Pyrrolizidine Alkaloid Profiles of the *Senecio cineraria* Group (Asteraceae)

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Z. Naturforsch. **62 c**, 467–472 (2007); received July 7/September 13, 2006

Alkaloid profiles of five *Senecio* species (Asteraceae), including *S. ambiguus* subsp. *ambiguus*, *S. ambiguus* subsp. *nebrodensis*, *S. gibbosus* subsp. *bicolor*, *S. gibbosus* subsp. *gibbosus*, and *S. gibbosus* subsp. *cineraria*, were studied. Eleven pyrrolizidine alkaloids were identified and their content was evaluated by GLC-MS and GLC analysis. Otosenine and florosenine were found to be the major alkaloids in all studied species. It is interesting that only *S. ambiguus* subsp. *nebrodensis* was characterized by a high content of the alkaloids jacobine, jacoline, jaconine, and jacozine.

Key words: Senecio cineraria Group, Pyrrolizidine Alkaloids, GLC-MS

Introduction

Pyrrolizidine alkaloids (PAs) encompass a diverse group of some 350 structures that are scattered among angiosperm species. The most prominent occurrences are the genera *Senecio* and *Eupatorium* (Asteraceae), several genera of the Boraginaceae, the genus *Crotalaria* (Fabaceae) and certain orchids such as *Phalaenopsis*. Reliable occurrences, sometimes only in single species, are documented for other families such as Apocynaceae, Celastraceae, Ranunculaceae, Proteaceae and Convolvulaceae (Hartmann, 1999; Hartmann and Ober, 2000). PAs are ester alkaloids consisting of a necine base moiety, esterified with a necic acid. They may occur as monoesters, openchain diesters, or macrocyclic diesters. In all *Senecio* species senecionine *N*-oxide was identified as the primary product of biosynthesis. It is synthesized in the roots and translocated into the shoots, where it is transformed into the species-specific PAs (Pelser *et al.*, 2005). Senecionine was proved to be incorporated into: (i) simple retronecine esters as seneciphylline; (ii) epoxides of retronecine esters as jacobine including jaconine, its product of chlorolysis, and jacozine; and (iii) epoxides of otonecine esters as otosenine and florosenine. PAs are part of the plant's constitutive defence against insect herbivores. However, since insects coevolved with plants, there also exist a number of insect species that have not only learned to cope with the defence barrier but are able to recruit PAs from their host plant and utilize them for their own defence against predators. These insects, which belong to unrelated taxa such as Lepidoptera, Coleoptera and Orthoptera, developed efficient morphological and biochemical adaptations to safely handle toxic PAs. In certain arctiid moths, PAs even greatly influence the mating behavior. Adult males signal females their alkaloid load by close-distance pheromones made from PAs. Females preferentially accept males with a high load of PAs for mating since they may receive more than a third of their PAs from males. These PAs of parental origin are utilized for protection of the eggs against predators (Hartmann, 1999). Plants and adapted insects maintain PAs in the state of the non-toxic *N*-oxides which are easily converted into the toxic tertiary alkaloid (free base) in the gut of an unadapted predator. In conclusion, PAs represent a complex, but effective plant defence system successfully adopted by specialized insects in the course of their evolutionary adaptation to PA-containing plants. Pyrrolizidine alkaloids are considered also to be important secondary metabolites largely on account of their biological activities including acute ones.

Although pyrrolizidine alkaloids are known for their hepatotoxicity, mutagenicity carcinogenicity, and teratogenicity (Green and Christie, 1961; Hirono *et al.*, 1978, 1979), biological activities of

0939–5075/2007/0700–0467 $ 06.00

some *Senecio* species have been reported (Kovach *et al.*, 1979; Schmeller *et al.*, 1997; El-Shazly *et al.*, 2002; Toma *et al.*, 2004; Tundis *et al.*, 2005a; Loizzo *et al.*, 2006).

Senecio is the largest and complex genus of the Asteraceae and more than 1500 species have been reported (Nordestam, 1977). In the Italian flora the *Senecio* genus is represented by 52 specific or infra-specific taxonomic units (Conti *et al.*, 2005); about 26% of these units are endemic. In order to continue our chemical studies of the genus *Senecio* (Tundis *et al.*, 2005b) in the present work we have investigated the qualitative and quantitative alkaloidal pattern of members of the *Senecio cineraria* group, including *S. ambiguus* (Biv.) DC. subsp. *ambiguus* (Biv.) DC., *S. ambiguus* (Biv.) DC. subsp. *nebrodensis* (Guss.) Peruzzi & N. G. Passal., *S. gibbosus* (Guss.) DC. subsp. *bicolor* (Willd.) Peruzzi, N. G. Passal. & Soldano, *S. gibbosus* (Guss.) DC. subsp. *gibbosus*, and *S. gibbosus* (Guss.) DC. subsp. *cineraria* (DC.) Peruzzi, N. G. Passal. & Soldano, by sensitive phytochemical methods (GLC, GLC-MS and NMR). Reviewing the current literature PAs were previously identified in *S. cineraria* and *S. ambiguus* (El-Shazly *et al*., 2002; Pelser *et al.*, 2005); no information could be obtained concerning the alkaloid content of *S. ambiguus* subsp. *nebrodensis*, *S. gibbosus* subsp. *bicolor*, and *S. gibbosus* subsp. *gibbosus.*

Materials and Methods

Plant material

Plants were identified and collected from their natural habitats in Southern and Central Italy by Dr. N. G. Passalacqua and Dr. L. Peruzzi of the Natural History Museum of Calabria and Botanical Garden of Calabria University, Italy. Plants were preserved as air-dried material and stored at room temperature until use. Voucher specimens (Table I) were deposited in the Herbarium, University of Calabria (CLU), Italy.

Alkaloid extraction

Air-dried aerial parts of each species were powdered and exhaustively extracted with methanol (3×5 l) at room temperature. Combined methanol solutions were concentrated under reduced pressure and dried. The residue was suspended in 200 ml of distilled water, acidified with 2.5% sulphuric acid, then stirred overnight at room temperature with Zn powder to reduce native or artificial PA *N*-oxides. Excess Zn was removed by filtration. The aqueous acidic solution was filtered and basified (NH_4OH to pH 10), then extracted with chloroform until no more alkaloids could be detected in the aqueous phase (TLC; silica gel; eluent CH_2Cl_2/MeOH/NH_4OH 85:14:1; detection by Dragendorff's reagent). The organic portions were combined, dried over anhydrous sodium sulphate and the solvent evaporated to dryness to obtain the alkaloid extracts (Table II).

GLC-MS analysis

In order to determine the composition of the alkaloid extracts analyses were carried out using a gas chromatograph system (Hewlett-Packard Co. model 6890) with a fused capillary column (30 m length; 0.25 mm i. d.; 0.25 μm film thickness; static phase methylsilicone SE-30) directly coupled to a selective mass detector (Hewlett Packard 5973). Electron impact ionization was carried out at an energy of 70 eV. Helium was used as carrier gas. Injector and detector were maintained at 250 °C and 280 °C, respectively. The analytical conditions were as follows: oven temperature was 5 min isothermal at 60 °C, then 60–280 °C at a rate of 16 °C/min, then held isothermal for 10 min. The mass range from 50 to 550 amu was scanned at a rate of 2.9 scans/s. For analysis, alkaloid fractions were dissolved in dichloromethane (ca. 1 mg/ml) and aliquots (1 μl) were directly injected. Identification of the alkaloids was based on the comparison of the mass spectral data on computer match-

Table I. Sources of the studied *Senecio* species and their voucher specimen numbers.

Species	Subspecies	Voucher specimen number	Location of collection
S. ambiguus	*ambiguus*	7744–7746	Nicolosi, Etna (Catania, Italy)
	nebrodensis	7747–7748	Serre di Quacella (Palermo, Italy)
S. gibbosus	*gibbosus*	4451	Acqualandrone (Messina, Italy)
	cineraria	4455	Castiglioncello (Livorno, Italy)
	bicolor	4454	Cefalù (Palermo, Italy)

Table II. Content and yield (% relative to dried plant material) of methanolic and alkaloid extracts of *Senecio cineraria* group species.

Species	Subspecies	Plant material [g]	Methanolic extract		Alkaloid extract	
			Content [g]	Yield (%) ± S.D.[a]	Content [g]	Yield (%) ± S.D.[a]
S. ambiguus	*ambiguus*	500	62.50	12.50 ± 0.013	1.62	0.32 ± 0.010
	nebrodensis	130	11.47	8.83 ± 0.025	0.16	0.13 ± 0.005
S. gibbosus	*gibbosus*	150	20.77	13.85 ± 0.017	0.27	0.18 ± 0.004
	cineraria	150	12.17	8.12 ± 0.032	0.25	0.16 ± 0.003
	bicolor	500	44.03	8.81 ± 0.024	1.02	0.20 ± 0.003

[a] S.D., Standard deviation ($n = 3$).

ing against Wiley 138 and NIST 98 (Table III). Identification was confirmed by determination of the retention index; the modified Van Den Dool and Kratz formula was used to calculate the retention index by interpolation between bracketing C_9-C_{31} *n*-alkanes (Tranchant, 1995).

Capillary GLC analysis

A Shimadzu model gc 17 gas chromatograph equipped with a FID detector and a capillary column (30 m length; 0.25 mm i. d.; 0.25 μm film thickness; static phase methylsilicone SE-30) and controlled by Borwin software was employed. The conditions of injection were the same as above. The carrier gas was nitrogen. For quantitative determinations, an external standard method was used to compare peak areas with the amounts of standards injected (the results given are the averages of four determinations each).

Alkaloid isolation

Alkaloid extracts of *S. ambiguus* subsp. *ambiguus* and *S. gibbosus* subsp. *bicolor* were repeatedly subjected to column chromatography on silica gel 60 (20–45 μm; Merck, Germany) with gradient systems of dichloromethane/methanol and to preparative TLC on layers of silica gel GF_{254} (20 × 20 cm glass-backed plates; Merck, Germany). Spots were visualized under UV light (254 and 365 nm) on a separate silica gel layer, that had been eluted simultaneously with the layers used for isolation, then sprayed with Dragendorff's reagent (eluent $CHCl_3$/MeOH/NH_4OH 85:14:1) and H_2SO_4 50% v/v. All solvents were of analytical grade and purchased from Merck (Germany). Senecionine (**1**, 6 mg), integerrimine (**2**, 4 mg), seneciphylline (**3**, 7 mg), otosenine (**4**, 347 mg), and florosenine (**5**, 376 mg) were isolated from an alkaloid extract of *S. gibbosus* subsp. *bicolor*. Otosenine (**4**, 912 mg) and florosenine (**5**, 322.4 mg) were also isolated from an alkaloid extract of *S. ambiguus* subsp. *ambiguus* as major components.

NMR measurements

Structure elucidation was carried out using spectroscopic methods employing a Bruker (Milano, Italy) model AC 300 spectrophotometer operating at 300 MHz (^{1}H NMR) and 75 MHz (^{13}C NMR). Spectra were recorded in DMSO-D6 (compound **1**) and $CDCl_3$ (compounds **2**, **3**, **4** and **5**). Chemical shifts were expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference. ^{1}H and ^{13}C NMR assignments were made using two-dimensional COSY, HSQC and HMBC.

Results and Discussion

The results of GLC-MS analysis of samples of flowering aerial parts of five *Senecio* species of the *Senecio cineraria* group are summarized in Table III. *S. ambiguus* subsp. *nebrodensis*, *S. gibbosus* subsp. *bicolor*, and *S. gibbosus* subsp. *gibbosus* have not been studied before.

It was found that the yields of alkaloids ranged between 0.13% for *S. ambiguus* subsp. *nebrodensis* and 0.32% for *S. ambiguus* subsp. *ambiguus* (Table II). The PAs contents were given as w/w % of dried alkaloid extract.

Five pyrrolizidine alkaloids (**1**–**5**) were isolated from the alkaloid extract of *S. gibbosus* subsp. *bicolor* and two PAs (**4**, **5**) from *S. ambiguus* subsp. *ambiguus* and their structures were determined by GLC-MS and NMR analyses. Compounds **1**, **2**, and **3** were identified as senecionine (Roeder and Bourauel, 1993), integerrimine (Roeder and Bourauel, 1993), and seneciphylline (Noorwala *et al.*,

Table III. Pyrrolizidine alkaloid contents of members of the *Senecio cineraria* group.

Alkaloid	RI[a]	$[M]^+$ (EI-MS)	Percentage of alkaloids (% ± S.D.[c])				
			S. ambiguus		*S. gibbosus*		
			ambiguus	*nebrodensis*	*gibbosus*	*cineraria*	*bicolor*
Senecionine[b]	2415	335	< 0.1	–	–	< 0.1	< 0.1
Seneciphylline[b]	2442	333	< 0.1	–	–	< 0.1	< 0.1
Integerrimine[b]	2486	335	< 0.1	–	–	< 0.1	< 0.1
Jacobine	2613	351	< 0.1	15.0 ± 0.027	–	< 0.1	< 0.1
Jacozine	2661	349	tr	10.6 ± 0.013	–	tr	tr
Jacoline	2682	369	0.25 ± 0.007	5.0 ± 0.004	–	–	–
Jaconine	2727	387	0.25 ± 0.005	39.2 ± 0.046	–	< 0.1	1.4 ± 0.009
Otosenine[b]	2889	381	56.3 ± 0.052	3.1 ± 0.013	39.2 ± 0.019	23.6 ± 0.035	34.0 ± 0.045
Florosenine[b]	3188	423	19.9 ± 0.021	tr	33.7 ± 0.025	32.4 ± 0.035	36.9 ± 0.024
Floridanine	3215	441	2.1 ± 0.011	tr	4.1 ± 0.012	1.9 ± 0.006	2.4 ± 0.006
Doronine	3262	459	–	tr	3.3 ± 0.007	1.4 ± 0.003	3.5 ± 0.004

[a] RI, Retention index in methylsilicone SE-30.
[b] Alkaloids isolated from *S. ambiguus* and *S. bicolor*.
[c] S.D., Standard deviation ($n = 4$). Percentage of alkaloids calculated with respect to alkaloid extract.
tr, Trace.
–, Not detected.

2000), while compounds **4** and **5** were identified as otosenine (Liu and Roeder, 1991) and florosenine (Urones *et al.*, 1988; Roeder, 1990), respectively. In addition, the alkaloid extract of studied representatives of the *Senecio cineraria* group were analyzed by GLC-MS and GLC. Altogether, eleven pyrrolizidine alkaloids (Fig. 1) were detected and unequivocally identified by their MS fragmentation patterns and retention indices. GLC-MS represents a powerful high-resolution method for the identification of underivatized PAs from biological sources (El-Shazly, 2002). EI-MS fragmentation patterns were in agreement with those reported in the literature (Krebs *et al.*, 1996; Noorwala *et al.*, 2000; Asres *et al.*, 2004). PA profiles of all *Senecio* species studied are characterized by a few major components with minor constituents. All the identified PAs were distinguished by a retronecine-like necine base (1,2 unsaturated) and by a 12-membered macrocyclic diester with a necic acid moiety derived from isoleucine. In relation to the mass fragmentation pattern, the ions at *m*/*z* 220, 136, 120, and 94 are characteristic.

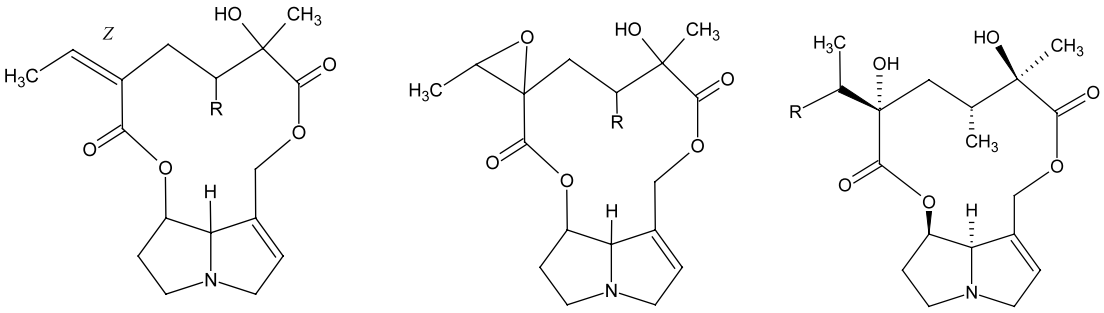
Otosenine (**4**) and florosenine (**5**) were the major alkaloids in all studied *Senecio* species except for *S. ambiguus* subsp. *nebrodensis*. Particularly, the amount of otosenine was significantly higher in *S. ambiguus* subsp. *ambiguus* (56.3%).

S. gibbosus subsp. *bicolor* and *S. gibbosus* subsp. *gibbosus* had significantly higher otosenine and florosenine contents. In fact, otosenine and florosenine contents were 39.2% and 33.7% for *S. gibbosus* subsp. *gibbosus* and 34.0% and 36.9% for *S. gibbosus* subsp. *bicolor*, respectively.

Comparative evaluation of PA patterns of *S. ambiguus* subsp. *nebrodensis* and other *Senecio* species analyzed in this work are clearly distinguished by their PAs contents. In fact, concerning *S. ambiguus* subsp. *nebrodensis* eight alkaloids were identified four of them, such as jacobine, jacoline, jaconine, and jacozine (Table III), were present with high percentage ranging from 5% to 39.2%. The alkaloids content of *S. ambiguus* subsp. *nebrodensis* was analyzed for the first time.

Previous studies on *S. cineraria* resulted in the identification of 13 alkaloids namely senecionine, seneciphylline, jacobine, otosenine, 9-methyl-didehydroretronecine, 5,6-dihydro-7,9-dimethoxy-7*H*-pyrrolizidine, senecivernine, integerrimine, jacoline, jaconine, usaramine, florosenine and doronine (El-Shazly, 2002). Our study demonstrated that the Italian *S. gibbosus* subsp. *cineraria* was characterized by 10 PAs while 9-methyl-didehydroretronecine, 5,6-dihydro-7,9-dimethoxy-7*H*-pyrrolizidine, jacoline and usaramine were not found. Like in *S. cineraria* growing in Egypt we have not found any trace of retrorsine (El-Shazly, 2002).

Senecio cineraria group pattern is different to that obtained by Pelser *et al.* (2005) and it seems to us that it is strongly affected by the ability of the



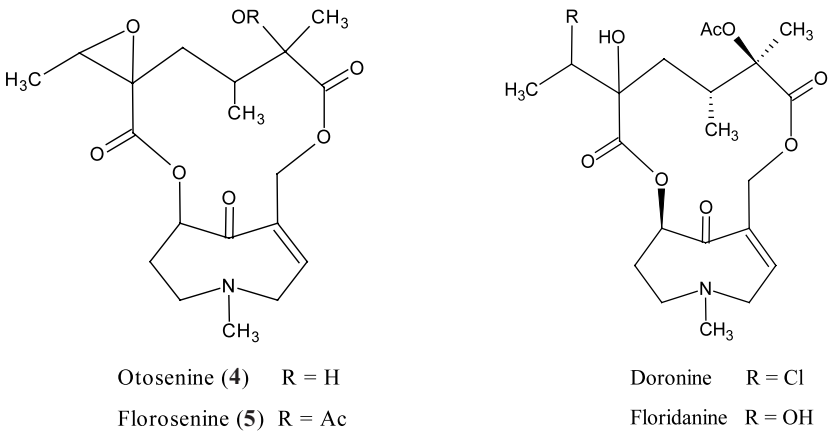


Fig. 1. Pyrrolizidine alkaloids from the *Senecio cineraria* group.

plants to gain and lose this class of chemical compounds on the base of local growing conditions as the lavic soil, for *S. ambiguus* subsp. *ambiguus,* or an humid and shallow habitat, for *S. gibbosus* subsp. *gibbosus.* Due to the important biological properties of PAs further research relating to the isolation of the active constituents and evaluation of activity are in progress in our laboratory.

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