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Detection of High Levels of Pyrrolizidine-*N*-oxides in the Endangered Plant *Cryptantha crassipes* (Terlingua Creek Cat's-eye) Using HPLC-ESI-MS

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ABSTRACT:

Introduction – A previous investigation of pyrrolizidine alkaloids produced by nine species of *Cryptantha* identified at least two chemotypes within the genus. Other research has postulated that pyrrolizidine-*N*-oxide concentrations increase as the growing conditions become harsher, particularly with respect to water availability. *Cryptantha crassipes* is an endangered plant with a very limited distribution range within a dry, harsh Texan ecosystem.

Objective – To determine the pyrrolizidine alkaloid (and their *N*-oxides) profile and concentrations in *Cryptantha crassipes*.

Methodology – Methanolic extracts of *Cryptantha crassipes* were partitioned into dilute sulphuric acid and the alkaloids concentrated using strong cation exchange, solid-phase extraction columns. Extracts were analysed using reversed-phase high-pressure liquid chromatography coupled to electrospray ionisation ion trap mass spectrometry.

Results – The *N*-oxides of lycopsamine and intermedine were the major pyrrolizidine alkaloids detected in *Cryptantha crassipes*. Smaller to trace amounts of other pyrrolizidine alkaloids observed were: the 7- and 3′-acetylated derivatives and the 1,2-dihydro analogs of lycopsamine-*N*-oxide and/or intermedine-*N*-oxide; a pair of unidentified *N*-oxides, isobaric with lycopsamine-*N*-oxide; and the *N*-oxides of leptanthine, echimiplatine, amabiline, echiumine and dihydroechiumine. Only trace amounts, if any, of the parent free base pyrrolizidine alkaloids were detected. The concentration of pyrrolizidine alkaloids was estimated to be 3–5% of the dry weight of milled leaves, or 10–50 times the levels previously reported for similar chemotypes.

Conclusions – The high levels of the *N*-oxides of lycopsamine and intermedine establish the genus chemotype of the endangered *Cryptantha crassipes* and support earlier data linking high levels of *N*-oxides to dry, harsh growing conditions.

Keywords: *Cryptantha crassipes*; pyrrolizidine; *N*-oxides; lycopsamine; HPLC-ESI-MS; SPE

Introduction

Cryptantha crassipes I. M. Johnston (Boraginaceae), commonly known as Terlingua Creek cat's-eye (Johnston, 1939), is an endemic strong perennial forb that is found in a very limited area within the Chihuahuan desert of Texas (Center for Plant Conservation, 2010). It grows across clay flats (*Geologic Atlas of Texas*, 1979) with low water potentials, limited precipitation and high temperatures, east of Terlingua Creek near Agua Fria Mountain (Correll and Johnston, 1979; Poole *et al.*, 2007). The environment has been termed a "moonscape" with plants widely spaced and very few other plant species adapted to grow in the presence of high levels of gypsum in the soil. All known populations of this species are located on privately owned land, subject to habitat destruction due to property development, mining, off-road vehicle use and grazing of cattle in the area (Texas Parks and Wildlife, 2009). *Cryptantha crassipes* was listed as an endangered species by the US Fish and Wildlife Service in 1991 (Poole *et al.*, 2007).

A previous investigation of six perennial (*C. cana*, *C. confertiflora*, *C. flava*, *C. thyrsiflora*, *C. virgata* and *C. virginensis*) and three annual (*C. fendleri*, *C. clevelandii* and *C. leiocarpa*) *Cryptantha* species, from the sections (or sub-genera) *Oreocarya* and *Krynitzkia* respectively, revealed a marked difference in the pyrrolizidine alkaloid levels as well as their qualitative profiles (Stermitz *et al.*, 1993). Although, in

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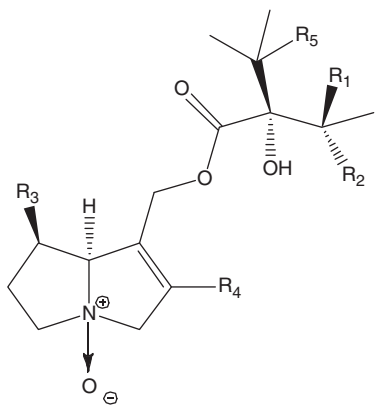
general, the pyrrolizidine alkaloid content of *Oreocarya* was different from that of the *Krynitzkia*-related species, there was no apparent consistent pattern of pyrrolizidine alkaloid production within each section. Most significantly, however, the *Oreocarya*-related species, with an estimated pyrrolizidine alkaloid content varying from about 0.1 to 0.3% dry weight of plant, were characterised by a major, and usually dominant, presence of lycopsamine (Fig. 1). The pyrrolizidine alkaloids were found to be present in the plants as the *N*-oxides since they were only revealed following zinc/acid reduction of the aqueous sulphuric acid soluble portion of the crude methanol extracts. A later investigation reported an association between *C. jamesii* and the six perennial species of the *Oreocarya* on the basis of the predominant presence of lycopsamine and a total alkaloid content ranging from 0.4 to 0.7% (Beck and Stermitz, 2002).

Waller and Nowacki (1978) have postulated that higher rates of alkaloid production by plants may be caused by stresses such as water deprivation, poor soils and pressure from herbivores. In a subsequent controlled environment study, water stress was shown to cause an increase in the concentrations of the pyrrolizidine alkaloids produced by *Senecio longilobus* (threadleaf groundsel) by up to 4.6 times (Briske and Camp, 1982). Results of a multi-season and multi-site study of several *Senecio* species also suggested that pyrrolizidine alkaloid levels increase in response to abiotic stress conditions such as drought and infertile soils (Molyneux and Johnson, 1984; Johnson *et al.*, 1985). The endangered *Cryptantha crassipes* is described as growing in "perhaps the most extreme environment in Texas" (Texas Parks and Wildlife, 2009). This consideration, combined with the well-known toxicity of 1,2-dehydropyrrolizidine alkaloids and *N*-oxides (Stegelmeier *et al.*, 1999), prompted this evaluation of the character and quantity of pyrrolizidine alkaloids in this species.



$R_1 = R_4 = R_5 = H$, $R_2 = OH$, $R_3 = OH$: lycopsamine-*N*-oxide

$R_1 = OH$, $R_2 = R_4 = R_5 = H$, $R_3 = OH$: intermedine-*N*-oxide

when $R_3 = OCOCH_3$: 7-acetyl derivatives

when R_1 or $R_2 = OCOCH_3$: 3'-acetyl derivatives

when R5 = OH : leptanthine/echimiplatine-*N*-oxides

when R_3 = angelyl i.e., $OCOC(CH_3)=CHCH_3$: echiumine-*N*-oxide and isomers

when $R_3 = H$: amabiline-*N*-oxide and isomers

when $R_4 = OH$: 2-hydroxy derivatives

Figure 1. Structures of some pyrrolizidine alkaloids in *Cryptantha crassipes*.

Experimental

Plant collections

With written permission from landowners, three collections of plant material for chemical analysis were made in July, September and October 2010, at times when *C. crassipes* was not in flower. Voucher specimens are housed at the Sul Ross State University herbarium: M. Williams 62, 23 Mar 2010; M. Williams 67, 28 Mar 2010; M. Williams 93, 30 July 2010; and M. Williams 94, 30 July 2010. Some of the voucher specimens documenting material collected for chemical analysis were collected from the same population earlier in the year when plants were in flower. The first collection (M. Williams 93) was for a screening study to detect the presence of pyrrolizidine alkaloids in fresh plant material. Three samples of leaves and one sample of roots were cut into small pieces (2–10 mm) and immediately immersed in methanol in separate vials. The second collection was of an entire plant specimen of *C. crassipes* (M. Williams 67) that was air-dried and divided into root, caudex and leaf material for qualitative and quantitative analysis. Extraction commenced about 5 days after collection. The third collection was for a confirmatory quantitative study of dried plant and involved clipping leaf clumps from mature *C. crassipes* in different locations across the very limited range in Brewster County, Texas, i.e. sample 106 (M. Williams 62), sample 107 (M. Williams 67) and sample 108 (M. Williams 94). The semi-dried plant material from these latter samples was milled to a fine powder and extracted with methanol about 4 days after collection.

Plant extractions

All plant samples (fresh or dry) were exhaustively extracted using methanol at room temperature (see representative example below). The methanolic extracts were then either analysed directly or were concentrated to dryness *in vacuo* and re-extracted with 0.05 M sulphuric acid. The resultant acid soluble portions were then either: (1) analysed directly; (2) further purified using strong cation exchange, solid-phase extraction (SCX SPE) columns before analysis; or (3) reduced to the free base pyrrolizidine alkaloids prior to analysis.

Representative plant extraction procedure. The leaves were removed from the stems of sample 106 and milled to a fine powder. An aliquot (572 mg) of the powder was shaken overnight with methanol (ca. 15 mL) at room temperature (20–22 °C) to yield, after filtration, a bright green methanol solution. The plant residue was re-extracted two more times with methanol (1 × 15 mL for 6 h and 1 × 15 mL for 16 h) such that the third methanol extract was colourless and HPLC-ESI-MS analysis showed only a trace of alkaloid in the extract. The three extracts were combined and diluted accurately to 100 mL with methanol.

Strong cation exchange, solid-phase extraction

The 0.05 M sulphuric acid soluble fraction of the dried combined methanolic extracts was applied to one or more appropriately sized and conditioned SCX SPE columns (Strata SCX 55 µm 70 Å, Phenomenex, Torrance, CA, USA) and the pyrrolizidine alkaloids and their *N*-oxides eluted as previously described (Colegate *et al.*, 2005; Boppré *et al.*, 2005, 2008).

Representative solid-phase extraction procedure. The methanol extracts from the root, crown and leaf material from the second plant collection were combined on the basis of their similar pyrrolizidine alkaloid profiles, and evaporated to dryness *in vacuo*. The dark green, gummy residue was extracted with 0.05 M sulphuric acid and filtered to yield an orange solution (75 mL) that was evenly loaded onto three conditioned (washed with 15 mL each of methanol and then 0.05 M sulphuric acid) SCX SPE columns (5 g/20 mL). The loaded columns were subsequently flushed with 0.05 M sulphuric acid (15 mL) and methanol

(15 mL) prior to elution of the alkaloids with 10% saturated ammoniated methanol. The latter was prepared by passing a slow flow of ammonia gas through methanol, cooled in an ice bath, for about 15 min. This saturated ammoniated methanol was then diluted 1:10 with methanol to yield the elution solvent. The entire colour was retained by the columns under aqueous acid conditions. An orange colour eluted with the methanol wash. A less intensely coloured fraction eluted with the 10% saturated ammoniated methanol, this colour front coinciding with detection of high pH using indicator paper. The first basic fractions from each column were combined and evaporated to dryness under a flow of nitrogen at about 35 °C, and the orange, gummy, alkaloid-rich residue (760 mg) was reconstituted in methanol.

Reduction of pyrrolizidine-*N*-oxides

The reduction of the *N*-oxides was achieved at one of three different stages using one of two methods. The stages were: (1) the crude methanol extract; (2) the 0.05 M sulphuric acid soluble fraction of the dried methanol extract; and (3) the dried and reconstituted ammoniated methanol fraction from the SCX SPE columns. The methods used were: (1) redox resin prepared by adding anion exchange resin (Amberlite IRA-410 CL, Sigma-Aldrich, St Louis, MO, USA) to a solution of indigocarmine (Fluka, Sigma-Aldrich, St Louis, MO, USA) in water (Colegate *et al.*, 2005) at ratios such that the aqueous phase remained blue. After filtration and washing with water the dark blue resin was activated (turns to golden orange) by treatment with 5% sodium dithionite solution in water; or (2) zinc/sulphuric acid.

Representative indigocarmine resin-based reduction procedure. Duplicate aliquots (5 mL) of the methanolic extracts of samples 106, 107 and 108, each made up to 100 mL as described in the representative plant extraction procedure, were shaken with dried, activated redox resin (ca. 1 g) for 3.5 h at room temperature (20–22 °C). The initial green colour of the solution changed to pale yellow and the resin darkened from golden orange to blue/black. The filtered reaction solutions were then analysed using HPLC-ESI-MS.

Representative zinc/sulphuric acid-based reduction procedure. The methanolic extracts of samples 106, 107 and 108 were combined (i.e. about 90 mL of methanolic solution from each sample left after the redox resin treatment) and evaporated to dryness *in vacuo*. The green residue was extracted with 0.05 M sulphuric acid (50 mL) and the filtered aqueous acid solution was made up to ca. 2 M sulphuric acid by addition of concentrated sulphuric acid (3 mL) and stirred with zinc dust at room temperature for 2 h. The pale red acid solution was filtered from the zinc and extracted with chloroform (50 mL). The acid solution was then made basic with ammonium hydroxide (until the initially precipitated zinc hydroxide redissolved and afforded an orange aqueous solution) and again extracted with chloroform (4 × 50 mL) to yield an almost colourless chloroform solution of the free base pyrrolizidine alkaloids.

HPLC-ESI-MS

Analytical samples were prepared by adding a small aliquot (e.g. 10 µL) of a larger sample into methanol (180 µL) containing a suitable internal standard (10 µL) to normalise detector responses. In this study the pyrrolizidine alkaloids lasiocarpine and/or heliotrine-*N*-oxide (both acquired from stocks held by the ARS Poisonous Plant Research Laboratory) were used as the internal standards. Separation was achieved using a Hewlett-Packard/Agilent Series 1100 HPLC system equipped with a Synergi Hydro Reversed Phase column (150 × 2 mm, 4 µm; Phenomenex, Torrance, CA, USA) protected by a guard cartridge (Security Guard Cartridge Kit fitted with an AQ C_{18} cartridge 4 × 2 mm; Phenomenex,

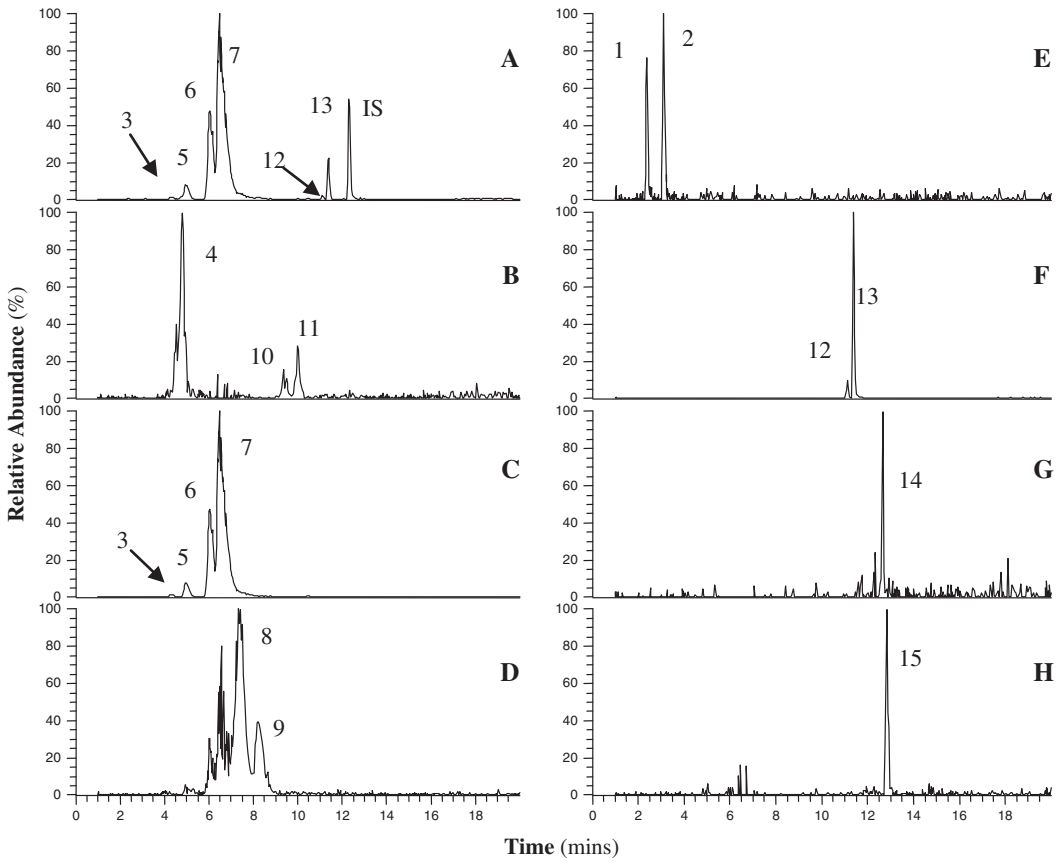


Figure 2. HPLC-ESI-MS base ion (*m/z* 200–800) chromatogram (A) and reconstructed ion chromatograms displaying *m/z* 300 (B), *m/z* 316 (C), *m/z* 318 (D), *m/z* 332 (E), *m/z* 358 (F), *m/z* 398 (G) and *m/z* 400 (H) for the SCX SPE-concentrated alkaloids isolated from *Cryptantha crassipes*.

Table 1. HPLC-ESI-MS/MS data for the pyrrolizidine-*N*-oxides detected in *Cryptantha crassipes*. Also shown are the data for the free base pyrrolizidine alkaloids derived from their respective *N*-oxides by reductive treatment with the indigocarmine-based redox resin. Molecular ion dimer adducts are only observed for the *N*-oxides

Peak number[a] and proposed identity	Molecular ion adduct (MH^+/$[2M + H]^+$) (*m/z*)		MS/MS[b] *m/z* (percentage abundance)
	N-oxide	Free base	
1 and **2** Echimiplatine and leptanthine	332/663		332 (7), 314 (100), 288 (3), 274 (14), 270 (50), 256 (21), 228 (8), 212 (2), 172 (26), 138 (3)
		316	316 (9), 298 (26), 254 (22), 240 (6), 236 (6), 212 (8), 196 (2), 156 (3), 138 (100), 120 (9), 116 (8), 94 (21)
3 and **5** Unknown	316/631		316 (12), 272 (5), 238 (2), 226 (2), 172 (100), 154 (2)
		300	300 (16), 256 (7), 238 (2), 156 (100), 138 (3)
6 and **7** Intermedine and lycopsamine	316/631		316 (83), 298 (5), 272 (21), 254 (2), 226 (16), 210 (3), 172 (100), 155 (7), 154 (6), 138 (16), 137 (4), 136 (8), 120 (1)
		300	300 (8), 256 (3), 210 (1), 156 (8), 138 (100), 120 (14), 94 (35)
8 and **9** 1,2-Dihydro intermedine and lycopsamine	318/635		318 (18), 300 (6), 274 (5), 174 (100), 157 (1), 156 (2)
		302	302 (100), 284 (11), 258 (12), 240 (1), 158 (42), 140 (22), 138 (1), 122 (12)
10 and **11** Amabiline-like	300/599		300 (100), 282 (4), 256 (28), 238 (3), 210 (15), 194 (2), 156 (98), 140 (1), 139 (12), 138 (4), 122 (2), 121 (3), 120 (10)
		284	284 (21), 240 (5), 194 (2), 140 (6), 122 (100), 120 (1), 94 (4)
12 7-Acetyl Intermedine and/or lycopsamine	358/715		358 (100), 340 (13), 316 (4), 314 (4), 298 (99), 280 (1), 268 (1), 214 (20), 180 (1), 172 (11), 155 (3), 154 (2), 138 (3), 137 (2), 136 (5)
		342	342 (2), 298 (1), 198 (2), 180 (81), 162 (4), 138 (4), 136 (1), 120 (100)
13 3′-Acetyl intermedine and/or lycopsamine	358/715		358 (76), 340 (16), 316 (6), 298 (100), 280 (1), 172 (20), 155 (2), 154 (3), 138 (6), 137 (1), 136 (4)
		342	342 (14), 300 (1), 282 (18), 198 (2), 187 (3), 180 (7), 156 (2), 138 (100), 136 (4), 120 (15)
14 Echiumine	398/n.o.[c]		398 (100), 380 (1), 354 (7), 338 (1), 283 (1), 254 (20), 236 (2), 220 (2), 120 (1), 118 (4)
15 1,2-Dihydro echiumine	400/799		400 (100), 382 (3), 356 (11), 310 (2), 294 (2), 284 (1), 256 (36), 222 (4), 220 (2)
		384	384 (2), 300 (3), 240 (2), 222 (45), 204 (7), 120 (100), 118 (2)

[a] Peak numbers refer to Fig. 2.
[b] Secondary fragmentation energy of 30%.
[c] not observed.

Torrance, CA, USA). Sample injections (2 µL) were eluted with a gradient flow (200 µL/min) in which the initial mobile phase composition of 10% acetonitrile in aqueous 0.1% formic acid was held for 2 min and then linearly increased to 70% acetonitrile over 8 min. This was held for a further 10 min before returning to the initial mobile phase composition and re-equilibration of the column. The column effluent was monitored using an ion trap mass spectrometer (LCQ Classic; Thermo-Finnigan, San Jose, CA, USA) in the electrospray (ESI), positive ion mode. The mass spectrometer response was tuned to a solution of heliotrine (acquired from stocks held by the ARS Poisonous Plant Research Laboratory) in methanol. The capillary temperature was 250 °C with a capillary voltage of 22 V. The source voltage was 4.5 kV at a source current of 80 µA. The sheath and auxiliary gas flows were set at a ratio of 70:20. Data-dependent MS/MS spectra were acquired in the second scan of a two-scan sequence in which ions identified in the first total ion scan were isolated and fragmented using either 30 or 35% applied dissociation energy.

Identification of alkaloids

The identification of the detected alkaloids was based on the mass spectrometry data and an assumption of a similarity to previously published data on other *Cryptantha* species. Lycopsamine was chromatographically compared to an authentic standard (>95% pure; Planta Analytica, Danbury, CT, USA; http://www.plantaanalytica.com) and all other identifications remain tentative until similar studies result in sufficient isolation to allow for unambiguous NMR spectroscopy analysis.

Quantitative analysis

After reduction of *N*-oxides, the peak areas in reconstructed ion chromatograms of free base pyrrolizidine alkaloids were quantitated against a calibration curve generated using authenticated lycopsamine and expressed in terms of dry weight of plant. Additionally, alkaloid content was estimated in the more traditional, gravimetric way by weighing the residue obtained after zinc/acid reduction of *N*-oxides and acid/base partitioning of the total alkaloidal fraction of a plant extract (as per Stermitz *et al.*, 1993).

Determination of moisture content. Accurately weighed aliquots (ca. 160 mg) of the milled leaf material from plant collection samples 106, 107 and 108 were placed in a warm (40–50 °C) oven and re-weighed after 24 and 48 h. Moisture content was estimated based upon the weight loss.

Generation of calibration curve. From a stock solution of lycopsamine in methanol (13.7 mg/100 mL) was prepared a series of dilutions that yielded a five-point calibration curve for 25.09, 11.16, 4.96, 2.2 and 0.98 µg/mL. Standard dilutions were analysed in duplicate and the lycopsamine peak area in each injection sample was normalised by division by the area of the internal standard. Excel-based regression analysis trend line fitting resulted in a best fit ($R^2 = 0.9999$) quadratic polynomial curve ($y = 12.92x^2 + 18.34x - 0.3856$, where y is lycopsamine concentration in µg/mL and x is the peak area divided by the area of the internal standard).

Results and Discussion

The first analyses were of small samples of leaves and roots from fresh plants collected and immediately immersed in methanol. The aim was an initial screen of the plants for the presence of pyrrolizidine alkaloids without any potential complicating degradation that might be initiated once the plant was picked or dried. The second collection was of a larger sample that was dried with the intent of quantitating alkaloids present and determining any significant degradation that might occur following collection and drying. The third collection of leaf samples from different locations within the very restricted distribution range of *C. crassipes* was required to confirm the relatively high pyrrolizidine-*N*-oxide content determined in the analysis of the second collection.

The HPLC-ESI-MS analyses were conducted either on crude extracts or on the SCX SPE semi-purified alkaloidal fractions. Where SCX SPE was used, HPLC-ESI-MS analysis showed that the alkaloids were effectively captured and were eluted very quickly with 10% saturated ammoniated methanol. Provided that columns were not overloaded, no SCX SPE fractions other than the first 10% ammoniated methanol fraction showed any pyrrolizidine alkaloids.

Across all the analyses from the three plant collections, the qualitative profiles of the alkaloids were very similar. The consistently major alkaloid (peak 7, Fig. 2A and C) was identified as lycopsamine-*N*-oxide (Fig. 1) based upon its protonated molecular ion adduct (MH^+ *m/z* 316), MS/MS data (Table 1, Fig. 3A) and comparison of the reduced sample with authenticated lycopsamine. As expected from previous reports of the ESI-MS of pyrrolizidine-*N*-oxide (Colegate *et al.*, 2005; Boppré *et al.*, 2005, 2008), there was a significant contribution of the protonated molecular ion dimer ($[2M + H]^+$) at *m/z* 631. The detection of lycopsamine-*N*-oxide as a major constituent clearly associates *C. crassipes* with the six perennial *Cryptantha* species belonging to the *Oreocarya* section analysed by Stermitz *et al.* (1993). The next major alkaloid (peak 6, Fig. 2A and C) is deduced to be the diastereoisomer intermedine-*N*-oxide (Fig. 1) based upon its ESI-MS (MH^+ *m/z* 316; $[2M + H]^+$ *m/z* 631), the exact similarity of the MS/MS data to those of lycopsamine-*N*-oxide and the expectation of its presence (Stermitz *et al.*, 1993). Assuming equal or very similar responses to the ESI-MS detector, the intermedine-*N*-oxide was usually present at about 30% of the lycopsamine-*N*-oxide. Lycopsamine and intermedine

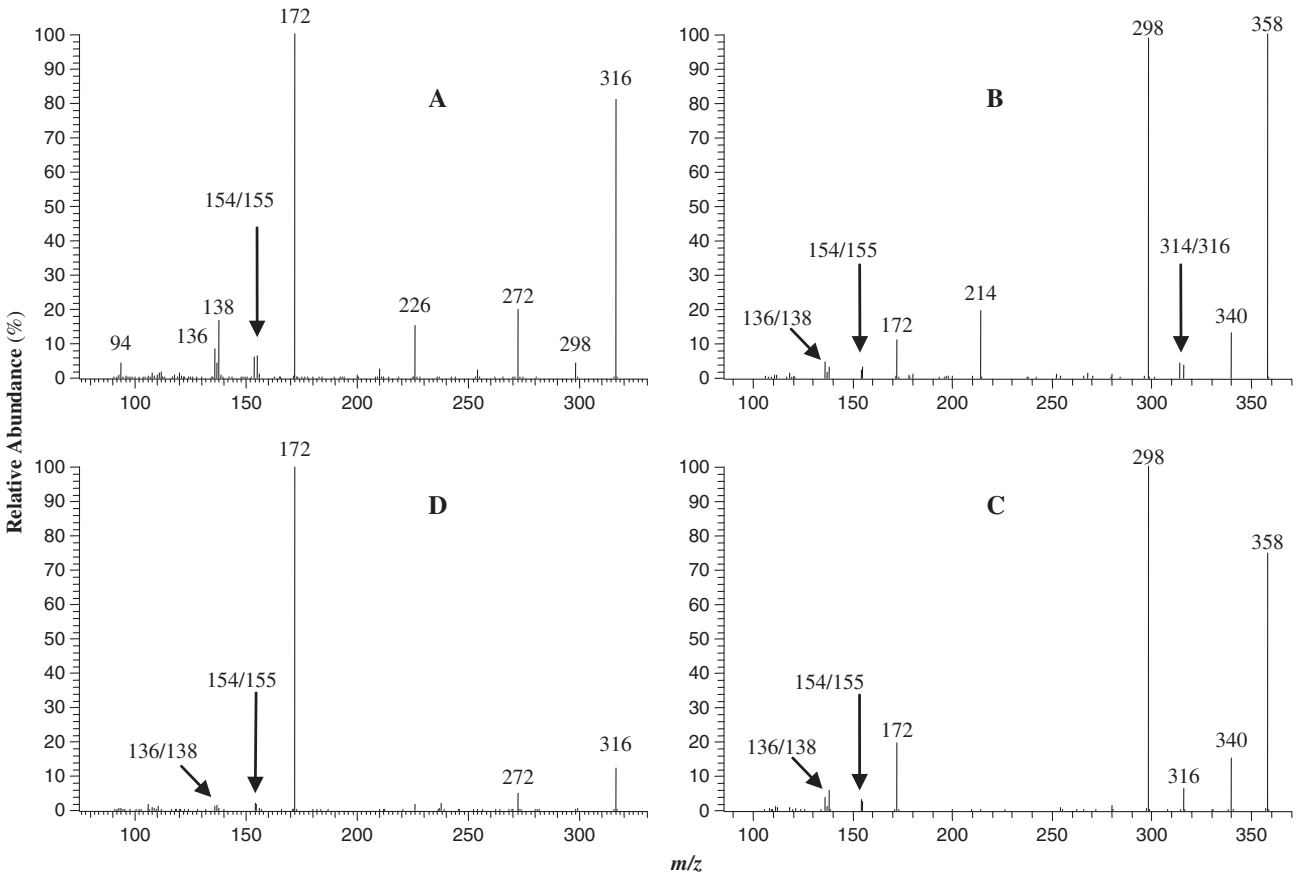


Figure 3. ESI ion trap MS/MS spectra for: (A) lycopsamine-*N*-oxide (peak 7, Fig. 2); (B) 7-acetyllycopsamine-*N*-oxide (peak 12, Fig. 2); (C) 3′-acetyllycopsamine-*N*-oxide (peak 13, Fig. 2); and (D) unidentified pyrrolizidine alkaloid-*N*-oxide (peak 5, Fig. 2).

were the only free base pyrrolizidine alkaloids detected in the extracts (peak 4, Fig. 2B).

Peaks 12 and 13 (Fig. 2A and F) showed MH^+ and $[2M + H]^+$ values (*m/z* 358 and 715) consistent with acetylated derivatives of lycopsamine (or intermedine)-*N*-oxide (Fig. 1). The MS/MS data (Table 1, Fig. 3B) for the smaller peak 12 included ions at *m/z* 314, 214 and 180 all consistent with 7-acetyllycopsamine-*N*-oxide (Fig. 1; Colegate *et al.*, 2005). The losses of 44 and 144 Da from the molecular ion adduct ($MH^+ \rightarrow$ *m/z* 314 and 214 respectively) are particularly indicative of the C9 esterification with trachelanthic acid (or one of its stereoisomers) such as with echiumine (7-angeloylintermedine) (Colegate *et al.*, 2005). The larger peak 13, on the other hand, only showing losses of 42, 60 and 186 Da ($MH^+ \rightarrow$ *m/z* 316, 298 and 172 respectively; Table 1, Fig. 3C), is the 3′-acetyllycopsamine (and/or intermedine)-*N*-oxide (Fig. 1) that was reported to be present in three of the six perennial *Cryptantha* species investigated by Stermitz *et al.* (1993). Based on the MS/MS data (Table 1), the elution order of the resultant parent free base alkaloids, following reduction of the *N*-oxides, was reversed with the 3′-acetyllycopsamine/intermedine (base ion *m/z* 138 consistent with a 7-hydroxy-1,2-dehydropyrrolizidine alkaloid) eluting slightly ahead of the 7-acetyllycopsamine/intermedine (base ion *m/z* 120 and an 81% relative abundance of *m/z* 180 characteristic of a 7-acetyl-1,2-dehydropyrrolizidine alkaloid).

The MS/MS data (Table 1, Fig. 3D) for the lower abundance *m/z* 316 ions (peaks 3 and 5; Fig. 2A and C) confirmed the difference between them and the isobaric *N*-oxides of lycopsamine and intermedine (peaks 6 and 7; Fig. 2A and C). Although the difference was clear with the *N*-oxides, it became more structurally significant when the *N*-oxides were reduced, resulting in a base ion of *m/z* 138 for lycopsamine (Fig. 4A) and *m/z* 156 for the unidentified alkaloids (Fig. 4B). Base ion formation at *m/z* 138 ion was also observed for some other lycopsamine diastereomers, i.e. intermedine, indicine and echninatine (the latter two acquired from the stocks of the ARS Poisonous Plant Research Laboratory). The trachelanthate character of the unidentified alkaloids was evident from the MS/MS data that showed the losses of 44 and 144 Da in common with lycopsamine. However, the increased abundance of the *m/z* 156 ion for the unidentified alkaloids suggests a significant difference that either facilitates the formation of this ion relative to *m/z* 138 or stabilises the *m/z* 156 ion against dehydration to the more usual *m/z* 138 ion characteristically observed for 7-hydroxy-1,2-dehydropyrrolizidine alkaloids (Fig. 5). Such stabilisation could suggest a structure such as 2-hydroxyamabiline (or one of its diastereomers; Fig. 5). However, this would be a new type of necine base and therefore, despite the contrary evidence of a consistently abundant *m/z* 138 ion provided by the analysis of lycopsamine, intermedine, indicine and echninatine, stereochemical differences in the acid would need to be considered. Such differences in the necic acid would need to facilitate the proton transfer from the acid to the necine base fragment and favour the fragmentation of the acid from the necine base at the ester oxygen and carbonyl carbon bond. This would then leave a more abundant *m/z* 156 ion rather than the *m/z* 138 that

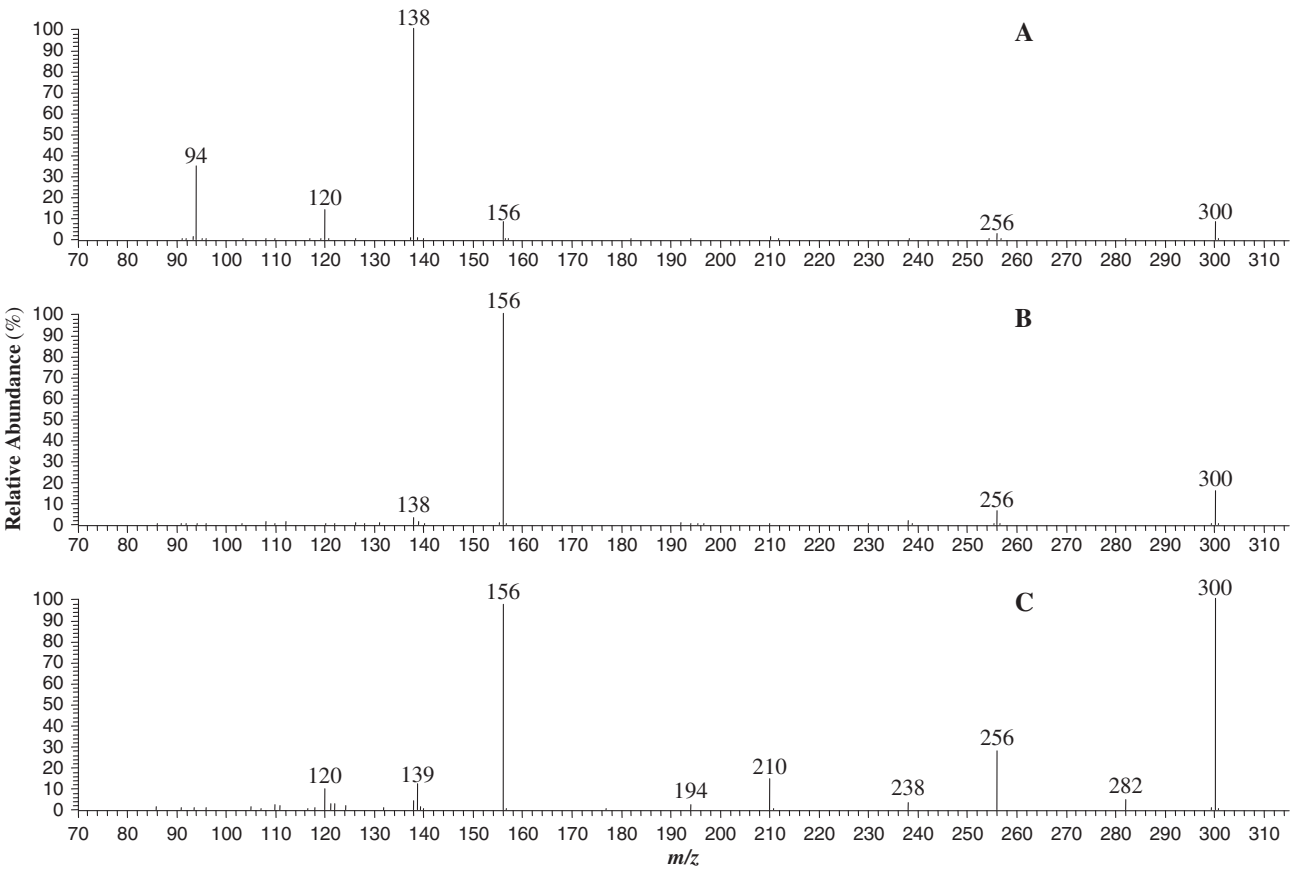


Figure 4. ESI ion trap MS/MS of *m/z* 300 for: (A) lycopsamine; (B) unidentified pyrrolizidine alkaloid; and (C) the putative amabiline-*N*-oxide (peak 11, Fig. 2).

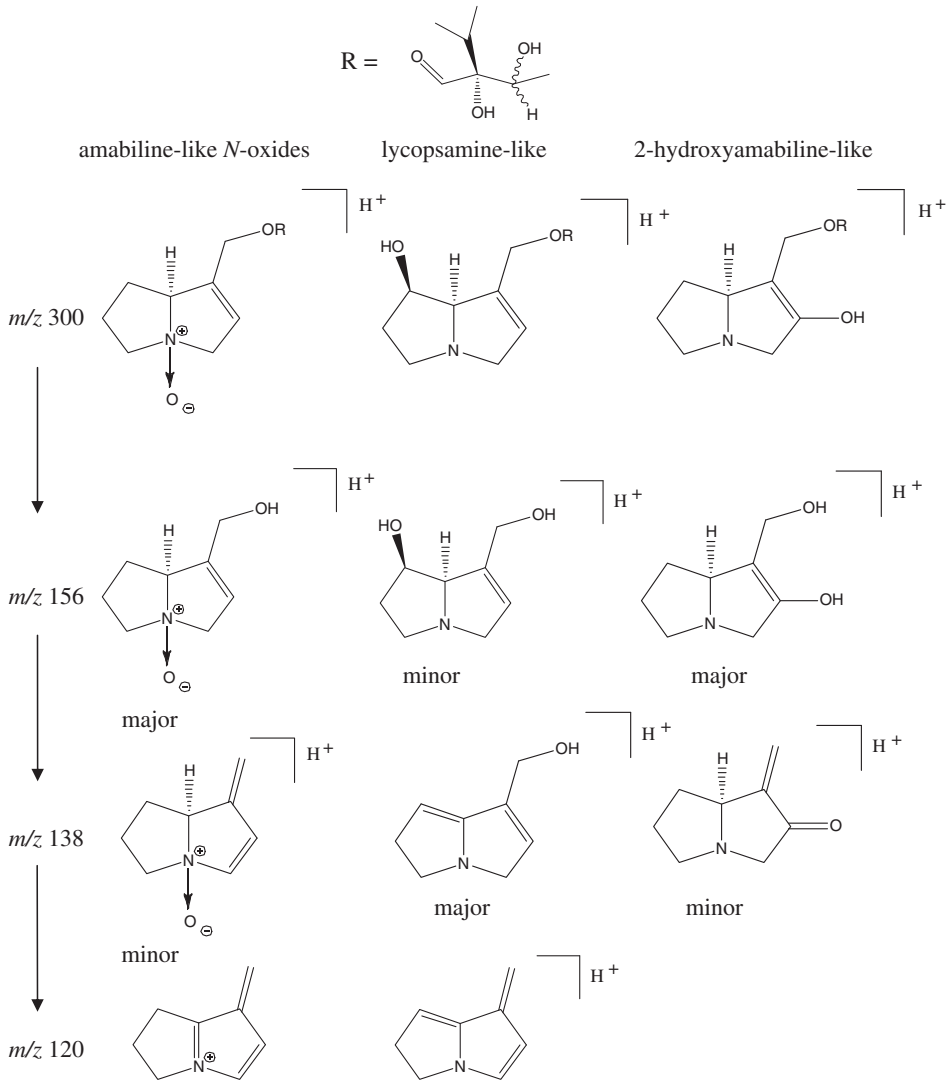


Figure 5. Fragmentation schemes for the ESI-MS/MS of *m/z* 300 for the putative amabiline-*N*-oxide, lycopsamine and the unidentified pyrrolizidine alkaloid shown in Fig. 4.

would result from fragmentation at the C9-ester oxygen bond. Another rational alternative would involve postulating a dehydrotrachelanthic acid esterified to 1-hydroxymethyl-2, 7-dihydroxypyrrolizidine. Such a necic acid is similar to sarracinic acid (necic acid on 2-hydroxysarracine in Fig. 6) while the 2-hydroxylpyrrolizidine necine base has been confirmed for several alkaloids, including rosmarinine (Roitman, 1983; Fig. 6). However, it might be expected that the *m/z* 156 ion derived from fragmentation of this molecule would readily dehydrate to form a more abundant *m/z* 138 as was observed for the putative 2-hydroxysarracine (Boppré *et al.*, 2008; Fig. 6). Isolation and purification of sufficient amounts of peaks 3 and/or 5 for NMR-based elucidation of the structure was beyond the scope of this present study due to the limited plant material available from this endangered species.

As appears to be fairly common with pyrrolizidine alkaloid-producing plants, the few predominating alkaloids produced in *C. crassipes* are accompanied by several alkaloids at minor to trace levels that are, in this present case, revealed mainly through reconstructed ion chromatograms from the MS data. The HPLC-ESI-MS and MS/MS data (Table 1) for minor peaks 1 and 2 (Fig. 2E) are the same as those recorded for leptanthine-*N*-oxide and echimiplatine-*N*-oxide (Fig. 1) that were detected in extracts of *Echium plantagineum*, another Boraginaceous plant (Colegate *et al.*, 2005). Reduction led to the formation of the corresponding free base alkaloids with MH^+ at *m/z* 316. Also in common with *Echium plantagineum*, but at a much lower concentration, was the observation, based on the MS/MS data, of echiumine-*N*-oxide (MH^+ *m/z* 398; peak 14, Fig. 2G). By contrast, Stermitz *et al.* (1993) only reported trace or minor amounts of echiumine from the three annual *Cryptantha* species investigated. A pair of alkaloids with MH^+ at *m/z* 300 and $[2M + H]^+$ at *m/z* 599 was observed (peaks 10 and 11, Fig. 2B). The MS/MS data of these (Table 1) again indicated an lycopsamine-type of alkaloid *N*-oxide showing a loss of 44 and 144 Da associated with the C9 trachelanthic acid substituent on echiumine and suggest an *N*-oxide of a 7-deoxylycopsamine-like alkaloid such as amabiline (Fig. 1) that has previously been reported from three of the six perennial *Cryptantha* species (Stermitz *et al.*, 1993). In support of this suggestion, the MS/MS data for the parent alkaloids (MH^+ *m/z* 284, Table 1) include a base ion at *m/z* 122 that can formally be derived by loss of trachelanthic acid from amabiline.

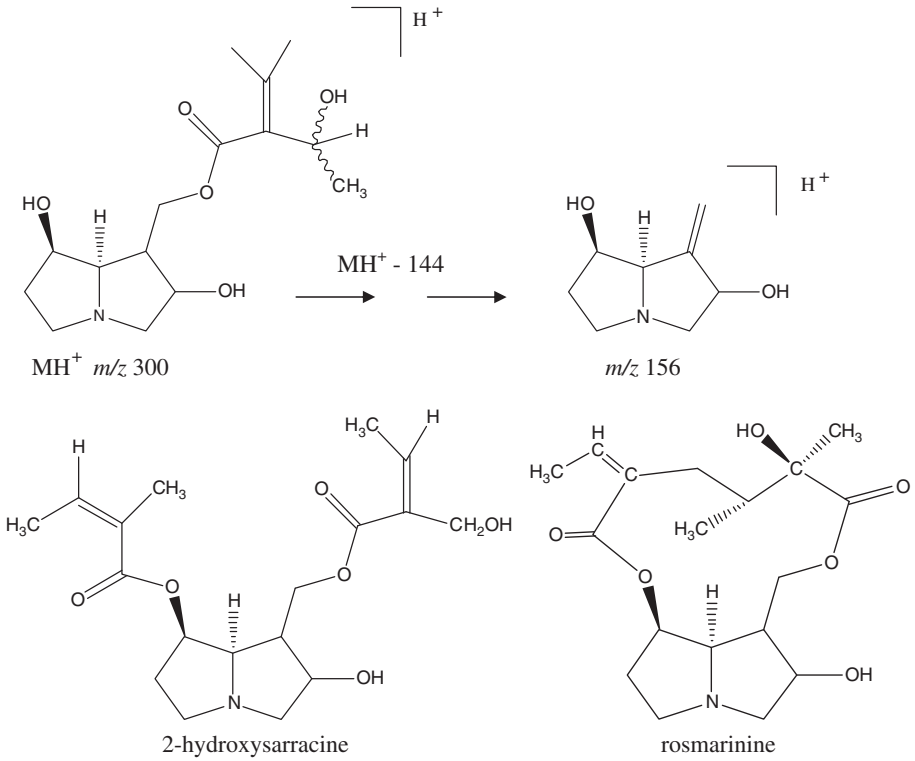


Figure 6. A potential 2-hydroxyplatynecine-based structure for the unidentified pyrrolizidine alkaloid (peak 5, Fig. 2A and C) isobaric with lycopsamine.

The MS/MS data for the *N*-oxides at peaks 8 and 9 (MH^+ *m/z* 318 and $[2M + H]^+$ *m/z* 635; Fig. 2D) indicate that these are the 1,2-dihydro analogues of lycopsamine and intermedine-*N*-oxides whereas peak 15 (MH^+ *m/z* 400 and $[2M + H]^+$ *m/z* 799; Fig. 2H) is tentatively proposed as the 1,2-dihydro analogue of echiumine-*N*-oxide (peak 14, Fig. 2G). These proposed structures are supported by the MS/MS data for the respective reduction products (Table 1) that are consistent with a saturated necine base, especially the ions at *m/z* 122 and 222 for reduced 8/9 and 15, respectively. However, despite MS/MS data supporting the tentative identification of peak 14 as echiumine-*N*-oxide (Fig. 1), due to only trace levels being present and therefore ion abundances being very low, no dimer ion peaks were observed to support the *N*-oxide character of this compound.

For quantitative purposes, the alkaloidal fraction was reduced to provide the free base pyrrolizidine alkaloids because the only relevant standard available was lycopsamine. The *N*-oxide reduction using the indigocarmine resin proceeded smoothly and HPLC-ESI-MS analysis confirmed complete reduction of the *N*-oxides, even in the presence of the crude methanol co-extractives. Under the HPLC conditions used, lycopsamine, intermedine and the unidentified alkaloid(s), all with MH^+ at *m/z* 300 (reduced from *m/z* 316), were not baseline-resolved and were therefore integrated together and quantitated under the assumption that they had very similar ESI-MS detector responses. In this way, quantitative estimates for the pyrrolizidine alkaloid content of *Cryptantha crassipes* leaves, taking into account the moisture content of 7 ± 0.7% determined by drying several samples of the milled plant, varied from 3.5 to 5% w/w dry plant. There was no major difference in estimated levels of pyrrolizidine alkaloids detected in the leaves harvested at different locations across the distribution range of *C. crassipes*. Because this estimate was 10–50 times the estimates previously reported for other *Cryptantha* species with similar pyrrolizidine alkaloid profiles using an alternative zinc/acid-based reduction method (Stermitz *et al.*, 1993), the quantitation was repeated with another sample of *C. crassipes* extract. Thus, the total alkaloid fraction was extracted from the crude methanol extract of *C. crassipes* into aqueous sulphuric acid, reduced and partitioned into acid, base and chloroform in the usual way. HPLC-ESI-MS monitoring of the zinc/acid reduction clearly showed the presence of the *N*-oxides in the 2 M acid solution prior to reduction, the complete reduction of the N-oxides, and the subsequent extraction into chloroform of the free base pyrrolizidine alkaloids from the basified aqueous solution after treatment with zinc/acid. The HPLC-ESI-MS (against the lycopsamine calibration curve) and gravimetric estimations of alkaloid content varied from 2 to 2.7%, lower than the redox resin reduction results but still an order of magnitude higher than previously reported for other *Cryptantha* species. The lower result may be due to physical and chemical (hydrolysis and polymerisation) losses consequent to the many process steps involved with the zinc/sulphuric acid reduction.

Qualitatively, the pyrrolizidine alkaloid content of *C. crassipes* aligns this perennial species with other perennial *Cryptantha* species investigated by Stermitz *et al.* (1993). However, the quantity of pyrrolizidine alkaloids, as their *N*-oxides, was much greater. This raises the possibility that the harsh environment, especially the lack of water, has resulted in the increased production of the pyrrolizidine-*N*-oxides that may, because of their zwitterionic-like charge separation, provide some form of osmoprotection within the plant, helping the cells to survive drought stress in a manner similar to the betaines

(Hanson *et al.*, 1994). Certainly, the levels of dehydropyrrolizidine alkaloids present in *C. crassipes* would render it readily toxic to grazing herbivores or even to humans if, for example, the plant was used medicinally.

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