166. A New Triterpene Saponin from *Heteropappus biennis* with an Unusual Carbohydrate Chain

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Dedicated to Prof. *W. Kubelka* on the occasion of his 60th birthday

(22.VI.94)

A new saponin, *O*-α-D-arabinopyranosyl-(1→6)-*O*-[α-L-rhamnopyranosyl-(1→2)]-*O*-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranosyl arjunolate (**1**) was isolated from the flowers of *Heteropappus biennis* (LDB.) TAMAMSCH. The structure was established mainly by a combination of 1D selective and 2D NMR techniques like COSY, TOCSY, ROESY, HMQC, and HMBC. Molecular-modelling calculations showed that the oligosaccharide chain is rather rigid. Six minimum structures are discussed.

1. Introduction. – *Heteropappus biennis* (LDB.) TAMAMSCH. is found as 'Lugtschung' in the Mongolian medicinal tract 'Baidur-Onbo' from the 17th century [1]. In the traditional Mongolian medicine, a tea is made from the above-ground parts of *Heteropappus biennis* against various diseases, especially against cold and bronchitis [2]. Three main saponins heteropappussaponins 5, 7, and 8, were obtained from these parts, and their isolation and structure elucidation were reported in a previous paper [3].

This paper deals with the isolation and identification of the fourth main saponin, heteropappussaponin 2 (**1**), with the lowest polarity and having completely different characteristics compared to the other main saponins: whereas the heteropappussaponins 5, 7, and 8 give green spots on TLC after detection with anisaldehyde reagent, **1** becomes blue. In contrast to heteropappussaponins 5, 7, and 8, nearly no haemolytic activity was found for **1** which is enriched in the flowering heads in comparison to stem and leaves.

2. Results and Discussion. – *Isolation and Structure of* **1**. Dried flowering heads of *Heteropappus biennis* were extracted with butan-1-ol. The saponins were precipitated from the extract by addition of Et_2O and purified by column chromatography over *Sephadex LH-20*. The saponin mixture was separated by MPLC on silica gel *60* giving pure **1**.

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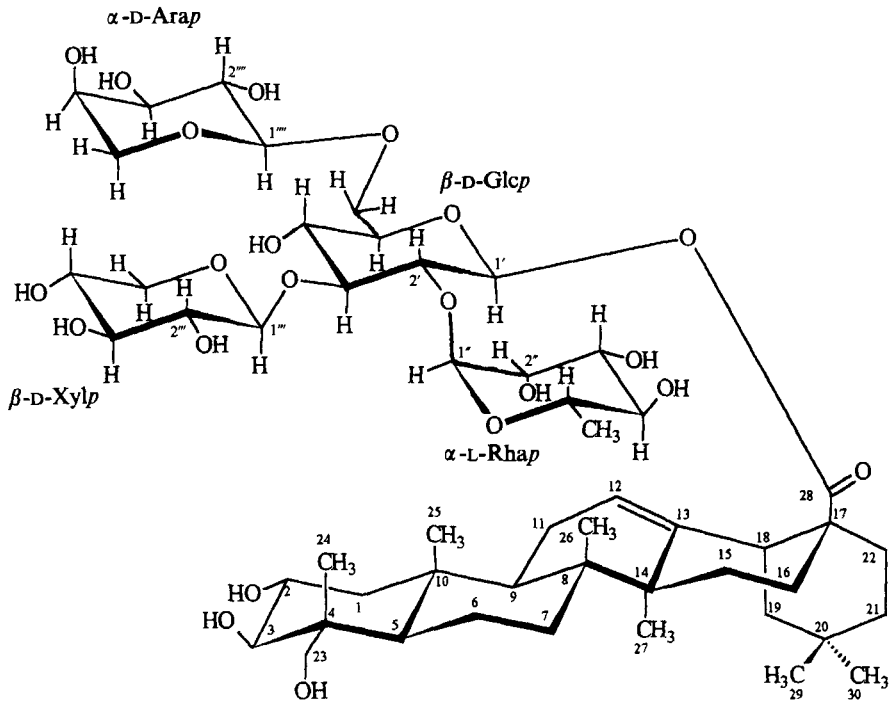

1 heteropappussaponin 2

The structure elucidation of the aglycone of **1** was achieved by a combined interpretation of HMBC, HMQC, TOCSY, and H,H-COSY measurements [4] [5]. The assignments and connectivities of protons were derived from a H,H-COSY experiment (see *Table 1*). These were confirmed by a TOCSY spectrum. Axially and equatorially oriented protons were assigned using the values of the vicinal-coupling constants. We could group the protons into 7 spin systems (CH_2(1)/H–C(2)/H–C(3), H–C(5)/CH_2(6)/CH_2(7), H–C(9)/CH_2(11)/H–C(12), CH_2(15)/CH_2(16), H–C(18)/CH_2(19), CH_2(21)/CH_2(22), CH_2(23)). Additionally 6 Me groups appeared in the ^{1}H-NMR spectrum. The connectivities between these spin systems were determined by long-range H,C couplings in a HMBC spectrum. From these, the constitution of the aglycone was derived. In our HMBC experiment, the delay was tuned to a coupling constant of 4 Hz, so that only two- and three-bond connectivities were detectable [6]. The relative configuration of the C-atoms bearing the Me groups could be determined by long-range couplings from the H,H-COSY spectrum. We observed several ^4J(H,H) and one ^5J(H,H) W-type couplings between aglycone protons (Me(26)/H_{ax}–C(7), Me(26)/H_{ax}–C(9), Me(26)/Me(27), Me(27)/H_{ax}–C(15), Me(29)/Me(30), Me(30)/H_{ax}–C(19), and Me(30)/H_{ax}–C(21)). Furthermore, a ^6J(H,H) 'through-space' coupling [7] (Me(24)/Me(25)) was found, representing a 1,3-*cis*-diaxial orientation between these Me groups. The relative configuration of C(18) could be derived by the characteristic proton shift of *H*–C(18) (δ 2.85) [5] [8], proving the *cis*-orientation to the carbonyl group C(28).

Table 1. *^{1}H- and ^{13}C-NMR Data of the Aglycone of* **1**, *the Arjunolic-Acid Moiety*

	δ (^{13}C) [ppm]	δ (^{1}H) [ppm][a])		δ (^{13}C) [ppm]	δ (^{1}H) [ppm][a])
CH_2(1)	48.0	0.95, 1.97	CH_2(16)	24.1	1.98, 1.68
H–C(2)	69.6	3.78	C(17)	48.0	
H–C(3)	78.6	3.55	H–C(18)	42.7	2.85
C(4)	44.0		CH_2(19)	47.3	2.63, 1.17
H–C(5)	48.4	1.34	C(20)	31.4	
CH_2(6)	19.1	1.32, 1.45	CH_2(21)	34.8	1.27, 1.11
CH_2(7)	33.4	1.54, 1.36	CH_2(22)	33.0	1.82, 1.55
C(8)	40.7		CH_2(23)	67.0	3.64, 3.32
H–C(9)	48.8	1.63	Me(24)	14.1	0.73
C(10)	39.0		Me(25)	17.8	0.97
CH_2(11)	24.6	1.89, 1.83	Me(26)	18.1	0.84
H–C(12)	123.3	5.23	Me(27)	26.3	1.07
C(13)	144.9		C(28)	177.5	
C(14)	43.0		Me(29)	33.5	0.81
CH_2(15)	29.1	1.69, 1.15	Me(30)	24.3	0.86

[a]) The first mentioned δ refers to the axial, the second to the equatorial H-atom.

The FAB-MS gave information about the molecular weight of the molecule and of the aglycone. A *retro-Diels-Alder* fragmentation of the aglycone ion led to a fragment *m/z* 247, as expected for olean-12-ene derivatives with 3 OH functions on rings A and/or B as well as a carboxylic group in ring D/E.

We could not obtain enough aglycone from the hydrolytic cleavage, to determine the optical rotation. Therefore, we assume the absolute configuration to be the same as given for arjunolic acid (= 2α,3β,23-trihydroxyolean-12-en-28-oic acid) in [9].

The determination of the structure of the complex carbohydrate chain was mainly based on modern NMR pulse experiments [4] [8]. The typical range of ^{13}C-NMR shifts of the anomeric C-atoms of monosaccharides between 90 and 110 ppm showed 4 signals, indicating 4 different monosaccharide units. H,H-COSY signals demonstrated the proton connectivities of the individual monosaccharides (see *Table 2*). The coupling constants were determined by 2D H,H-COSY and 1D TOCSY experiments. Using *Gaussian* pulses, the transitions of the anomeric protons of the individual monosaccharide units were selectively excited, and then the magnetization was transferred within one monosaccharide residue to H–C(2), H–C(3), H–C(4), H–C(5), and in case of Glc*p* to CH_2(6) depending on the mixing time used. The C-atoms were identified by correlations in a HMQC spectrum. A HMBC experiment proved the sequence of the carbohydrate chain. It showed 4 interglycosidic cross-peaks connecting the anomeric protons of one sugar unit with the linkage-site C-atom of the adjacent unit (*H*–C(1'''')(Ara)/C(6')(Glc), *H*–C(1''')(Xyl)/C(3')(Glc), *H*–C(1'')(Rha)/C(2')(Glc), and *H*–C(1')(Glc)/C(28)(aglycone)). The ROESY spectra (1D, 2D) confirmed the sequence of the carbohydrate chain and the positions of the sugar connectivity. In addition, ROE cross-peaks were found between not directly bonded monosaccharide units. This behaviour is not very common in oligosaccharides. When the transition of the anomeric proton *H*–C(1''') of the xylose residue was selectively excited, correlations to *H*–C(1'') and *H*–C(2'') of the not directly bonded rhamnose could be observed in the 1D ROESY spectrum (see *Fig. 1* and *Exper. Part*), proving that xylose and the rhamnose residue were close in space.

Table 2. *^{1}H- and ^{13}C-NMR Data of the Oligosaccharide Chain of* **1**

	δ (^{13}C) [ppm]	δ (^{1}H) [ppm]	J(H,H) Values[a] [Hz]		δ (^{13}C) [ppm]	δ (^{1}H) [ppm]	J(H,H) Values[a] [Hz]
β-D-Glucopyranose				β-D-Xylopyranose			
H–C(1′)	95.0	5.70	$^3J(1',2') = 7.3$	H–C(1‴)	105.0	4.62	$^3J(1''',2''') = 7.3$
H–C(2′)	77.3	3.90	$^3J(2',3') = 8.9$	H–C(2‴)	74.9	3.45	$^3J(2''',3''') = 8.9$
H–C(3′)	86.8	3.86	$^3J(3',4') = 9.6$	H–C(3‴)	78.0	3.54	$^3J(3''',4''') = 9.0$
H–C(4′)	69.7	3.72	$^3J(4',5') = 9.1$	H–C(4‴)	71.0	3.65	$^3J(4''',5''') = 9.9$
H–C(5′)	77.3	3.68	$^3J(5',6'a) = 5.4$	CH_2(5‴)	67.2	3.34, 3.96	$^3J(4''',5'''eq) = 5.3$
			$^3J(5',6'b) = 2.6$				$^2J(5'''ax,5'''eq) = 11.6$
CH_2(6)	69.6	4.15, 3.79	$^2J(6'a,6'b) = 11.7$				
				α-D-Arabinopyranose			
α-L-Rhamnopyranose				H–C(1⁗)	104.9	4.45	$^3J(1'''',2'''') = 6.6$
H–C(1″)	101,9	5.54	$^3J(1'',2'') = 1.5$	H–C(2⁗)	72.4	3.81	$^3J(2'''',3'''') = 8.6$
H–C(2″)	72.2	4.18	$^3J(2'',3'') = 3.3$	H–C(3⁗)	74.4	3.70	$^3J(3'''',4'''') = 3.5$
H–C(3″)	72.5	3.88	$^3J(3'',4'') = 9.4$	H–C(4⁗)	69.2	3.90	$^3J(4'''',5''''ax) = 4.6$
H–C(4″)	73.8	3.63	$^3J(4'',5'') = 9.4$	CH_2(5⁗)	66.4	3.53, 3.94	$^3J(4'''',5''''eq) = 3,4$
H–C(5″)	70.7	3.89	$^3J(5'',6'') = 6.1$				$^2J(5''''ax, 5''''eq) = 10.6$
Me(6″)	18.7	1.34					

[a]) ΔJ(H,H) = 0.1 Hz.

The identification of the sugar units and determination of their sequence was also confirmed by chemical degradation. Acid hydrolysis of **1** gave arjunolic acid, glucose, rhamnose, arabinose, and xylose, all identified by cochromatography with authentic reference substances. Their absolute configuration (D or L) was determined by GC/MS

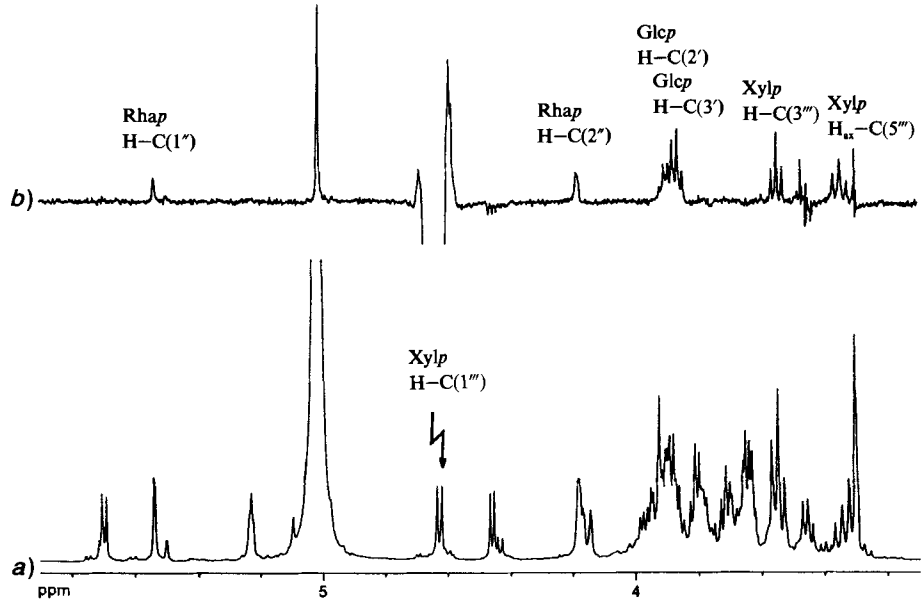

Fig. 1. a) *500-MHz ^{1}H-NMR Spectrum of heteropappussaponin 2* (1) *in CD_3OD* (sugar region) *and* b) *1D-ROESY spectrum obtained by selective excitation of H–C(1‴) of the xylopyranose unit.* Correlations to H–C(1″)(Rha*p*), H–C(2″)(Rha*p*), H–C(2′)(Glc*p*), H–C(3′)(Glc*p*), H–C(3‴)(Xyl*p*), and H–C($5''_{ax}$)(Xyl*p*) are observed.

after reaction with (−)-(*R*)-butan-2-ol according to [10]. The linkage positions of the monosaccharide residues were identified by GC/MS after hydrolysis of the permethylated saponin and trimethylsilylation of the sugar derivatives [11].

Molecular-Modelling Calculations. Since NOE signals between not directly linked sugar residues, which are usually rare, appeared in ROESY measurements, we used these as constraints to compute the three-dimensional arrangement of these units, although molecular-modelling calculations on oligosaccharides are hampered due to the great flexibility of the sugar chain generally [12]. A typical conformational analysis of oligosaccharides determines the variation in energy for all mutual orientations of monosaccharide residues. Because of the great flexibility, the minimum regions in this so-called relaxed maps are very broad.

Therefore, we combined experimental data from NMR measurements with computational methods like molecular mechanics and molecular dynamics. We used ROE measurements which were added to the force field as constraints and served to exclude energetical similar conformers which were not consistent with the NMR data. Potential-energy calculations served as basis to exclude energetical unreasonable NMR structural solutions.

The potential surface was examined as a function of the glycosidic torsion angles ϕ and Ψ for both the glucose-rhamnose and glucose-xylose linkages. The rotations around the glycosidic bonds were described by the dihedral angles ϕ and Ψ. ϕ was the torsion angle defined by the anomeric proton, anomeric C-atom, glycosidic O-atom, and glycosidic C-atom. Ψ represented the dihedral angle defined by the anomeric C-atom, glycosidic O-atom, glycosidic C-atom, and glycosidic proton (see **1**). Relaxed potential energy maps with experimental constraints were calculated as a function of the dihedral angles ϕ_{Rha}/Ψ_{Rha} and ϕ_{Xyl}/Ψ_{Xyl}. Eight distance constraints to the force field took into account the experimental data from ROESY measurements. The optimal value for ϕ_{Rha} was *ca.* −40° and the optimal values for Φ_{Xyl} were *ca.* 180° and *ca.* 40°. Without using constraints, the minimum regions of ϕ_{Rha} and Φ_{Xyl} varied over the whole range. Although the minimum regions for Ψ_{Rha} and Ψ_{Xyl} were shown to be very broad, favoured values could be deduced.

All possible combinations of these optimal values led to 6 different starting structures for further minimization (see *Table 3*). As shown there, the value for ϕ_{Rha} was nearly the same in all 6 minimized structures (*ca.* −50°). For Ψ_{Rha}, ϕ_{Xyl}, and Ψ_{Xyl}, there were two

Table 3. *Minimized Structures of Different Starting Structures*[a])

Starting structure				Minimized structure				Energy [kcal/mol]	Minimum
ϕ_{Rha}	Ψ_{Rha}	ϕ_{Xyl}	Ψ_{Xyl}	ϕ_{Rha}	Ψ_{Rha}	ϕ_{Xyl}	Ψ_{Xyl}		
−40	180	170	0	−44.0	−176.8	176.7	−0.6	34.2	I
−40	180	40	0	−41.6	179.5	48.9	3.9	33.4	II
−40	180	180	180	−43.6	177.9	−173.5	−159.1	36.9	III
−40	10	170	0	−56.2	−23.6	156.4	9.4	35.6	IV
−40	10	40	0	−66.0	−46.6	53.0	9.8	32.5	V
−40	10	180	180	−54.0	48.3	−173.7	−158.5	37.4	VI

[a]) All possible combinations of the dihedral angles ϕ and Ψ due to minima positions were used as starting points for a further minimization to find more precise values for these dihedral angles. Six distance constraints were added to the force field. The minimizations were carried out with conjugated gradient, until the rms derivative was less than 0.001 kcal/Å.

optimal values detected, 180°/−40°, 180°/40°, and −160°/0°, respectively. It had to be considered that a point in the two-dimensional map represented an entire family of points in $(3n-2)$ dimensional space. Therefore, a minimum in conformational space was represented by a small but unspecific area around a point in the conformational map. The global minimum was defined by $\phi_{Rha} = -66°$, $\Psi_{Rha} = -46.6°$, $\phi_{Xyl} = 53.0°$ and $\Psi_{Xyl} = 9.8°$ (see *Table 3* and *Fig. 2*, minimum V).

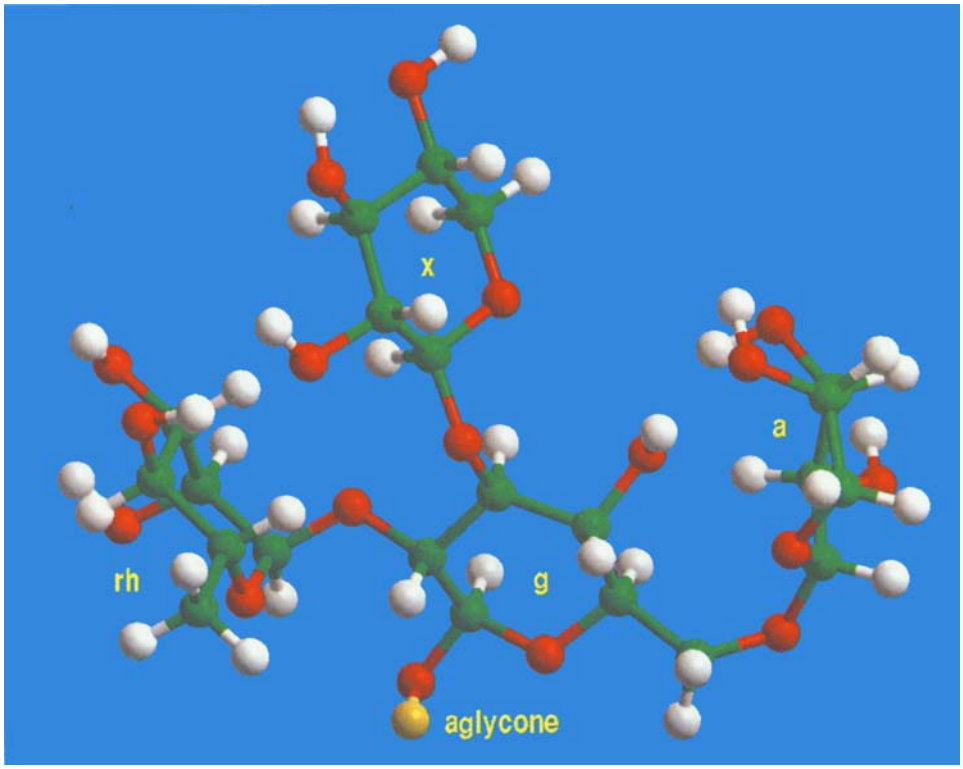

Fig. 2. *Molecular structure of the oligosaccharide chain* (minimum V). Sugar abbreviations: g, β-D-glucopyranose; rh, α-L-rhamnospyranose; x, β-D-xylopyranose; a, α-L-arabinopyranose.

The other 5 minimized structures differed mainly in the orientation of xylose and rhamnose to each other. There were only a few, defined angels Ψ and ϕ possible. All 6 minimized structures had similar energy content and were, therefore, connected by a dynamic equilibrium.

Experimental Part

Plant Material, Extraction, and Isolation. The above-ground parts of *Heteropappus biennis* (LDB.) TAMAMSCH. were collected in August 1990 and 1991, 150 km north of Ulan-Bator, Mongolia, and were air-dried. The identification of the material was carried out by Dr. *D. Santschir*, Academy of Science, Institute of Botany, Mongolia. Voucher specimen are deposited in the herbarium of the Fachbereich Pharmazie, Humboldt University

Berlin, Germany. Air-dried flowers from *Heteropappus biennis* (200 g) were extracted twice with 45% EtOH/H_2O (2 l), and the EtOH, was removed. The aq. soln. was extracted twice with butan-1-ol and the org. phase evaporated. The residue was dissolved in MeOH and the saponins were precipitated by addition of Et_2O to give 3.1 g of saponin extract. A further purification was carried out by column chromatography (*Sephadex LH-20*, MeOH): 1.4 g of saponin mixture. Medium-pressure liquid chromatography (MPLC; *Büchi* glass column (320 mm × 36 mm), silica gel *60 H TLC* (*Merck*; 15 μm), $CHCl_3$/MeOH/H_2O 62.5:32:5.5) yielded 40 mg of **1**.

Aglycone of **1**. Saponin **1** was hydrolysed on the TLC plate by exposing it to conc. HCl soln. for 20 min. The sapogenin was identified by TLC (silica gel *60*, *Merck*), $CHCl_3$/MeOH 9:1, using authentic arjunolic acid as reference and detection with anisaldehyd reagent (AcOH/MeOH/conc. H_2SO_4/anisaldehyd 10:85:5:0.5): blue spot of arjunolic acid.

Sugar Units of **1**. The saponin **1** was hydrolysed as described above. The carbohydrates were identified as glucose, xylose, arabinose, and rhamnose by cochromatography with authentic reference sugars using TLC (silica gel *60* (*Merck*), BuOH/AcOEt/Me_2CO/H_2O 5:5:5:1).

Absolute Configuration of the Sugars [10]. At 37°, saponin **1** (2 mg) was hydrolysed with β-glucuronidase (*Sigma*, No. G 0751; 2 mg) from *Helix pomatia* in H_2O (1 ml) for 5 h. The H_2O was removed and the sugars reacted with (−)-(*R*)-butan-2-ol (*Merck*, No. 818400; 0.45 ml) and conc. HCl (0.05 ml; 15 h, 100°) to give the respective but-2-yl glycosides that were identified by GC/MS using appropriate reference substances. The sugars were identified as D-glucose, D-arabinose, D-xylose, and L-rhamnose. GC: fused-silica capillary column, *SE 54 CB* (*Macherey & Nagel*), 50 m × 0.25 i.d., film 0.45 μm; mobile phase, He 5.0, *ca.* 2.5 ml/min, split 1:10; oven temp. 130–190° with rate 0.3°/min and 190–270° with rate 3°/min.; injector and interface temp. 270°. MS: ion-source temp. 180°, 70 eV, vacuum $3 \cdot 10^{-5}$ Torr; scan 50–500 amu/2 s; mass chromatography with fragment 175.

Linkage Positions of the Sugars. Permethylation of the saponin with the *Hakamori* method, hydrolysis with *Kiliani* mixture, trimethylsilylation with hexamethyldisilazane (HMDS) and chlorotrimethylsilane in dried pyridine. Identification of the sugar derivatives by GC/MS using reference data [11]. Mol.-wt. determination by EI- and CI-MS. EI-MS: see [11]. CI-MS: NH_3, vacuum $7 \cdot 10^{-5}$ Torr, 200 eV, scan 50–700 amu/2 s. The sugars were analysed as 2,3,4-tri-*O*-methyl-1-*O*-(trimethylsilyl)xylopyranose (terminal), 2,3,4-tri-*O*-methyl-1-*O*-(trimethylsilyl)arabinopyranose (terminal), 2,3,4-tri-*O*-methyl-1-*O*-(trimethylsilyl)rhamnopyranose (terminal), and 4-*O*-methyl-1,2,3,6-tetrakis-*O*-(trimethylsilyl)glucopyranose.

NMR Spectroscopy. *Bruker AMX-500* Spectrometer (^1H, 500.13 MHz; ^{13}C, 125.76 MHz); data processing with an *Aspect X32* computer by using UXNMR software; 5-mm reverse probe head; solvent CD_3OD/(D_5)pyridine 3:2, temp. 303 K; MeOH signal as internal standard (δ(H) 3.3, δ(C) 49.0); 90° H-pulses: ^1H 9.6–10 ms, ^{13}C 10.3 ms; WALTZ ^1H-decoupling pulse 112 ms; GARP ^{13}C-decoupling pulse 60 ms; MLEV-17 pulse 20.5 ms.

COSY: 45° mixing pulse. TOCSY: phase-sensitive using TPPI, mixing time 138 ms (100 MLEV-17 cycles plus 2 trim pulses of 2.5 ms each). ROESY: phase-sensitive using TPPI, spin lock cw pulse (250 ms). HMQC: phase-sensitive using TPPI, BIRD sequence, GARP-decoupled. HMBC: phase-sensitive using TPPI; delay tuned to long-range couplings (J(C,H) = 4 and 7 Hz): 125 and 71 ms.

The selective experiments were performed with 90°-*Gaussian* soft pulses (truncation level 1%, 80–82 dB, 55–70 ms). The delays for an optimal magnetization transfer were adjusted to $1/J$. 1D-ROESY: spin lock cw pulse (250 ms). 1D-TOCSY: mixing time 85 ms (60 MLEV-17 cycles plus 2 trim pulses of 2.5 ms each).

Molecular-Modelling Calculations. The calculations were performed on a *Silicon Graphics Indigo* using the software-package INSIGHT II (version 2.1.0)/DISCOVER (version 2.9) from *Biosym*. For the calculations we used *Homan*'s force field for oligosaccharides [13], which was incorporated into the AMBER force field of *Kollman et al.* [14]. The force field uses parameters, charges and *van der Waals* parameters derived for monosaccharides by *Ha et al.* [15]. In all calculations, a dielectric constant ε of 32 was used. The minimization was performed by a conjugate gradient method, until the root mean square gradient was less than 0.001 kcal/Å. The pcl 4 parameters in the discover program was set to 0.5 according to the manual. Minimization was performed with the addition of constraints to the force field. The minimized structures were very similar to the starting structures in spite of the fact that Ψ_{Rha} changes drastically during minimization.

Heteropappussaponin 2 (= O-α-D-*Arabinopyranosyl-(1→6)*-O-[α-L-*rhamnopyranosyl-(1→2)*]-O-[β-D-*xylopyranosyl-(1→3)*]-β-D-*glucopyranosyl Arjunolate*; **1**). White powder. M.p. 312–315° (uncorr.) $[\alpha]_D^{20} = -3.3$ (c = 0.35, 70% MeOH/H_2O). NOE's: H–C(2′)/H–C(1″), H–C(2′)/H–C(1‴), H–C(3′)/H–C(1″) and H–C(1‴),

CH_2(6′)/H–C(1‴″), H–C(1″)/H–C(1‴), H–C(2″)/H–C(1‴). FAB-MS (*MAT 8500* (*Finnigan*), matrix glycerol, negative ions (Cs)). 1059 ([M – H]$^-$), 927 ([M – H – pentose]$^-$), 649 ([aglycone–H + glucose]$^-$), 487 ([aglycone – H]$^-$), 247 [aglycone – $C_{14}H_{23}O_3$]$^-$, *retro-Diels-Alder* fragment).

The HPLC and GC/MS instruments were financed by the support of the Austrian *Bundesministerium für Wissenschaft und Forschung*. We are grateful to the *Fonds der chemischen Industrie* and the *Deutsche Forschungsgemeinschaft* for financial assistance.

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