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Triterpenoid saponins of *Bellis perennis*.

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Summary: Ten saponins have been isolated from the deacylated saponin mixture of *Bellis perennis* L. The structures of these saponins can be divided into two groups that show differences both in the aglycone and the carbohydrate moieties bound at C-28. The first group consists of glycosides of polygalacic acid with tri- or tetrasaccharide moieties consisting mainly of deoxyhexoses and a second group of glycosides of bayogenin or asterogenic acid which possess glucose as major carbohydrate moiety and one xylose or rhamnose unit. From the most polar fraction of the alkaline hydrolysate obtained from the underground parts of *Bellis perennis* one saponin possessing only glucose residues has been obtained. On the basis of a sugar methylation analysis and NMR spectral data, the structure was established as 3-*O*-ß-D-glucopyranosyl-2ß,3ß,23-trihydroxyolean-12-en-28-oic acid 28-*O*-ß-D-glucoyranosyl-(1→2)-[ß-D-glucopyranosyl(1→6)]-ß-D-glucopyranoside.

Key words: *Bellis perennis*, Asteraceae, triterpenoid saponins, bayogenin, asterogenic acid, polygalacic acid.

Bellis perennis L., the common daisy, is a small perennial herb that is native to almost the whole of Europe. It is used especially in folk medicine in the treatment of various diseases, such as rheumatism, and as an expectorant [1]. In previous papers we have reported the isolation and structure elucidation of nine saponins from the alkaline hydrolysates of the major saponin fractions obtained from the underground and aerial parts of *B. perennis* [2-6]. TLC of the saponin mixtures indicated that there were still saponins in the nonpolar and polar part which have not as yet been identified. In this concluding study, we have investigated the nonpolar and polar saponin fractions obtained from the underground parts. The results of these studies are reported in this paper which resolves the question as to the identity of these final compounds. Additionally, results obtained in former studies are summarised.

Two compounds (**1** and **2**) were obtained from the nonpolar part of the saponin fraction [3] by repeated column chromatography on silica gel as detailed in the experimental part. On the basis of the ESI-MS, ^{1}H and ^{13}C NMR and COSY spectra compound **1** was identified as polygalacic acid (2ß,3ß,16α,23-tetrahydroxyolean-12-en-28-oic acid) and compound **2** as 1-*O*-butyl-rhamnopyranoside.

Compound **3** was obtained from the polar fraction by repeated column chromatography on silica gel. The COSY spectrum showed three characteristic spin systems indicative of a 2ß,3ß-hydroxy-olean-12-ene type aglycone: H-12 (δ 5.38) - H-11A,B (δ 2.08, 1.99) - H-9 (δ 1.60); H-3 (δ 4.40) - H-2 (δ 3.69) - H-1A,B (δ 2.10, 1.27); H-18 (δ 2.89) - H-19A,B (δ 1.74, 1.27). The presence of four anomeric

proton signals indicated that **3** was a triterpenoid glycoside. Sugar component analysis afforded glucose only. The electrospray ionisation mass spectra showed a molecular ion at m/z 1135 $[M-H]^-$ in the negative ion mode and at m/z 1159 $[M+Na]^+$ in the positive ion mode. MS-MS of the ion at m/z 1159 gave an ion at m/z 509 $[hex+hex+hex+Na]^+$ as most intense daughter ion. These data indicated that **3** was a bisdesmosidic saponin with an aglycone of 488 Dalton molecular weight. These data also showed that the trisaccharide unit was attached to the aglycone by an ester linkage while the remaining hexose was attached by an *O*-glycosidic linkage. Comparison of the ^{13}C NMR data with those of saponins previously obtained from *B. perennis* indicated that 3,28-glycosylated bayogenin (2ß,3ß,23-trihydroxyolean-12-en-28-oic acid) was the aglycone of **3**. A poor signal/noise ratio prevented rigorous assignment of the ^{13}C NMR data of the carbohydrate moiety. However, as glucose was the only carbohydrate component, the structure of the carbohydrate moiety could be identified on the basis of the methylation analysis data alone. In this experiment terminal glucose and 2,6-linked glucose were obtained in a 3:1 ratio. Thus, the trisaccharide attached to the aglycone in position 28 has a glucopyranosyl(1→2)-[glucopyranosyl(1→6)]-glucopyranoside. H-1–H-2 coupling constants of about 8 Hz indicated that all glucose units were bound ß-glycosidically. On the assumption that glucose was present in the pyranose form as the common D-enantiomer, the structure of compound **3** named bellissaponin BS9 was 3-*O*-ß-D-glucopyranosyl-2ß,3ß,23-trihydroxyolean-12-en-28-oic acid 28-*O*-ß-D-glucoyranosyl-(1→2)-[ß-D-glucopyranosyl(1→6)]-ß-D-glucopyranoside.

As mentioned above, studies presented in this paper are based on the finding that TLC indicated that saponins are also present in the nonpolar and polar parts of the deacylated saponin mixture obtained from *B. perennis*. In the present study we have shown by isolation and structure elucidation of compounds from the nonpolar fraction of underground parts that the saponin-like behaviour is caused by the presence of the aglycone, polygalacic acid, and an O-alkylated sugar. In contrast, a true saponin (**3**) was obtained from the polar part. However, this compound was isolated in very small amounts although intense spots were present in the TLC. These findings indicated that the majority of the polar part of the saponin fraction consisted of compounds of other structural types displaced into the saponin fraction.

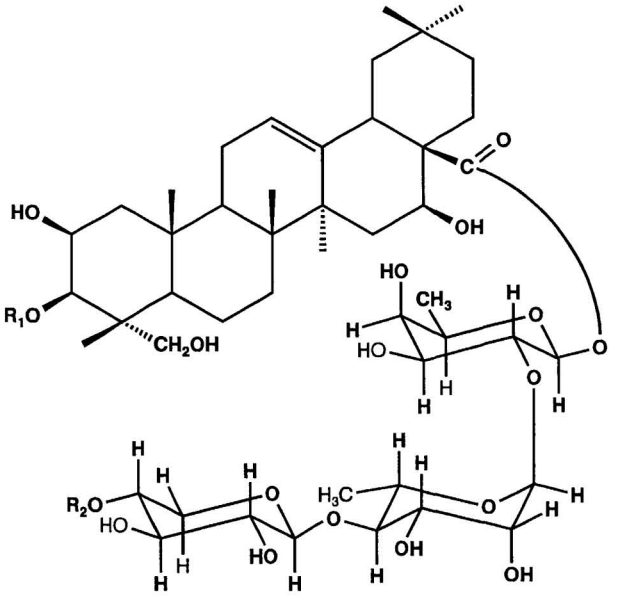
As discussed previously [2], saponins of *B. perennis* can be divided in two groups: glycosides of polygalacic acid with a linear sugar chain attached to C-28 as major saponins and glycosides of bayogenin or asterogenic acid with a branched sugar chain with a central glucose attached to C-28 as minor saponins (Figure). Although glucose was the predominant sugar in all of the glycosides of bayogenin, bellissaponin BS9 is the first compound obtained from the whole *Bellis* genus which contained only glucose and, in addition, is a novel triterpenoid saponin. It is closely related to the three other bayogenin-containing saponins (BS5 – BS7) [6] found in *B. perennis* where a terminal rhamnose or xylose moiety has been substituted for a glucose unit found in BS9.

Materials and methods.

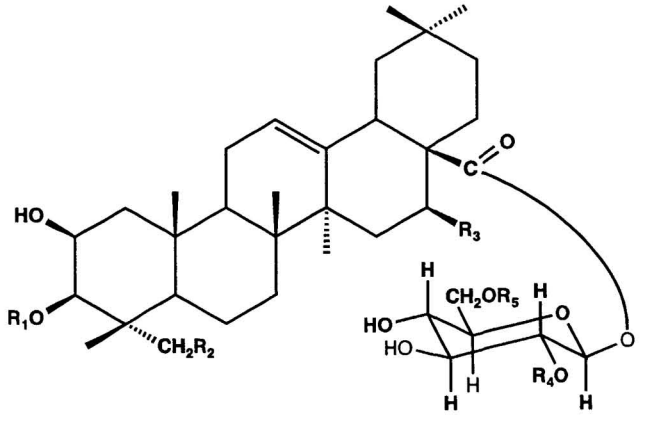
General experimental procedures

^1H and ^{13}C NMR spectra were recorded at 300° K on Bruker DPX-300 (compounds **1** and **2**) or DMX-600 NMR spectrometers (compound **3**). MS spectra were obtained on a Finnigan TSQ 700 equipped with a Finnigan electrospray source [ESIMS and MS-MS] and a Finnigan CGQ ion trap mass spectrometer (GC-MS). CC was performed on Diaion HP-20 (Mitsubishi Chemicals) and on

Figure: Structures of deacylsaponins of *Bellis perennis* L.



Compound	R_1	R_2
Bellissaponin BS1	α-L-rhap	α-L-rhap
Bellissaponin BS2	ß-D-glcp	ß-D-glcp
Bellissaponin BS3	ß-D-glcp(1-->3)-ß-D-glcp	α-L-rhap
Besysaponin C_{12}	α-L-rhap	H



Compound	R_1	R_2	R_3	R_4	R_5
Bellissaponin BS4	ß-D-glcp	H	OH	α-L-rhap	ß-D-glcp
Bellissaponin BS5	ß-D-glcp	OH	H	ß-D-xylp	ß-D-glcp
Bellissaponin BS6	ß-D-glcp	OH	H	α-L-rhap	ß-D-glcp
Bellissaponin BS7	α-L-rhap	OH	H	ß-D-glcp	ß-D-glcp
Bellissaponin BS8	α-L-rhap	OH	H	H	ß-D-glcp
Bellissaponin BS9	ß-D-glcp	OH	H	ß-D-glcp	ß-D-glcp

silica gel 60, 0.063-0.2 μm (Merck), preparative HPLC on LiChrosorb RP-18 (Merck), 250 × 10 mm I. D., 7μm, flow rate 8.00 mL/min, UV-detection at 206 nm. TLC was carried out on silica gel 60 plates (Merck) with $CHCl_3$-MeOH-H_2O, 7:4:1 (solvent system I) or EtOAc-MeOH-H_2O, 100:17:13 (solvent system II). Anisaldehyde-H_2SO_4 (anisaldehyde 0.5 mL, H_2SO_4 5.0 mL, HOAc 10.0 mL, MeOH 100 mL) was used as the visualisation reagent.

Plant material

Plants were collected during May 1987 in a meadow located at Templin (Brandenburg, Germany). The material was dried at 50-60 °C. A voucher specimen is deposited at the herbarium of the Institute of Pharmacy, Humboldt-University, Berlin (number Scho-1).

Isolation

A crude glycoside mixture was obtained from the MeOH-extract of the underground parts of *B. perennis* by n-BuOH extraction and ether precipitation as described previously [3]. The crude glycoside mixture was separated by Sephadex LH-20 column chromatography using MeOH as solvent into a major and a minor saponin fraction. An aliquot of 1.45 g of the minor saponin fraction was chromatographed on 150 g silica gel with CH_2Cl_2-MeOH (7:0.2→0-100%). Fractions having identical composition on TLC (solvent system I) were combined. Finally, they were purified on LiChrolut RP-18 extraction columns (Merck) using H_2O-MeOH (0→100%) to give 4 mg of compound **1** and 8 mg of compound **2**.

Another aliquot of 1.46 g of the minor saponin fraction was subjected to Diaion HP-20 CC and eluted successively with water, 50% MeOH and methanol. The methanol eluate was concentrated to dryness and chromatographed on 140 g silica gel 60 with EtOAc-MeOH-H_2O (110:17:13) as mobile phase. Fractions showing one major spot at R_f 0.04 on TLC (solvent system II) were further purified by reversed phase HPLC with MeOH-H_2O (55:45) to give 8 mg of compound **3**.

Quantitation of the Component Monosaccharides

Monosaccharides were analysed as the corresponding methyl glycosides after methanolysis and trimethylsilylation by GC/MS [7]. Monosaccharide ratios were determined by electronic integration of all relevant peaks of the total ion current chromatogram. GC conditions and instrument set-up were identical to those used for the methylation analysis (see below). Glucose: R_t = 14.24 and 14.34 min.

Methylation analysis of the sugar constituents

Aliquots of each compound (10 μg) were dissolved in 150μl of DMSO and methylated according to the method of Hakomori [8]. Purification of the permethylated sample, hydrolysis using trifluoroacetic acid and, reduction using $NaBH_4$ and acetylation using Ac_2O were performed as described [9]. All GC/MS analysis were performed on a Finnigan GCQ ion trap mass spectrometer running in the positive-ion EI mode equipped with 30 m capillary column. GLC conditions: Column DB-5 (J&W Scientific Inc., Folsom, CA, 30 m x 0.32 μm i.d., film thickness 0.1 μm,), temperature program, 3 min 80 °C, 10 °C per min to 300 °C. The respective partially methylated alditol acetates were identified by comparison with standard compounds, their characteristic EIMS fragments and their retention times: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (R_t = 13.29 min), 1,2,5,6-tetra-*O*-acetyl-3, 4-di-*O*-methylglucitol (R_t = 15:43 min).

Compound 1 was obtained as a white powder, TLC Rf 0.89 (solvent system I), ESIMS (negative ion mode) m/z 503 $[M-H]^-$, (positive ion mode) m/z 527 $[M+Na]^+$, ^1H NMR (CD_3OD) δ 5.38 (t, H-12),

4.41 (dd, H-16), 4.11 (ddd, H-2), 3.71 (d, J = 4.7 Hz, H-3), 3.54 (d, J = 11.0 Hz, H-23A), 3.29 (d, J = 11.0 Hz, H-23B), 3.18 (dd, J = 3.8/14.0 Hz, H-18), 2.18 (dd, H-19A), 2.11 (dd, J = 3.0/14.5 Hz, H-1A), 2.00 (H-11A,B), 1.96 (H-15A), 1.65 (H-9), 1.39 (H-15B), 1.18 (H-1B), 1.05 (d, H-19B), 1.38, 1.33, 1.02, 0.95, 0.92, 0.91 (6 x H_3, H_3-24, -25, -26, -27, -29, -30), ^{13}C NMR δ 45.4 (C-1), 72.3 (C-2), 73.6 (C-3), 43.2 (C-4), 51.0 (C-5), 18.9 (C-6), 33.8 (C-7), 41.0 (C-8), 37.9 (C-10), 24.6 (C-11), 122.8 (C-12), 145.8 (C-13), 42.7 (C-14), 36.1 (C-15), 75.8 (C-16), 42.8 (C-18), 31.3 (C-20), 36.6 (C-21), 33.6 (C-22), 67.6 (C-23), 14.1 (C-24), 18.4 (C-25), 17.7 (C-26), 27.7 (C-27), 33.6 (C-29), 26.3 (C-30).

Compound 2 was obtained as an amorphous substance , TLC Rf 0.90 (solvent system I), ^{1}H NMR (D_2O) δ 4.69 (d, J = 1.6 Hz, H-1), 3.81 (dd, J = 1.7/3.6 Hz, H-2), 3.66 (dd, J = 3.6/9.4 Hz, H-3), 3.40 (t, J = 9.4 Hz, H-4) 3.61 (dd, J = 9.4/6.2 Hz, H-5) 1.29 (d, J = 6.2 Hz, H_3-6), 3.73 (m, H-1A'), 3.46 (m, H-1B'), 1.60 (m, H-2'), 1.45 (m, H-3'), 0.98 (t, H_3-4'), ^{13}C NMR δ 101.6 (C-1), 72.5 (C-2), 72.4 (C-3), 74.0 (C-4), 69.7 (C-5), 18.0 (C-6), 68.2 (C-1'), 32.8 (C-2'), 20.5 (C-3'), 14.2 (C-4').

Compound 3 was obtained as an amorphous powder, TLC Rf 0.04 (solvent system II), ESIMS (negative ion mode) m/z 1135 $[M-H]^-$, (positive ion mode) m/z 1159 $[M+Na]^+$, 997 $[M-162+Na]^+$, MS-MS of 1159: m/z 673 $[M-162-162-162+Na]^+$, 509 $[162+162+162+Na]^+$, MS-MS of 997: m/z 673 $[M-162-162-162+Na]^+$, 509 $[162+162+162+Na]^+$, ^{1}H NMR (CD_3OD), aglycone: δ 5.37 (H-12), 4.40 (H-3), 3.69 (H-2), 2.87 (H-18), 2.08 (H-11A), 2.10 (H-1A), 1.99 (H-11B), 1.74 (H-19A), 1.60 (H-9), 1.27 (H-1B), 1.27 (H-19B), 1.31, 1.22, 1.02, 1.00, 0.98, 0.80 (6 x H_3, H_3-24, -25, -26, -27, -29, -30), anomeric protons of glucose: δ 5.60 (d, J = 7.8 Hz), 4.90 (d, J = 7.6 Hz), 4.53 (d, J = 7.7 Hz), 4.49 (d, J = 8.0 Hz), ^{13}C NMR: δ 43.6 (C-1), 71.2 (C-2), 83.8 (C-3), 42.1 (C-4), 48.3 (C-5), 18.1 (C-6), 33.2 (C-7), 40.2 (C-8), 49.5 (C-9), 36.5 (C-10), 25.1 (C-11), 123.1 (C-12), 144.1 (C-13), 42.1 (C-14), 29.5 (C-15), 23.9 (C-16), 47.2 (C-17), 41.5 (C-18), 46.1 (C-19), 30.8 (C-20), 34.0 (C-21), 32.2 (C-22), 64.7 (C-23), 14.0 (C-24), 17.2 (C-25), 17.2 (C-26), 24.1 (C-27), 178.5 (C-28), 33.6 (C-29), 26.8 (C-30).

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