Experimental

1. Stereoisomers of 4-fluoroglutamate

Individual stereoisomers were prepared by resolution of racemates through diastereomeric salts [6].

2. Enzyme preparation and chemicals

GAD from *Escherichia coli* (Sigma, 4.5 mg prot./ml suspension, 45 units/mg prot.) was used as the source of the enzyme.
The protein content was determined following the method of Lowry [8].
Chemicals used in GAD assay: L-1-^{14}C-Glutamate (Radiochemical Centre Amersham, specific activity 1.65 MBq · mmol^{-1}), pyridoxal-5′-phosphate (Koch-Light Labs.), 1,4-dithiothreitol (DTT, Merck), Bray's scintillation cocktail(Spolana, Neratovice). All chemicals used were at least of analytical grade.

3. Enzyme assay

The incubation mixture contained the enzyme, substrate L-glutamate-1-^{14}C, coenzyme pyridoxal-5′-phosphate, 1,4-dithiothreitol and the tested compound similarly as in [10] and was buffered to pH 5.0 (pH optimum for GAD from *E. coli*). The mixture was incubated at 37 °C for 30 min and the radioactivity of $^{14}CO_2$ liberated from the mixture and trapped by means of 0.1 ml of 30% KOH was measured in a dioxane scintillation cocktail using a 1219 Rackbeta scintillation counter LKB Wallac (Radioisotope Laboratory, Faculty of Pharmacy, Charles University, Hradec Králové).

Acknowledgement: This work was supported by the Charles University Grant 126/96.

References

1 Martin, D. L.; Rimvall, K.: J. Neurochem. **60**, 395 (1993)
2 Tunnicliff, G.: Int. J. Biochem. **22**, 1235 (1990)
3 Unkeless, J. C.; Goldman, P.: Mol. Pharmacol. **6**, 46 (1970)
4 Hudlický, M.: Coll. Czech. Chem. Commun. **26**, 1414 (1961)
5 Unkeless, J. C.; Goldman, P.: Mol. Pharmacol **7**, 293 (1971)
6 Tolman, V.; Šimek, P.: 30th Conference "Advances in organic, bioorganic and pharmaceutical chemistry", 6–8 November 1995, Liblice, Czech Rep., Abstracts p. 71.
7 Netopilová, M.; Dršata, J.; Ulrichová, J.: Pharmazie **51**, 589 (1996)
8 Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J.: J. Biol. Chem. **193**, 265 (1951)

Received March 3, 1999
Accepted April 27, 1999

Prof. Dr. Jaroslav Dršata
Charles University Prague
Faculty of Pharmacy
50005 Hradec Králové
Czeck Republic

Department of Pharmacognosy and Botany[1], Pharmaceutical Faculty, Comenius University, Bratislava, Slovak Republic and Institute of Organic Chemistry and Biochemistry[2], Academy of Sciences of the Czech Republic, Prague, Czech Republic

Triterpenoid saponins from *Cynara cardunculus* L.

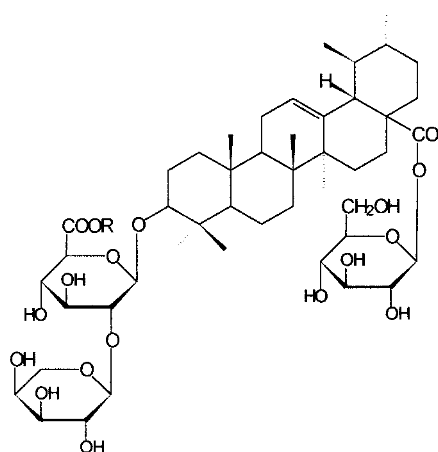
P. Mučaji[1]; D. Grančai[1], M. Nagy[1], M. Buděšínský[2] and K. Ubik[2]

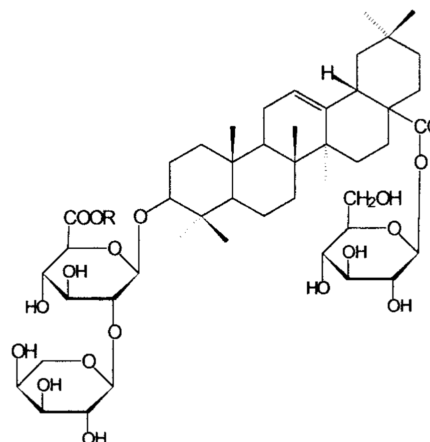
Cynara cardunculus L. (Asteraceae) is a plant occasionaly cultivated for the leaves which are blanched and eaten as a vegetable (cardoon). In folk medicine it is often used for the treatment of hepatic insufficiency [1]. Previous chemical investigation of involucral bracts of this plant established the occurence of sterols and triterpenoids [2], cynarine and coumarins [3–4] and flavonoids [5–6]. From the roots of *Cynara cardunculus* L. seven ursane-type saponins (cynarasaponins A–G) and three oleanane-type saponins (cynarasaponins H–J) have been isolated [7]. Now, we describe the isolation and identification of the triterpenoid saponins cynarasaponin A (**1b**) and cynarasaponin H (**2b**) and its methyl esters (**1a, 2a**) from the involucral bracts of this plant.
NMR spectra of the sample A (**1a** + **2a**) show the mixture of two structurally similar compounds in a ratio of ca. 43 : 57. The character of spectra indicates the presence of three saccharide units and a triterpenoid as an aglycone. Heavy overlap of proton multiplets in the ^{1}H NMR spectrum makes its detailed analysis difficult. On the other hand, the ^{13}C NMR spectrum shows resolved peaks for the most of carbon atoms. The comparison of our ^{13}C NMR data (Table) with literature data [7] proves that two components correspond to the methyl esters of cynarasaponin A and cynarasaponin H (**1a** + **2a**) containing oleanolic and ursolic acid, respectively, as aglycone. MS (see Experimental) are in agreement with the proposed structures.
Also NMR spectra of the sample B (**1b** + **2b**) showed the mixture of two components in a similar ratio (ca. 45 : 55). The ^{1}H and ^{13}C NMR spectra are very similar to those of the above discussed sample A (see Table). The only significant difference – the absence of a methoxy group signal – allowed to suggest the structures of cynarasaponin A and cynarasaponin H (**1b** + **2b**) as components of the mixture. The change from $COOCH_3$ to COOH is accompanied by a downfield shift of corresponding carbonyl signal in the ^{13}C NMR spectrum (from $\delta \sim 170.5$ to ~ 176.5). The suggested structure was supported by MS results. Standard FAB-MS and linked scans showed that the aglycone is substituted by both one hexose and by a chain consisted of one pentose and glucuronic acid.
Additional evidence for structures **1a** + **2a** and **1b** + **2b** provided the MS and NMR spectra of the aglycone, prepared by hydrolysis of sample B with HCl (see Experimental). Its EI-MS is in a good agreement with known spectra of oleanolic and ursolic acid [8]. The ^{1}H NMR spectrum has confirmed a mixture of ursolic and oleanolic acid in the ratio 45 : 55 (for data – see Experimental) by comparison with ^{1}H NMR spectra of authentic samples.

Experimental

1. Equipment

MS were measured on a ZAB-EQ mass spectrometer (Micromass, Manchester, U.K.) using both electron ionization (EI) for aglycon and fast atom bombardment (FAB) ionization with a magic bullet matrix and Xe at 8 kV as a bombarding gas. Daughter ion linked scans at B/E = const. and parent





1b R=H (cynarasaponin A)

1a R=CH_3

2b R=H (cynarasaponin H)

2a R=CH_3

Table: ^{13}C NMR data of the mixtures of cynarasaponin A and H (1b + 2b) and of their methyl esters (1a + 2a) in d_5-pyridine

Position	Sample A (**1a** + **2a**)	Sample B (**1b** + **2b**)	Position	Sample A (**1a** + **2a**)	Sample B (**1b** + **2b**)
	Aglycone moiety			Saccharide units	
1	39.20; 38.80	~39.59	**Glc:**		
2	26.76; 26.70	26.67	**1′**	95.80; 95.83	95.84; 95.81
3	89.36	89.16; 89.13	**2′**	74.13; 74.20	74.15
4	39.43; 39.60	~39.59	**3′**	79.26; 79.38	79.29; 79.41
5	55.95; 55.91	55.96; 55.91	**4′**	71.30; 71.20	71.29; 71.19
6	18.56	18.52	**5′**	78.91; 78.93	78.96
7	33.62; 33.21	~33.20	**6′**	62.40; 62.30	62.38; 62.29
8	40.20; 39.98	40.17; 39.96	**Glc-UA:**		
9	48.09	48.05	**1″**	105.46; 105.42	105.44; 105.39
10	36.90; 37.01	36.97	**2″**	83.37; 83.32	83.69; 83.62
11	23.85; 23.72	23.47	**3″**	76.82	76.58
12	126.20; 122.93	126.26; 122.99	**4″**	73.71	73.84
13	138.49; 144.24	138.50; 144.25	**5″**	77.40	77.81
14	42.59; 42.22	42.58; 42.21	**6″**	170.57	176.42
15	28.74; 28.33	28.72; 28.32	**COOMe**	52.22; 52.19	–
16	24.75; 23.49	24.75; 23.80	**Ara:**		
17	48.48; 47.10	48.46; 47.08	**1‴**	106.72; 106.69	106.86; 106.82
18	53.41; 41.83	53.41; 41.81	**2‴**	72.92	73.13
19	39.60; 46.29	39.19; 46.26	**3‴**	74.30	74.29
20	39.60; 30.84	39.42; 30.83	**4‴**	69.19	69.23
21	30.89; 34.08	30.83; 34.06	**5‴**	67.08	67.09
22	36.84; 32.61	36.86; 32.61			
23	27.98	28.02			
24	15.79; 15.63	15.78; 15.62			
25	16.50; 16.48	16.51			
26	17.45; 17.54	17.43; 17.51			
27	23.85; 26.19	23.82; 26.19			
28	176.38; 176.60	176.63			
29	17.71; 33.21	17.70; 33.20			
30	21.38; 23.72	21.36; 23.70			

ion linked scans at B^2/E = const., respectively, were used to confirm some fragmentation pathway. NMR spectra were recorded on a FT NMR spectrometer Varian UNITY-500 (^{1}H at 500 MHz; ^{13}C at 125.7 MHz frequency) in d_5-pyridine and/or d_4-methanol. 2D-COSY spectra were measured for the structural assignment of coupled protons. The "attached proton test" ^{13}C NMR spectra were used to distinguish signals of CH_3, CH_2, CH and quarternary carbon atom.

For CC silica gel (Silpearl Kavalier Votice) was used. TLC was carried out on UV 254 or UV 366 plates and silica gel $60F_{254}$ glass plates (Merck).

2. *Plant material*

Involucral bracts of *Cynara cardunculus* L. were collected in the Medicinal Plants Garden in Bratislava. A voucher specimen was deposited at the Pharmaceutical Faculty, Comenius University, Bratislava.

3. *Extraction and isolation*

Fresh involucral bracts (12 kg) were cut into pieces and repeatedly extracted with EtOH at room temperature. The concentrated EtOH extract (326.5 g) in aqueous suspension was partitioned successively with $CHCl_3$ (45 g), EtOAc (15 g) and *n*-BuOH (71.5 g), leaving a residual H_2O extract (195 g). A part of the *n*-BuOH fraction (15 g) was dissolved in MeOH and subjected to CC over silica gel (600 g) using EtOAc/MeOH mixtures of increasing polarity as eluents. A total of 370 fractions (150 ml) were collected. Fractions showing similar TLC profiles were pooled to provide 13 combined fractions. Fractions 7–11 was chromatographed over Sephadex LH-20 column (60 g) using i-PrOH and i-PrOH/MeOH mixtures in different ratio to obtain 105 Fractions (100 ml) which were combined according to TLC to 9 fractions. fraction 2 was further separated by silica gel CC using $CHCl_3/MeOH/C_6H_6$ (7 : 2 : 1) mixture. Total of 190 fractions (2 ml)

were collected with an automatic fraction collector. From this separation, sample A (compounds **1a**, **2a**) and sample B (compounds **1b**, **2b**) were isolated.

4. Spectroscopic characteristics

Sample A (**1a** + **2a**): Standard FAB-MS, m/z (% rel. int.): 963 (6) $[M + Na]^+$, 801 (3) $[M + Na - Glc]^+$, 475 (6), 439 (3) $[aglycone + H - H_2O]^+$, 413 (55), 391 (100). Daughter ion linked scan for precursor $[M + Na]^+$ at m/z 963 has shown m/z 801 $[M + Na - Glc]^+$ and m/z 755 $[M + Na - Glc - HCOOH]^+$. ^{13}C NMR data – see Table.

Sample B (**1b** + **2b**): Standard FAB-MS, m/z (% rel. int.): 949 (23) $[M + Na]^+$, 787 (6) $[M + Na - Glc]^+$, 641 (5) $[M + Na - Ara - GlcA]^+$, 623 (6), $[M + Na - Ara - GlcA - H_2O]^+$, 439 (100) $[aglycone + H - H_2O]^+$. Daughter ion linked scan for precursor $[M + Na]^+$ at m/z 949 has shown m/z 641 $[M + Na - Ara - GlcA]^+$ and for precursor $[aglycone + H - H_2O]^+$ at m/z 439 has shown 421 $[aglycone + H - 2H_2O]^+$, 393 $[Aglycone + H - H_2O - HCOOH]^+$. Parent ion scan for daughter $[aglycone + H - H_2O]^+$ at m/z 439 has given m/z 457 $[aglycone + H]^+$ which is not visible in the standard spectrum. ^{13}C NMR data – see Table.

Sample B aglycone: El-MS, m/z (% rel. int.): $M^{+\cdot}$ 456 (3), 248 (100), 207 (21), 203 (46), 189 (13), 133 (17). ^1H NMR (CD_3OD, ursolic acid/oleanolic acid, 45 : 55): 5.23 t/5.24 t (H-12); 3.16 dd/3.14 dd (H-3); 2.20 bd/2.85 bdd (H-18); 1.120 /1.162 s (Me-26); 0.977 s/0.974 s (23-Me); 0.965 d/0.942 s (29.Me); 0.961 s/0.946 s (27-Me); 0.886 d/0.909 s (Me-30); 0.850 s/0.818 s (Me-25); 0.779 s/0.778 s (Me-24).

5. Acid hydrolysis

Sample B (10 mg) was dissolved in MeOH (2 ml) and refluxed with 11% HCl for 3 h. The aglycone was extracted with $CHCl_3$, purified on the silica gel column (eluent C_6H_6 : acetone 95 : 5) and used for the MS and ^1H NMR spectra.

Acknowledgement: This work was supported by Scientific Grant Agency of Ministry of Education of Slovak Republic. Project No. 1/5212/98.

References

1 Čižmář, J.: Lidové lékařství v Československu, vol. 2, p. 184, Melantrich, Brno 1946 (Traditional medicine in Czechoslovakia)
2 Grančai, D.; Nagy, M.; Suchý, V.; Ubik, K.: Farm. Obzor **61**, 577 (1992)
3 Grančai, D.; Nagy, M.; Suchý, V.; Novomeský, P.: Fitoterapia **65**, 282 (1994)
4 Grančai, D.; Nagy, M.; Mučaji, P.; Suchý, V.; Ubik, K.: Farm Obzor **63**, 447 (1994)
5 Grančai, D.; Nagy, M.; Suchý, V.; Ubik, K.: Farm. Obzor **62**, 31 (1993)
6 Grančai, D.; Nagy, M.; Mučaji, P.; Ubik, K.: Farm. Obzor **64**, 255 (1996)
7 Shimizu, S.; Ishihara, N.; Umehara, K.; Miyase, T.; Ueno, A.: Chem. Pharm. Bull. **36**, 2466 (1988)
8 McLafferty, F. W.: Wiley Registry of Mass Spectral Data, 6th Ed., 1995, Spectra No. 208397 and 208378

Received February 11, 1999
Accepted April 10, 1999

Mgr. Pavel Mucaji, PhD
Dept. of Pharmacognory
Odbojarov 10
832 32 Bratislava
Slovak Republic