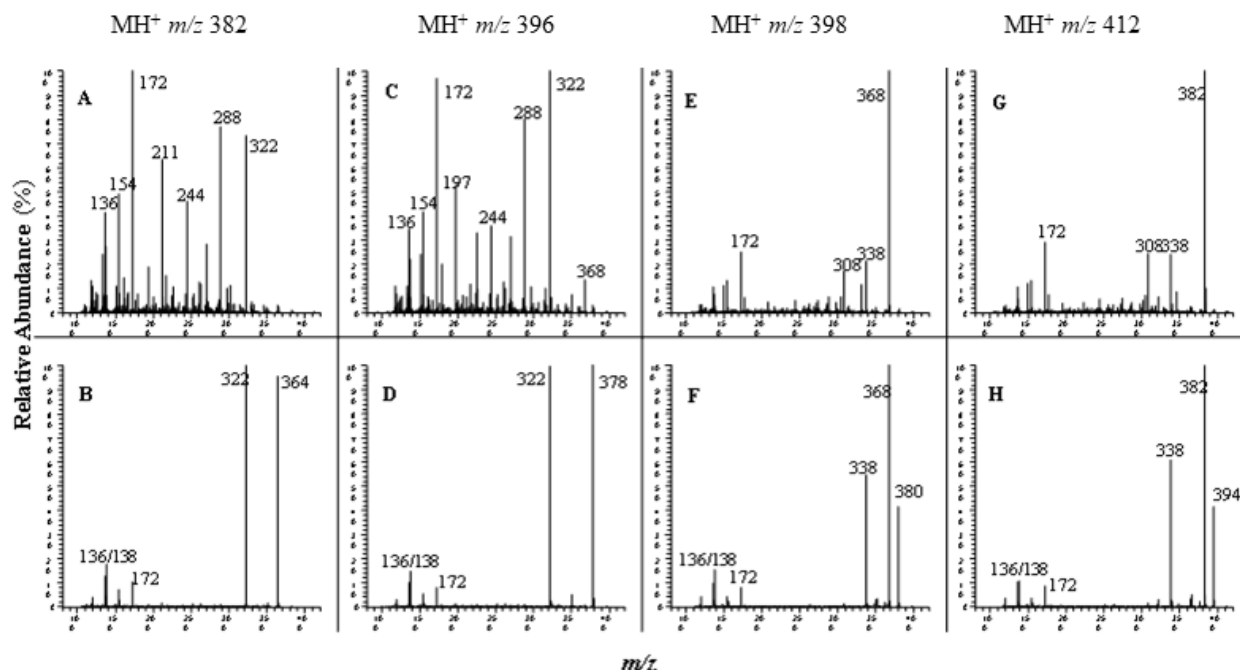


Figure 8. MS/MS fragmentation spectra for the ethanol (EtOH) and methanol (MeOH) addition products of riddelline-*N*-oxide (RNO) and seneciophylline-*N*-oxide (SphNO) (table 1). A, SphNO-MeOH (1); B, SphNO-MeOH (2); C, SphNO-EtOH (1); D, SphNO-EtOH (2); E, RNO-MeOH (1); F, RNO-MeOH (2); G, RNO-EtOH (1); H, RNO-EtOH (2). *m/z*, mass to charge ratio.

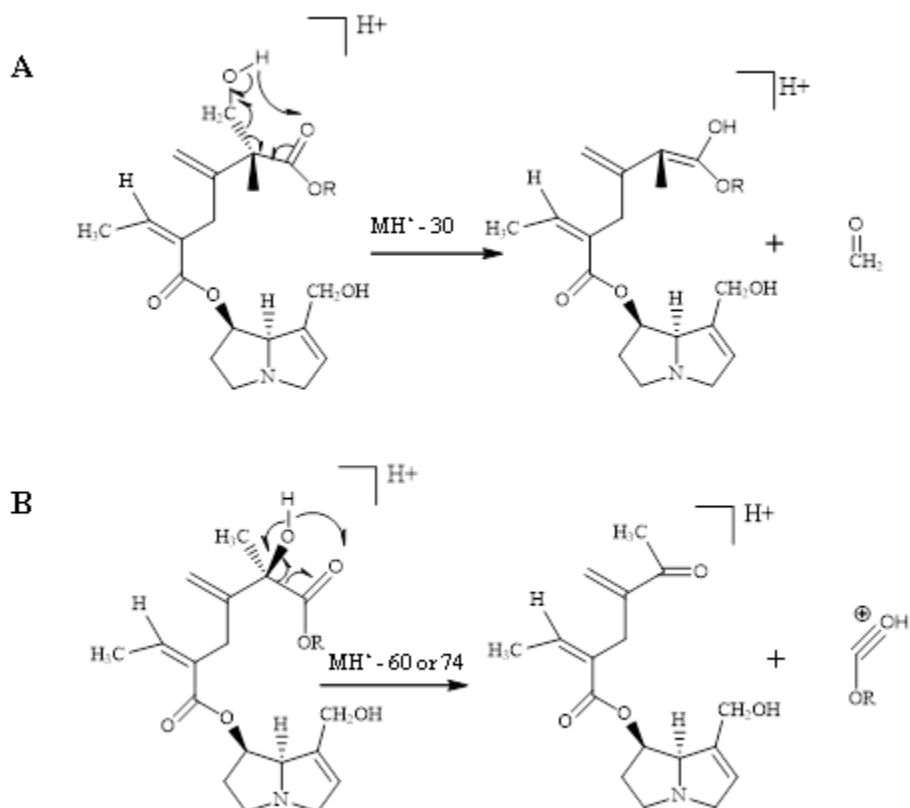


Figure 9. Proposed initial MS/MS fragmentation for A, methanol and ethanol addition products with riddelline and its *N*-oxide and B, methanol and ethanol addition products with seneciophylline and its *N*-oxide.

Acknowledgments

USDA-ARS provided research internships for WR and NB. NIH/ODS funded SMC.

References

- Boppré M, Colegate SM, Edgar JA, and Fischer OW. 2008. Hepatotoxic pyrrolizidine alkaloids in pollen and drying-related implications for commercial processing of bee pollen. *Journal of Agricultural and Food Chemistry* 56:5662-5672.
- Bull LB, Culvenor CCJ, and Dick AT. 1968. *The Pyrrolizidine Alkaloids: Their Chemistry, Pathogenicity and Other Biological Properties*. North Holland Publishers, Amsterdam, The Netherlands.
- Colegate SM and Gardner DR. 2008. LC-MS of alkaloids: qualitative profiling, quantitative analysis, and structural identification. In E Fattorusso, O Tagliabatella-Scafati, eds., *Modern Alkaloids*, pp. 369-408. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.
- Colegate SM, Gardner DR, Joy RJ, et al. 2012. Dehydropyrrolizidine alkaloids, including monoesters with an unusual esterifying acid, from cultivated *Crotalaria juncea* (Sunn Hemp cv. 'Tropic Sun'). *Journal of Agricultural and Food Chemistry* 60:3541-3550.
- Edgar JA, Colegate SM, Boppré M, and Molyneux RJ. 2011. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. *Journal of Food Additives and Contamination* 28:308-324.
- Edgar JA, Molyneux RJ, and Colegate SM. 2015. Pyrrolizidine alkaloids: potential role in the etiology of cancers, pulmonary hypertension, congenital anomalies and liver disease. *Chemical Research in Toxicology* (accepted December 6, 2014), DOI: 10.1021/tx500403t.
- IARC. 2014. International Agency for Research on Cancer Monographs on the Evaluation of Carcinogenic Risk to Humans. <http://monographs.iarc.fr/ENG/Classification/> (last accessed November 25, 2014).
- Molyneux RJ, Gardner DR, Colegate SM, and Edgar JA. 2011. Pyrrolizidine alkaloid toxicity in livestock: a paradigm for human poisoning? *Journal of Food Additives and Contamination* 28:293-307.
- Molyneux RJ, Johnson AE, Olsen JD, and Baker DC. 1991. Toxicity of pyrrolizidine alkaloids from Riddell groundsel (*Senecio riddellii*) to cattle. *American Journal of Veterinary Research* 52:146-151.
- Pedersen E and Larsen E. 1970. Mass spectrometry of some pyrrolizidine alkaloids. *Organic Mass Spectrometry* 4:249-256.
- Pessoa CRM, Pessoa AFA, Maia LA, et al. 2013. Pulmonary and hepatic lesions caused by the dehydropyrrolizidine alkaloid-producing plants *Crotalaria juncea* and *Crotalaria retusa* in donkeys. *Toxicol* 71:113-120.

Submitted: January 1, 2015

Revised: January 12, 2015

Accepted: January 15, 2015