Pyrrolizidine alkaloids in overwintering monarch butterflies *(Danaus plexippus)* from Mexico[1]

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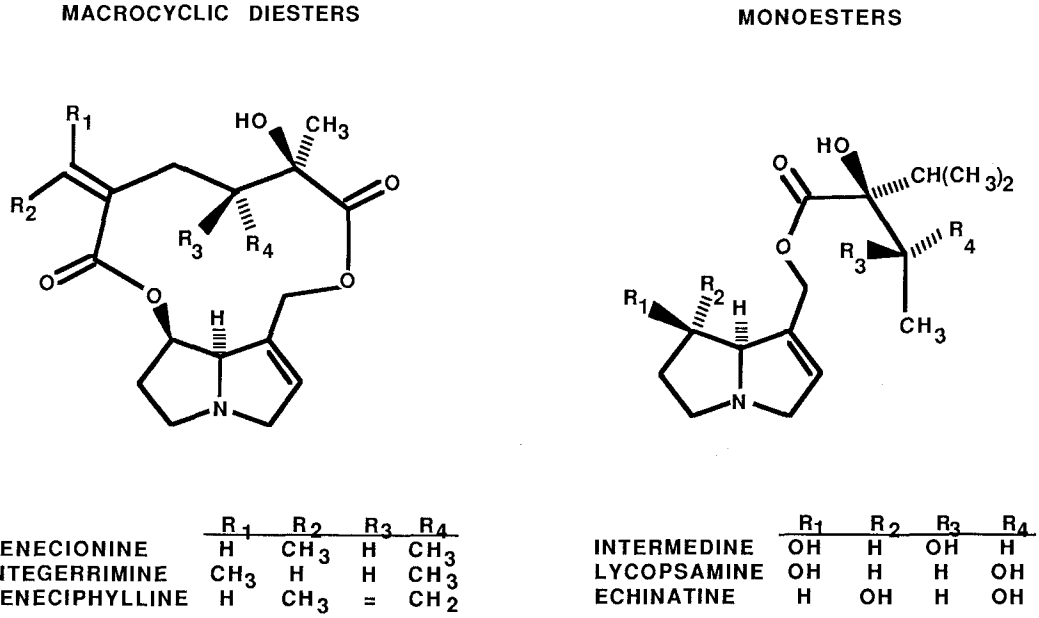
Summary. North American populations of the monarch butterfly, *Danaus plexippus,* have been found to contain pyrrolizidine alkaloids and their N-oxides. Analytical methods (TLC, GC, and GC/MS) have been developed to isolate, quantitate, and structurally elucidate the alkaloids. Examples of at least two classes of pyrrolizidine alkaloids have been identified, the macrocyclic diesters, senecionine, integerrimine, and seneciphylline, and the monoesters, echinatine, intermedine, and lycopsamine.

Key words. Pyrrolizidine alkaloids; macrocyclic diesters; monoesters; dihydropyrrolizines; Lepidoptera; *Danaus plexippus;* monarch butterflies; overwintering; Asteraceae; Boraginaceae; ecological chemistry.

Pyrrolizidine alkaloids (PAs) or their pheromone metabolites, dihydropyrrolizines, have been found in many species of the lepidopteran subfamily Danainae, tribe danaini[2–9]. These butterflies actively sequester and store the 1,2-dehydropyrrolizidine alkaloids from PA containing plant families, such as the Asteraceae (Compositae), Boraginaceae, Apocynaceae, and Fabaceae (Leguminosae)[2, 3, 5–13]. The alkaloid is acquired by imbibing either damaged parts of plants or plant exudates, such as nectar[6–10, 12, 13]. Our study reports the first finding of PAs occurring in North American populations of the monarch butterfly, *Danaus plexippus* L. Several populations of *D. plexippus* have previously been analyzed for PAs[7, 9] or their dihydropyrrolizine metabolites[4–6]. Both sexes have been shown to sequester PAs[7, 9, 12]. In the only previous analysis of North American monarchs, Meinwald et al.[4] looked unsuccessfully for the dihydropyrrolizine metabolites, but not their source compounds, the PAs. In no case have PA derived dihydropyrrolizine pheromone components been discovered in monarchs[4–6], although they are present in all other danaid species examined which had prior access to PAs or PA containing plants[2, 3, 5, 6, 8]. This suggests that in monarch butterflies, PAs may serve an alternative function, perhaps one of defense[7–11].

The butterflies used in our study are part of the major global population which breeds in eastern North America, migrates to Mexican overwintering sites in the fall, and returns again in the spring[14, 15]. Our butterfly specimens were collected at overwintering site 'Alpha'[14, 15], located in the Sierra Chincua, Michoacan, Mexico[15]. Live butterflies were collected in mid-December, 1982 and mid-January, 1983. The butterflies were either maintained whole, separated by sex, and placed six to a vial in methanol, or their wings were removed, dried in a forced draft oven at 60 °C, bagged, and kept frozen until analyzed at Davis, California. Control butterflies from northern California were raised as larvae on various Californian *Asclepias* species (*A. eriocarpa* Benth., *A. speciosa* Torr., *A. fascicularis* Dcne, in A. DC.), and the adults were frozen immediately following emergence.

Pooled samples of six butterflies were macerated and thoroughly extracted with methanol. Half of the methanol extract was reduced in a Serdoxit resin column[16] (Serva Fine Biochemicals, Inc., USA) to convert any proportion of PAs present as N-oxides to tertiary amines. The alcohol was removed under reduced pressure, the residue taken up in aqueous 2 N H_2SO_4, and the solution extracted four times with chloroform to remove neutral and acidic components. The acid solution was then made alkaline with ammonium hydroxide (pH 10.5), extracted four times with chloroform, saturated with NaCl, and extracted twice more with chloroform. The chloroform extract was dried over Na_2SO_4 and



	R1	R2	R3	R4
SENECIONINE	H	CH_3	H	CH_3
INTEGERRIMINE	CH_3	H	H	CH_3
SENECIPHYLLINE	H	CH_3	=	CH_2

	R1	R2	R3	R4
INTERMEDINE	OH	H	OH	H
LYCOPSAMINE	OH	H	H	OH
ECHINATINE	H	OH	H	OH

Figure 1. 1,2-Dehydropyrrolizidine alkaloids identified from *Danaus plexippus.*

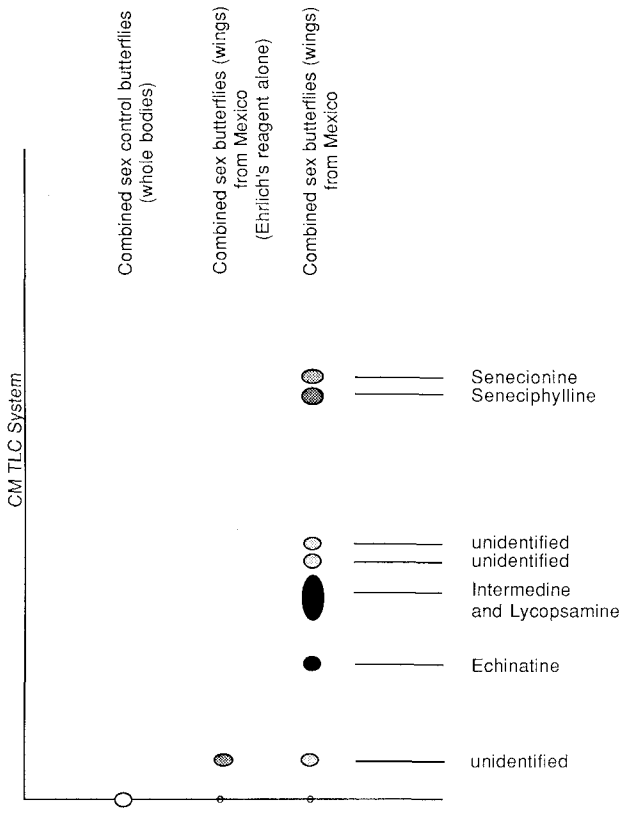


Figure 2. Redrawn thin-layer chromatographic channels showing typical PA profiles from control monarch whole bodies and site Alpha monarch wings. These channels are from plates developed two times in chloroform-methanol (80:20). Detection was with chloranil followed by Ehrlich's reagent except where noted. The most heavily shaded spots were the most intense in the original chromatograms.

evaporated to leave a residue representative of one half of the total alkaloid present in the sample. The other half of the methanol extract was evaporated and the methanol soluble material taken up in aqueous 2 N H_2SO_4, extracted with chloroform to remove impurities, made alkaline, and the alkaloids extracted with chloroform as above. This alkaloid containing residue represented the proportion of free tertiary amine present in the sample. The basic aqueous solution left behind after free amine extraction was neutralized. The water was evaporated and the water soluble material taken up in methanol, reduced with Serdoxit resin, and evaporated. Methanol soluble material was taken up in aqueous 2 N H_2SO_4, made alkaline, and extracted with chloroform as above, This alkaloid containing residue represented the proportion of reduced N-oxide present in the sample.

Identification of the alkaloids was based on comparison of thin-layer chromatographic (TLC) retardation factors (R_fs), gas chromatographic (GC) retention times, and characteristic mass spectra (MS) with those of authentic compounds[1]. The amount of individual PAs in each sample was determined from the height of the gas chromatographic peaks produced by injection of a known proportion of the sample compared with injections of known quantities of an external pyrrolizidine alkaloid standard, monocrotaline.

Thin-layer chromatography was performed on silica gel plates (0.25 mm thick) using two different systems, a lithium chloride activated plate with a solvent of chloroform: methanol (80:20)[17] (CM), and an untreated plate with a solvent of chloroform:methanol:ammonia (84.5:14.5:0.5)[18] (CMA). Detection was with chloranil[19] followed by modified Ehrlich's reagent[20].

Gas chromatography was performed on a Hewlett-Packard 5710A using a nitrogen-phosphorus thermionic selective detector (NP-TSD) and a 30 m × 0.32 mm DB-1 WCOT fused silica capillary column (J & W Scientific, U.S.A.) with a 0.25-μm film thickness. Column temperature was held constant at 220 °C, split injection was used with a split ratio of 50:1, and the helium carrier gas flow was 1.5 ml/min. GC/MS was performed on a Kratos MS 25 using a 15 m × 0.53 mm J & W Scientific DB-5 WCOT fused silica megabore column with a 1.0-μm film thickness, and temperature programmed from 170 °C to 270 °C at 8 °C/min. Electron impact ionization was used with energies of 70 eV.

In agreement with previous findings[2,6,7,9,10] the control butterflies without access to PA-containing plants had no detectable amounts of PAs or their metabolites.

Butterflies captured at site Alpha contained chemicals from at least two classes of PAs, macrocyclic diesters and monoesters (fig. 1). These same components were found in both whole body samples and, in much lesser quantities, in wings. The CMA TLC solvent system resolved four alkaloid spots from the butterfly sample, while the CM solvent system resolved seven spots (fig. 2). R_fs coincided with the monoesters echinatine and the diastereomeric pair lycopsamine and intermedine, and with the macrocyclic diesters of seneciphylline and the geometric isomers senecionine and integerrimine. One spot in a combined sex wing sample, $R_f = 0.05$ in the CM solvent system, gave a characteristic rose colored response to Ehrlich's reagent alone[21]. This indicates the presence of a dihydropyrrolizine component[5,21]. Although this compound has not yet been identified, its presence is the first indication that dihydropyrrolizines might occur in *D. plexippus*[4-6]. Compounds of this type are used as pheromones in other danaid species[2,3,5,6,8,10].

GC analysis (fig. 3) confirmed the presence of the PA monoester echinatine, and established the presence of both intermedine and lycopsamine. GC analysis also confirmed the presence of the PA macrocyclic diester seneciphylline, and established the presence of both senecionine and, in a few unquantitated samples, integerrimine. GC/MS confirmed the structural identities of echinatine, intermedine, lycopsamine, seneciphylline, senecionine, and integerrimine. Quantitation was based only on the five major identified GC peaks (table). Other potential peaks of interest were present only sporadically and in smaller amounts.

The ratio of N-oxides to free amines varied among the samples. Five out of six male samples and three out of six female samples showed more free amine (> 70%) than N-oxide when separate analyses of the two classes was attempted. One female sample showed equal amounts of each type, while the remainder had more N-oxide than free amine (> 60% N-oxide). Further study is needed to determine whether this is representative of butterflies in the population, or was the result of variable recoveries in the analytical method. Difficulties in achieving a mass balance accounting for the total alkaloid material present by summing the N-oxide and free amine fractions precludes making any further observations at this time. It is suspected that losses of material during the additional extraction steps involved in the more elaborate direct measurement of N-oxides have caused this discrepancy.

In ten out of twelve samples monoesters were the predominant class, comprising more than 90% of the PAs (fig. 4). Echinatine was the most abundant monoester in male monarchs while in five out of six female samples lycopsamine was the most abundant monoester (fig. 5). Senecionine was the most common macrocyclic diester, occurring in seven out of twelve samples, while seneciphylline was the most abundant when present (table).

This storage discrepancy between the amount of monoester and macrocyclic diester found in the monarch butterfly

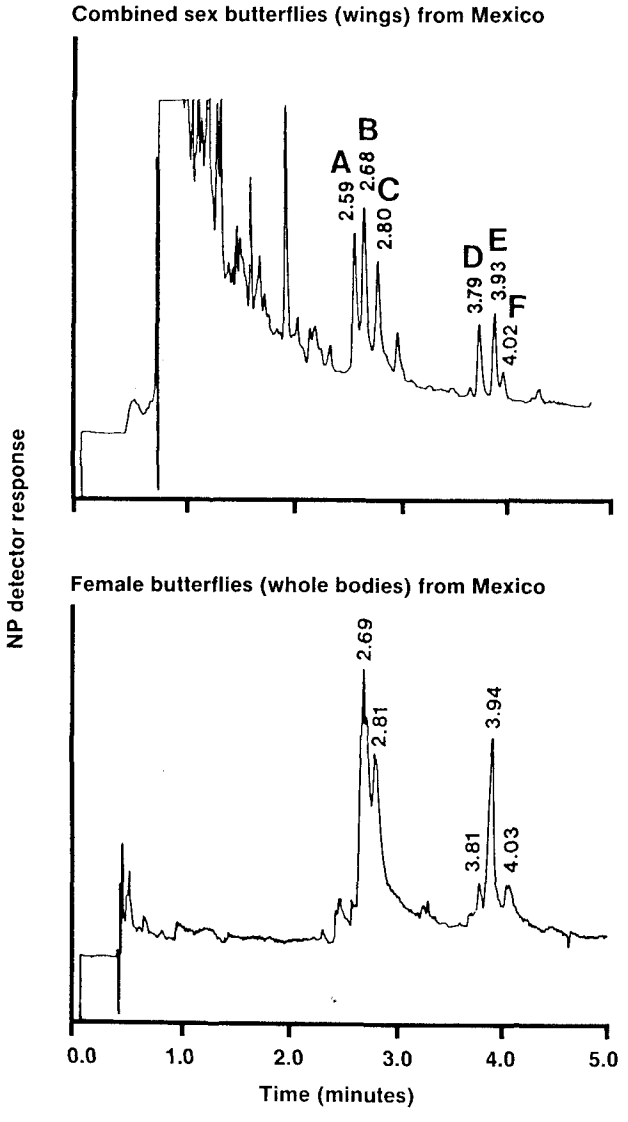


Figure 3. Gas chromatograms typical of those obtained from alkaloid extracts of site Alpha populations of *Danaus plexippus* using conditions described in text. Identified components with their respective retention times, in minutes, are: (A) intermedine, 2.59 (B) lycopsamine, 2.68–2.69, (C) echinatine, 2.80–2.81, (D) senecionine, 3.79–3.81, (E) seneciphylline, 3.93–3.94, and (F) integerrimine, 4.02–4.03.

could reflect differences between the two classes of PAs. The butterfly might store the more hydrophilic monoesters easier than the macrocyclic diesters[7]. Alternatively, the plant sources for the PAs may have been predominantly monoester containing plants, such as species in the Boraginaceae or more likely the conspicuous fall blooming composite species in the Eupatorieae tribe[11,22]. For instance, on their southward fall migration through the state of Texas to Mexico[15], the monarchs could encounter over sixty species of monoester-containing plants, including over fifty species within the Eupatorieae tribe[23]. During the same period of the year and in the same area, only ten species of macrocyclic diester-containing composites in the Senecioneae tribe are reputed to be actively growing[11,22,23]. Since the butterflies actively imbibe very large amounts of nectar on their southward journey[15], particularly as they approach the overwintering site, it is plausible that PAs are sequestered at that time.

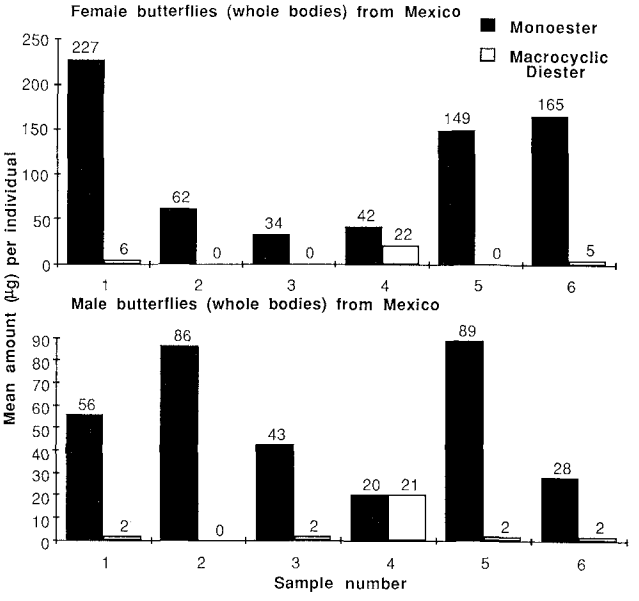


Figure 4. Total amount of pyrrolizidine alkaloid in site Alpha monarch whole bodies by sex as described by chemical class of PA, monoester versus macrocyclic diester. Values represent mean amounts, in µg, per individual per sample.

The occurrence of fall growing PA-containing plants at the Mexican overwintering site has not been fully investigated. It is possible that the butterflies acquire at least some of the PAs on-site before cold weather limits biological activity of both insects and plants.

There was a tendency for female butterflies to contain more alkaloid than male butterflies, 119 µg ± 77 and 59 µg ± 25, respectively. However, the values were quite variable for butterflies of the same sex in both absolute and relative amounts of the five quantitated PAs which effectively precludes definite conclusions about differences between the sexes. In addition the pooled sampling technique prevented

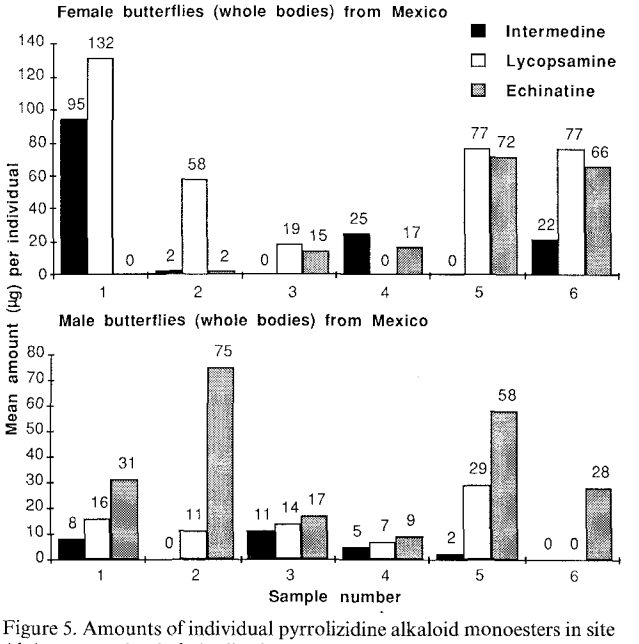


Figure 5. Amounts of individual pyrrolizidine alkaloid monoesters in site Alpha monarch whole bodies by sex as described by compound. Values represent mean amounts, in µg, per individual per sample.

The amounts and distribution of identified pyrrolizidine alkaloid free amines in site Alpha monarch butterflies from Mexico

Alkaloid		Male (μg)	Female (μg)
Intermedine	-frequency[a]	4/6	4/6
	-range[b]	trace[c]-11	trace-95
Lycopsamine	-frequency	5/6	5/6
	-range	7–29	19–132
Echinatine	-frequency	6/6	5/6
	-range	9–75	trace-73
Senecionine	-frequency	4/6	3/6
	-range	trace-10	4–6
Seneciphylline	-frequency	2/6	1/6
	-range	trace-11	18
Total alkaloid	-frequency	6/6	6/6
	-range	30–91	34–233

Quantitation by GC method using conditions described in text; [a]six sampling units each containing six individuals; [b]mean amount (μg) per individual; [c] < 2 μg.

any comparisons among individuals; one individual heavily laden with PA in a given sample could bias results for that sample.

It has been suspected that PA storage by adult danaid butterflies has been for a purpose other than to aid reproduction[2, 3, 7–11]. As has been well documented, danaid butterflies sequester and store cardenolides from larval food sources[2, 3, 24]. These cardenolides can act as effective predator deterrents[3, 10].

Pyrrolizidine alkaloids have also been suggested as predator defense agents against both vertebrate[10, 13, 25, 26] and invertebrate predators[10, 26, 27], as PAs are purported to be distasteful to these predators[3, 10, 26, 27]. Alternatively, PAs alone might not be effective deterrents but could complement the insect's other unpalatable toxic compounds[28]. Thus it has been sugested that an alternating dual PA-cardenolide based defense exists in danaid butterflies that has evolved through evolutionary time[8–11]. This concept is based upon PA supplementation of cardenolides which their larval host plants, *Asclepias* species, offer only in unreliable supply[26, 29]. The finding of PAs in North American monarchs links them to other danaid species in support of the dual chemical hypothesis. The butterflies collected at site Alpha originate as late summer brood populations stretching from the Great Plains to the Atlantic Coast which all eventually converge on the Mexican highlands. The constancy of identified PAs exhibited in this population suggests common sources of PAs. Larger increases in stored adult lipids as the butterflies approach the overwintering sites[15] suggest that they may obtain these from plants found along the fall migration routes. Further study will be required to identify these sources, and to understand individual storage and sequesteration variability as influenced by plant source, PA type, and butterfly sex and age.

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