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Hepatotoxic Pyrrolizidine Alkaloids in Pollen and Drying-Related Implications for Commercial Processing of Bee Pollen

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Using HPLC-ESI-MS, several saturated and 1,2-dehydropyrrolizidine alkaloids were detected, mainly as their *N*-oxides, in fresh pollen collected from flowers of the pyrrolizidine alkaloid-producing plants *Echium vulgare*, *E. plantagineum*, *Senecio jacobaea*, *S. ovatus*, and *Eupatorium cannabinum*, and/or pollen loads from bees (bee pollen) that foraged on those plants. A major alkaloidal metabolite in *S. ovatus* was tentatively identified, using its mass spectrometric data and biogenic considerations, as the previously unreported, saturated alkaloid, 2-hydroxysarracine. Heating had very little effect on the 1,2-dehydropyrrolizidine alkaloids and their *N*-oxides from a variety of sources. Considered in conjunction with international concerns about the adverse effects of these alkaloids, the results strongly indicate a need for monitoring pollen supplies intended for human consumption, at least until conditions for processing and/or selection are clearly defined such as to significantly reduce the hepatotoxic (and potentially carcinogenic and genotoxic) pyrrolizidine alkaloid content of bee pollen.

KEYWORDS: Pyrrolizidine alkaloid; pollen; bee pollen; *Senecio*; *Echium*; *Eupatorium*

INTRODUCTION

Pollen and pollen-based bee products are increasingly being recommended and used as dietary supplements (*1, 2*). However, previous research has clearly identified the presence of toxic 1,2-dehydropyrrolizidine alkaloids in fresh pollen collected from the flowers of the pyrrolizidine alkaloid-producing plant *Echium vulgare* L. (common viper's bugloss or blue borage) (*3*).

The 1,2-dehydropyrrolizidine alkaloids, and their *N*-oxides, are a diverse class of monoester, open chain diester, and macrocyclic diester compounds that can be hepatotoxic, pneumotoxic, genotoxic, and carcinogenic to mammals (*4*). The *N*-oxides are mammalian hepatic metabolites associated with detoxification (elimination) of the pyrrolizidine alkaloids. However, previous reports reviewed by Mattocks (*5*) indicate that orally administered pyrrolizidine-*N*-oxides can be as toxic to rats as their parent free base pyrrolizidine alkaloids. It was suggested that this toxicity occurs primarily as a result of intragut reduction of the *N*-oxides to their parent free bases (ref *5* and references therein). This latter point was highlighted by the observation that the median lethal dose (LD_{50}) for retrorsine-*N*-oxide was 250 mg/kg or 48 mg/kg for intraperitoneal or oral administration, respectively (*6*). This oral LD_{50} for retrorsine-*N*-oxide is very similar to the reported intraperitoneal LD_{50} of 34 mg/kg for the retrorsine free base (*6*). More recently, it has been shown that, in vitro (rat and human liver microsomes) and in vivo (rats), the pyrrolizidine-*N*-oxides can result in similar profiles of hepatic DNA adducts related to the potential for genotoxicity and carcinogenicity (*7, 8*). As a food and pharmaceutical safety measure, some countries have established or have recommended very low levels of oral and topical exposure to pyrrolizidine alkaloids and their *N*-oxides (*9, 10*).

The major effect of dietary 1,2-dehydropyrrolizidine alkaloids on humans is reported to be hepatic veno-occlusive disease (*11, 12*). There have been reports of widespread, acute intoxication of humans that have ingested herbs and food that contain pyrrolizidine alkaloids either as natural metabolites or as contaminants (*13*). There have also been reports of fatal liver disease in human fetuses, neonates, and infants as a result of the mothers chronically ingesting low levels of pyrrolizidine alkaloids or the infant being exposed to the alkaloids in herbal remedies (*14, 15*).

It has been speculated that contamination with pyrrolizidine alkaloid-containing pollen may be the reason for the pyrrolizidine alkaloids found in certain honeys and that the high content of pyrrolizidine alkaloids in some pollens may also be an issue for the commercial use of bee pollen-based products as food and food additives (*3*). Therefore, when considering possible factors in the cause of liver disease of unknown etiology, low levels of dietary pyrrolizidine alkaloids and their *N*-oxides, including those in honey and bee pollen, should be taken into

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Figure 1. (**A**) A honeybee (*Apis mellifera*) with its two loads (arrowed) of purple colored pollen collected from *Echium* sp. (**B**) Close up of the pollen basket with an *Echium* pollen load (arrowed) on the hindleg of a bee.

consideration, especially in cases where exposure to 1,2-dehydropyrrolizidine alkaloids is suspected but no ingestion of herbal products was recorded (*16, 17*).

The current study was aimed at determining the toxic pyrrolizidine alkaloid profile and content of freshly collected pollen from *Senecio ovatus* (wood ragwort), *S. jacobaea* (ragwort, tansy ragwort or common ragwort), *Echium plantagineum* (Paterson's curse, salvation Jane, Riverina bluebell, blue weed, purple bugloss, or purple viper's bugloss), and *Eupatorium cannabinum* (hemp agrimony), and/or pollen loads from bees that foraged on those plants. The pyrrolizidine alkaloid content of bee-collected pollen from *Echium vulgare* was compared to a previous report of the pollen collected directly from the plant (*3*). Furthermore, the effect of drying time and temperature on the stability of some pure pyrrolizidine-*N*-oxides and their free bases, the suite of alkaloids in an extract of *Echium plantagineum*, and the pyrrolizidine alkaloids in pollen collected from bees foraging on *Echium plantagineum* or *Senecio ovatus* was also investigated.

MATERIALS AND METHODS

Collection of Pollen. Flowering *Senecio jacobaea* L., *S. ovatus* (P. Gaertn., Mey. et Scherb.) Willd., previously known as *S. fuchsii* C. C. Gmel. and *S. nemorensis* L (*18*)., and *Eupatorium cannabinum* L. were unambiguously identified (MB) in the vicinity of Freiburg i. Br., Germany. Voucher specimens (06−380, 06−384, and 06−405, respectively) were deposited with the Herbarium of the Forstzoologisches Institut, Albert-Ludwigs-Universität, Freiburg. The pure pollen from *S. jacobaea* and *S. ovatus*, free of any other contaminating plant parts, was gently rinsed from anthers that were carefully inserted into the tip of a Pasteur pipet containing *n*-hexane (*3*). Honeybees (*Apis mellifera)* were captured while they were visiting flowers of *S. ovatus* and *E. cannabinum*, whereas both honeybees and solitary bees (*Andrena* sp.) were captured while they were visiting flowers of *S. jacobaea*. The honeybee collects and retains pollen into pollen baskets with the aid of regurgitated fluids (**Figure 1**). The resultant conglomerates of pollen were gently plucked from the captured bees as discrete loads. The solitary bee, however, does not need the regurgitated glue but retains the pollen on special hairs. Some of the pollen from the solitary bees was gently brushed off and the residual pollen, resistant to the gentle brushing, was then separately collected when washed off the bee using *n*-hexane. Alternatively, the *S. jacobaea* pollen from the solitary bees was washed off with *n*-hexane immediately, without the preceding brushing stage. By contrast, in New South Wales, Queensland, and Western Australia, pollen loads were dislodged from honeybees, foraging in the vicinity of late season *Echium plantagineum* L., as they entered the hive via a commercially used pollen trap. The purple *E. plantagineum* pollen loads were manually separated from all other colors.

Heat Treatment of Bee-Collected Pollen, Pure Alkaloids, and an Extract of *Echium plantagineum*. Prior to alkaloid extraction, samples of pollen loads (20−40 discrete pollen loads), freshly collected from bees that had foraged on *S. ovatus*, were placed in open, glass vials and heated in an oven at 37, 40, or 60 °C for 24, 48, 72, or 84 h.

Similarly, the residues obtained from evaporating aliquots (1 mL) of methanol solutions of purified senecionine or its *N*-oxide (10 and 100 μg/mL) and integerrimine or its *N*-oxide (10 μg/mL), under a stream of nitrogen, were placed in an oven at 58 ± 2 °C for 72 h. After heat treatment, the dried residues were reconstituted in methanol (1 mL) and analyzed using LCMS as usual.

Six equal aliquots of a methanolic extract of *E. plantagineum* were evaporated to dryness under a stream of nitrogen. Three of the dried residues were placed in an oven at 56 °C, while three were kept at 4 °C. After 72 h, the dried residues were reconstituted into equal volumes of methanol and analyzed in duplicate using LCMS.

Two samples (ca. 1−2 g) of the purple *E. plantagineum* pollen loads, one sample each from New South Wales and Western Australia, were placed in open, glass vials in an oven at 56−60 °C for about 100 h.

Finally, two samples (0.234 and 2.0 g) of *E. plantagineum* pollen loads, collected and sun-dried on a window-sill (ca. 25 °C) for 5 h in Queensland, were extracted without further heat treatment.

Extraction of Pollen. Essentially as previously described (*3*), pollen or pollen load samples (usually about 0.150 g) were mixed with 0.05 M H_2SO_4 (4−7 mL), vortexed vigorously for 2−3 min to completely disrupt the pollen loads, and then gently agitated (inversion mixing) for about 16 h at room temperature (23 °C). Because of the unexpectedly low level of pyrrolizidine alkaloids in the pollen loads collected by bees from late season *Echium plantagineum*, samples of about 1.5 to 1.9 g were vortexed and mixed with 0.05 M sulfuric acid (15 mL) for about 30 h. After mild centrifugation of the aqueous acid−pollen mixtures, the pale yellow or, in the case of the *Echium plantagineum* pollen, the purple/pink-colored supernatant from a pollen extract was decanted. The residue was resuspended in fresh acid and followed immediately by centrifugation to provide a rinsing supernatant that was added to the main extract.

Each aqueous acid extract of a pollen sample was applied (0.8 μm syringe filter) to a separate stack of two strong cation exchange (SCX) solid phase extraction (SPE) cartridges (Phenomenex Strata SCX, 500 mg/3 mL) that had been conditioned with methanol followed by 0.05 M H_2SO_4 according to the manufacturer's advice. The stacked cartridges were then separated, providing a top and bottom cartridge that were individually processed henceforth. Lack of detection of pyrrolizidine alkaloids from the bottom cartridge confirmed complete capture of extracted alkaloids by the top cartridge. Each cartridge was washed with water (3 mL) and then methanol (3 mL) before any retained alkaloids were eluted with ammoniated methanol (methanol saturated at 0−4 °C with ammonia gas, 6−10 mL). The ammoniated methanolic eluates were 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 (0.5−1.5 mL) to yield the analytical stock sample that was kept at −10 °C until analyzed.

Pyrrolizidine Alkaloid Standards. Authenticated (NMR, MS) samples of heliotrine, lasiocarpine, senecionine, and integerrimine, and their *N*-oxides, and sarracine were obtained from the collection of the CSIRO Livestock Industries' Plant Toxins Research Group.

Preparation and Use of Redox Resin. An indigocarmine-based redox resin was prepared and used, to confirm the *N*-oxide character of extracted compounds, as previously described (*19*) with the exception that analytical samples were exposed to the redox resin for only about 4 h at 37 °C.

HPLC-ESI-MS Analysis. Samples were analyzed essentially as previously described (*3*) using a ThermoFinnigan Surveyor autosampler and liquid chromatography system employing an acetonitrile/water/0.2% formic acid gradient elution of a C18 reverse phase column (Phenomenex Aqua C18, 2.1 × 150 mm, Lane Cove, Australia) coupled to a ThermoFinnigan LCQ ion trap electrospray ionization mass spectrometer.

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

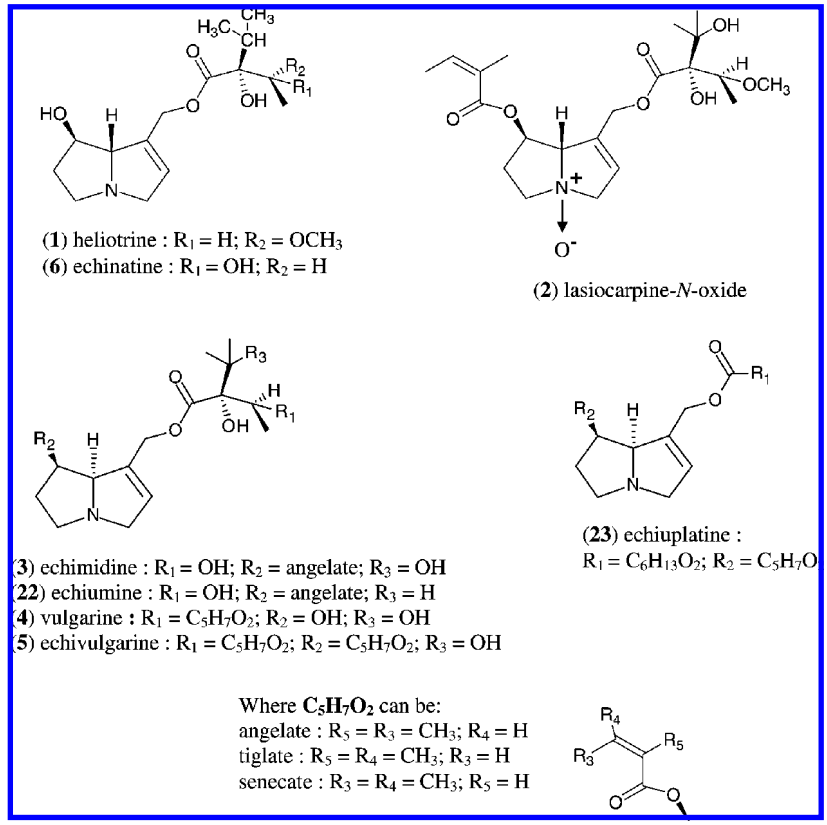

Figure 2. Chemical structures for the internal standard, heliotrine; the calibration standard, lasiocarpine-*N*-oxide; and pyrrolizidine alkaloids from *Echium vulgare* (**Figure 3A**), *Echium plantagineum*, and *Eupatorium cannabinum* (**Figure 3B**) pollen.

heliotrine (**1**) (**Figure 2**) (20 μg/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. The pyrrolizidine alkaloid nature of eluted compounds was indicated by the characteristic fragmentations observed in the MS/MS experiments (*3, 19*). The identities were suggested on the basis of the correlation of the MS data (MH^+ and ion fragments) with pyrrolizidine alkaloids expected to be present and, in some cases, with chromatographic coelution with authenticated standards. For previously undescribed alkaloids, since it was not possible in this MS study to differentiate between the various configurational or stereochemical isomers of the necic acids and the necine bases that constitute the spectrum of pyrrolizidine alkaloids, tentative structures were proposed on the basis of the MS/MS data and biogenic considerations that assumed a similarity of unknown pyrrolizidine alkaloids to those that were or have been positively identified in the same plant.

For quantitation estimates, a reconstructed ion chromatogram (RIC) was generated displaying only the MH^+ for a specific pyrrolizidine alkaloid or its *N*-oxide and the area under the peak integrated and adjusted relative to the internal standard. In the absence of calibration standards for each identified alkaloid, the concentrations of the pyrrolizidine alkaloids/*N*-oxides were estimated from a six-point calibration curve ($R^2 = 0.999$) over the range 0.5−25 μg/mL of lasiocarpine-*N*-oxide (**2**) (purity >99% based on NMR and LCMS). The choice of calibration standard can have significant effects on the final estimates of alkaloids. For example, upon the basis of the relative slopes of the curves, using lasiocarpine-*N*-oxide as the calibration standard provides a more conservative estimate of the pyrrolizidine alkaloids and their *N*-oxides compared to the calibration curves generated using senecionine, heliotrine, heliotrine-*N*-oxide or lasiocarpine (*20, 21*).

Alternatively, to enhance sensitivity in cases where levels of the alkaloids are low, selective reaction monitoring (SRM) MS experiments were set up to specifically identify heliotrine (the internal standard; *m/z* 314 − 138 − 120) and echimidine (*m/z* 398 − 336 − 220 − 120) and its *N*-oxide (*m/z* 414 − 396 − 352 − 254) in the extracts of the *Echium plantagineum* pollen loads. The areas under the peaks observed in RICs displaying the specific SRMs were used to estimate the level of echimidine and its *N*-oxide present before and after heating.

RESULTS AND DISCUSSION

Consistent with a previous report of the concentrations and profile of pyrrolizidine alkaloids detected in pollen collected directly from isolated anthers of *Echium vulgare* (*3*), SCX-SPE and subsequent LC-ESI-MS analyses of pollens collected directly from flowers of *Senecio ovatus*, *S. jacobaea*, and *Eupatorium cannabinum*, and/or pollen loads from bees foraging on these plants, and from bees visiting flowering *Echium vulgare*, revealed significant concentrations of the *N*-oxides of pyrrolizidine alkaloids (**Figure 3**). The concentrations of the pyrrolizidine alkaloids, normalized against heliotrine (**1**) (**Figure 2**) as an internal standard, were expressed as equivalents of lasiocarpine-*N*-oxide (**2**) (**Figure 2**) by comparison to a calibration curve generated using lasiocarpine-*N*-oxide. While this is a useful way to compare relative amounts of pyrrolizidine alkaloids in different pollen samples, it should be recognized that such quantitation, relying as it does on a specific calibrant, may not reflect the actual concentrations due to differences in the relative responses of individual alkaloids under the MS conditions used. Nonetheless, the high (parts per million, ppm) levels of 1,2-dehydropyrrolizidine alkaloids found in the pollen in this study far exceeded the low levels (low parts per billion, ppb) considered tolerable for human consumption in several countries (*9, 10*).

Analysis of Pollen. *Echium vulgare.* In a sample of 8 pollen loads, collected from 4 bees (2 loads per bee), the total level of pyrrolizidine alkaloids present was estimated at 350 ± 50 μg equivalents of lasiocarpine-*N*-oxide/g pollen loads. Somewhat lower than the level (8000−14000 μg equivalents of lasiocarpine-*N*-oxide/g pollen) of pyrrolizidine alkaloids found in

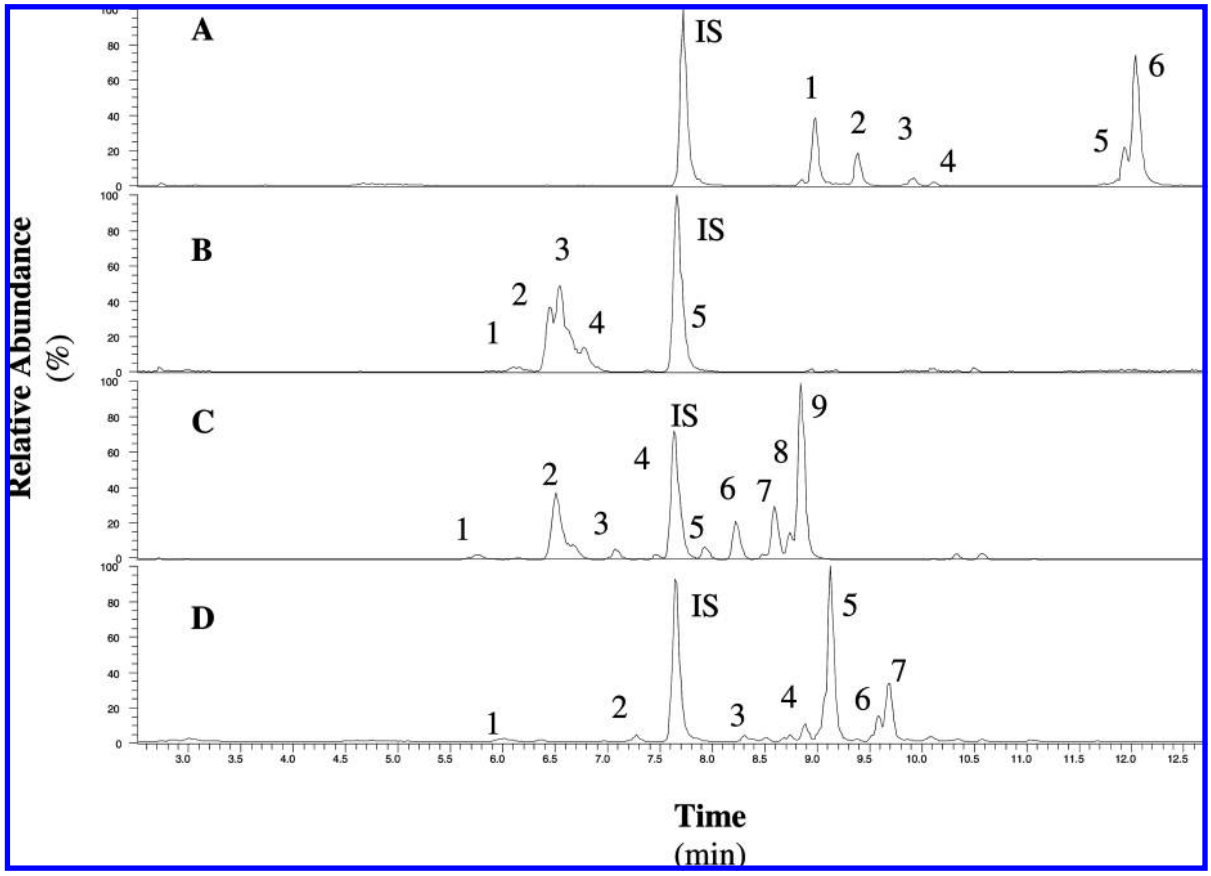

Figure 3. Base ion (*m/z* 200−500) HPLC-ESI-MS chromatograms for (**A**) pollen loads from bees foraging on *Echium vulgare*; (**B**) pollen loads from bees foraging on *Eupatorium cannabinum*; (**C**) pollen, hexane-washed from anthers of *Senecio jacobaea*; and (**D**) pollen loads from bees foraging on *Senecio ovatus*. The internal standard (IS) was heliotrine (**1**). The peak numbers for chromatograms **A**−**D** are referred to in the text, and their structural assignments shown in **Figures 2**, **4**, and **8**.

pure pollen collected directly from *E. vulgare* flowers (*3*), the difference reflects the dilution effect of the regurgitate glue used by the bees to form the pollen loads. As expected, HPLC-ESI-MS analysis of the *Echium vulgare* pollen loads (**Figure 3A**) showed the same suite of major alkaloids (**Figure 2**) that was previously observed in pollen taken directly from the plant (*3*). Between different bee-pollen samples, the relative levels of echimidine-*N*-oxide (**3**-NO) (peak 1, MH^+ *m/z* 414) and vulgarine-*N*-oxide (**4**-NO) (peak 2, MH^+ *m/z* 414) could be reversed, but, in all cases, echivulgarine-*N*-oxide (**5**-NO) (peak 6, MH^+ *m/z* 496) was the major pyrrolizidine alkaloid detected. Low levels of acetylechimidine-*N*-oxide (peak 3, MH^+ *m/z* 456) and acetylvulgarine-*N*-oxide (peak 4, MH^+ *m/z* 456) were easily observed. Echivulgarine (**5**) (peak 5, MH^+ *m/z* 480) was the major parent pyrrolizidine alkaloid observed, but echimidine (**3**) and vulgarine (**4**) (both with MH^+ *m/z* 398) could easily be visualized and quantitated using RICs that displayed this ion.

Eupatorium cannabinum. In a sample of 20 pollen loads from 10 bees, the total levels of pyrrolizidine alkaloids were estimated at 120 ± 20 μg equivalents of lasiocarpine-*N*-oxide/g pollen loads collected from bees that foraged on *Eupatorium cannabinum*. The MS identifications were consistent with previous reports of isomeric, echinatine-like pyrrolizidine alkaloids produced by this plant (*22, 23*) and mainly comprises three isobaric *N*-oxides (peaks 2−4, MH^+ *m/z* 316) (**Figure 3B**). The similarity of the MS/MS spectra for these isobaric peaks and for authenticated echinatine-*N*-oxide (**6**-NO) (**Figure 2**) clearly indicated that they were related isomers. There was a smaller presence (**Figure 3B**) of the corresponding free bases (e.g., peak 1, MH^+ *m/z* 300) and an acetylated echinatine (or isomer)-*N*-oxide (peak 5, MH^+ *m/z* 358). The latter coeluted with the internal standard (heliotrine) and was revealed by the use of a RIC displaying *m/z* 358. The observation of an ion at *m/z* 172 (rather than at *m/z* 180) in the MS/MS data for this acetylated pyrrolizidine-*N*-oxide indicated that the acetylation occurs on the C9 ester functionality of the *N*-oxide rather than on the free C7 hydroxyl (*19*). Further investigation using RICs also revealed the presence of minor peaks in some samples with MS/MS data consistent with the acetylated analogue of echinatine or its isomers (MH^+ *m/z* 342).

Senecio jacobaea. A previous report that deals with the evolution of oligolecty in bees also briefly compared the pyrrolizidine alkaloid content of *S. jacobaea* stems, leaves, flowers, and pollen (*24*). Extracted alkaloids were reduced to the parent free bases and estimated at about 180 μg senecionine equivalents/g pollen using GC-MS.

In the current study, estimates of total pyrrolizidine alkaloid content, expressed as equivalents of lasiocarpine-*N*-oxide, ranged from up to 800 μg/g pollen collected directly from the flowers to about 100 μg/g pollen collected from 17 solitary bees. The *S. jacobaea* pollen (both pure pollen and pollen loads) (**Figure 3C**) was shown to be from an erucifoline chemotype of the plant by the major presence of the *N*-oxides of erucifoline (**7**-NO) (peak 2, MH^+ *m/z* 366), seneciphylline (**8**-NO) (peak 6, MH^+ *m/z* 350), and senecionine (**9**-NO) (peak 9, MH^+ *m/z* 352), but no or very little jacobine or its *N*-oxide (**Figure 4**) (*25, 26*). Also readily observed in the pollen and pollen load extracts (**Figure 3C**) were peaks consistent with the *N*-oxides of eruciflorine (**10**-NO) (peak 3, MH^+ *m/z* 368), retrorsine (**11**-NO) (peak 4, MH^+ *m/z* 368, coelutes with the internal standard

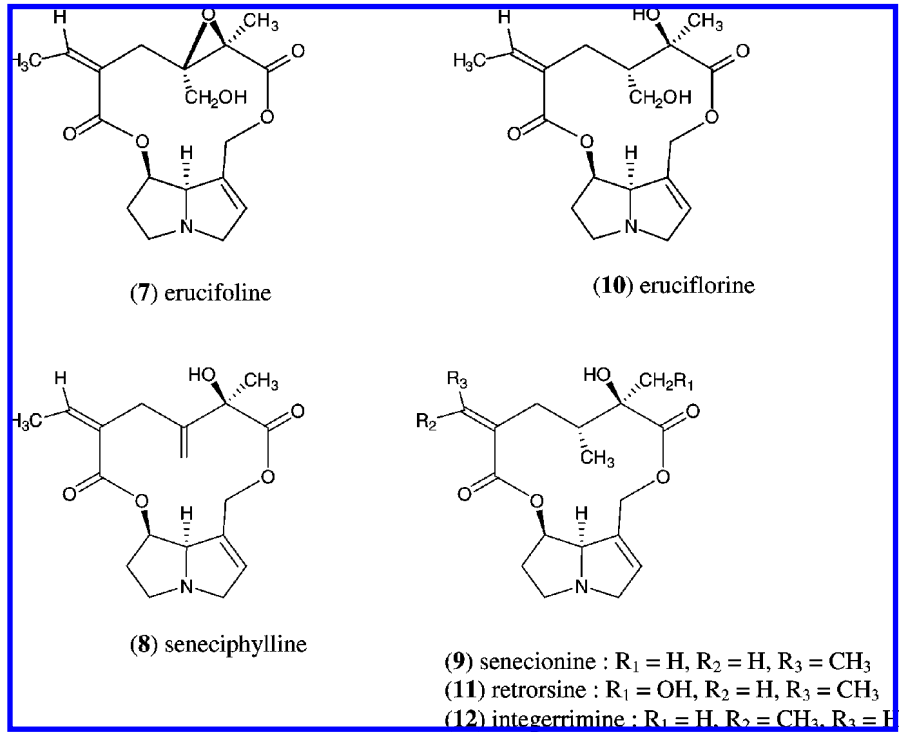

Figure 4. Chemical structures for the pyrrolizidine alkaloids from *Senecio jacobaea* pollen (**Figure 3C**).

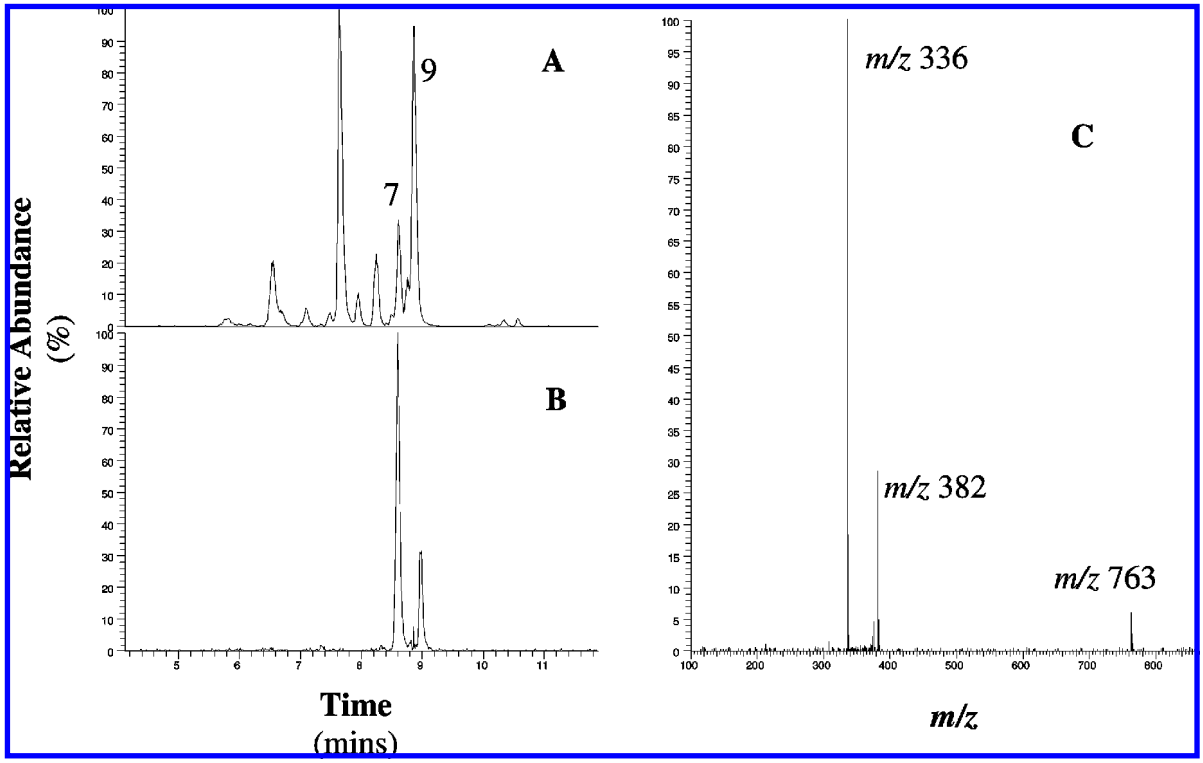

Figure 5. Visualizing hidden pyrrolizidine alkaloids. (**A**) Base ion (*m/z* 200−500) HPLC-ESI-MS chromatogram for *Senecio jacobaea* pollen extract (**Figure 3C**); (**B**) the reconstructed ion chromatogram from **A** displaying *m/z* 382; and (**C**) the mass spectrum of the major peak in **B** showing a putative pyrrolizidine-*N*-oxide (MH^+ *m/z* 382 and $[2M + H]^+$ *m/z* 763) hidden beneath peak 7 (*m/z* 336) for senecionine.

but revealed using a RIC displaying *m/z* 368), and integerrimine (**12**-NO) (peak 8, MH^+ *m/z* 352) (**Figure 4**). Corresponding peaks were observed directly or using RICs for the parent, free base pyrrolizidine alkaloids of the *N*-oxides, i.e., erucifoline (**7**) (peak 1, MH^+ *m/z* 350), seneciphylline (**8**) (peak 5, MH^+ *m/z* 334), senecionine (**9**) (peak 7, MH^+ *m/z* 336), integerrimine (**12**) (MH^+ *m/z* 336), and eruciflorine (**10**) and retrorsine (**11**) (both with *m/z* 352 and eluting slightly earlier than their respective *N*-oxides). Another example of using RICs to reveal hidden pyrrolizidine alkaloids that are potentially novel is shown in **Figure 5**. The larger peak 7 of senecionine (*m/z* 336) is hiding a fairly significant, coeluting peak with an MH^+ ion at *m/z* 382 and a $[2M + H]^+$ ion at *m/z* 763. While more extensive elucidation of this structure was not within the scope of this study, the available data tantalisingly suggest an *N*-oxide, due to the large dimeric molecular ion adduct (*19*), of a methoxylated

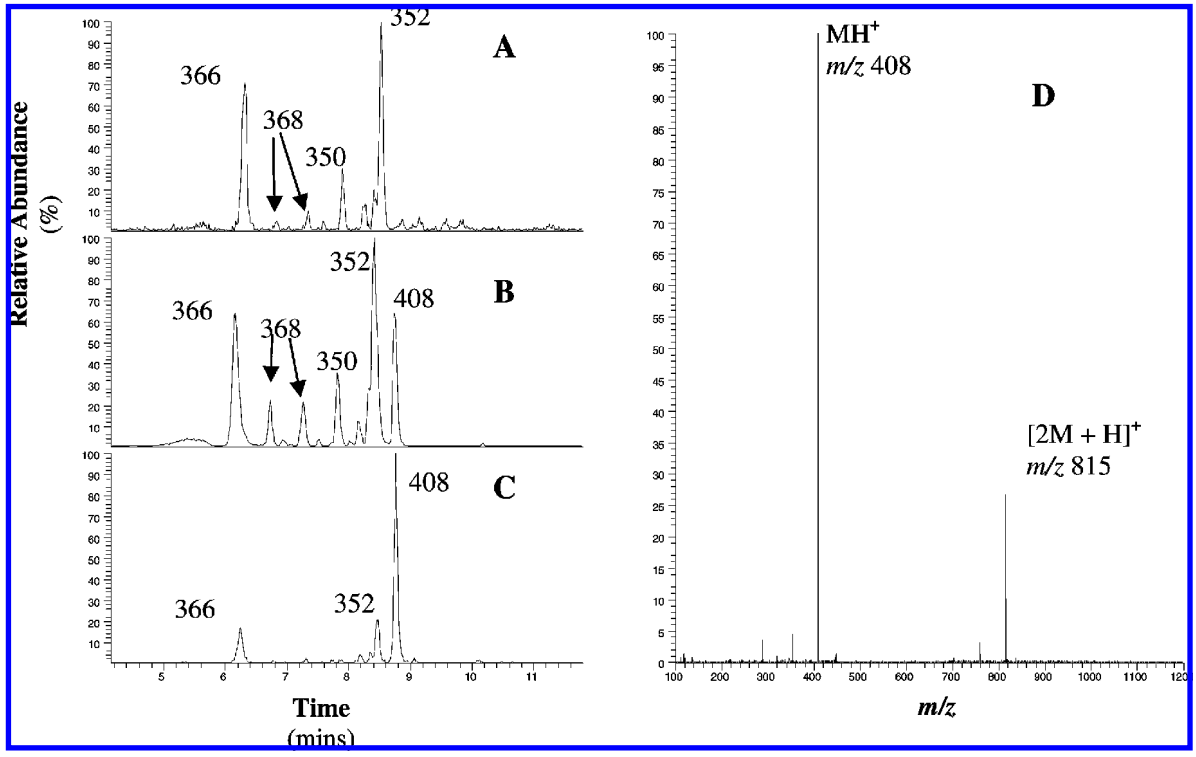

Figure 6. The HPLC-ESI-MS base ion (*m/z* 320−500) chromatograms of the alkaloidal extracts of (**A**) pollen, (**B**) flowers, and (**C**) leaves of *Senecio jacobaea*. Also shown is (**D**) the ESI mass spectrum for the late-eluting peak present in leaves and flowers. The MH^+ and the presence of the dimer ion adduct ($[2M + H]^+$) are consistent with an acetylated derivative of erucifoline-*N*-oxide (*m/z* 366, peak 1) (**Figure 3C**).

senecionine (or isomer). The minor peak in the RIC (**Figure 5B**) did not show a corresponding dimeric molecular ion adduct.

An incidental but interesting observation during this study was the presence, in the extract of flowers or leaves from *S. jacobaea*, of a major peak eluting after senecionine-*N*-oxide (*m/z* 352) (**Figure 6**). The abundant dimeric molecular ion adduct (*m/z* 815) and observation of the redox resin reduction product of *m/z* 392 confirmed its *N*-oxide character. The molecular ion adduct (*m/z* 408) is consistent with an acetylated derivative of erucifoline-*N*-oxide (**7**-NO) (or of its isomers). Full structural characterization was beyond the scope of this present study, but the lack of appearance in the pollen may be of ecological interest.

Senecio ovatus. The total pyrrolizidine alkaloid concentrations observed in *Senecio ovatus* pollen (**Figure 3D**) varied from 155 ± 20 μg equivalents of lasiocarpine-*N*-oxide/g pollen collected directly from the flowers down to 70 ± 20 μg equivalents of lasiocarpine-*N*-oxide/g pollen loads collected from bees. The latter was based upon analysis of a sample of 28 pollen loads collected from 14 bees. As with the other pollens, the pyrrolizidine alkaloids were present mainly as their *N*-oxides as demonstrated by the treatment with the redox resin (**Figure 7**). In keeping with previous reports of alkaloids isolated from *S. fuchsii* or *S. nemorensis* (*27*), the significant presence of platynecine-based pyrrolizidine alkaloids (a fully saturated pyrrolizidine alkaloid core, in contrast to the 1,2-unsaturated core of the retronecine and heliotridine-based pyrrolizidine alkaloids) was indicated by the observation of the fragment ion at *m/z* 122 instead of *m/z* 120 observed with the 1,2-unsaturated hepatotoxic pyrrolizidine alkaloids (*19*). There were several, low-abundance peaks (e.g., peaks 1 and 3) (**Figure 3D**) for which the MS and MS/MS data were consistent with simple monoesters of platynecine and retronecine (and their *N*-oxides) such as the previously identified fuchsisenecionine (**13**) (**Figure 8**) (*28*). Sarracine-*N*-oxide (**14**-NO) (**Figure 8**) (peak 7, MH^+ *m/z* 354) (**Figure 3D**) was identified by coelution and MS/MS data comparison of its indigocarmine-based, resin-reduced product (*m/z* 338) with an authenticated sample of sarracine. A similarity in the MS/MS data (**Table 1**) and elution times suggested that peak 6 (MH^+ *m/z* 352) (**Figure 3D**) is the 1,2-unsaturated analogue of peak 7, i.e., triangularine-*N*-oxide (**15**-NO) (**Figure 8**). To support this, a strong loss of 115 Da, indicative of esterification with sarracinic acid, from the molecular ion adduct was in agreement with the electron impact ionization (EI) mass spectrum and chemical ionization mass spectrum (CIMS) reported for triangularine isolated from *S. triangularis* (*29*) and the CIMS of the C7 senecioyl configurational isomer of triangularine isolated from *S. cacaliaster* (*30*). The most prominent peak (MH^+ *m/z* 370) in all extracts of *S. ovatus* pollen and pollen loads (and in leaf and flower material; unpublished data) did not correspond to any previously identified pyrrolizidine alkaloids from *S. fuchsii* or *S. nemorensis* (*27*). HPLC-ESI-MS and ESI-MS/MS comparison of the indigocarmine-based, redox resin-reduction product (MH^+ *m/z* 354) of peak 5 (**Figure 3D**) with rosmarinine (**16**) (2-hydroxyplatyphylline, MH^+ *m/z* 354) (**Figure 8**), which has been reported from several other *Senecio* species, clearly differentiated the two on the basis of retention time and the presence (rosmarinine) or absence (reduced peak 5) of an $MH^+ - 28$ ion that is characteristic of macrocyclic pyrrolizidine alkaloids possessing a C12 hydroxyl substituent (*21*). The similarity of the MS/MS data (**Table 1**) for peaks 5, 6, and 7 in **Figure 3D** indicated a close relationship. In particular, the observation of ($MH^+ -$ 82/100) and ($MH^+ -$ 98/116) ions indicated a series of open chain diesters with angelic and sarracinic acids, respectively. The MS data for peak 5 are consistent with an additional hydroxylation relative to sarracine-*N*-oxide (**14**-NO) (peak 7). The position of this hydroxylation was indicated by observation of an abundant ion at *m/z* 138 in the ESI-MS/MS spectra of peak 5 and its redox resin reduction product. This ion can be

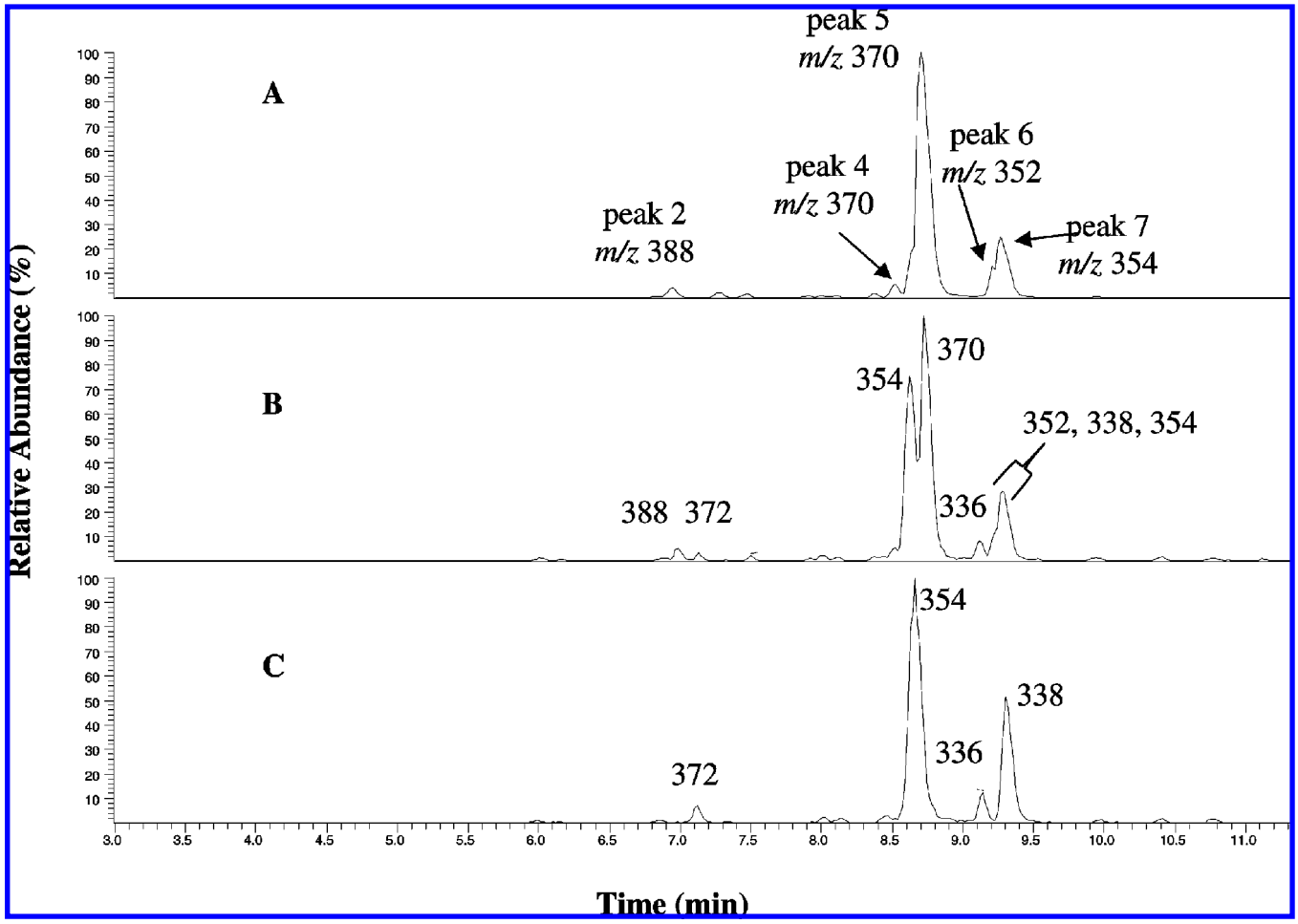

Figure 7. HPLC-ESI-MS base ion (*m/z* 200−500) chromatograms for an extract of *Senecio ovatus* pollen loads: (**A**) untreated extract from the pollen loads and (**B**) the partially reduced and (**C**) the fully reduced extract after treatment with the indigocarmine-based redox resin. The peaks are numbered in accord with **Figure 3D** and are annotated with the *m/z* of the MH^+ ion.

rationalized as the 1,2-hydrated analogue of the *m/z* 120 ion characteristically observed for 1,2-dehydropyrrolizidine alkaloids (*19*), thereby indicating hydration of the 1,2 olefinic center of triangularine-*N*-oxide (**15**-NO) (peak 6) (**Figure 3D**) to yield 2-hydroxysarracine-*N*-oxide (**17**-NO) (**Figure 8**) (peak 5). This co-occurrence of the 1,2-unsaturated alkaloid, triangularine (**15**) (peak 6), with its 1,2-dihydro and 1,2-hydrated derivatives, sarracine (**14**) (peak 7) and the putative 2-hydroxysarracine (**17**) (peak 5) (**Figure 3D**), respectively, is consistent with other such observations including the co-occurrence of the similarly related senecionine (**9**), platyphylline (**18**), and rosmarinine (**16**) (**Figure 8**) (*29*). The MS/MS data (**Figure 9**) for the minor peak 4 (MH^+ *m/z* 370) (**Figure 3D**) are very similar to those of the isobaric major peak 5 including the ion at *m/z* 122 indicative of the fully saturated platynecine base. The fragment losses of 100/82 Da in both compounds are indicative of an angelate (or configurational isomer) as an esterifying acid. However, the very strong ions at *m/z* 140 and 238 in the mass spectrum of peak 4 are significant differences to the mass spectrum of peak 5 and indicated that the extra hydroxylation (relative to sarracine-*N*-oxide) occurs on the esterifying acid rather than the platynecine base. This was supported by the observation of losses of 132 Da (*m/z* 370 − 238) and 114 Da (*m/z* 352 − 238) that imply esterification by a hydroxylated sarracinic acid, perhaps yielding a hydroxysarracine (**19**-NO) (**Figure 8**).

In further support of this structure, the MS/MS data for the minor peak 2 in **Figure 3D** (MH^+ *m/z* 388) (**Table 1**) suggested an additional hydroxylation resulting in a dihydroxy derivative of sarracine (**20**-NO) (**Figure 8**) analogous to the previously reported dihydroxy derivative of triangularine (**21**) (**Figure 8**) (*31*).

Echium plantagineum. Fresh pollen loads were harvested from bees that had been foraging in the vicinity of *E. plantagineum* growing in Western Australia, New South Wales, and in Queensland. In all cases, the collection was made late in the season (November 2007), and the collectors reported that the pollen loads scraped from the bees were smaller and perhaps drier than usual. With estimated concentrations of 28 ± 15 μg equivalents of lasiocarpine-*N*-oxide/g pollen loads from Queensland and 6 ± 1 μg equivalents of lasiocarpine-*N*-oxide/g pollen loads from New South Wales and Western Australia, the levels of pyrrolizidine alkaloids found were unexpectedly low, albeit still in the ppm range rather than the very low ppb range required by some countries (*9, 10*). However, as expected from analysis of other tissues from *E. plantagineum* (*19*), echimidine-*N*-oxide (**3**-NO) (MH^+ *m/z* 414) was the predominant alkaloid detected with a minor contribution from echiumine-*N*-oxide (**22**-NO) (MH^+ *m/z* 398) and echiuplatine-*N*-oxide (**23**-NO) (MH^+ *m/z* 398) (**Figure 2**).

Effect of Heating Pollen. Initial studies of heating samples of pollen loads collected from *S. ovatus* resulted in a temperature and time-dependent decrease in the levels of the *N*-oxides and their parent pyrrolizidine alkaloids relative to the unheated samples. While temperatures below 40 °C did result in minor differences in alkaloid content, a temperature of between 40 and 60 °C, maintained in this study for 84 h, was required for

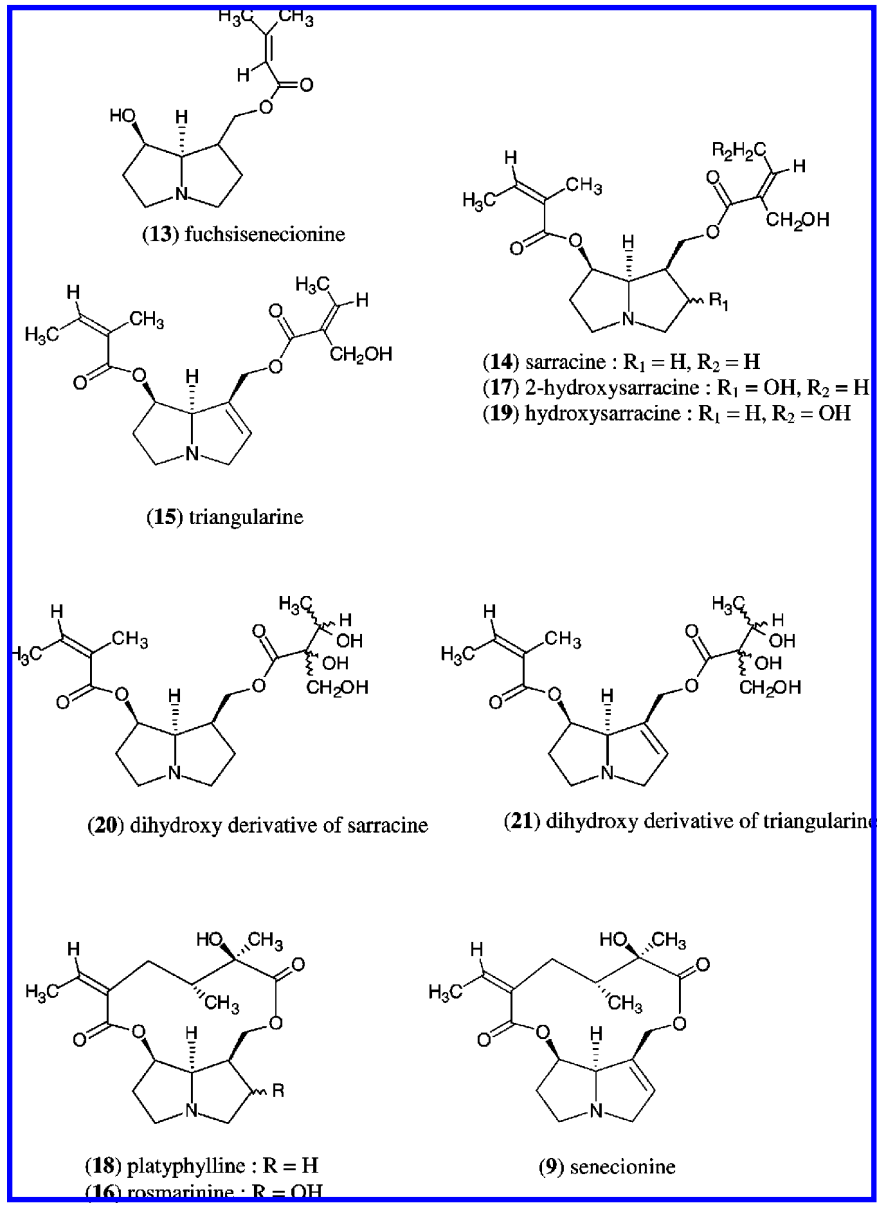

Figure 8. Chemical structures for the pyrrolizidine alkaloids from *Senecio ovatus* pollen.

more significant degradation of the *N*-oxides in particular, resulting in a relative dominance of the parent pyrrolizidine alkaloid free bases over their *N*-oxides (**Figure 10** and **Table 2**). The weight loss as a result of heating did not correlate with the effect on the level of pyrrolizidine alkaloids or their *N*-oxides (**Table 2**).

Apart from triangularine (**15**) and its *N*-oxide, the major alkaloids in *Senecio ovatus* are fully saturated, i.e., without the 1,2 double bond required for hepatotoxicity. Therefore, for comparison, duplicate samples of some macrocyclic diester and open chain diester 1,2-dehydropyrrolizidine alkaloids (and their *N*-oxides) were either untreated or heated in a similar way to ascertain their relative stability to heating.

Senecionine (**9**) and its stereoisomer integerrimine (**12**), and their *N*-oxides are macrocyclic diester hepatotoxic pyrrolizidine alkaloids usually, but not exclusively, produced by *Senecio* spp. (*32*). There was no apparent effect on the senecionine or integerrimine free bases when heated at about 56 °C for 72 h. Similar treatment of the *N*-oxides indicated a significant reduction (up to about 50%) in the *N*-oxide level and a relative increase in the respective free base forms. This effect was confirmed when 5 replicates each of senecionine and senecionine-*N*-oxide were either left unheated or heated at 56 °C for 96 h. Again, there was no effect on senecionine (at 10 or 100 μg) as a result of heating the dried samples; however, there was a clear reduction of up to about 50% in the level of senecionine-*N*-oxide (at both 10 and 100 μg). On heating the *N*-oxide, there was a production of the parent free base senecionine, not equivalent to the loss of senecionine-*N*-oxide but still resulting in levels more than double the level of contaminating senecionine in the unheated samples of senecionine-*N*-oxide.

To examine the effect of heating on some open chain diester 1,2 unsaturated hepatotoxic pyrrolizidine alkaloids, triplicate aliquots of a methanolic extract of *Echium plantagineum* (*19*) were evaporated to dryness and either heated or left untreated. Heating the dried residues had little effect on the relative levels of the *N*-oxides or the parent pyrrolizidine alkaloids as indicated by the effects on the major alkaloids present, i.e., echimidine-*N*-oxide (**3**-NO) and echiumine-*N*-oxide (**22**-NO) (**Figure 2**).

At this stage of the study, it seemed that the apparently greater degree of degradation/reduction of the pyrrolizidine alkaloids from *S. ovatus* pollen was either due to the major alkaloids being saturated or the pollen matrix facilitating reduction of the

Table 1. HPLC-ESI-MS/MS Data for Some Pyrrolizidine-*N*-oxides Extracted From *Senecio ovatus* Pollen and Free Bases Derived by Reduction of Their Respective *N*-Oxides

peak number[a] (suggested structure[b])	molecular ion adduct (*m*/*z*) natural product	molecular ion adduct (*m*/*z*) reduction product	MS-MS[c] (*m*/*z*, % abundance)
2 (**20**)	388		388(2), 370(6), 340(8), 326(80), 306(2), 288(20), 256(40), 254(75), 238(28), 236(20), 220(24), 140(100), 138(5), 122(20), 120(10)
		372	372(60), 328(5), 310(100), 290(2), 272(55), 240(62), 222(42), 140(9), 122(20)
4 (**19**)	370		370(20), 352(30), 288(10), 270(20), 254(75), 238(90), 222(10), 220(20), 156(15), 155(20), 140(100), 138(35), 122(65), 120(20), 108(3)
		354	354(<0.5), 336(13), 272(60), 256(22), 254(15), 238(20), 222(1), 220(4), 174(5), 156(36), 138(100), 122(1), 120(11), 108(2)
5 (**17**)	370		370(21), 352(3), 288(18), 270(15), 254(100), 238(4), 236(20), 172(4), 156(29), 155(33), 154(8), 138(51), 137(10), 136(10), 122(10), 120(6), 118(2), 108(1)
		354	336(15), 272(100), 256(25), 254(24), 238(15), 236(3), 222(1), 220(4), 174(5), 156(40), 138(94), 120(14), 118(1), 108(2)
6 (**15**)	352		352(50), 334(90), 270(25), 254(95), 252(20), 237(100), 236(25), 220(15), 218(45), 190(5), 174(45), 154(45), 136(55), 120(30), 108(12), 106(12)
		336	336(<0.5), 332(<1), 254(28), 238(5), 236(1), 220(14), 174(1), 138(10), 120(100)
7 (**14**)	354		354(75), 336(35), 272(25), 254(40), 238(75), 222(10), 220(45), 156(3), 140(100), 139(100), 138(35), 122(90), 120(33), 108(10)
		338	338(100), 256(15), 238(40), 222(60), 140(5), 122(30)

[a] Peak numbers refer to **Figure 3D**. [b] **Figure 8**. [c] Secondary fragmentation energy of 35%.

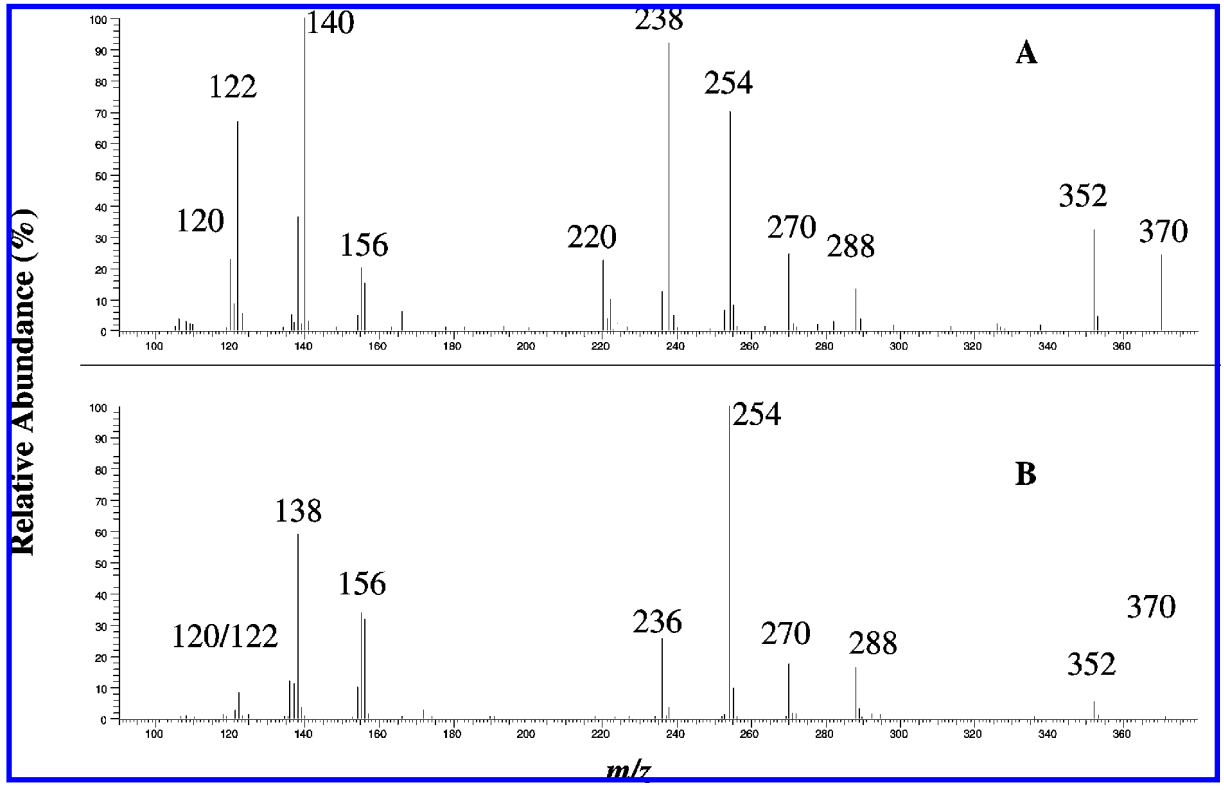

Figure 9. HPLC-ESI-MS/MS on *m*/*z* 370 for (**A**) peak 4 (**Figure 3D**) and (**B**) peak 5 (**Figure 3D**) showing a differential fragmentation indicative of significant structural differences. Mass spectrum **A** is consistent with platynecine esterified with an angelic acid isomer and a hydroxylated sarracinic acid, whereas mass spectrum **B** is consistent with a hydroxylated platynecine esterified with an angelic acid isomer and sarracinic acid.

N-oxide and/or degradation. Therefore, the effect of heat on pollen loads collected from bees that had foraged on *Echium plantagineum* and therefore containing hepatotoxic 1,2 unsaturated pyrrolizidine alkaloids was also investigated (**Table 3**). The effect of heating on quite large samples of pollen loads (ca. 1.5 g), collected from New South Wales and from Western Australia, resulted in about a 12% weight loss and caused a degradation of the *N*-oxide content of up to 30% with a concomitant increase (150−190%) in the level of the parent pyrrolizidine alkaloid free bases relative to the unheated samples. In general, analysis of the heated *E. plantagineum* pollen loads indicated that the total pyrrolizidine alkaloid content might decrease slightly, although more samples would be needed to confirm this tentative observation. Nevertheless, it is clear that there is no drastic change in hepatotoxic pyrrolizidine alkaloid content upon drying/heating the pollen loads for up to 100 h at 56−60 °C.

While a significant decrease in pyrrolizidine alkaloid *N*-oxide levels is observed on heating pollen, it does seem from the data that at least one pathway of heat-induced *N*-oxide degradation is reduction to the parent free base, thereby maintaining total levels of 1,2-dehydropyrrolizidine alkaloids and, presumably,

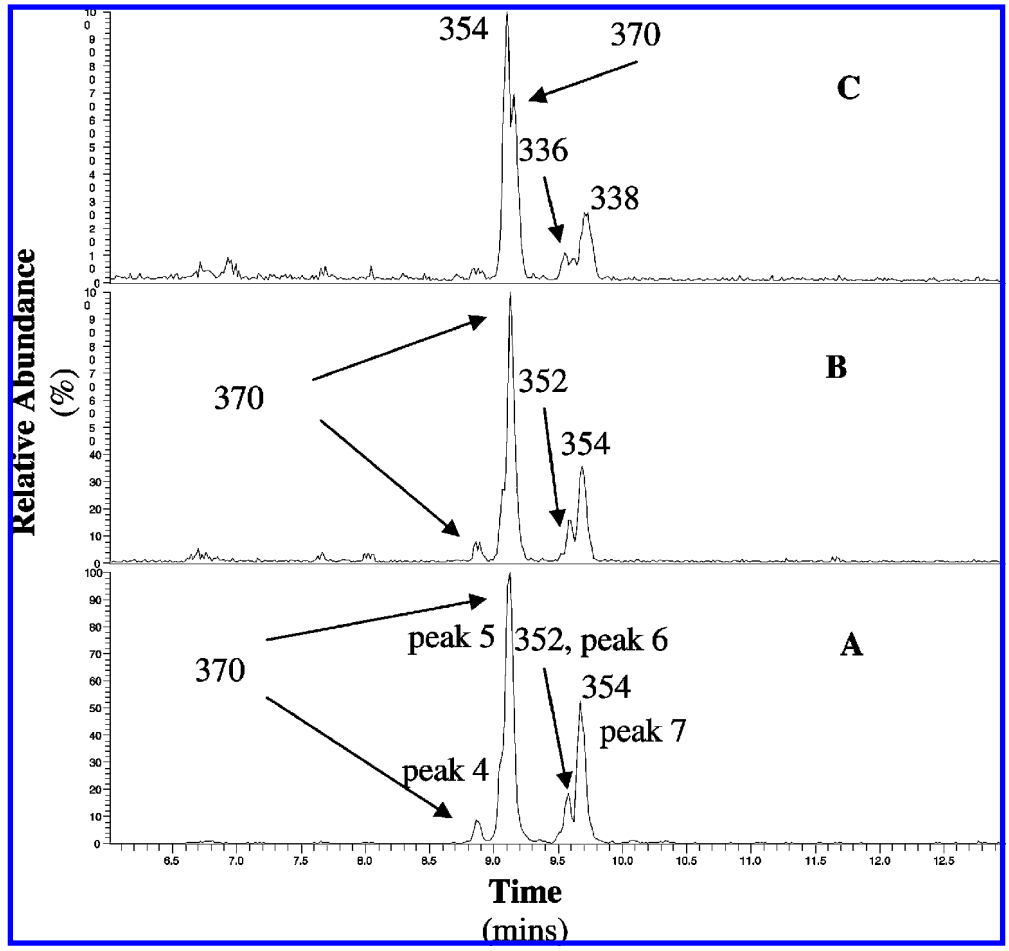

Figure 10. Effect of heating on the pyrrolizidine alkaloid content of *Senecio ovatus* pollen loads. Peaks are numbered in accord with **Figure 3D** and are labeled with the molecular ion adduct (MH^+, *m*/*z*), and relative abundances are shown in **Table 2**. (**A**) Unheated pollen showing peak labels consistent with **Figure 3D**. (**B**) Pollen heated at 35 °C for 72 h. (**C**) Pollen heated at 60 °C for 84 h.

Table 2. Effect of Heating on the Weight, the Total Pyrrolizidine Alkaloid Level, and the Amounts of the *N*-Oxides Relative to Their Respective Parent Free Bases for *Senecio ovatus* Pollen Loads

treatment	weight loss on drying (%)	total pyrrolizidine alkaloid content (μg/g fresh weight pollen loads)	change relative to unheated pollen loads (%)	ratio of *N*-oxide (*m*/*z* 370) to parent free base (*m*/*z* 354)
none	na*	80	na	3.5−4.4
35 °C for 72 h	20	55	30	3.4
40 °C for 84 h	15	28	65	3.2−3.7
60 °C for 84 h	14	20	75	0.7−0.4

Table 3. Quantitative HPLC-ESI-MS Analysis of Pollen Loads (Unheated and Heated) Collected From Bees Foraging on *Echium plantagineum* in Queensland (Qld.), New South Wales (NSW), and Western Australia (WA)[a]

	concentration (μg of lasiocarpine-*N*-oxide equivalents/g pollen loads)				
pollen source	echimidine-*N*-oxide	echimidine	echiumine-*N*-oxide	echiumine	total free base and *N*-oxide
Qld	10.9	1.03	0.8	nd[b]	12.8
NSW	3.8	0.96	0.1	nd	4.8
NSW (heated)	3.3	2.8	nd	nd	6.0
WA	6.3	0.93	0.22	0.08	7.5
WA (heated)	4.3	2.6	nd	0.25	7.1

[a] Ion responses were normalized against a heliotrine (**1**) internal standard. [b] None detected.

the same level of hazard to consumers. Furthermore, the results from this study support the suggestion that the presence of pyrrolizidine alkaloids and their *N*-oxides in certain types of honey are due to contamination with pollen (*3*). Moreover, commercial bee pollen (also called pollen granules, pollen nuggets, or pollen pellets) might be unsafe for human consumption if it originates from plants producing pyrrolizidine alkaloids. This is of particular concern where there is potential for selective use of bee pollen derived from pyrrolizidine alkaloid-producing plants or by specific subsections of the population, e.g., children.

Considered along with the international concern about dietary and topically applied 1,2-dehydropyrrolizidine alkaloids, the data suggest a program for monitoring hepatotoxic pyrrolizidine alkaloid levels in commercially available bee pollen for human consumption.

Importantly, because the nature of the degradation products from the pyrrolizidine alkaloids and their *N*-oxides in pollen are undefined, it is not known whether the slight degradation observed in this study and perhaps the more extensive degradation that might be achieved in developments of this current research is concomitant with a reduction in the potential toxicity to consumers.

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LITERATURE CITED

(1) Kroyer, G.; Hegedus, N. Evaluation of bioactive properties of pollen extracts as functional dietary food supplement. *Innovative Food Sci. Emerging Technol.* **2001**, *2*, 171–174.

(2) Food and Agricultural Organization of the United Nations. Value-Added Products from Beekeeping. *FAO Agric. Serv. Bull.* No. 124, **2001**.

(3) Boppré, M.; Colegate, S. M.; Edgar, J. A. Pyrrolizidine alkaloids of *Echium vulgare* honey found in pure pollen. *J. Agric. Food Chem.* **2005**, *53*, 594–600.

(4) 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.

(5) Mattocks, A. R. *Chemistry and Toxicology of Pyrrolizidine Alkaloids*. Academic Press: London, 1986.

(6) Mattocks, A. R. Acute hepatotoxicity and pyrrolic metabolites in rats dosed with pyrrolizidine alkaloids. *Chem.-Biol. Interact.* **1972**, *5*, 227–242.

(7) 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.

(8) Wang, Y.-P.; Yan, J.; Fu, P. P.; Chou, M. W. Human liver microsomal reduction of pyrrolizidine alkaloid N-oxides to form the corresponding carcinogenic parent alkaloid. *Toxicol. Lett.* **2005**, *155*, 411–420.

(9) European Food Safety Authority. Opinion of the scientific panel on contaminants in the food chain on a request from the European Commission related to pyrrolizidine alkaloids as undesirable substances in animal feed. *EFSA J.* **2007**, *447*, 1−51.

(10) Edgar, J. A.; Roeder, E.; Molyneux, R. J. Honey from plants containing pyrrolizidine alkaloids: A potential threat to health. *J. Agric. Food Chem.* **2001**, *50*, 2719–2730.

(11) Larrey, D. Hepatotoxicity of herbal remedies. *J. Hepatol.* **1997**, *26*, 47–51.

(12) Prakash, A. S.; Pereira, T. N.; Reilly, P. E. B.; Seawright, A. A. Pyrrolizidine alkaloids in human diet. *Mutat. Res.* **1999**, *443*, 53–67.

(13) 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.

(14) Rasenack, R.; Müller, C.; Kleinschmidt, M.; Rasenack, J.; Wiedenfeld, H. Veno-occlusive disease in a fetus caused by pyrrolizidine alkaloids of food origin. *Fetal Diagn. Ther.* **2003**, *18*, 223–225.

(15) Zuckerman, M.; Steenkamp, V.; Stewart, M. J. Hepatic veno-occlusive disease as a result of a traditional remedy: confirmation of toxic pyrrolizidine alkaloids as the cause, using an *in vitro* technique. *J. Clin. Pathol.* **2002**, *55*, 676–679.

(16) Sergi, C.; Beedgen, B.; Linderkamp, O.; Hofmann, W. J. Fatal course of veno-occlusive disease of the liver (endophlebitis hepatica obliterans) in a preterm infant. *Pathol. Res. Pract.* **1999**, *195*, 847–851.

(17) Seibold-Weiger, K.; Vochem, M.; Mackensen-Haen, S.; Speer, C. Fatal hepatic veno-occlusive disease in a newborn infant. *Am. J. Perinatol.* **1997**, *15*, 107–111.

(18) Herborg, J. *Senecio ovatus* (P. Gaertn., Mey. & Scherb.) Willd., der korrekte name für *Senecio fuchsii* C.C. Gmelin. *Wildenowia* **1985**, *15*, 183–186.

(19) 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.* **2005**, *16*, 108–119.

(20) Betteridge, K.; Cao, Y.; Colegate, S. M. An improved method for extraction and LCMS analysis of pyrrolizidine alkaloids and their *N*-oxides in honey: application to *Echium vulgare* honeys. *J. Agric. Food Chem.* **2005**, *53*, 1894–1902.

(21) Colegate, S. M.; Gardner, D. R. LC-MS of Alkaloids: Qualitative Profiling, Quantitative Analysis, And Structural Identification. In *Modern Alkaloids*; Fattorusso, E., Taglialatela-Scafati, O., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2008; pp 369−408.

(22) Hendriks, H.; Balraadjsing, W.; Huizing, H. J.; Bruins, A. P. Investigation into the presence of pyrrolizidine alkaloids in *Eupatorium cannabinum* by means of positive and negative ion chemical ionization GC-MS. *Planta Med.* **1987**, *53*, 456–461.

(23) Edgar, J. A.; Lin, H. J.; Kumana, C. R.; Ng, M. M. T. Pyrrolizidine alkaloid composition of three Chinese medicinal herbs *Eupatorium cannabinum*, *E. japonicum* and *Crotalaria assamica*. *Am. J. Chinese Med.* **1992**, *20*, 281–288.

(24) Budde, J.; Reckert, A.; Sporer, F.; Wink, M.; Eltz, T.; Lunau, K. Contributions to the evolution of oligolecty in solitary bees of the genus *Andrena*. *Entomologie heute* **2004**, *16*, 191–200.

(25) Macel, M.; Vrieling, K.; Klinkhamer, P. G. L. Variation in pyrrolizidine alkaloid patterns of *Senecio jacobaea*. *Phytochemistry* **2004**, *65*, 865–873.

(26) Witte, L.; Ernst, L.; Adam, H.; Hartmann, T. Chemotypes of two pyrrolizidine alkaloid-containing Senecio species. *Phytochemistry* **1992**, *31*, 559–565.

(27) Roeder, E. Medicinal plants in Europe containing pyrrolizidine alkaloids. *Pharmazie* **1995**, *50*, 83–98.

(28) Roeder, E.; Wiedenfeld, H. Isolierung und strukturaufklarung des alkaloids fuchsisenecionin aus *Senecio fuchsii*. *Phytochemistry* **1977**, *16*, 1462–1464.

(29) Roitman, J. N. The pyrrolizidine alkaloids of *Senecio triangularis*. *Aust. J. Chem.* **1983**, *36*, 1203–1213.

(30) Roeder, E.; Wiedenfeld, H.; Britz-Kirstgen, R. Pyrrolizidine alkaloids from *Senecio cacaliaster*. *Phytochemistry* **1984**, *23*, 1761–1763.

(31) Roeder, E.; Wiedenfeld, H.; Schraut, R. Pyrrolizidine alkaloids from *Alkanna tinctoria*. *Phytochemistry* **1984**, *23*, 2125–2126.

(32) Bull, L. B.; Culvenor, C. C. J.; Dick, A. T. In *The Pyrrolizidine Alkaloids*; North Holland Publishing Company: Amsterdam, 1968.

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