

Pyrrolizidine Alkaloids of *Echium vulgare* Honey Found in Pure Pollen

MICHAEL BOPPRÉ,[†] STEVEN M. COLEGATE,^{*,‡} AND JOHN A. EDGAR[‡]

Plant Toxins Research Group, CSIRO Livestock Industries, Private Bag 24, Geelong, Victoria 3220, Australia, and Forstzoologisches Institut, Albert-Ludwigs-Universität, D-79085 Freiburg i.Br., Germany

The pyrrolizidine alkaloids previously identified in floral honey attributed to *Echium vulgare* (Boraginaceae) have been detected (8000–14 000 ppm) in pure pollen collected from the anthers of *Echium vulgare*. Pyrrolizidine alkaloids and/or their *N*-oxides were isolated from the aqueous acid extracts of pollen by use of strong cation-exchange, solid-phase extraction and identified by liquid chromatographic/mass spectrometric (LCMS) analysis. The pyrrolizidine alkaloids in the pollen are present mainly as the *N*-oxides. In addition to seven previously described pyrrolizidine alkaloids and/or their *N*-oxides (echimidine, acetylicheimidine, uplandicine, 9-*O*-angelylretronecine, echiuplatine, leptanthine, and echimiplatine), one unidentified (echivulgarine), but previously found in honey, and two previously undescribed (vulgarine and 7-*O*-acetylvulgarine) pyrrolizidine alkaloids and/or their *N*-oxides were identified in the pollen. Tentative structures for these unidentified pyrrolizidine alkaloids are proposed on the basis of the mass spectrometric data and biogenetic considerations. The implications of these results for identifying the source and subsequent concentrations of pyrrolizidine alkaloids in honeys and commercial bee pollen are briefly discussed.

KEYWORDS: Pyrrolizidine alkaloids; pyrrolizidine-*N*-oxides; pollen; honey; solid-phase extraction; food safety; LCMS; *Echium vulgare*; Boraginaceae

INTRODUCTION

The 1,2-dehydropyrrolizidine alkaloids and their *N*-oxides are a diverse class of secondary metabolites that are well established as hepatotoxins in animals and humans (1, 2). In some cases pyrrolizidine alkaloids exert extrahepatic effects resulting in pneumotoxicity, genotoxicity, and carcinogenicity. However, the major effect of dietary pyrrolizidine alkaloids on humans is reported to be hepatic veno-occlusive disease (3). The genotoxic and carcinogenic effects of orally administered pyrrolizidine-*N*-oxides have been shown (4) to be equal to those of their parent pyrrolizidine alkaloids despite the fact that they are also readily excretable (detoxifying), hepatic metabolites of the parent pyrrolizidine alkaloids.

These hazardous 1,2-dehydropyrrolizidine alkaloids have been identified in floral honeys attributed to plants in the Boraginaceae (*Heliotropium* spp., *Echium* spp.) and the Asteraceae (*Senecio* spp.) (5–9), all known pyrrolizidine alkaloid-producing plant genera (1). The presence of the toxic pyrrolizidine alkaloids in honey presents a food safety concern for consumers and regulatory authorities (8–10).

The flowers of pyrrolizidine alkaloid-producing plants contain high concentrations of a suite of pyrrolizidine alkaloids, and/or

their *N*-oxides, characteristic of the plant (11, 12). The pyrrolizidine alkaloids are transferred to the honey by bees visiting the flowers to forage nectar and pollen. This investigation of pure pollen harvested from *Echium vulgare* was undertaken to provide evidence for the pollen as a potential source of the pyrrolizidine alkaloids that were detected in honey attributed to *E. vulgare*.

MATERIALS AND METHODS

Pyrrolizidine Alkaloid Standards. Authenticated (NMR, MS) samples of heliotrine, lasiocarpine-*N*-oxide, senecionine, echimidine, echiumine, lycopsamine, and intermedine were obtained from the collection of the CSIRO Livestock Industries' Plant Toxins Research Group. A natural mixture of *Echium*-type pyrrolizidine-*N*-oxides was provided by an extract of *Echium plantagineum* flowers (13).

Pollen Collections. Pure pollen, free of any other contaminating plant parts, was collected in the vicinity of Freiburg i. Br., Germany, from fresh flowers of *Echium vulgare* that had been protected from visitation by insects by a net covering. Individual anthers were carefully inserted into the tip of a Pasteur pipet containing *n*-hexane (200 μ L). Thus exposed to the *n*-hexane, the pollen drifted away from the anthers into the hexane. Evaporation of the *n*-hexane yielded the pollen sample ready for weighing and extraction.

Extraction of Pollen. Two pollen samples (50.8 and 47.8 mg) were each mixed with 0.05 M H₂SO₄ (5 mL), vortexed vigorously for 2–3 min, and then gently agitated for about 4 h. After mild centrifugation, the supernatant was decanted off and the residues were reextracted with the acid in the same way. The two consequent extracts, per sample,

* Corresponding author: tel 61-3-52275739; fax 61-3-52275555; e-mail steve.colegate@csiro.au.

[†] Forstzoologisches Institut.

[‡] CSIRO Livestock Industries.

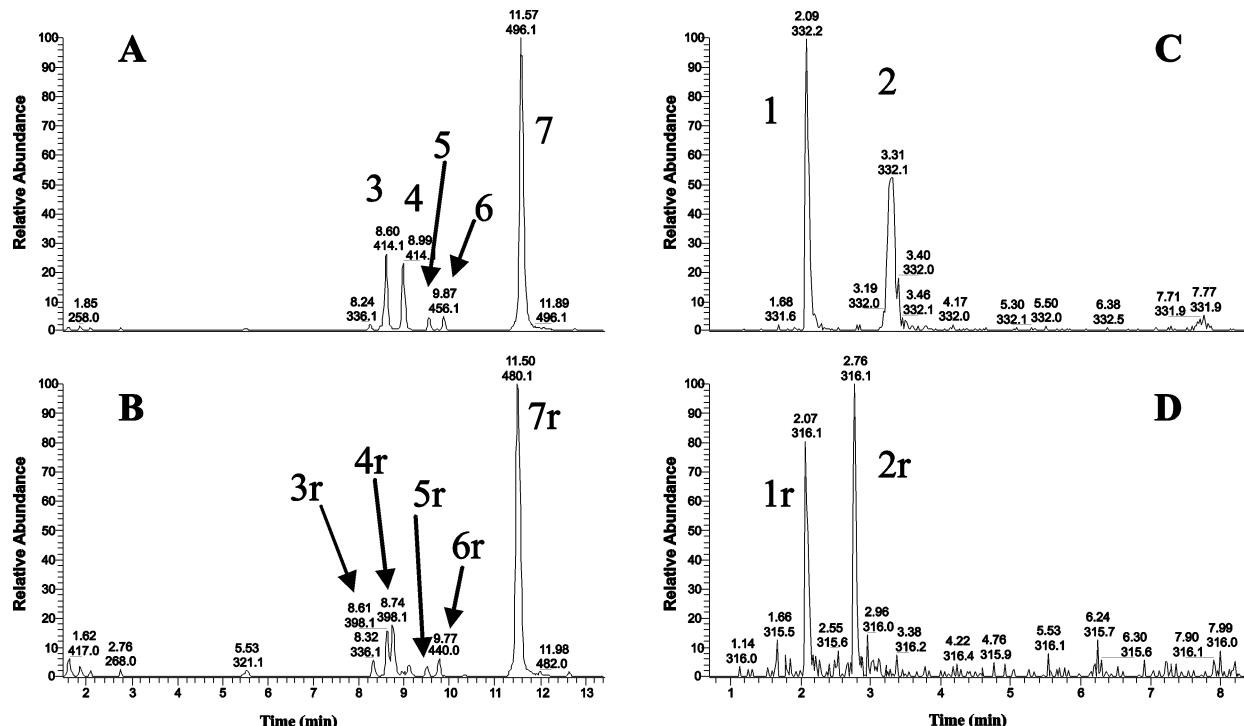


Figure 1. HPLC-ESI-MS base ion (200–500) chromatograms of (A) extract of pure pollen from *Echium vulgare* showing the presence of pyrrolizidine-*N*-oxides and (B) extract treated with the indigocarmine redox resin showing the parent tertiary pyrrolizidine alkaloids. (C) Reconstructed ion chromatogram from panel A displaying *m/z* 332 for leptanthine-*N*-oxide and echimiplatine-*N*-oxide. (D) Reconstructed ion chromatogram from panel B displaying *m/z* 316 for leptanthine and echimiplatine. The peak numbers shown are referred to in the text.

were combined, filtered through a $0.45\mu\text{m}$ GHP Acrodisc 13 mm syringe filter (Gelman Laboratory, Pall Corporation, East Hills, NY), and applied to separate strong cation-exchange, Strata SCX (500 mg/3 mL) solid-phase extraction cartridges (Phenomenex) that had been conditioned with methanol followed by 0.05 M H_2SO_4 . Each cartridge was then washed with water (3 mL) and then methanol (3 mL) before being eluted with ammoniated methanol (methanol saturated at 0–4 °C with ammonia gas, 6 mL). The ammoniated methanolic eluate was immediately evaporated to dryness under a flow of nitrogen in a heating block at 30–40 °C. The residue from each sample was reconstituted into methanol (1 mL) to yield the analytical stock sample that was kept at –10 °C until analysis.

Preparation and Use of Redox Resin. The indigocarmine-based redox resin was prepared and used as previously described (13) with the exception that samples were exposed to the redox resin for only about 2–4 h at 37 °C.

HPLC-ESI-MS Analysis. Samples were analyzed by use of a ThermoFinnigan Surveyor autosampler and liquid chromatography system coupled to a ThermoFinnigan LCQ ion-trap mass spectrometer. Following sample injection onto a 150×2.1 mm i.d. Aqua C18 reverse-phase column (Phenomenex), protected by a guard cartridge of equivalent adsorbent, the column was eluted with a gradient flow (200 $\mu\text{L}/\text{min}$) of 0.1% formic acid in water (mobile phase A) into 0.1% formic acid in acetonitrile (mobile phase B). Mobile phase A was held at 93% for 5 min before decreasing in a linear fashion to 30% over 15 min. A reequilibration phase restored the mobile phase to 93% A in preparation for the next sample injection. A methanolic solution of heliotrine was directly infused into the ESI source to optimize the MS response. The mass spectra were collected in the positive-ion mode with a capillary temperature and voltage of 225 °C and 43 V, respectively. The source voltage was 4.5 kV at a source current of 80 μA . The sheath and auxiliary nitrogen gas flows ratio was 70:3. Data-dependent MS/MS spectra were recorded in the second scan of a two-scan sequence. Thus, nominated ions observed in the first scan (100–1200 mass units, mu) were isolated in the ion trap and then dissociated in the second scan with a generic dissociation energy of 35%. This dissociation energy was not necessarily optimal for each pyrrolizidine

alkaloid/*N*-oxide. For ESI-MS/MS analysis, an aliquot (20 μL) of the sample solution was diluted (1:10) with acetonitrile/0.1% aqueous formic acid (50/50 v/v) and directly infused into the ESI chamber at a flow rate of 5 $\mu\text{L}/\text{min}$. Selected daughter ions from initial MS/MS fragmentation were isolated in the ion trap for further fragmentation.

An analytical sample was prepared by diluting an aliquot (80 μL) of the sample stock solution with an aliquot (20 μL) of a solution of senecionine (5 $\mu\text{g}/\text{mL}$) or heliotrine (20 $\mu\text{g}/\text{mL}$) as an internal standard to normalize the injection process for the LCMS. An aliquot (2 μL) of the analytical sample was injected onto the HPLC column.

Identification and Quantitation of Pyrrolizidine Alkaloids/*N*-Oxides. Pyrrolizidine alkaloids were identified in the analytical samples by comparison of their HPLC retention times and ESI mass spectra with those of authenticated pyrrolizidine alkaloid standards. Where standard pyrrolizidine alkaloids were not available, the pyrrolizidine character of eluted compounds was indicated by the characteristic fragmentations observed in the MS/MS experiments (13). For quantitation purposes, reconstructed ion chromatograms (RICs) were generated, displaying only the molecular ion adduct ($[\text{M} + \text{H}]^+$) for specific pyrrolizidine alkaloids/*N*-oxides and the area under the peak was integrated. The peak area was adjusted relative to the internal standard and the concentration of the pyrrolizidine alkaloid/*N*-oxide was estimated from a six-point calibration curve over the range 0.5–25 $\mu\text{g}/\text{mL}$ lasiocarpine-*N*-oxide with a coefficient of determination (R^2) of 0.9978.

Effectiveness of Extraction. The effectiveness of the solid-phase extraction (SPE) method for capturing and recovering pyrrolizidine alkaloids/*N*-oxides was demonstrated by spiking pollen samples with known amounts of pyrrolizidine alkaloids and their *N*-oxides, either as pure, authenticated compounds or as a total, crude extract of *Echium plantagineum* flowers (13). The capture and recovery effectiveness from spiked pollen was compared directly with the recoveries from processing acidic (0.05 M sulfuric acid) or methanolic solutions of the same pyrrolizidine alkaloids and *N*-oxides, that is, no exposure to the pollen.

Comparison of Methanol and *n*-Hexane *Echium vulgare* Extracts. Samples of dried, milled whole plant (500 mg) were vigorously vortex-mixed (2 min) with methanol (5 mL) or *n*-hexane (5 mL). The samples

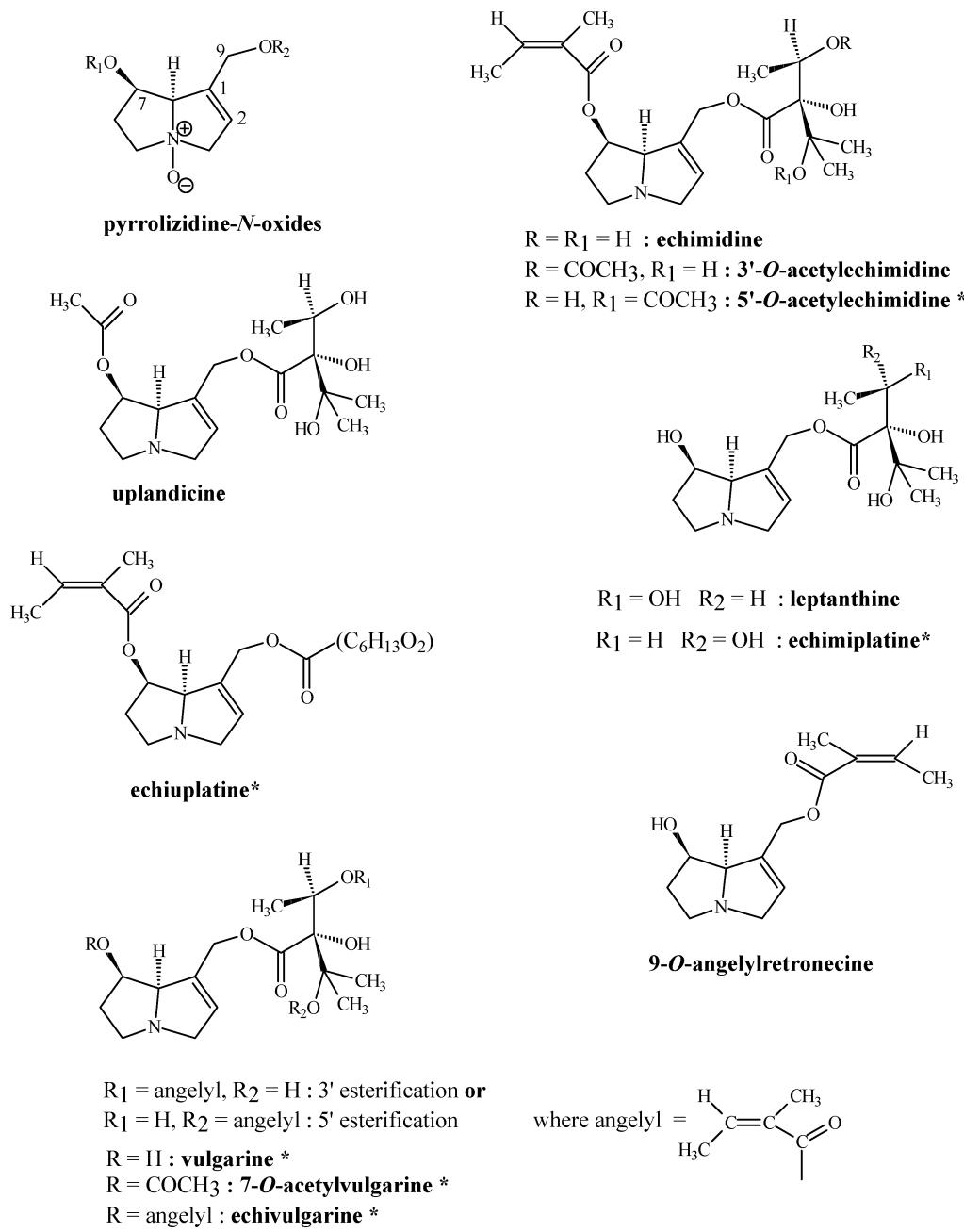


Figure 2. Structures of pyrrolizidine alkaloids and associated N-oxides isolated from *Echium vulgare* pollen. Structures marked with asterisks are tentative, based upon mass spectrometric fragmentations, and are supported by expected HPLC retention times and biosynthetic considerations.

Table 1. MS/MS Data for the Five Major Pyrrolizidine-N-oxides and Their Respective Tertiary Nitrogen Pyrrolizidine Alkaloids

peak	[M + H] ⁺	pyrrolizidine alkaloid/N-oxide	m/z (% relative abundance)
7	496	echivulgarine-N-oxide	478 (19), 396 (32), 378 (2), 352 (15), 338 (100), 254 (10), 220 (2)
7r	480	echivulgarine	462 (35), 418 (10), 398 (10), 380 (90), 336 (40), 322 (100), 220 (70)
6	456	acetyl vulgarine-N-oxide	438 (21), 356 (33), 338 (3), 312 (15), 298 (100), 214 (14), 180 (2), 178 (2)
6r	440	acetyl vulgarine	422 (40), 380 (15), 358 (5), 340 (65), 296 (42), 282 (85), 180 (100)
5	456	acetylechimidine-N-oxide	438 (16), 396 (31), 378 (2), 352 (15), 338 (100), 254 (7)
5r	440	acetylechimidine	422 (24), 380 (60), 336 (25), 322 (100), 220 (40)
4	414	vulgarine-N-oxide	396 (16), 338 (2), 314 (37), 296 (2), 270 (10), 256 (100), 172 (20), 138 (3), 136 (2)
4r	398	vulgarine	380 (18), 336 (5), 316 (5), 298 (40), 254 (15), 240 (52), 138 (100), 120 (14)
3	414	echimidine-N-oxide	396 (100), 370 (3), 356 (15), 352 (78), 338 (18), 312 (2), 310 (2), 254 (22), 220 (2), 120 (1)
3r	398	echimidine	380 (8), 336 (30), 316 (10), 220 (60), 138 (5), 120 (100)

^a Data for other alkaloids detected have been reported previously (13).

were then centrifuged and the supernatants were filtered to remove any fine plant particulates. The solvents were evaporated under a stream of nitrogen and the residues were reconstituted in methanol (500 μ L) for HPLC-ESI-MS analysis.

RESULTS AND DISCUSSION

Analysis of honey attributed to *Echium vulgare* has shown the presence of several pyrrolizidine alkaloids totalling about

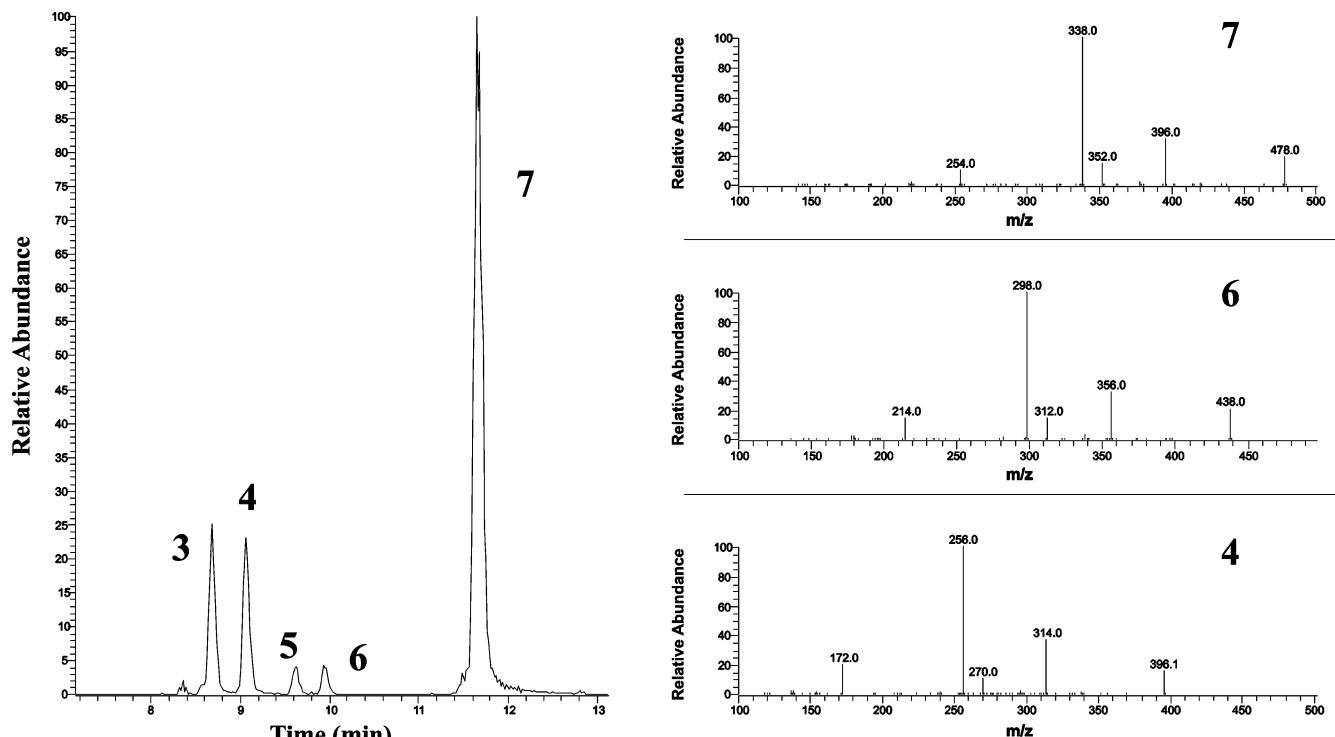


Figure 3. (Left) HPLC-ESI-MS base ion (200–500) chromatogram (7–13 min) of the extract of pure pollen from *Echium vulgare* showing the major pyrrolizidine-*N*-oxides: echivulgarine-*N*-oxide (peak 7), 7-*O*-acetylvulgarine-*N*-oxide (peak 6), acetylechimidine-*N*-oxide (peak 5), vulgarine-*N*-oxide (peak 4) and echimidine-*N*-oxide (peak 3). Also shown (right) are the MS/MS spectra for peaks 7, 6, and 4 illustrating the structural relationship between them.

1220 µg/kg of honey (ppb) (9). The method of analysis, in contrast to this present study, involved a zinc/acid reduction step so that only the tertiary nitrogen pyrrolizidine alkaloids, including the intrinsic free-base, pyrrolizidine alkaloids and those resulting from the reduction of their respective *N*-oxides, were detected. In addition to echimidine, 3'-*O*-acetylechimidine and uplandicine, an unidentified pyrrolizidine alkaloid ($[M + H]^+$ 480) was observed.

While the presence of pyrrolizidine alkaloids in honeys has been well documented (5–9), their source is undefined. The pyrrolizidine alkaloids could originate from nectar, as is currently assumed, but another potential source is pollen, which is an integral component of honey (14).

Pollen free of any other plant tissue was analyzed to investigate the question whether pollen per se contains pyrrolizidine alkaloids/*N*-oxides that could potentially contribute the pyrrolizidine alkaloids found in honey. To avoid contamination of pollen with other plant-derived parts, individual pollen-loaded anthers were rinsed very gently with *n*-hexane within the tip of a Pasteur pipet. Because of their lack of solubility in *n*-hexane, pyrrolizidine alkaloids and their *N*-oxides were not expected to be simultaneously extracted from the anthers during the short duration of collection of the pollen from the plant. This expectation was substantiated by briefly extracting dry, milled *E. vulgare* whole plant with *n*-hexane or methanol and analyzing the extracts with HPLC-ESI-MS. As expected, the methanolic extract showed a high level of a suite of pyrrolizidine alkaloid *N*-oxides, similar in profile but different in relative amounts to those subsequently extracted from the pollen (data not shown). The *n*-hexane extract on the other hand did not show any sign of the pyrrolizidine alkaloids or their *N*-oxides.

The hexane-washed pollen from *E. vulgare* anthers was purple in color and the acid extracts of the pollen samples were purple/pink in color. This color was trapped on the SPE columns but turned black/blue when basified with the ammoniated

methanol during the elution step of the SPE process. HPLC-ESI-MS analysis of the ammoniated methanol eluate revealed that the pyrrolizidine alkaloids occurred mainly as their *N*-oxides (Figure 1A), as was also observed with the pyrrolizidine alkaloids from *E. plantagineum* (13). Under the ESI-MS conditions used, the *N*-oxide character was indicated by the appearance (5–10% relative abundance) of a dimeric molecular ion adduct ($[2M + H]^+$), absent in the ESI mass spectrum of the parent tertiary pyrrolizidine alkaloids. Confirmation of *N*-oxide character was obtained by mixing a methanolic solution of the extracts with indigocarmine-based redox resin for about 2–4 h at 37 °C and then reanalyzing the solution by HPLC-ESI-MS (Figure 1B). The formation of new peaks eluting slightly earlier than the putative *N*-oxides, with molecular ion adducts 16 mu less than the *N*-oxides and no evidence of dimeric molecular ion adducts, strongly supported the pyrrolizidine alkaloid/*N*-oxide relationship. Where possible, chromatographic and mass spectrometric comparison with authenticated pyrrolizidine alkaloids then confirmed the identities. Where authenticated standards were not available, tentative structural assignments were based upon the MS/MS fragmentations and biogenetic considerations that assumed a structural similarity within the same plant genus.

Thus, analysis of the *E. vulgare* pollen revealed the presence of the *N*-oxides (Figure 2) of leptanthine, echimiplatine, echimidine, and acetylechimidine (peaks 1 or 2, 2 or 1, 3, and 5, respectively, in Figure 1A). Trace levels of uplandicine, 9-*O*-angelylretronecine, and echiuplatine (Figure 2) were also revealed by RICs displaying their specific, protonated molecular ions ($[M + H]^+$) and supported by the retention times and MS/MS spectra as previously described (13). Observation of these seven *N*-oxides is analogous to those identified in *E. plantagineum* (13). However, peaks labeled 4 ($[M + H]^+$ 414), 6 ($[M + H]^+$ 456), and 7 ($[M + H]^+$ 496) (Figure 1A) were not readily identified by comparison with known pyrrolizidine

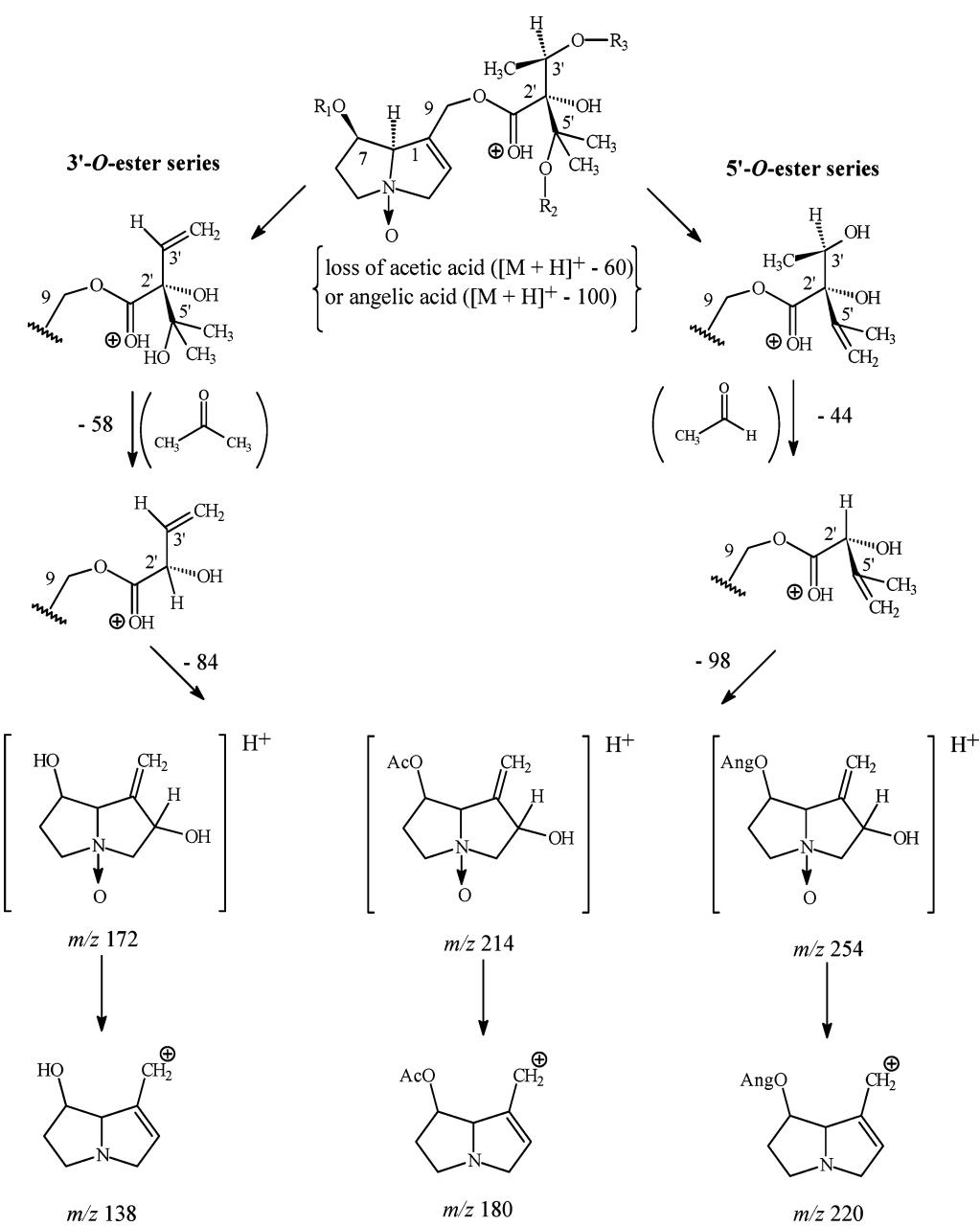


Figure 4. Rationalization of the MS/MS (Table 1) and MS/MS/MS (Table 2) data for echivulgarine-N-oxide ($R_1 = \text{angelyl}$), 7-O-acetylvulgarine-N-oxide ($R_1 = \text{acetyl}$), vulgarine-N-oxide ($R_1 = \text{H}$), and acetylechimidine-N-oxide ($R_1 = \text{angelyl}$). The 3'-O-ester series is represented by $R_3 = \text{angelyl}$ or acetyl and $R_2 = \text{H}$, while the 5'-O-ester series is represented by $R_3 = \text{H}$ and $R_2 = \text{angelyl}$ or acetyl .

alkaloids. As with all the other pyrrolizidine alkaloids isolated, their *N*-oxide character was indicated by the presence of a significant (10% relative abundance) dimer peak and by reductive treatment to form their parent tertiary pyrrolizidine alkaloids. Peak 7 ($[M + H]^+ m/z 496$, $[2M + H]^+ m/z 991$) is of particular note in that its reduction product (peak 7r, Figure 1B), with an $[M + H]^+$ at $m/z 480$, is the same pyrrolizidine alkaloid as was observed in the *E. vulgare* honey (9). The MS/MS data (Table 1) for the major pyrrolizidine-*N*-oxides (peaks 3–7, Figure 1A) and their respective, reduced tertiary alkaloids (peaks 3r–7r, Figure 1B) provided structural information that, combined with biogenic assumptions of basic structural and stereochemical equivalence with other pyrrolizidine alkaloids isolated from *Echium* spp., allowed tentative structures to be proposed for the unknown alkaloids. The MS/MS spectra (Table 1; Figure 3) for the three unidentified pyrrolizidine-*N*-oxides, peaks 4, 6, and 7, indicated a related series of compounds

differing in the functionality at C7. The observation of fragment ions at $m/z 172$, 214, and 254 for peaks 4, 6, and 7, respectively, supported a series of pyrrolizidine-*N*-oxides with no esterification or esterification with acetic acid or angelic acid (or one of its configurational isomers) at the C7 hydroxyl (Figure 4) (13). This was reinforced by the MS/MS spectra of the reduced analogues that showed major fragment ions at $m/z 138$, 180, and 220, consistent with the same C7 derivatization (13). The observation of an $[M + H]^+ - 100$ ion at $m/z 314$, 356, and 396 for peaks 4, 6, and 7, respectively, indicated the loss of an angelic acid moiety (or one of its configurational isomers) (13) that has to be present on the C9 esterifying acid. This would yield the C5'-*O* or C3'-*O*-angelyl (or one of its configurational isomers) analogues of echimidine-*N*-oxide (to give peak 7), uplandicine-*N*-oxide (to give peak 6), and leptanthine-*N*-oxide (and/or echimiplatine-*N*-oxide) (to give peak 4). These compounds appear to be previously unreported and are hence given

Table 2. MS³ and MS⁴ Data for the Five Major Pyrrolizidine-N-oxides

peak	pyrrolizidine-N-oxide	isolated ions (<i>m/z</i> , % dissociation energy)	<i>m/z</i> (% relative abundance)
7	echivulgarine-N-oxide	496, 35; 478, 35	478 (100), 378 (80)
		496, 35; 396, 35	378 (10), 352 (100), 338 (32), 254 (40)
		496, 35; 338, 30	338 (53), 254 (100), 220 (6)
		496, 35; 352, 30	352 (6), 254 (100)
6	acetylulgarine-N-oxide	456, 35; 356, 30	356 (5), 338 (8), 312 (100), 298 (25), 252 (2), 214 (25)
		456, 35; 356, 30; 298, 30	298 (30), 280 (6), 252 (45), 214 (100)
		456, 35; 356, 30; 312, 30	312 (5), 214 (100)
5	acetylechimidine-N-oxide	456, 35; 438, 35	438 (15), 378 (100), 254 (2), 220 (4)
		456, 35; 396, 32	396 (3), 378 (8), 352 (100), 338 (25), 292 (2), 254 (25), 220 (2)
4	vulgarine-N-oxide	456, 30; 396, 30; 352, 30	352 (6), 254 (100)
		414, 32; 314, 30	314 (2), 296 (5), 270 (100), 256 (35), 210 (4), 172 (30), 138 (2)
3	echimidine-N-oxide	414, 33; 396, 33	396 (20), 378 (4), 352 (100), 254 (50), 220 (5), 120 (3)
		414, 33; 396, 33; 352, 30	352 (20), 294 (1), 254 (100), 220 (1), 120 (2)
		414, 33; 356, 33	356 (100), 338 (14), 312 (40), 310 (100), 294 (20), 220 (8), 120 (6)

the trivial names echivulgarine-N-oxide (peak 7, **Figure 1**), 7-*O*-acetylulgarine-N-oxide (peak 6, **Figure 1**), and vulgarine-N-oxide (peak 4, **Figure 1**).

Following an initial loss of 100 mu (loss of angelic acid) or 60 mu (loss of acetic acid), the MS/MS fragmentation pattern (**Table 1**; **Figure 3**) observed for the *N*-oxides of vulgarine, 7-*O*-acetylulgarine, and echivulgarine is completely analogous to the MS/MS spectrum of the acetylechimidine-*N*-oxide (peak 5, **Table 1**), indicating a similarity in position of esterification on the C9 acid by angelic or acetic acid. A previous report (15) has described the identification of 3'-*O*-acetylechimidine in *E. vulgare* based upon NMR data that showed a deshielding of the 3' and 4' carbons and protons. However, in this present study, the MS/MS (**Table 1**) and MS/MS/MS (**Table 2**) analysis of these compounds (*N*-oxides and their respective tertiary bases following treatment with the redox resin) indicated the presence of the 3' and 5' acetyl or angelyl esters (**Figure 4**). Thus, after the initial loss of the 3'- or 5'-*O* esterifying acid, there is a cleavage of 58 mu (acetone) or 44 mu (acetaldehyde), respectively, from the unesterified branch of the C9 acid. This is followed by the loss of the remaining portion of the C9 acid with a concomitant transfer of a water equivalent (OH, H) to the pyrrolizidine-*N*-oxide core (loss of 84 or 98 mu, **Figure 4**). The MS/MS/MS data (**Table 2**) clearly show the sequential losses indicated in Figure 4. The MS/MS data for the parent, tertiary pyrrolizidine alkaloids (**Table 1**) follow the pattern of their *N*-oxides except that there is no transfer of a water equivalent to the core pyrrolizidine after the losses of the 3'- or 5'-*O* esterifying acid (100 or 60 mu) and the associated C9 acid fragments (44 or 58 mu) (**Figure 4**). This results in fragment losses of 102 or 116 mu instead of 84 or 98 mu, respectively, observed with the *N*-oxides (**Figure 4**). For example, whereas echivulgarine-*N*-oxide ([M + H]⁺ 496) loses angelic acid (*m/z* 496 to 396) and then acetone (*m/z* 396 to 338) or acetaldehyde (*m/z* 396 to 352) before the loss of 84 or 98 mu, respectively, (*m/z* 338 or 356 to 254), its parent tertiary alkaloid, echivulgarine ([M + H]⁺ 480), loses angelic acid (*m/z* 480 to 380) and then acetone (*m/z* 380 to 322) or acetaldehyde (*m/z* 380 to 336) before the loss of 102 or 116 mu, respectively, (*m/z* 322 or 336 to 220).

As further evidence for the presence of both 3'-*O* and 5'-*O* esters, the loss of 58 mu (acetone) or 44 mu (acetaldehyde) from a common parent ion, that is, *m/z* 396 for echivulgarine-*N*-oxide, *m/z* 356 for 7-*O*-acetylulgarine-*N*-oxide, or *m/z* 314 for vulgarine-*N*-oxide, is in contrast to the multiple MS fragmentations observed for echimidine-*N*-oxide, which does not have any esterification at 3'-*O* or 5'-*O*. In this latter case a sequential loss of these subunits is observed as loss of 58 or 44

Table 3. Estimated Amounts (in Descending Order) of Individual Pyrrolizidine Alkaloids/*N*-Oxides Extracted from Pure Pollen of *Echium vulgare*^a

pyrrolizidine alkaloid/ <i>N</i> -oxide	[M + H] ⁺ <i>m/z</i>	concn of pyrrolizidine alkaloids/ <i>N</i> -oxides, ppm (% of total)	
		sample 203A (50.8 mg)	sample 203B (47.8 mg)
echivulgarine- <i>N</i> -oxide	496	6875 (84.0)	11 633 (83.0)
vulgarine- <i>N</i> -oxide	414	644 (8.0)	913 (7.0)
echimidine- <i>N</i> -oxide	414	440 (5.4)	1146 (8.2)
acetylechimidine- <i>N</i> -oxide	456	116 (1.4)	96 (0.7)
7- <i>O</i> -acetylulgarine- <i>N</i> -oxide	456	67 (0.8)	88 (0.6)
echivulgarine	480	56 (0.7)	77 (0.6)
acetylechimidine	440	8 (0.1)	9 (0.06)
leptanthine- <i>N</i> -oxide	332	9 (0.1)	12 (0.09)
echiplatine- <i>N</i> -oxide	332	7 (0.1)	11 (0.09)
total		8222	13 985

^a Amounts are expressed as parts per million (ppm) of lasiocarpine-*N*-oxide equivalents. Structures of the pyrrolizidine alkaloids/*N*-oxides are shown in Figure 2. The *N*-oxides of uplandicine, 9-*O*-angelylretronecine, and echiuplatine are not listed because they occur in trace amounts only.

mu from *m/z* 414 to yield ions at *m/z* 356 or 370, respectively, followed by reciprocal loss of 44 or 58 mu to afford an ion at *m/z* 312. It is not clear from this study whether the data reflect the inseparable presence of both isomers within the pollen or a transesterification artifact (transfer of esterifying acid from 3'-*O* to 5'-*O*) of the ESI-MS process. As with echivulgarine, 7-*O*-acetylulgarine, and vulgarine, further work is required to isolate and purify larger quantities of the acetylechimidine identified in this investigation to allow unambiguous determination of their structures by 1D and 2D NMR experiments.

Individual pyrrolizidine alkaloids have different response factors under the ESI-MS conditions and therefore, without generating calibration curves for each individual alkaloid isolated, the quantitation can only be estimated. In this study the estimated quantities of the various pyrrolizidine alkaloids/*N*-oxides (**Table 3**) are expressed in terms of lasiocarpine-*N*-oxide equivalents. In both samples of pure pollen analyzed, the newly described echivulgarine-*N*-oxide (84%) was the major pyrrolizidine alkaloid present, followed by vulgarine-*N*-oxide (8%) and echimidine-*N*-oxide (7%).

The very high levels of pyrrolizidine alkaloids found in the pollen, that is, parts per million in pollen (**Table 3**) vs parts per billion in honeys (9), indicate that pollen has potential to be a significant contributor of pyrrolizidine alkaloids to honey. Therefore, if contamination of honey with pollen can be reduced,

then a potential for avoiding or reducing pyrrolizidine alkaloids in honey exists. Further, commercial bee pollen, used as food supplements, might contain pyrrolizidine alkaloids at unsafe levels.

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LITERATURE CITED

- (1) Stegelmeier, B. L.; Edgar, J. A.; Colegate, S. M.; Gardner, D. R.; Schoch, T. K.; Coulombe, R. A.; Molyneux, R. J. Pyrrolizidine alkaloid plants, metabolism and toxicity. *J. Nat. Toxins* **1999**, *8*, 95–116.
- (2) Huxtable, R. J. Human health implications of pyrrolizidine alkaloids and herbs containing them. In *Toxicants of Plant Origin, Volume 1 Alkaloids*, Cheeke, P. R., Ed.; CRC Press: Boca Raton, FL, 1989; pp 41–86.
- (3) Prakash, A. S.; Pereira, T. N.; Reilly, P. E. B.; Seawright, A. A. Pyrrolizidine alkaloids in human diet. *Mutat. Res.* **1999**, *443*, 53–67.
- (4) Chou, M. W.; Wang, Y.-P.; Yan, J.; Yang, Y.-C.; Beger, R. D.; Williams, L. D.; Doerge, D. R.; Fu, P. P. Riddelliine *N*-oxide is a phytochemical and mammalian metabolite with genotoxic activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. *Toxicol. Lett.* **2003**, *145*, 239–247.
- (5) Deinzer, M. L.; Thompson, P. A.; Burgett, D. M.; Isaacson, D. L. Pyrrolizidine alkaloids: Their occurrence in honey from tansy ragwort (*Senecio jacobaea* L.). *Science* **1977**, *195*, 497–499.
- (6) Culvenor, C. C. J.; Edgar, J. A.; Smith, L. W. Pyrrolizidine alkaloids in honey from *Echium plantagineum* L. *J. Agric. Food Chem.* **1981**, *29*, 958–960.
- (7) Crews, C.; Startin, J. R.; Clarke, P. A. Determination of pyrrolizidine alkaloids in honey from selected sites by solid-phase extraction and HPLC-MS. *Food Addit. Contamin.* **1997**, *14*, 419–428.
- (8) Edgar, J. A.; Roeder, E.; Molyneux, R. J. Honey from plants containing pyrrolizidine alkaloids: A potential threat to health. *J. Agric. Food Chem.* **2002**, *50*, 2719–2730.
- (9) Beales, K. A.; Betteridge, K.; Colegate, S. M.; Edgar, J. A. Solid-phase extraction and LCMS analysis of pyrrolizidine alkaloids in honeys. *J. Agric. Food Chem.* **2004**, *52*, 6664–6672.
- (10) Food Standards Australia New Zealand. Pyrrolizidine alkaloids in food: A toxicological review and risk assessment. Technical Report Series No. 2, 2001; <http://www.foodstandards.gov.au/miareleasespublications/technicalreportseries/index.cfm> (accessed 20th June 2004).
- (11) Hartmann, T.; Zimmer, M. Organ-specific distribution and accumulation of pyrrolizidine alkaloids during the life history of two annual *Senecio* species. *J. Plant Physiol.* **1986**, *122*, 67–80.
- (12) Biller, A.; Boppré, M.; Witte, L.; Hartmann, T. Pyrrolizidine alkaloids in *Chromolaena odorata*. Chemical and chemoeconomic aspects. *Phytochemistry* **1994**, *35*, 615–619.
- (13) Colegate, S. M.; Edgar, J. A.; Knill, A. M.; Lee, S. T. Solid-phase extraction and LCMS profiling of pyrrolizidine alkaloids and their *N*-oxides: a case study of *Echium plantagineum*. *Phytochem. Anal.* **2004**, in press.
- (14) Crane, E., Ed. *Honey, a Comprehensive Survey*; William Heinemann Ltd.: London, 1976.
- (15) El-Shazly, A.; Sarg, T.; Ateya, A.; Abdel Aziz, S.; El-Dahmy, L.; Witte, L.; Wink, M. Pyrrolizidine alkaloids from *Echium setosum* and *Echium vulgare*. *J. Nat. Prod.* **1996**, *59*, 310–313.

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