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Pyrrolizidine alkaloids from *Anchusa strigosa* and their antifeedant activity

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Abstract

The pyrrolizidine alkaloid (PA) content of flowers, leaves, and roots of *Anchusa strigosa* (Boraginaceae) was analysed by ESI–LC–MS. Six PAs, including two new natural compounds, were detected, characterized by NMR spectroscopy, and quantified in each plant organ. The results indicated that the highest total concentration of PAs was in the leaves (23.63 mg/g of dried part), followed by the flowers (19.77 mg/g), and finally by the roots (1.80 mg/g). All PAs isolated were subjected to *Spodoptera exigua* and *Pieris brassicae* larvae. Feeding activity by both herbivore species using a bioassay was inhibited up to circa 75% depending on PA and applied concentration.

Keywords: *Anchusa strigosa*; Boraginaceae; Pyrrolizidine alkaloids; ESI–MS; Antifeedant activity

1. Introduction

The pyrrolizidine alkaloids (PAs) represent a class of natural compounds that are widespread in the family Boraginaceae, Leguminoseae, Asteraceae, and some genera of the family Apocynaceae. They are detected in some human foods, cattle forage, phytopharmaceuticals, cosmetics, and herbal teas. PAs demonstrate hepatotoxic, pulmotoxic, antimitotic, mutagenic, carcinogenic effects, and 1,2-unsaturated PAs can interact with neuron signal transduction (Mattocks, 1986; Hartmann and Witte, 1995; Schmeller et al., 1997; Prakash et al., 1999). Also in developing countries, characteristic liver diseases, causing high mortality, such as cirrhosis and primary tumors occur due to occasional or continued consumption of medicinal PAs containing plants (Roeder, 1995). In addition, PAs are assumed to be powerful plant defence compounds against insects and vertebrate herbivores (Van Dam et al., 1995). Some specialist herbivores that are adapted to PAs utilize them as feeding and oviposition cues (Boppré, 1986; Macel and Vrieling, 2003), and sequester them for their own defence and/or as pheromones (Boppré, 1986). Adapted insects store plant-derived PAs preferably as non-toxic *N*-oxides (Cheeke, 1994; Hartmann et al., 1999). Thus, for pharmacological and ecological reasons it is valuable to investigate the presence of these alkaloids and their *N*-oxides even at low concentration in plant species.

In this paper, we carry out chemical and biological studies of *Anchusa strigosa* Bank et Sol (Boraginaceae) to investigate its qualitative and quantitative PAs composition and their antifeedant activity. Previously,

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we reported the results of a phytochemical study on *A. strigosa* roots in which we isolated a number of PAs esters (Braca et al., 2003). *A. strigosa*, a plant widely distributed in the Mediterranean region (Feinbrun-Dothan, 1978), is used in local folk medicine. The root decoction is used as a diuretic, analgesic, and for treatment of stomach ulcers (Said et al., 2002), the flower decoction is used for analgesic, sedative, sudorific, and diuretic remedies (Al-Khalil, 1995), and finally the leaf cataplasm is used as an anti-inflammatory remedy or is applied externally for skin diseases and wounds (Al-Douri, 2000).

Here, we analyse the qualitative and quantitative composition of PAs in flowers, leaves, and roots of *A. strigosa* using ESI–LC–MS. Two new PAs were isolated from the leaf extract and were characterized by spectroscopical methods. The antifeedant activity of all isolated compounds towards the generalist herbivore *Spodoptera exigua* and the cabbage specialist *Pieris brassicae* was also evaluated. PAs are not recorded from the Brassicaceae and hence we expected that *P. brassicae* should be more sensitive to PAs than generalist *S. exigua*, known to feed on a large variety of plant species with very different secondary metabolite profiles including PAs.

2. Results and discussion

The phytochemical study of *A. strigosa* leaves led to the isolation of six PAs: **1**–**3** and **6** were previous isolated in a phytochemical investigation of the plant roots (Braca et al., 2003), while **7** and **8** were new natural compounds (see Fig. 1). The spectral data of compounds **1**–**3** and **6** are identical to those reported in our previous study (Braca et al., 2003).

Compound **7** showed a $[M + H]^+$ at *m/z* 314 (corresponding to $C_{16}H_{27}NO_5$ by means of ^{13}C-, ^{13}C-DEPT, MS, and elemental analysis), with a base peak at *m/z* 138 and the ion series, *m/z* 296, 270, 224, 156, 120, and 94, which are characteristic for 1,2-unsaturated monoester pyrrolizidine alkaloids with a necic acid esterified at position C-9 and a hydroxyl group on C-7 (El-Shazly

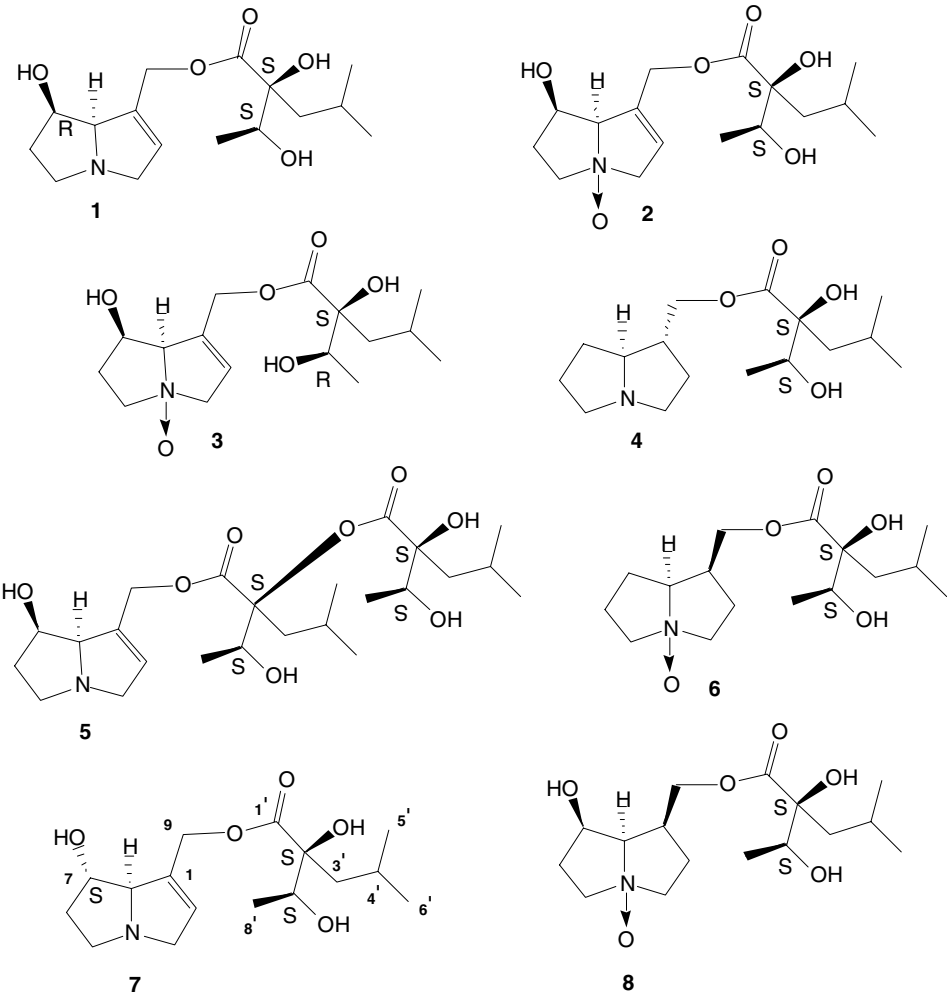

Fig. 1. Structure of PAs isolated from *Anchusa strigosa*.

et al., 1996). The NMR spectra (see Table 1) were in agreement with the presence of heliotridine as a necic base (Chamberlin and Chung, 1983). The ^{1}H NMR spectrum of **7** showed also proton signals at δ 0.87 (3H, *d*, J = 6.5 Hz, H-5′), 0.99 (3H, *d*, J = 6.5 Hz, H-6′), 1.82 (1H, *m*, H-4′), 3.78 (1H, *m*, H-7′), 1.74 (1H, *m*, H-3′a), 1.79 (1H, *m*, H-3′b), 1.15 (3H, *d*, J = 6.5 Hz, H-8′), attributed, with the help of HSQC data to two isopropyl methyl groups, two methine signals, one methylene, and one methyl signal, respectively, corresponding to the necic part of the alkaloid. Key correlation peaks in the HMBC experiment led us to confirm the presence of 2-hydroxy-2-(1-hydroxyethyl)-4-methyl-pentanoyl ester unit and to establish the C-9 esterification of heliotridine skeleton (cross peak between H_2-9 and C-1′). The absolute configuration of the esterifying acid 1,2-diol was determined with the following method. First, in order to obtain the relative configuration of C-2′ and C-7′, the acetonide derivative was prepared and the ^{1}H NMR chemical shift of the acetonyl methyl was observed. The ^{1}H NMR signals of the acetonyl methyl groups appeared as two separated three-proton singlets at δ 1.38 and 1.52, demonstrating the presence of the 1,2-diol as *erythro* (Gu et al., 1994). Thus, the structure of **7** was identified as heliotridine 2*S*-hydroxy-2*S*-(1*S*-hydroxyethyl)-4-methyl-pentanoyl ester.

Compound **8** displayed molecular formula $C_{16}H_{29}NO_6$ by means of MS, ^{13}C-, ^{13}C-DEPT NMR data, and elemental analysis. Its ESI–MS revealed a molecular ion at *m*/*z* 332 $[M + H]^+$, a base peak at *m*/*z* 174 and several fragments at *m*/*z* 314, 288, 156, and 112. The ^{1}H and ^{13}C NMR spectra of **8** (see Table 1) suggested the presence of a saturated *N*-oxide necine base with the same necic acid of **7**. The necine base of **8** was identified as platynecine by comparison of its spectral data with those reported in the literature (Mody et al., 1979). Therefore, compound **8** was identified as platynecine *N*-oxide 2*S*-hydroxy-2*S*-(1*S*-hydroxyethyl)-4-methyl-pentanoyl ester.

Table 1
^{1}H and ^{13}C NMR data of compounds **7**–**8** (600 MHz, CD_3OD)[a]

Position	**7**		**8**	
	δ_H	δ_C	δ_H	δ_C
1		134.0	2.22 *m*	37.3
2a	5.95 *br s*	123.5	1.90 *m*	30.6
2b			2.33 *m*	
3a	3.70 *dd* (16.1, 1.6)	62.0	3.30 *m*	73.0
3b	4.18 *br d* (16.1)		3.89 *m*	
5a	3.10 *m*	55.9	3.61 *m*	70.6
5b	2.64 *m*		3.67 *m*	
6a	2.10 *m*	35.0	2.07 *m*	35.8
6b			2.14 *m*	
7	4.52 *br d* (2.0)	70.8	4.52 *m*	70.1
8	4.60 *br s*	79.4	3.84 *m*	91.5
9a	4.85 *br s*	62.2	4.29 *dd* (10.4, 4.9)	67.3
9b			3.85 *dd* (10.6, 4.9)	
3′a	1.74 *m*	45.0	1.76 *m*	44.5
3′b	1.79 *m*		1.70 *m*	
4′	1.82 *m*	25.2	1.82 *m*	25.3
5′	0.87 *d* (6.5)	23.1	0.87 *d* (5.5)	23.3
6′	0.99 *d* (6.5)	24.4	0.98 *d* (5.5)	24.8
7′	3.78 *m*	73.9	3.78 *m*	73.9
8′	1.15 *d* (6.5)	17.4	1.15 *d* (5.5)	17.4

[a] Coupling constant (J in Hz) are in parentheses.

To evaluate the phytochemical profile of PAs present in the extracts of flowers, leaves, and roots of *A. strigosa*, LC–ESI–MS analyses were carried out in total scan modality. Then, to increase the sensitivity and selectivity of these analyses, the SIM modality was chosen. The LC–SIM–MS Base Peak Chromatograms of the methanolic extract of each organ (flowers, leaves, and roots) are shown in Fig. 2. The retention time (t_R), $[M + H]^+$, and MS–MS fragments of the individual peaks are listed in Table 2. The analyses of the fragmentation pattern of peaks A–I showed the characteristic fragment ions of a saturated or unsaturated necic unit, either as an *N*-oxide or as a free base. For compounds **1** and **7** the peak at *m*/*z* 156 was formed after Mc Lafferty rearrangement, and possesses the structure of a 1-methylene-7,9-dihydroxypyrrolizidine. Major fragmentation in the MS^2 spectrum occurred after loss of the H_2O molecule (*m*/*z* 296). Further ions were typical of an unsaturated necine with a free hydroxyl group at C-7 (*m*/*z* 138, 120, 94). In the MS^1 and MS^2 spectra of compounds **2** and **3** (*N*-oxide derivatives), fragments were typically higher than 16 amu compared to those measured for compounds **1** or **7** (free base derivatives). The fragmentation pattern of compound **8** showed 2 amu more than that of compound **2**, due to the saturated necine base. Compound **6** had a fragmentation pattern similar to that of **2**, differing by 16 amu, due the absence of the 7-OH group. The loss of 158 amu in all compounds could be correlated to the necic acid residue. To identify each peak in the LC–MS chromatogram, a reference methanol solution of pure compounds isolated from leaves (see Section 3) was prepared and injected in LC–MS, maintaining the same condition as with the crude methanolic extracts. In this way peaks B, C, D, E, G, and I were identified as compounds **2**, **3**, **8**, **6**, **1**, and **7**, respectively. The compounds corresponding to peaks A, F, and H were not purified from the plant material, and they remained unidentified. From the LC–MS–MS spectra we can hypothesize that peak F is an acetyl derivative of compound **2** or **3**, while peak H is a saturated derivative of compound **1**, **7** or **6**, and peak A is an isomer of compound **2** or **3**. The profile LC–SIM–MS of PAs from all extracts did not show significant qualitative differences, except for the absence of peak I (compound **7**) in the roots, and the presence of peak F in the flowers. From a quantitative point of view the differences are more substantial (Table 3). The quantitative analysis showed that the organs

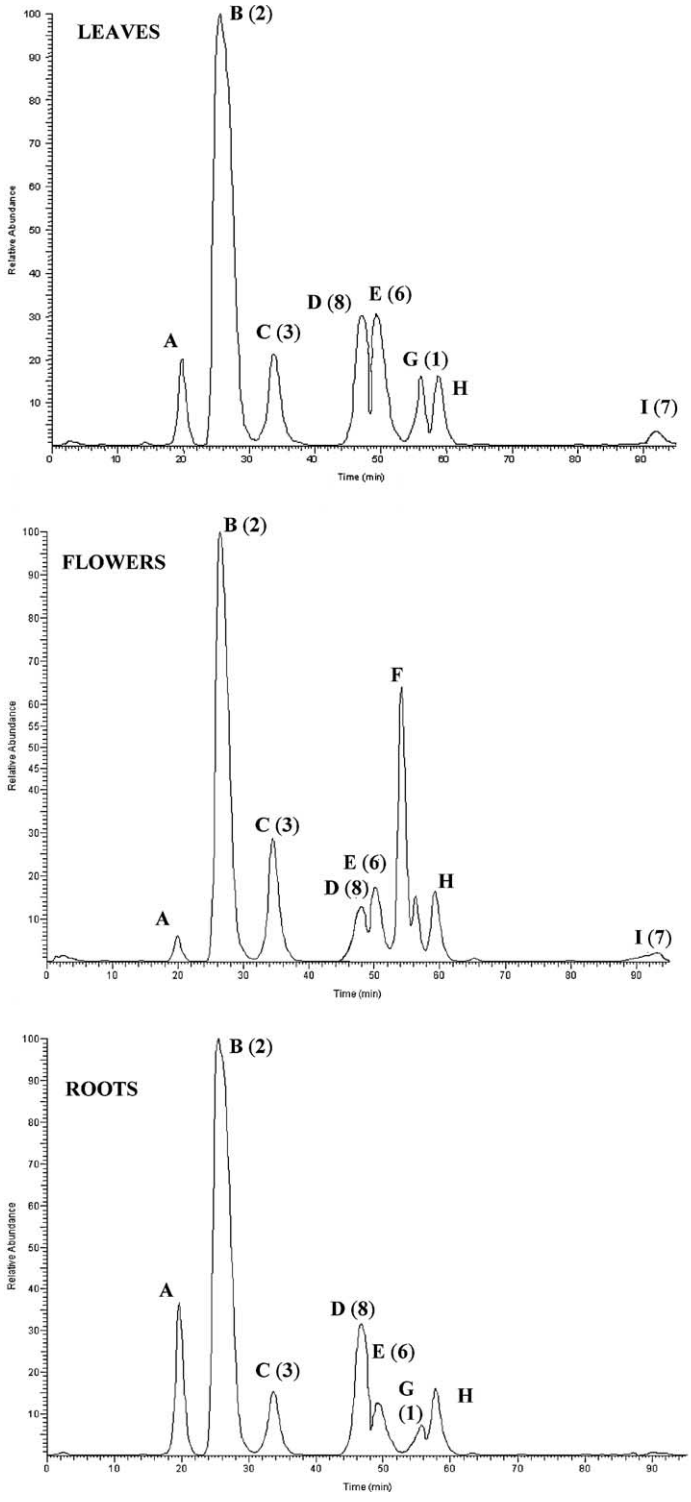

Fig. 2. LC–SIM–MS chromathograms of methanolic extracts of leaves, flowers, and roots of *A. strigosa*.

richest in PAs are the leaves, followed by flowers, while the PAs content of the roots is tenfold lower than the other organs. The *N*-oxide derivatives are the most abundant in all organs. The main differences between leaves and flowers were due to the major amount in compounds **6** and **8**.

All PAs isolated from the *A. strigosa* leaves (**1**–**2** and **4**–**8**) were subjected to free choice feeding assays using the generalist lepidopteran larva of *S. exigua* and specialist lepidopteran larva of *P. brassicae*. Compounds **4** and **5** were previously isolated from the roots of *A. strigosa* collected in a different year and region of Jor-

Table 2
t_R $[M + H]^+$, MS–MS fragments of PAs from flowers, leaves, and roots of *Anchusa strigosa*

Peak	PAs	t_R (min)	$[M + H]^+$ *m/z*	MS–MS fragments (*m/z*)
A	Unidentified	19.36	330	312, 286, 240, 172, 154, 138, 110, 94
B	**2**	24.88	330	312, 286, 268, 240, 172, 154, 138, 110, 94
C	**3**	31.30	330	312, 286, 240, 172, 154, 138
D	**8**	42.15	332	314, 288, 174, 156, 112
E	**6**	47.19	314	296, 270, 224, 156, 138
F	Unidentified	54.73	372	354, 312, 172
G	**1**	56.07	314	296, 270, 224, 156, 138, 94
H	Unidentified	60.00	316	298, 272, 158, 140, 110
I	**7**	94.21	314	296, 270, 224, 156, 138, 120, 94

Table 3
Amounts of PAs present in different parts of *Anchusa strigosa*

Peak	Flowers	Leaves	Roots
A	Trace	Trace	0.14
B(**2**)	14.55	14.15	1.19
C(**3**)	0.70	0.69	Trace
D(**8**)	0.35	4.12	0.47
E(**6**)	0.70	3.16	Trace
F	3.48	nd	nd
G(**1**)	Trace	Trace	Trace
H	Trace	Trace	Trace
I(**7**)	Trace	1.51	nd
Total amount PAs	19.77 (1.9%)[a]	23.63 (2.4%)	1.80 (0.18%)

nd = not detected.
[a] mg/g of dried vegetal material (% PAs/dried vegetal material).

dan (Braca et al., 2003). Out of all choice experiments conducted *P. brassicae* selected the control over the PA-lined disk in 13 out of 21 tests ($\chi^2 = 1.19$, Df = 1, NS). *S. exigua* preferred the control disk over the PA-lined disk in 20 out of 27 tests ($\chi^2 = 6.25$, Df = 1, $p < 0.01$) (see Table 4). These results demonstrate the overall antifeedant activity of the PAs against *S. exigua*. When individual PAs were tested at higher concentrations, PAs **1** and **2** reduced feeding by *P. brassicae* by 52% and 68% compared to the control. Feeding of *S. exigua* was significantly reduced by 76%, 61%, and 54% for the higher concentrations of PAs **5**, **1**, and **2**, respectively. From Table 4, it is clear that the antifeedant effect is concentration dependent. Significant feeding effects are only found at higher PA concentrations, although such effects are less pronounced for *P. brassicae*. The highest PA concentration tested is comparable to concentration found in the plant. PAs **4**, **6**, and **8**, lacking the 1,2-double bond, showed no deterrent activity, which is in line with the observation that the 1,2-double bond is the structural feature that is converted by cellular cytochrome P450s into a toxic moiety (Hartmann et al., 1999). The larvae of the cabbage specialist *P. brassicae*, which never encounter PAs in their natural diet, were not more deterred by PAs than the generalist *S. exigua* (Table 4). No synergistic effects were detected in a feeding assay with *S. exigua* (data not shown).

The coupling of liquid chromatography (LC) with ion trap mass spectrometry (MS) provides a substantial gain in terms of selectivity and allows simultaneous determination of PAs and their corresponding *N*-oxides, in a single chromatographic run without the requirement of a reduction step. In our work, six PAs were detected, characterized, and quantified in flowers, leaves, and roots of *A. strigosa*. The plant seems to preferentially allocate the PAs to aerial parts, which may results from the important role of defence played by these secondary metabolites. This role is confirmed by results of our antifeedant test.

3. Experimental

3.1. General procedures

Optical rotations were measured on a Perkin–Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. Elemental analysis was obtained from a Carlo Erba 1106 elemental analyzer. UV spectra were recorded on a Perkin–Elmer-Lambda 12 spectrophotometer. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^1H and at 150.86 MHz for ^{13}C, using the UXNMR software package was used for NMR experiments. Column chromatographies were performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden); HPLC separations were conducted on a Shimadzu LC-8A (Shimadzu Corporation, Kyoto, Japan) series pumping system equipped with a Waters R401 refractive index detector and Shimadzu injector and with a Waters μ-Bondapak C_{18} column (Waters, Milford, MA). TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); compounds were detected by $Ce(SO_4)_2$/H_2SO_4 (Sigma–Aldrich, Milano, Italy) solution and/or with Dragendorff's reagent.

3.2. Plant material

The flowers, leaves, and roots of *A. strigosa* Banks et Sol were collected at Al-Jubaiha (Amman), Jordan,

Table 4
Average amount eaten of cabbage disks lined with different of amounts of PAs (treatment) or solvent (control) by *Pieris brassicae* and *Spodoptera exigua* in a choice tests

PA	Conc. in μg/cm^2	*Pieris brassicae*				*Spodoptera exigua*			
		N[a]	Average area eaten in mm^2		p[b]	N	Average area eaten in mm^2		p[b]
			Control	Treatment			Control	Treatment	
1	10					13	20	7.85	0.037
	2	20	28.4	16.5	0.141	20	23.5	14	0.083
	0.4	20	37.25	18	0.015				
	0.08	20	12.65	37.65	0.017				
2	10	20	15.15	4.85	0.015	25	6.04	2.76	0.012
	2	20	5.85	14.4	0.102	25	8.72	6.52	0.25
	0.4	20	12.95	6.5	0.161	25	8.6	5.6	0.266
	0.08	20	16.15	14.1	0.811	25	12.52	15.2	0.595
	0.01	20	14.5	5.6	0.112				
4	10					11	15.18	12.72	0.363
	2	20	25.35	26.65	0.868	20	17.25	11.5	0.091
	0.4	20	22.8	17.3	0.437	20	13.1	13.15	0.984
	0.08	20	12.85	22.1	0.119				
5	10	19	8.05	4.58	0.215	25	20.32	4.72	0
	2	20	8.05	3.85	0.061	25	22.6	9.08	0
	0.4	19	14.63	7.32	0.334	25	18.84	13.04	0.136
	0.08	18	10.83	14.44	0.509	25	13.44	9.04	0.047
	0.01	20	14.75	16.7	0.753				
6	10	20	22.5	13.65	0.326	25	12.2	9.08	0.337
	2	15	26.87	17.2	0.506	20	8.45	13.5	0.173
	0.4	20	23.3	5.1	0.025	25	10.12	8.44	0.518
	0.08	20	16.25	22.5	0.55	25	6.52	10.76	0.081
	0.01	16	14.6	20.3	0.586				
7[c]	11.3					20	76.05	50.85	0.107
	2.25					19	71.05	76.79	0.742
	0.45					18	79.5	75.22	0.776
	0.09					19	63.74	61.79	0.904
	0.01					19	59.89	63.47	0.748
8[c]	11.3					11	28.18	17.9	0.315
	2.25					20	34.2	30	0.651
	0.45					20	21.05	24.9	0.565
	0.09					20	38.05	26.05	0.065
	0.01					19	28.1	24.2	0.461

[a] N, number of replicate tests.
[b] p, p value of paired t-test.
[c] In choice test only one larvae was used.

in April 2003 and identified by Dr. A. Bader, Al-Zaytoonah Private University of Jordan, Amman. A voucher specimen (No. 7093) is deposited at Herbarium Horti Botanici Pisani (Nuove Acquisizioni), Pisa, Italy.

3.3. Extraction and isolation

The air dried powdered leaves of *A. strigosa* (180 g) were defatted with *n*-hexane and successively extracted for 48 h with $CHCl_3$, and MeOH, by exhaustive maceration (3 × 2 l), to give 3.6 g, and 34.0 g of the respective residues. The chloroform extract was chromatographed on a silica gel flash column (6 × 16 cm), eluting with chloroform followed by increasing concentrations of MeOH (between 1% and 50%) in $CHCl_3$; the following volumes of solvents were used: 2.5 l of $CHCl_3$, 2.5 l of $CHCl_3$:MeOH 9:1, 4.0 l of $CHCl_3$:MeOH 1:1, 2 l of $CHCl_3$:MeOH 2:3, 2.0 l of MeOH. Fractions of 30 ml were collected, analysed by TLC (silica gel plates, in $CHCl_3$ or mixtures $CHCl_3$–MeOH 99:1, 98:2, 97:3, 9:1, 8:2), and grouped into 10 groups. Group 5 was subjected to RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (2.5:7.5) to yield pure compounds **2** (20 mg, t_R = 18.0 min), **3** (1.5 mg, t_R = 26.8 min), **8** (4.0 mg, t_R = 31.5 min), and **6** (8.3 mg, t_R = 40.4 min). Group 7 was separated with the same RP-HPLC method with MeOH–H_2O (2:3) to yield pure compounds **1** (1.5 mg, t_R = 25 min) and **7** (4.0 mg, t_R = 42 min).

3.4. Heliotridine 2S-hydroxy-2S-(1S-hydroxyethyl)-4-methyl-pentanoyl ester (7)

Yellow-orange oil, $[\alpha]_D^{25}$: +14.3° (*c* 0.1, MeOH); UV λ_{max} (MeOH) 285, 233sh nm; ESIMS: *m/z* 314 $[M + H]^+$, see Table 2; elemental analysis: C, 61.29%; H, 8.69%; N, 4.46%; O, 25.56%. Calcd. for $C_{16}H_{27}NO_5$, C, 61.32%; H, 8.68%; N, 4.47%; O, 25.53%; ^1H and ^{13}C NMR: see Table 1.

3.5. Platynecine N-oxide 2S-hydroxy-2S-(1S-hydroxyethyl)-4-methyl-pentanoyl ester (8)

Orange oil, $[\alpha]_D^{25}$: +3.0° (*c* 0.1, MeOH); UV λ_{max} (MeOH) 282, 235sh nm; ESIMS: *m/z* 332 $[M + H]^+$, see Table 2; elemental analysis: C, 57.90%; H, 8.85%; N, 4.23%; O, 29.02%. Calcd. for $C_{16}H_{29}NO_6$, C, 57.97%; H, 8.83%; N, 4.24%; O, 28.99%; ^1H and ^{13}C NMR: see Table 1.

The NMR data of all other PAs match the literature reports: retronecine 2*S*-hydroxy-2*S*-(1*S*-hydroxyethyl)-4-methyl-pentanoyl ester (**1**), retronecine *N*-oxide 2*S*-hydroxy-2*S*-(1*S*-hydroxyethyl)-4-methyl-pentanoyl ester (**2**), retronecine *N*-oxide 2*S*-hydroxy-2*S*-(1*R* -hydroxyethyl)-4-methyl-pentanoyl ester (**3**), and supinidine *N*-oxide 2*S*-hydroxy-2*S*-(1*S*-hydroxyethyl)-4-methyl-pentanoyl ester (**6**) (Braca et al., 2003).

3.6. Preparation of acetonide derivatives

A suspension of compounds **7**–**8** (2.0 mg) in THF (2.0 ml) was separately treated with 2,2-dimethoxypropane (0.5 ml), followed by a catalytic amount of anhydrous *p*-TsOH at 25 °C. After 1 h of stirring, a few drops of Et_3N were added, and the mixture was concentrated in vacuo. The residue was partitioned between $CHCl_3$ and a saturated solution of $NaHCO_3$ and the chloroform part was concentrated in vacuo, affording the corresponding acetonides (Gu et al., 1994); their ^1H NMR spectra were recorded.

3.7. LC–ESI–MS

LC–ESI–MS analyses: the analyses were performed using a Surveyor LC pump, a Surveyor Autosampler, coupled with LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with Xcalibur 3.1 software. Analyses were performed using a 3.9 mm × 30 cm i.d., μ-Bondapak C-18 column (Waters Corporation, Milford, MA, USA). The eluent was a mixture of methanol (solvent A) and water (solvent B). The solvent gradient was as follows: 0–45 min from 15% (A) to 25% (A); 45–90 min from 25% (A) to 100% (A). Elution was performed at flow rate of 1.0 ml/min with a splitting system of 200 μl to MS detector. The total running time was 100 min and MS data were recorded from 0 to 90 min. The volume of the injection was 20 μl for all the samples (standard of calibration and extracts of all plant organs). All analyses were performed in SIM on positive ion mode. The ionization conditions were optimized, and the following parameters were retained: capillary temperature, 280 °C; capillary voltage, 40 V; tube lens offset, 55 V; sheath gas flow rate, 60; auxiliary gas flow rate 6.0; source voltage, 3.5 kV. N_2 was used as sheath and auxiliary gas.

Sample preparation for LC–SIM–MS study: Representative quantities (10 g) of plant flowers, leaves, and roots were dried, powdered, and extracted at room temperature. Each dried part was defatted with *n*-hexane (4 × 100 ml) and successively extracted for 48 h with MeOH, by exhaustive maceration (4 × 100 ml). Yields of methanol extracts were 3.48, 2.29, and 0.28 g of flowers, leaves, and roots, respectively. 20 μl of methanolic solutions (5 mg/ml) of each residue was injected for analysis. Triplicate injections were made for each extract. In order to detect the alkaloid composition of the samples, the sample preparation and HPLC analytical condition was adjusted to avoid any detectable degradation of these compounds during the performance.

Calibration, quantification, and statistical analyses: Compound **2** was selected as external standard of calibration. Standard curve calibration was prepared over a concentration range of 1–500 μg/ml with six different concentration levels (1, 10, 100, 200, 300, and 500 μg/ml). Triplicate injections were made for each level and a weighed linear regression was generated and the curve of calibration was obtained using concentration (μg/ml) with respect to area obtained from integration of the SIM base peak extracting the value of area of each standard for $[M + H]^+$. The relation between variables was analysed using linear simple correlation. The linear regression of the external standards, R^2, was 0.997. For the quantification of the compounds a GraphPad Software, Prism 3.0 was used. The concentration of the PAs was expressed as mg/10 g of dried plant organ and % dry mass of PAs in the dried plant.

3.8. Herbivores

S. exigua Hubner (Lepidoptera, Noctuidae) larvae were reared on an artificial diet (Aerts et al., 1992) at 25 °C/16 h light, 18 °C/8 h dark and 70% relative humidity. *S. exigua* is a polyphagous leaf-chewing herbivore (Heath, 1983) and is known to be sensitive to several types of pyrrolizidine alkaloids (Aerts et al., 1992). Larvae of *P. brassicae* Linnaeus (Lepidoptera, Pieridae) are specialist feeders on the cabbage family. They were reared on white cabbage leaves (*Brassica oleracea* var. *alba*) at room temperature.

3.9. Choice tests

Feeding experiments with single PA: PAs were dissolved in methanol and applied on cabbage disks of Ø 1.9 cm. For each PA, five solutions of different concentrations were used: 10 μg/cm^2 which is comparable to the mean PA level of leaves of *A. strigosa* and the following dilutions 2.0, 0.4, 0.08, 0.01 μg/cm^2. PAs **1** and **4** were tested at only three concentrations because we possessed limited amounts of these PAs. Two disks were placed on a moist filter paper in every transparent plastic cups (9 cm diam., 7 cm high) and offered to 1–2 caterpillars at 2–4 instar. Every disk was painted with 20 μl of PA solution; control cabbage disks were treated with only methanol. The larvae were allowed to feed overnight, and the amount of cabbage eaten was measured to the nearest mm^2. Experiments were repeated 15–25 times, depending on availability of caterpillars and PAs. PAs **7** and **8** were tested only for *S. exigua*.

Feeding experiments with PA mixtures: To test if PAs showed synergistic activity, PAs were tested as mixtures of two PAs (1:1 PA **1**/PA **4**, 1:1 PA **2**/PA **6**) in choice test with *S. exigua*. We also applied each single PA to the cabbage disks in the same concentration of mixtures (2.0 μg/cm^2).

3.10. Statistical analysis

Statistics were performed in SPSS 8.0 (SPSS Inc., 1998). Differences in amount of cabbage disk eaten by caterpillars were analyzed with a paired *t*-test for each PA concentration. Results of tests for synergistic effects were analysed with ANOVA.

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