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Triterpene glycosides from transformed root cultures of *Astragalus mongholicus* BGE

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Abstract. Transformed root cultures of *Astragalus mongholicus* Bge. have been established by infecting of sterile grown plants with *Agrobacterium rhizogenes* strains LBA 9402 and ATCC 15834. A promoting effect of acetyl syringone on the *Agrobacterium*-mediated hairy root (HR) initiation was observed. The transformed root clones were assayed for opines by electrophoresis and TLC. All of them were positive for agropine/mannopine. It was shown, that *Astragalus*-hairy roots (HR) of the transformed cultures produce the same kind of saponins in considerable amounts as those of intact plants. The triterpene saponins have been isolated from the methanol extract of the HR and identified as cycloartane derived saponins. On the basis of analytical data obtained by mass spectrometry (NI-FAB-MS, EI-MS), and homo- and heteronuclear NMR experiments (DEPT, DQF-COSY, HOHAHA, ROESY, HMQC, HMBC), the structures of three triterpenoid glycosides were determined as astragaloside I, II, III [1,2]. A complete assignment of the ^{1}H- and ^{13}C-NMR data is given.

1. Introduction

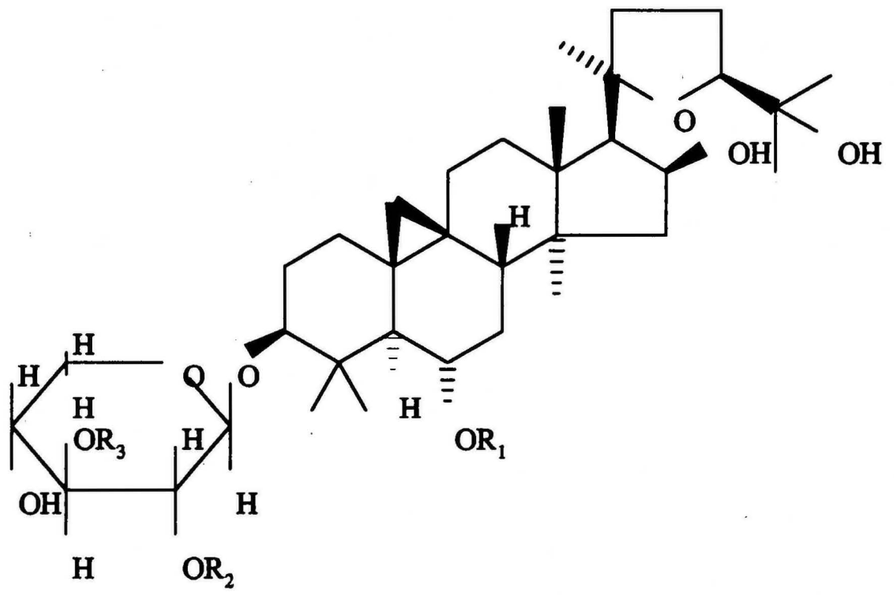
The saponins formed by *Astragalus* species are of great pharmaceutical interest. *Astragalus mongholicus* Bge. (*Fabaceae*) is a famous traditional Chinese medicinal plant. The roots of this plant, called *Huangqi*, have been widely used in China as an immunostimulant, tonic, antiperspirant or diuretic [3]. Since the properties of *Astragalus mongholicus* and *Astragalus membranaceus* are somewhat similar to those of the more expensive herb ginseng (*Panax ginseng*), they are used as a substitute for that species. Due to the increased demand for this plant and the limited spread in northeastern China, the Korean Peninsula and Japan, we have used hairy root cultures *in vitro* for production of secondary metabolites.

In this paper we describe the establishment of hairy root (HR) cultures of *Astragalus mongholicus* Bge., the isolation of triterpene glycosidic constituents, and the identification of the three major compounds F1, F2, F3 (Fig. 1) as astragaloside I, II, III [1,2] especially with 2D-NMR experiments. A complete assignment of the ^{1}H- and ^{13}C-NMR data is given.

2. Results and Discussion

Astragalus mongholicus Bge. was transformed by co-culture of the leaf-discs with *Agrobacterium rhizogenes* strains LBA 9402 and ATCC 15834. The addition of acetyl

syringone to the liquid medium of *Agrobacterium* resulted in increased transformation frequencies of *Astragalus mongholicus* Bge. The transformation was confirmed by the detection of opines produced in the HR. The opines were extracted and identified by paper electrophoresis and TLC (detection: Trevelyan reagent [4]). The tests of all transformed root clones were positive for agropine/mannopine, showing that the HR contained the appropriate enzymes from the plasmids responsible for opine synthesis. The roots of the parent plant did not synthesize opines.



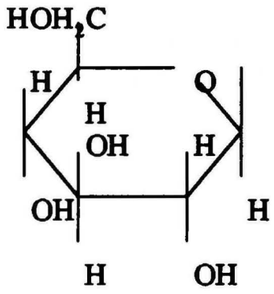
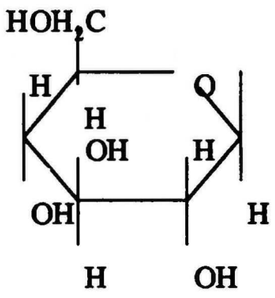
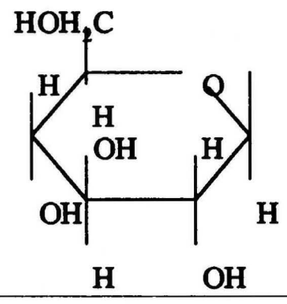
	R_1	R_2	R_3
F1 = astragaloside I		CH_3CO	CH_3CO
F2 = astragaloside II		CH_3CO	H
F3 = astragaloside III	H		H

Figure 1. Structures of saponins F1, F2, F3.

The crude saponin fractions (M1 and M2) derived from the methanol extract of HR were repeatedly chromatographed on silica gel columns to yield compounds F1 (120 mg), F2 (40 mg), F3 (60 mg). The compounds F1, F2, and F3 were identified as the saponins astragaloside I, II, III, respectively by spectroscopical means and degradation studies. After

heterogeneous acid hydrolysis of F1, F2, and F3 the carbohydrates xylose and glucose could be identified in each case by TLC.

NI-FAB-mass spectra exhibited $[M - H]^-$-ions with *m/z* 867 for F1, *m/z* 825 for F2, and *m/z* 783 for F3. For F3 a daughter scan of *m/z* 783 showed the daughter ions *m/z* 621 $[M - H - C_6H_{10}O_5]^-$, *m/z* 489 $[M - H - C_6H_{10}O_5 - C_5H_8O_4]^-$, and *m/z* 161 $[C_6H_{10}O_5 - H]^-$. A direct loss of xylose was not detected which was an indication that glucose was connected to xylose and not to the aglycone. EI-mass spectra of the three compounds showed mainly the fragment ions of their common aglycone cycloastragenol. Some characteristic ions are given in Table 1.

Structural information for F1, F2, and F3 was mainly obtained by 2D-NMR of their solutions in methanol-d_4. HOHAHA and DQF-COSY were used for identification of proton spin systems and evaluation of coupling constants, DEPT and HMQC for carbon assignments, while HMBC gave information about the surrounding of quarternary carbons. Proton distances were estimated by ROESY to gain informations about the absolute configuration of the different chiral centers. Based upon these experiments full assignment of proton and carbon resonances for the three compounds has been achieved as given in Table 2.

Identification of the aglycone: An AB-system ($|J|$ = 4.1-4.5 Hz) in the region 0.2-0.6 ppm of the proton spectrum, the proton singlets of seven methyl groups in the region 0.8-1.4 ppm, and the correspondence of carbon resonances with reference data [5,6] indicated cycloastragenol [5] as the common aglycone of the three compounds.

The absolute configuration of the chiral centers in rings A-D could be determined by the positions of the corresponding methine protons or methyl groups relative to C-19 (α: opposite, β: same side of the ring system). In the ROESY spectrum of compound F3, H-19b (which is the endo proton of the methylene bridge in cycloastragenol) shows intensive NOE cross peaks with H-6, H-8, H-30, and a weaker cross peak with H-18. No cross peak for H-19b/H-5 is observed. Therefore, H-6, H-8, and H-18 were assigned as β while H-5 had to be assigned as α. For ring A a cross peak for H-30/H-2a and NOE cross peaks for each combination of H-5, H-3, and H-1b are observed. Hereby H-3 could be assigned as α, standing in an axial position. The absolute configurations in ring C and D were defined by the NOE cross peaks for H-18/H-19b, H-18/H8, and cross peaks of H-28 with H-7a, H-11a, H-12b, H-15b, H-16, H-17. It follows that H-28, H-16, and H-17 are α-configured.

Table 1
EI-MS, typical fragment ions of the saponins F1, F2, F3.

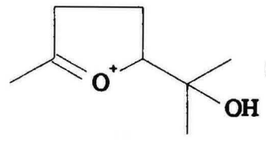
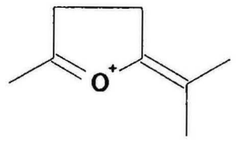
m/z	fragment
604	$[M - C_6H_{12}O_6]^{+\bullet}$ (F3)
586	$[M - C_6H_{12}O_6 - H_2O]^{+\bullet}$ (F3), $[M - C_6H_{12}O_6 - CH_3COOH]^{+\bullet}$ (F2)
472	$[\text{cycloastragenol} - H_2O]^{+\bullet}$
454	$[\text{cycloastragenol} - 2\ H_2O]^{+\bullet}$
439	$[\text{cycloastragenol} - 2\ H_2O - {}^{\bullet}CH_3]^{+}$
413	$[\text{cycloastragenol} - H_2O - {}^{\bullet}C(CH_3)_2OH]^{+}$
395	$[\text{cycloastragenol} - 2\ H_2O - {}^{\bullet}C(CH_3)_2OH]^{+}$
143	 (base ion)
125	

Table 2a. the aglycone

NMR data of the saponins F1(astragaloside I), F2 (astragaloside II), F3 (astragaloside III) in CD_3OD;

		proton resonances			carbon resonances		
atom	a)	F1	F2	F3	F1	F2	F3
1a	(β)	1.24	1.52	1.54	32.8	32.3	33.6
1b	(α)	1.53					
2a	(β)	1.63	1.63	1.69	29.8	30.3	30.6
2b	(α)	1.92	1.91	1.95			
3	(α)	3.18	3.14	3.22	90.0	90.2	90.5
4		-	-	-	42.4	42.8	43.3
5	(α)	1.60	1.59	1.36	52.6	53.2	54.6
6	(β)	3.51	3.51	3.46	79.9	80.1	69.5
7a	(α)	1.57	1.57	1.36	34.8	35.2	39.0
7b	(β)	1.91	1.90	1.46			
8	(β)	1.84	1.85	1.79	46.3	46.8	48.6
9		-	-	-	21.7	22.1	21.9
10		-	-	-	29.5	29.9	30.6
11a	(β)	1.32	1.32	1.21	26.7	27.0	27.0
11b	(α)	1.91	1.91	2.03			
12a	(β)	1.58	1.58	1.64	33.8	34.1	34.1
12b	(α)	1.66	1.66	1.67			
13		-	-	-	45.6	46.0	45.9
14		-	-	-	46.7	47.1	47.0
15a	(β)	1.38	1.38	1.40	45.8	46.2	46.8
15b	(α)	2.03	2.03	1.95			
16	(α)	4.64	4.64	4.65	74.2	74.7	74.6
17	(α)	2.35	2.35	2.35	58.4	58.9	59.1
18	(β)	1.24	1.24	1.26	21.2	21.4	22.0
19a	(exo)	0.26	0.25	0.38	29.6	29.7	32.2
19b	(endo)	0.57	0.57	0.54			
20		-	-	-	88.1	88.4	88.4
21	(α)	1.20	1.19	1.20	28.4 b)	28.4	28.5
22a	(α)	1.63	1.63	1.64	35.1	35.5	35.5
22b	(β)	2.61	2.61	2.60			
23a	(α)	2.01	2.01	2.03	26.6	26.8	26.8
23b	(β)	2.03	2.03	2.03			
24	(α)	3.75	3.75	3.75	82.2	82.6	82.6
25		-	-	-	72.2	72.5	72.4
26		1.11	1.11	1.12	26.6	26.6	26.7
27		1.25	1.25	1.25	27.5	27.6	27.6
28	(α)	1.00	1.00	0.99	20.1	20.2	20.5
29	(α)	1.19	1.18	1.31	28.1 b)	28.5	28.7
30	(β)	0.89	0.89	1.01	16.5	16.6	16.5

δ (ppm) relative to TMS; a) in brackets: configuration relative to C19; b) assignment might be exchanged.

TABLE 2b. the sugar moeities

NMR data of the saponins F1(astragaloside I), F2 (astragaloside II), F3 (astragaloside III) in CD_3OD; **b:** the sugar moieties

	proton resonances						carbon resonances		
atom-no.	F1	$^3J_{(i,i+1)}$	F2	$^3J_{(i,i+1)}$	F3	$^3J_{(i,i+1)}$	F1	F2	F3
1'	4.54	7.8	4.40	7.9	4.41	6.8	104.5	105.2	106.6
2'	4.77	9.6	4.70	9.5	3.56		73.2	75.7	81.0
3'	4.96	9.4	3.42	9.3	3.51		76.5	76.2	77.9
4'	3.69		3.52		3.48		68.8	71.3	71.1
5'a	3.30		3.19		3.18		66.2	66.9	66.5
5'b	3.89		3.83		3.84				
1"	4.31	7.8	4.31	7.8	4.65	7.7	104.2	104.9	104.7
2"	3.16		3.16		3.23		75.1	75.7	76.2
3"	3.31		3.32		3.35		78.1	78.6	77.9
4"	3.27		3.26		3.25		71.3	71.8	71.8
5"	3.24		3.24		3.25		77.0	77.7	78.3
6"a	3.64		3.64		3.63		62.7	63.0	63.1
6"b	3.83		3.83		3.83				
CH_3-Ac2'	2.00		2.08		-		21.0	21.3	-
CO-Ac2'							171.2	171.9	-
CH_3-Ac3'	2.03		-		-		20.9	-	-
CO-Ac3'							171.9	-	-

δ (ppm) relative to TMS; J (Hz); ') xylose; ") glucose.

The configuration at C-20 and C-24 was the remaining problem. H-24 shows NOE cross peaks with H-26, H-27 and H-21. The latter excludes the combinations 20-R/24-R and 20-S/24-S. A decision between 20-R/24-S and 20-S/24-R was possible based on the additional NOE cross peaks for H-21/H-17, H-21/H-22a, H-18/H-22a, H-18/H-22b, H-27/H-16 and on the absence of a cross peaks between H-17/H-22b and H-16/H-21. A 20-R/24-S configuration is in good agreement with these data when a nearly staggered anti-conformation of H-17 and C-22 is assumed. Hydrogen bridges between HO-16 and O-20 or HO-16 and HO-25 may stabilize this conformation. Contrary to this no conformation can be reconciled with the ROESY data when a 20-S/24-R configuration is assumed. The ROESY data of F1 and F2 allow the same conclusions.

Identification of the sugar components: The ^3J(H,H) coupling constants (Table 2b) and the comparison of the carbon resonances with reference data for methyl aldosides [7] confirm that each of the saponins F1-F3 has a xylopyranosyl and a glucopyranosyl moiety. In each case the chemical shifts of the anomeric carbons, NOE cross peaks of H-1' with H-3', H-5' and of H-1" with H-3", H-5", as well as ^3J(H-1',H-2'), ^3J(H-1",H-2") values in the range of *trans* couplings correspond to a β-D stereochemistry of the pyranosyl moieties.

For the saponin F3 a glycosidic linkage of the xylopyranosyl moiety to C-3 of the aglycon was deduced from a long range correlation H-1'/C-3 and NOE cross peaks of H-1' with H-3 and H-29 in the ROESY spectrum. Compared to the reference value (C-2 74.0 ppm) given for methyl-β-D-xylopyranoside [7] the carbon resonance of C-2' in F3 (81 ppm) is shifted by about 7 ppm to lower field. NOE's for H-1"/H-2', H-1"/H-3' and a cross peak for H-1"/C-2' in the HMBC revealed a glycosidic linkage of the glucopyranosyl moiety to C-2' of xylose.

For the saponin F2 a long range correlation H-1'/C-3 and NOE's for H-1'/H-3, H-1'/H-29 proved a glycosidic linkage of the xylopyranosyl moiety to C-3 of the aglycon exactly as for F3. Compared to F3 no glycosidation shift is observed for C-2' of xylose. Instead, C-6 of the aglycone is shifted about 10.6 ppm to lower field and a cross peak for H-1"/C-6 is found in the HMBC. Together with NOE cross peaks of H-1" with H-6, H-7b, H-29 all these data point to a glycosidic linkage of the β-D-glucopyranosyl moiety to C-6 of the aglycone. The proton and the carbon spectrum of F2 show the additional signals of an acetyl group. The relatively large frequency found for the proton resonance of H-2' (4.70 ppm) reveals that the xylopyranosyl moiety is acetylated at O-2'.

NMR data of the saponin F1 are similar to those of F2, but show an additional acetylation at the O-3' position of the xylopyranosyl moiety. The position of the acetyl group was deduced from the shift of the proton resonance of H-3' from 3.42 ppm (F2) to 4.96 ppm (F1), and a weak cross peak for H-3'/CO-(Ac3') in the HMBC.

3. Experimental

Plant material: Seeds of *Astragalus mongholicus* Bge. were surface sterilized (15 min) with Domestos (10%) and incubated on a MS solid medium [11] to obtain sterile plants. The formation of hairy roots was induced by co-cultivation of wounded leaf segments from sterile grown plants with the *Agrobacterium rhizogenes* strains LBA 9402 and ATCC 15834. These strains were incubated overnight in the presence of acetyl syringone (10 µl) in a YMB medium [12]. The explants were cultured on MS medium, with 1 g/l casaminoacids, 2% sucrose and solidified with 0.9% agar, without phytohormones (DoH). After 3 days, the explants were transferred to DoH solid medium supplemented with 500 mg/l sodium cefotaxim (Claforan^R, Hoechst AG, Frankfurt).

Culture condition: All cultures were kept at 25°C in the dark. A profusion of roots appears at the site of inoculation within 3-4 weeks. The roots were cultivated in 300 ml Erlenmeyer flasks containing 100 ml DoH medium on a gyratory shaker at 100 rpm in the dark. The roots were subcultured every 4 weeks.

Saponin extraction: After a growth period of about 28 days the root material (1480g FW) was filtered, lyophilized (54.920 g DW) and worked up as follows. The lyophilized roots were extracted 3 times with methanol (4.5 l) until the methanol extract became colourless. After concentrating i.v. the saponins were precipitated with acetone (1.5 l) under stirring. The precipitate (10.69 g) was dissolved in methanol and chromatographed on a silica gel column with chloroform/methanol/acetone/water 80:50:10:10 (v/v) and 16 fractions of 25 ml each were collected. The fractions 4-6 were re-chromatographed in the same way. From the newly collected fractions, 3-6 were re-chromatographed in the same way once more. The now collected fractions 1-5 were chromatographed with the lower phase of a mixture of chloroform/methanol/water 10:3:1 (v/v). In this way F1 (120 mg), F2 (40 mg) and (after further purification by preparative TLC using the same solvent system) F3 (60 mg) were obtained.

Heterogenous acid hydrolysis: A solution of F1 (15 mg), F2 (5 mg), and F3 (10mg), respectively, in ethanol (2.6 ml) was mixed with 10% HCl (2ml) and toluene (5.7 ml) and refluxed for 6 hrs. The reaction mixture was poured into ice water and extracted with ethyl acetate. This extract was washed and worked up as usual. From the aqueous layer after neutralisation the sugars were identified as xylose and glucose by TLC (acetonitrile/water/ acetic acid, 85:14:1 v/v).

Mass spectrometry: NI-FAB mass spectra were obtained with a FINNIGAN MAT HSQ 30 equipped with an ION TECH LTD. FAB gun (Xe). The saponins were dissolved in methanol. m-Nitro benzyl alcohol was used as FAB matrix. EI mass spectra were measured with a FINNIGAN MAT 212.

NMR spectroscopy: All NMR experiments were carried out with solutions of the compounds F1, F2, and F3 in methanol-d_4 at 298 K. MLEV17 HOHAHA [8] and ROESY [9] with CW-spin lock were performed on a BRUKER AMX 500 using UXNMR installed on an ASPECT X32 for acquisition and processing. The 2D-experiments were run in the phase sensitive mode using the time proportional phase increments scheme (TPPI) [10]. For each spectrum 512 experiments with 2048 data points and 8 scans were acquired. The spectral widths were 3012 Hz (6.02 ppm) in both dimensions. Mixing times were 40 ms for HOHAHA and 250 ms for ROESY. Time domain data in both dimensions were multiplied by a $\pi/3$ shifted squared sine-bell and zero filled to obtain 2048 x 512 real data points after Fourier transformation.

DEPT, DQF-COSY, inverse HMQC and HMBC were performed on a BRUKER AC 300 using DISNMR installed on an ASPECT 3000 for acquisition and processing. Acquisition parameters of the not decoupled HMBC were 512 experiments with 2048 data points, 4 dummy scans, and 48 scans per experiment. Spectral widths were 2000 Hz (6.66 ppm) in F2, and 12800 Hz (170 ppm) in F1. A 80 ms delay ($J_{C,H}$ = 6.3 Hz) was used for the evolution of long range couplings. Time domain data were multiplied by a sine-bell in both dimensions and zero filled to obtain a matrix of 2048 x 1024 real data points after Fourier transformation. A BIRD-sequence with GARP-decoupling during acquisition was used for the HMQC. The acquisition parameters were 128 experiments with 2048 data points, 4 dummy scans, 48 scans per experiment, and spectral widths of 2000 Hz (6.66 ppm) in F2 and 8390 Hz (111 ppm) in F1. A delay of 3.45 ms ($^1J_{C,H}$ = 145 Hz) was used for the evolution of direct ^1H-^{13}C couplings. Time domain data were multiplied in both dimensions by a $\pi/3$ shifted squared sine bell and zero filled to obtain a matrix of 2048 x 512 real data points after Fourier transformation.

4. Acknowledgements

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Abbreviations

BIRD, Bilinear Rotation Decoupling;
DEPT, Distortionless Enhancement by Polarisation Transfer;
DQF-COSY, Double Quantum Filtered Correlated Spectroscopy;
EI, Electron Impact;
HMBC, Heteronuclear Multiple-Bond Correlation;
HMQC, Heteronuclear Multiple-Quantum Correlation;
HOHAHA, Homonuclear Hartmann-Hahn;
HR, Hairy Root;
NI-FAB-MS, Negative Ion Fast Atom Bombardment Mass Spectrometry;
ROESY, Rotating Frame Nuclear Overhauser Effect Spectroscopy;
TLC, Thin Layer Chromatography.