Alkaloids from the Roots of *Senecio macedonicus* Griseb

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The new alkaloids 7-,9-diangeloylplatynecine (**1**) and 8-episarracine N-oxide (**2**), were isolated and identified from the roots of *Senecio macedonicus*. Another one, 8-epineosarracine was detected by GC/MS analyses of the crude alkaloid mixture. The cytotoxicity and biological activity of the alkaloids were tested on normal murine spleen lymphocytes and P3U1 mouse myeloma.

Introduction

Senecio is the most important genus of the tribe *Senecioneae* (*Asteraceae*) and includes about 1500 species widespread all over the world (Tahtajian *et al.*, 1981). In the Bulgarian flora genus *Senecio* is represented by 16 species. One of them, *Senecio macedonicus* is a Balkan endemit (Andreev *et al.*, 1992). Up to now the following mono- and open chain diester pyrrolizidine alkaloids (PAs) were isolated and identified: 7-angeloylretronecine, 9-angeloylretronecine, 7-angeloylplatynecine, 9-angeloylplatynecine and mixture of sarracine and neosarracine (Christov *et al.*, 1997).

Most of the plants containing 1,2-unsaturated PAs are toxic for humans and domestic animals due to liver transformation into reactive alkylating agents (Witte *et al.*, 1993). At the same time there are almost no data about the physiological action of the mono- and open chain diester saturated PAs. That is why except for the phytochemical study of the roots of *S. macedonicus* the aim of this study was to test some of the above-mentioned alkaloids for toxicity and their influence on normal and myeloma lymphocyte proliferation.

Material and Methods

General

The CIMS and EIMS were obtained on a Varian MAT 311A spectrometer. The GC/MS analyse was performed with GC Hewlett Packard 6890 plus MS detector Hewlett Packard 5973. Column: HP 5-MS, (30 m × 0.25 mm i.d df = 0.25 μM) conditions: injector 250 °C, temp. programme 100° (2 min) –280 °C, 5 °C/min, isoterma at 280 ° for 20 min; split ratio 1:50, carrier gas: He const. flow 0.9 ml/min, mass range 35–700; sample 1 mg crude alkaloid mixture (CAM). The NMR spectra were measured in $CDCl_3$ on a Bruker DRX 250 spectrometer with TMS as internal standard using standard Bruker software. Column chromatography (CC): neutral Al_2O_3, Brockmann II, and mobile phase acetone, acetone/MeOH gradient; TLC: aluminium sheets, silica gel 60 F_{254} (Merck), bands detected under UV light or by *Dragendorff* reagent; Preparative TLC: 20 × 20 cm plates with silica gel GF_{254} Merck 1 mm thickness, mobile phase acetone: 25%NH_4OH (2:0.3 v/v).

Plant material

The roots of *S. macedonicus* were collected in October 2000 in the region not far from Sofia in West Stara Planina mountain. A voucher specimen was deposited at the Herbarium of the Institute of Botany, Bulgarian Academy of Sciences SOM.

Extraction and Isolation

Dried and powdered plant material (200 g) was extracted exhaustively with EtOH. The combined

EtOH extracts after evaporation to dryness were acidified with 5% HCl, filtered and extracted with $CHCl_3$. The aqueous acid solution was stirred with 10 g Zn dust (24h), then filtered and made alkaline with 25%NH_4OH to pH 9. The alkaline solution was extracted with $CHCl_3$ to afford CAM. Pure alkaloids ware obtained after CC of CAM and preparative TLC of mix fractions obtained from CAM (see *General*).

7,9-Diangeloylplatynecine(**1**): 0.92 mg oil. CIMS *m/z* (%) rel. int. 322[M+1 100]$^+$. EIMS *m/z* (%) rel. int. 321[M$^+$ 1], 238(3), 221(33), 138(100), 122(65), 108(17), 95(54), 82(61), 69(16), 55(78). ^{1}H NMR (see Table I).

8-Episarracine N-oxide (**2**): 4 mg oil. ^{1}H NMR (Table I). ^{13}C NMR (62.8MHz, $CDCl_3$), δ(ppm): 166.3s,165.1s (C17,C11), 142.9d (C19), 141.8d (C13), 131.7s,125.4s (C12,C18), 85.3d (C8), 73.7d (C7), 69.1t (C3), 67.0t (C5), 64.5t (C15), 61.2t (C9), 37.6d (C1), 32.5t (C6), 28.3t (C2), 20.7q (C21), 16.0q,15.7q (C14,C20). **2** was reduced with Zn/HCl to 8-episarracine (**3**), which was identified by EIMS *m/z* (%), rel. int. 337[M$^+$ 1], 237 (27), 222 (30), 140 (68), 138 (100), 122 (84), 108 (8), 95 (70), 82 (68), 55 (14) and NMR (Table I).

Biological activity

Cytotoxicity test: 10^5 murine spleen lymphocytes or 10^5 P3U1 myeloma cells were resuspended in 200 μl RPMI 1640 medium, containing 10% calf serum and incubated with serial dilutions of the compounds starting at 10 μg per ml assay for the spleen lymphocytes or at 400 ng per ml assay for the myeloma cells. After 24 h at 37 °C, 5% CO_2 and 95% humidity the viability of the cells was evaluated using the Trypan Blue exclusion test.

Lymphocyte proliferation test. 10^6 murine spleen lymphocytes or myeloma cells per milliliter were resuspended in RPMI 1640 medium, containing 10% fetal calf serum and plated in 96 well tissue culture plates. The alkaloids were added to the cultures at concentrations of 16 ng, 8 ng, 4 ng or 2 ng per ml. For PHA stimulation 5 μg/ml of the mitogen Phytohemagglutinin -PHA/DIFCO Lab. (Detroit, USA) was added to the cultures. After incubation of 96 h at 37° C, 5% CO_2 and 95% humidity 1 μCi (0.04 MBq) ^{3}H thymidine was added to each well (200. μl). All the samples were in triplicates. The samples were counted in scintillation counter (Beckman) and the results shown as mean ± standard deviation. As controls we used the same number of cells resuspended in the same medium.

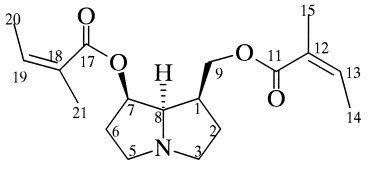
Results and Discussion

The new PAs 7-,9-diangeloylplatynecine (**1**) and 8-episarracine N-oxide (**2**) together with the already known: 7-angeloylplatynecine, 9-angeloylplatynecine, mixture of sarracine and neosarracine (1:3 w/w) were isolated and identified from the roots of *S. macedonicus*.

The structure of **1** was solved after detailed study of MS, ^{1}H NMR spectra and comparisons with spectral data of similar structures (sarracine, neosarracine, N-methyl-O^7, O^9-diangeloyl-1-hydroxylplatynecinium chloride and s. o.).

The CIMS of **1** showed an ion with *m/z* 322(100) [M+1]$^+$. The M$^+$ at *m/z* 321(1), as well as the characteristic fragments at *m/z* 221(33) obtained after losing of angelic, tiglic or senecioic acid attached at C-7 and C-9; 138(100), 122(65), 95(54), 82(61), diagnostic for acyclic diesters of saturated necine base, defined this alkaloid as an O^7, O^9 substituted PA (Grue *et al.*, 1993; Witte *et al.*, 1993).

The ^{1}H NMR spectrum supported the structure of a saturated O^7, O^9 substituted PA ester of an-



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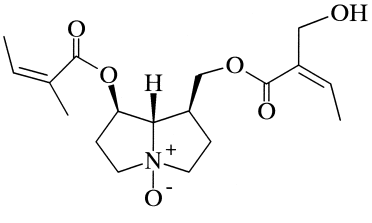

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Fig. 1.

gelic acid. Two olefinic protons H-13 and H-19 at δ 6.10 and 6.16 respectively, combined with four methyl groups at 2.02, 1.97, 1.92 and 1.88 indicated that two angelic moieties are present. The O^7,O^9 substitutions followed from the signals, of H-7, H-8, H-9_d, H-9_u resp. at δ 5.43, 3.96, 4.38 (1H, dd, J = 11.0, 7.3 Hz), 4.19 (1H, dd, J = 11.0, 8.3 Hz). The sum of the coupling constans in H-7 as measured by width at half height (ΣJ_7) was 8.3 Hz and corresponded to platynecine type of the necine base. The other two possibilities for the necine–hastanecine and turneforcidine are respectively ΣJ_7 12.07 and 14.1 Hz (Aasen *et al.*, 1969; Roeder and Lin, 1991). All these data defined **1** as 7,9-diangeloylplatynecine.

Alkaloid **2** was proved by ^{1}H, ^{13}C NMR spectra including 2D H-H and C-H correlations, reduction of **2** to it's corresponding base and comparisons of all spectral data with that of similar substances.

The ^{1}H NMR of **2** contained two olefinic protons of H-13 at δ = 6.35 (1H, q J = 7.2 Hz) and H-19 at δ 6.23 (1H, qq J = 7.0, 1.5 Hz) coupling with H-14 at δ = 2.03 (3H, d J = 7.2 Hz) and H-20 at δ = 2.02 (3H, d J = 7.0 Hz) resp. and a singlet for H-15 at δ = 4.25 (2H,s), which corresponded for acyclic diesters of sarracinic and angelic acids. The strong downfield chemical shifts of the protons H-3_d, H-3_u, H-5_d, H-5_u, H-7, H-8 resp. at δ = 4.3 (1H,m), δ = 4.15 (1H,m), δ = 4.5 (1H,m), δ = 3.85 (1H,m), δ = 5.81 (1H,bs), δ = 4.8 (1H,m) were characteristic for N-oxide. The ΣJ_7 is 12.2 Hz.

The ^{13}C NMR spectrum exhibited signals at 131.7, 125.4 for the olefinic C-12, C-18 and 16.0 (C-14), 64.5 (C-15), 15.7 (C-20), 20.7 (C-21) which confirmed the sarracinic and angelic moiety.

2 was reduced with Zn/HCl to it's corresponding base 8-episarracine (**3**). The latter was identified by MS and ^{1}H NMR spectra. MS of **3** contained M^+ at *m/z* 337(1), and fragments at *m/z* 237(27), 222(30), 138(100), 122(84), 82(68). The ^{1}H NMR spectrum of **3** is similar to that of sarracine and differed only by the chemical shifts of some of the protons (see Table I). The ΣJ_7 is 10.3 Hz, while for sarracine it is 6.5 Hz. All these data confirmed the structure of **2** as 8-episarracine N-oxide.

The phytochemical investigations were supplemented after a small part of CAM was subjected to GC/MS analyses (Table II).

The results from GC/MS analysis corresponded with our phytochemical investigations. Besides the already described alkaloids for this species, other three: 7,9-diangeloylplatynecine, 8-episarracine and 8-epineosarracine could be identified by their MS and R_t.

In our previous phytochemical investigations 7-,9-diangeloylplatynecine was identified in other two studies, from overground parts and stems and was not found in leaves and flowers, while 8-epi-

Table I. ^{1}H NMR data for compounds **1**, **2** and **3** in $CDCl_3$, δ in ppm, (J in Hz).

Atom	**1**	**2**	**3**
1	2.99 m	2.98 m	3.14 m
2	2.2–2.3*m	2.1*; 2.5 m	2.05–2.25*
3u	2.85*m	4.15 m	2.95*m
3d	3.65*m	4.3*m	3.72*m
5u	2.85*m	3.85*m	2.95*m
5d	3.65*m	4.5*m	3.72 m
6	2.2–2.3*m	2.3*m; 2.9 m	2.05–2.25*
7	5.43bs; $\nu_{1/2}$8.3	5.81bs; $\nu_{1/2}$12.2	5.63bs; $\nu_{1/2}$10.3
8	3.96 m	4.8 m	4.03 m
9u	4.19dd (11.0,8.3)	4.4dd (11.0,6.5)	4.15 m
9d	4.38dd (11.0,7.3)	4.4*	4.39dd (11.0,7.6)
11			
12			
13	6.10 m	6.35q (7.2)	6.34 bq (8.4)
14	1.97 m	2.03[a] d (7.2)	1.98 m
15	1.88 m	4.25s	4.15s
17			
18			
19	6.16qq (7.25,1.4)	6.23qq (7.0,1.5)	6.19qq (8.4,1.0)
20	2.02 m	2.02[a] d (7.0)	1.98 m
21	1.92 m	1.91d (1.5)	1.88t(1)

- * Overlapped signals. The coupling constants not observed.
- [a] The values could be interchanged.

Table II. Retention time and mass spectral data of pyrrolizidine alkaloids identified from the roots of *S. macedonicus* by GC/MS analyses.

Alkaloid	Retention time [min]	$[M]^+$	Characteristic ion *m/z* [%]
9-Angeloylretronecine	19.00	237(2)	55(13),80(16),83(8),93(100),108(7),126(8),138(37),154(19),193(6),219(1)
7-Angeloylplatynecine	19.48	239(1)	55(16),68(4),82(100),108(10),122(5),139(85),156(70)
9-Angeloylplatynecine	20.35	239(7)	55(20),67(4),82(100),95(93),108(3),122(10),139(11),156(8),221(13)
7-,9-Diangeloylplatynecine	26.01	321(1)	55(28),67(3),82(50),95(30),108(6),122(37),138(100),221(10),236(1)
Sarracine	30.26	337(1)	55(18),82(45),95(28),108(5),122(46),138(100),140(35),222(20),237(14),254(2)
Neosarracine	30.45	337(1)	55(16),82(35),95(36),108(5),122(27),138(100),140(28),222(16),237(2),254(1)
8-Episarracine	30.67	337(1)	55(17),82(41),95(36),108(6),122(35),138(100),140(36),222(23),237(16),254(1)
8-Epineosarracine	30.90	337(1)	55(23),82(56),95(36),108(8),122(57),138(100),140(46),222(23),237(16),254(1)

sarracine and 8-epineosarracine were presented only in the roots. (Christov, *unpubl. data*).

Some members of the group of pyrrolizidine alkaloids have been tested for biological activity on murine lymphocytes and found to be toxic and with suppressive effect on the proliferation (Deyo, *et al.*, 1994; Deyo and Kerkvliet, 1991; Deyo and Kerkvliet, 1990). As could be seen from Tables III, IV the 9-angeloylplatynecine and the mixture of sarracine and neosarracine were not toxic for normal mouse spleen lymphocytes at doses lower than 250 ng/ml and for mouse myeloma P3U1 cells at doses lower than 200 ng/ml. We next performed a test of these alkaloids on the proliferation of normal and myeloma mouse lymphocytes. As Table V shows the 9-angeloylplatynecine and mixture of sarracine and neosarracine had a stimulatory effect on unstimulated mouse spleen cells compared to the control. Applied to PHA stimulated spleen lymphocytes the mixture from sarracine and neosarracine had a prominent costimulatory effect on the lymphocyte proliferation (Table V). It was very important to test these alkaloids also on pathologically dividing cells to find out whether they have the same stimulatory effect. We tested 9-angeloylplatynecine and the mixture of sarracine and neosarracine on murine myeloma cells P3U1 and as Table VI shows they did not exhibit such a stimulatory effect. We may conclude that the 9-angeloylplatinecine and the mixture of sarracine and neosarracine alkaloids could be considered as potential immunomodulators.

Table III. Cytotoxicity of 9-angeloylplatynecine and mixture of sarracine and neosarracine (1:3 w/w) on normal mouse spleen lymphocytes (in percent viable cells).

Alkaloids	10 μg	1 μg	500 ng	250 ng	125 ng	62 ng	31 ng	16 ng	8 ng	4 ng	2 ng	1 ng
9-Angeloyl platinecine	48	–	28	71	76	59	55	56	58	73	74	75
Mixture of sarracine and neosarracine	23	78	74	70	77	85	82	–	95	95	94	95

The samples were diluted in 96° ethanol and the viability of the control-cells in 96° ethanol was 70%.

Table IV. Cytotoxicity of 9-angeloylplatynecine and mixture of sarracine and neosarracine (1:3 w/w) on mouse myeloma P3U1 (in percent viable cells).

Alkaloid	400 ng	200 ng	100 ng	50 ng	25 ng	12.5 ng	6.25 ng	3.12 ng	1.06 ng	0.53 ng
9-Angeloyl platynecine	68	88	89	94	92	95	93	94	96	95
Mixture of sarracine and neosarracine	91	92	92	94	85	88	90	85	80	80

The samples were diluted in 96° ethanol and the viability of the control-cells in 96° ethanol was 70%.

Table V. Effect of 9-angeloylplatinecine and mixture of sarracine and neosarracine (1:3 w/w) on the proliferation of unstimulated and PHA stimulated mouse spleen lymphocytes (in cpm).

	Unstimulated		PHA stimulated	
Dose in ng per 10^5 cells in 200 μl	9-Angeloyl platynecine	Mixture of sarracine and neosarracine	9-Angeloyl platynecine	Mixture of sarracine and neosarracine
8	11197 ± 1230	8633 ± 1154	4711 ± 789	5400 ± 698
4	10226 ± 894	11216 ± 1562	5908 ± 697	12087 ± 1348
2	13216 ± 1457	13421 ± 1896	11224 ± 2569	34870 ± 2698
	control 5159 ± 594		control 13082 ± 1456	

Table VI. Effect of 9-angeloylplatynecine and mixture of sarracine and neosarracine (1:3 w/w) on the proliferation of mouse myeloma P3U1 (in cpm).

Dose in ng per 10^5 cells in 200 μl	9-Angeloylplatynecine	Mixture of sarracine and neosarracine
16	16670 ± 2596	27372 ± 3654
8	29091 ± 3695	36916 ± 3254
4	37883 ± 3789	42385 ± 1892
2	36573 ± 5962	43927 ± 4966
	Control 44532 ± 5896	

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