Secondary metabolites of *Stenactis annua* L.

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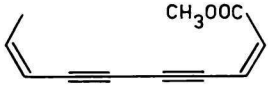
Matricaria ester, α-spinasterol, pyromeconic acid, and apigenin were isolated from flowers of *Stenactis annua*. Leaves were shown to contain dicaffeoylquinic acid, erigeroside, and pyromeconic acid. A procedure for determination of erigeroside was worked out; its content in dry leaves was found to be 6 mass %. Nitrogen-containing analogues of erigeroside were synthesized and their cytotoxicity was evaluated on leukemia P-388 cells; the highest activity in this test was shown by apigenin.

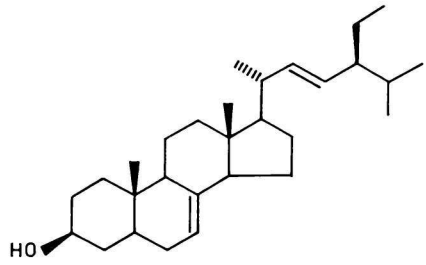
In our preceding paper [1] we reported a noticeable effect of matricaria ester *I* on leukemia P-388 cells. This compound shows also allelopathic [2] and insecticide effects [3] and therefore, this might be one of the factors conditioning the considerable ecosystem expansion of plants of the *Asteraceae* family to which *Stenactis annua* L. (NEES) belongs [4]. This was the reason why we focused our attention on identification of further secondary metabolites of this plant in addition to the already known ester *I*.

Fresh flowers of *S. annua* were extracted with dichloromethane; the concentrated extract afforded matricaria ester *I* [1] and α-spinasterol (*II*) [5, 6] by chromatographic purification and crystallization. Chromatography of the methanolic extract yielded compound *III*, identical with pyromeconic acid (4-hydroxy-4*H*-pyran-4-one) already identified in flowers of some plants of the *Erigeron* genus [7] and a flavonoid equal to apigenin [8, 9].

The dried ground leaves of *S. annua* extracted with dilute ethanol were concentrated and crystallized to give compound *V*; its mass spectral fragmentation pattern resembled that of pyromeconic acid (*III*). Tetraacetate *VI*, prepared by acetylation of *V*, revealed fragmentation of an acetylated hexose in its molecule. This information backed by the ^{1}H and ^{13}C NMR data (Table 1) allowed to ascribe the structure of 3-(β-D-glucopyranosyloxy)-4*H*-pyran-4-one to compound *V* The glucoside *V* was isolated from leaves of *Erigeron ramosus* [10] and denominated erigeroside. Mother liquor after removal of erigeroside (*V*) was chromatographed on a silica gel-packed column; work-up of the main fraction gave compound *VII*. Its mass spectrum displaying peaks at $m/z = 336$



I



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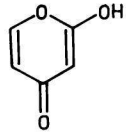
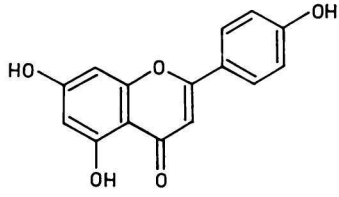


III



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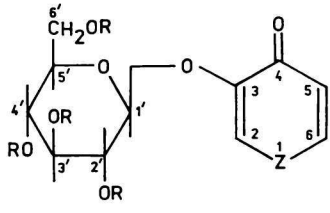
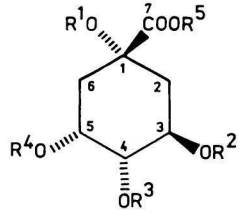


V	R = H	Z = O
VI	R = $COCH_3$	Z = O
XI	R = H	Z = NH
XII	R = H	Z = N—NH_2



	R^1	R^2	R^3	R^4	R^5	R^6
VII	H	Cf	H	Cf	H	H
VIII	CH_3CO	Cf	CH_3CO	Cf	H	CH_3CO
IX	H	Cf	H	Cf	CH_3	CH_3
X	H	Cf	Cf	H	CH_3	CH_3

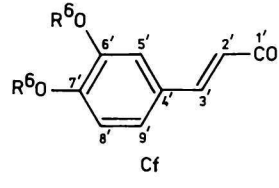


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Table 1

^{13}C NMR chemical shifts δ (CD_3OD, relative to TMS) of 4*H*-pyran-4-one and 4(1*H*)-pyridone derivatives

Position of carbon	*III*	*V*	*XI*	*XII*
2	140.0	147.1	131.5	126.2
3	146.3	147.3	146.9	147.6
4	173.2	177.0	171.6	173.3
5	114.2	116.9	115.8	116.7
6	155.4	158.9	141.6	137.1
1′	—	102.4	102.6	102.6
2′	—	74.0	73.9	74.0
3′	—	76.3	76.4	76.3
4′	—	70.4	70.6	70.5
5′	—	77.5	77.6	77.5
6′	—	61.6	61.8	61.7

Table 2

^{1}H NMR chemical shifts δ(CD_3OD, relative to TMS) and coupling constants (J/Hz) of esters of (-)-quinic acid *VII* and *VIII*

Position of hydrogen	*VII*	*VIII*
2a	2.20 (m)	2.42 (m)
2b	2.00 (m)	2.00 (m)
3	5.20 (m)	5.64 (m)
4	3.87 (dd, 6.7, 3.2)	5.17 (dd, 9.8, 3.4)
5	5.25 (m)	5.65 (m)
6a	2.20 (m)	2.74 (m)
6b	2.00 (m)	2.20 (m)
2′	6.23, 6.19 (d, 15.9)	6.33, 6.40 (d, 15.9)
3′	7.51, 7.48 (d, 15.9)	7.65, 7.59 (d, 15.9)
5′	7.08, 7.07 (d, 1.6)	7.38, 7.36 (d, 1.6)
8′	6.81, 6.80 (d, 8.1)	7.23, 7.20 (d, 8.0)
9′	7.00, 6.98 (dd, 8.1, 1.6)	7.42, 7.39 (dd, 8.0, 1.6)

(M − 180), 180, 163, 136, and 110 indicated the presence of caffeic acid in the structure. The ^{1}H NMR spectrum of compound *VII* (Table 2) disclosed couples of signals in the δ = 6—8 region the multiplicity of which corresponded to two caffeoyl residues. This presumption was proved by ^{13}C NMR data [11, 12]. Hydrolysis of compound *VII* and acetylation of the hydrolyzate yielded caffeic acid diacetate and (-)-quinic acid tetraacetate; the latter was previously isolated from the mixture of saccharides obtained from the pericarp of horse chestnut

Table 3

^{13}C NMR chemical shifts δ (CD_3OD, relative to TMS) of esters of (-)-quinic acid *VII*, *IX*, and *X*

Position of carbon	*VII*	*IX**	*X**
1	72.7	75.0	75.6
2	36.1	38.7	39.4
3	70.8	71.7	67.1
4	68.0	70.4	74.9
5	70.9	72.6	69.1
6	34.9	35.8	37.3
7	175.4	174.8	174.4
1′	166.1, 165.6	166.8, 167.1	166.4, 166.8
2′	115.9, 115.9	115.4, 115.4	115.2, 115.2
3′	145.1, 144.7	145.7, 145.9	145.5, 145.8
4′	125.8, 125.7	127.4, 127.5	127.2, 127.4
5′	114.8, 114.3	110.1, 110.2	109.7, 109.7
6′	145.6, 145.6	149.6, 149.6	149.2, 149.2
7′	148.2, 148.3	151.6, 151.6	151.3, 151.3
8′	121.4, 121.2	122.8, 122.8	122.9, 122.9
9′	114.9, 114.9	111.3, 111.3	110.0, 110.0

* Taken from Ref. [13], measured in $CDCl_3$.

Table 4

Effect of compounds isolated and erigeroside analogues on the incorporation of precursors of nucleic acids and proteins synthesis into the leukemia P-388 cells

Compound*	Inhibition of incorporation/%		
	^{14}C-Valine	^{14}C-Thymidine	^{14}C-Uridine
I	55.9	57.7	56.7
III	+35.0	+33.0	12.1
IV	45.0	61.0	96.0
V	+62.0	+38.0	+5.0
VII	+34.0	+12.0	+1.0
XI	+6.0	+22.0	+18.0
XII	+7.0	+21.0	+13.0

* Concentration 100 $\mu g\,cm^{-3}$, + — incorporation increase.

seeds [13]. Comparison of chemical shifts of ^1H NMR (Table 2) and ^{13}C NMR (Table 3) spectra of compounds *VII* and *VIII* with those of derivatives of quinic acid *IX* and *X* [14] enabled us to ascribe the structure of 3,5-dicaffeoylquinic acid to compound *VII*.

4*H*-Pyran-4-ones react with hydrazine to yield pyrazole derivatives [15].

Erigeroside (*V*) easily reacted with hydrazine and amines, too. Heating of *V* with ammonium hydroxide afforded 3-(β-D-glucopyranosyloxy)-4(1*H*)-pyridone (*XI*). This compound could theoretically exist in an enol form, *i.e.* as glucosylated 4-hydroxypyridine; nevertheless, the presence of a signal of the carbonyl group conjugated with double bonds ($\delta = 177.0$) in the ^{13}C NMR spectrum of *XI* excluded this possibility. Erigeroside (*V*) reacted with hydrazine to give 1-amino-3-(β-D-glucopyranosyloxy)-4(1*H*)-pyridone (*XII*).

Erigeroside (*V*) was determined in the drug by liquid chromatography. It was found that between 0.005—1.0 mg cm^{-3} the dependence of the erigeroside concentration on the peak area was linear with the regression coefficient $r = 0.998$. The experimental accuracy estimated by iterative determination of erigeroside was (5.49 ± 0.27) %, standard deviation $s = 0.22$, relative standard deviation $s_r = 4.05$ % (for $n = 7$, $\alpha = 0.05$). The amount of erigeroside in samples of leaves gathered in various locations varied within 4.1 and 5.8 mass %, stems contained 1.2 to 2.1 mass % of erigeroside; on the other hand, compound *V* was not found in flowers.

Cytotoxic effect of compounds isolated from *Stenactis annua* L. and of their synthetic analogues was tested on leukemia P-388 cells [16]. The greatest inhibitory effect on incorporation of precursors of proteins and nucleic acids synthesis were shown by apigenin (*IV*) and matricaria ester *I*. While the ester *I* had approximately equal effect on the synthesis of DNA and RNA, apigenin (*IV*) suppressed the synthesis of RNA more remarkably. The remaining compounds under investigation did not disclose an inhibitory effect, but on the contrary, stimulated the incorporation of precursors into the leukemia cells.

Experimental

Melting points were measured on a Kofler micro-hot stage, the UV and IR spectra were recorded with Specord UV VIS (Zeiss, Jena) and Perkin—Elmer, model 983 spectrophotometers, respectively. Mass spectra were taken with a Jeol JMS 100 D apparatus at ionization electron energy 70 eV, the ^1H and ^{13}C NMR spectra were run with a Bruker AM 300 instrument operating at 300 and 75 MHz, respectively, tetramethylsilane being the reference substance. Erigeroside was estimated by means of liquid chromatography using 150 mm × 3 mm column packed with Separon SGX C18 7 μm, mobile phase methanol—water ($\varphi_r = 5:95$); flow rate of the mobile phase 0.4 cm^3 min^{-1}, detector wavelength 254 nm. Silufol UV_{254} sheets were used for thin-layer chromatography in the following systems: toluene—ethyl acetate ($\varphi_r = 95:5$, S_1), chloroform—methanol ($\varphi_r = 9:1$, S_2), chloroform—methanol—water ($\varphi_r = 14:6:0.6$, S_3).

Isolation of flower constituents from S. annua

Fresh flowers (200 g) of *S. annua* were extracted with dichloromethane in a Soxhlet apparatus for 5 h. The extract was concentrated, the residue was chromatographed on a silica gel-packed column by elution with hexane—ethyl acetate (φ_r = 1 1) and the fraction of $R_f = 0.75$ (S_1) was concentrated and crystallized from hexane. Yield = 125 mg of compound *I*, which, according to m. p. and spectral data was identical with matricaria ester [1]. Work-out of the fraction of $R_f = 0.38$ (S_2) and crystallization from methanol yielded compound *II*, m. p. = 171—172 °C, m. p. of the acetate = = 180—182 °C, [α](D, 20 °C, ϱ = 10 g dm^{-3}, chloroform) = −3° ^{1}H NMR spectrum ($CDCl_3$), δ: 5.13 (dd, 1H, C-23—H), $J_{22,23}$ = 15.2 Hz, $J_{23,24}$ = 8.3 Hz; 5.03 (dd, 1H, C-22—H), $J_{20,22}$ = 8.3 Hz; 5.16 (m, 1H, C-7—H); 3.59 (m, 1H, C-3—H); 1.02 (d, 3H, C-21—H), $J_{20,21}$ = 6.6 Hz; 0.81 (s, 3H, C-19—H); 0.55 (s, 3H, C-18—H). Mass spectrum, *m/z* (I_r/%): 412, M^+ (40), 397 (8), 394 (10), 369 (8), 299 (12), 271 (100), 255 (80).

The remaining drug after extraction with dichloromethane was taken into methanol, the extract was concentrated and the residue was chromatographed over silica gel by elution with chloroform—methanol (φ_r = 4 : 1) to afford compound *III* from fraction of $R_f = 0.48$ (S_3). Yield = 180 mg, m. p. = 114—115 °C (diethyl ether—acetone, φ_r = 1 1). For $C_5H_4O_3$ (M_r = 112.1) w_i(calc.): 53.58 % C, 3.60 % H; w_i(found): 53.45 % C, 3.67 % H. IR spectrum (KBr), $\tilde{\nu}$/cm^{-1}: 3414 (O—H), 3003, 2927 (C—H), 1630 (C=C, C=O), 1566, 1457. ^{13}C NMR spectrum is given in Table 1. Mass spectrum, *m/z* (I_r/%): 112, M^+ (100), 86 (2), 84 (18), 71 (21), 69 (20), 58 (19), 55 (40).

Crystallization of the residue after removal of solvents from fraction of $R_f = 0.18$ (S_3) furnished the flavonoid *IV* (37 mg) identical according to melting point, thin-layer chromatography, UV and IR spectra with apigenin.

Isolation of erigeroside (V)

Dried ground leaves of *S. annua* (100 g) were successively extracted with diethyl ether (250 cm^3) and ethanol (700 cm^3). The ethanolic extract was evaporated to 50 cm^3; acetone was added (50 cm^3) and the crystallized compound *V* (3.9 g) was filtered off; m. p. = 196—197 °C. For $C_{11}H_{14}O_8$ (M_r = 274.2) w_i(calc.): 48.18 % C, 5.14 % H; w_i(found): 48.02 % C, 5.22 % H. IR spectrum (KBr), $\tilde{\nu}$/cm^{-1}: 3317 (O—H), 2977, 2926 (C—H), 1656, 1638 (C=O, C=C). ^{1}H NMR spectrum (CD_3OD), δ: 8.32 (d, 1H, C-2—H), $J_{2,6}$ = 0.8 Hz; 8.09 (dd, 1H, C-6—H), $J_{5,6}$ = 5.6 Hz; 6.52 (d, 1H, C-5—H); 4.73 (dd, 1H, C-1′—H), $J_{1',2'}$ = 7.4 Hz; 3.92 (dd, 1H, C-6′—H_A), $J_{6'A,6'B}$ = 12 Hz, $J_{5',6'A}$ = = 2.0 Hz; 3.66 (dd, 1H, C-6′—H_B), $J_{5',6'B}$ = 6.1 Hz; 3.2—3.5 (m, 4H, C-2′—H, C-5′—H). Mass spectrum, *m/z* (I_r/%): 165 (3), 144 (3), 126 (2), 112 (100), 86 (8), 84 (20), 71 (30), 69 (32).

Mass spectrum of erigeroside tetraacetate (*VI*), *m/z* (I_r/%): 368 (1), 331 (29), 270 (6), 256 (3), 211 (7), 169 (100), 127 (20), 109 (80).

3,5-Dicaffeoylquinic acid (VII)

Mother liquor after removal of erigeroside (*V*) was concentrated and chromatographed over silica gel applying gradient elution with chloroform—methanol (φ_r = 9:1—1:2). Fraction of R_f = 0.38 (S_3) afforded compound *VII*. For $C_{25}H_{24}O_{12}$ (M_r = 516.5) w_i(calc.): 58.19 % C, 4.69 % H; w_i(found): 58.02 % C, 4.52 % H. ^{1}H and ^{13}C NMR spectral data are listed in Tables 2 and 3. Mass spectrum, m/z (I_r/%): 336 (4), 313 (2), 298 (4), 282 (5), 186 (8), 180 (7), 163 (12), 145 (12), 136 (100), 110 (44).

3-(β-D-Glucopyranosyloxy)-4(1H)-pyridone (XI)

Erigeroside (*V*, 200 mg) was heated in methanol—ammonium hydroxide (φ_r = = 3:1, 10 cm^3) for 2 h, the mixture was concentrated and the residue was crystallized from water to furnish compound *XI*. Yield = 112 mg, m. p. = 259—260 °C. For $C_{11}H_{15}NO_7$ (M_r = 273.2) w_i(calc.): 48.35 % C, 5.53 % H, 5.12 % N; w_i(found): 48.25 % C, 5.42 % H, 5.02 % N. IR spectrum (KBr), $\tilde{\nu}$/cm^{-1}: 3365 (O—H, N—H), 2955, 2899 (C—H), 1628 (C=C). ^{1}H NMR spectrum (CD_3OD), δ: 7.72 (d, 1H, C-2—H); 7.63 (dd, 1H, C-6—H), $J_{2,6}$ = 1.3 Hz, $J_{5,6}$ = 7.0 Hz; 6.42 (d, 1H, C-5—H); 4.66 (d, 1H, C-1′—H), $J_{1',2'}$ = 7.4 Hz; 3.76 (dd, 1H, C-6′—H_A), $J_{6'A,6'B}$ = 12.0 Hz, $J_{5',6'A}$ = 2.0 Hz; 3.49 (dd, 1H, C-6′—H_B), $J_{5',6'B}$ = 6.1 Hz; 3.2—3.5 (mm, 4H, C-2′—H, C-5′—H). ^{13}C NMR spectrum is presented in Table 1. Mass spectrum, m/z (I_r/%): 111 (100), 110 (6), 94 (9), 83 (24), 73 (18), 69 (12).

1-Amino-3-(β-D-glucopyranosyloxy)-4(1H)-pyridone (XII)

Erigeroside (*V*, 200 mg) was heated in the mixture hydrazine hydrate (80 %)—ethanol (φ_r = 1 10, 10 cm^3) for 1 h, the material was concentrated and the residue was crystallized from water. Yield = 190 mg of substance *XII*, m. p. = 240 °C (decomp.). For $C_{11}H_{16}N_2O_7$ (M_r = 288.2) w_i(calc.): 45.84 % C, 5.60 % H, 9.71 % N; w_i(found): 45.70 % C, 5.65 % H, 9.62 % N. IR spectrum (KBr), $\tilde{\nu}$/cm^{-1}: 3342 (N—H, O—H),2921, 2879 (C—H), 1648, 1617 (C=C). ^{1}H NMR spectrum (CD_3OD), δ: 7.72 (d, 1H, C-2—H), $J_{2,6}$ = 2.5 Hz; 7.64 (dd, 1H, C-6—H), $J_{5,6}$ = 7.4 Hz; 6.30 (d, 1H, C-5—H); 4.63 (d, 1H, C-1′—H), $J_{1',2'}$ = 7.4 Hz; 3.73 (dd, 1H, C-6′—H), $J_{6'A,6'B}$ = 12.1 Hz, $J_{5',6'A}$ = 2.0 Hz; 3.49 (dd, 1H, C-6′—H_B), $J_{5',6'B}$ = 6.1 Hz; 3.2—3.5 (m, 4H, C-2′—H, C-5′—H). ^{13}C NMR spectrum is listed in Table 1. Mass spectrum, m/z (I_r/%): 186 (21), 159 (27), 144 (18), 126 (58), 111 (100), 83 (38), 73 (81).

Determination of erigeroside (V)

Dried ground leaves of *S. annua* (700 mg) were heated in methanol—water (φ_r = = 1 1, 50 cm^3) for 1 h. The suspension was filtered, the solid remaining on the filter was

three times washed with the extraction mixture (10 cm^3), the filtrates were combined and diluted with water to 100 cm^3 A portion of this solution (7 μdm^3) was injected into the chromatograph. The reference solution contained erigeroside (8.5 mg) in 25.00 cm^3 of the extraction mixture mentioned above.

References

1. Proksa, B., Uhrín, D., and Fuska, J., *Pharmazie 41,* 703 (1986).
2. Kobayashi, A., Morimoto, S., Shibata, Y. Yamashita, K., and Numata, M., *J. Chem. Ecol. 6,* 119 (1980).
3. Binder, R. G., Chan, B. G., and Elliger, C. A., *Agric. Biol. Chem. 43,* 2467 (1979).
4. Tronvold, G. M., Nestvold, M., Holme, D., Sörensen, J. S., and Sörensen, N. A., *Acta Chem. Scand. 7,* 1375 (1953).
5. King, L. C. and Ball, C. D., *J. Am. Chem. Soc. 64,* 2488 (1942).
6. Abraham, R. J. and Monasterios, J. R., *J. Chem. Soc., Perkin Trans. 2 1974,* 662.
7. Imai, K. and Mayama, T. J., *J. Pharm. Soc. Jpn. 73,* 128 (1953).
8. Šorm, F., Čekan, Z., Herout, V., and Rašková, H., *Chem. Listy 46,* 308 (1952).
9. Van Loo, D., DeBruyn, A., and Buděšínský, M., *Magn. Reson. Chem. 24,* 879 (1986).
10. Plouvier, V., *Compt. Rend. 258,* 1099 (1964).
11. Gering-Ward, B. and Junior, P., *Planta Med. 55,* 75 (1989).
12. Soicke, H., Al-Hassan, G., and Görler, K., *Planta Med. 54,* 175 (1988).
13. Proksa, B., Vadkerti, A., and Belan, J., *Pharmazie 46,* 135 (1991).
14. Timmermann, B. N., Hoffmann, J. J., Jolad, S. D., Schram, K. H., Klenck, R. F., and Bates, R. B., *J. Nat. Prod. Lloydia 46,* 365 (1983).
15. Ainsworth, C. and Jones, R. G., *J. Am. Chem. Soc. 76,* 3172 (1954).
16. Fuska, J., Miko, M., and Drobnica, Ľ., *Neoplasma 18,* 631 (1971).

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