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Separation and structural analysis of some saponins from *Quillaja saponaria* Molina

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Abstract

A fraction of saponins from *Quillaja saponaria* Molina, QH-B, was fractionated by consecutive separations on three different reverse-phase HPLC systems. Eight compounds were isolated and the structures of these were elucidated mainly by sugar analysis and NMR spectroscopy. The structures consisted of a quillaic acid substituted with two different trisaccharides at C-3, β-D-Gal*p*-(1→2)-[α-L-Rha*p*-(1→3)]-β-D-Glc*p*A and β-D-Gal*p*-(1→2)-[β-D-Xyl*p*-(1→3)]-β-D-Glc*p*A, and a tetra- or pentasaccharide at C-28, β-D-Xyl*p*-(1→4)-[β-D-Glc*p*-(1→3)]-α-L-Rha*p*-(1→2)-β-D-Fuc*p* and β-D-Api*f*-(1→3)-β-D-Xyl*p*-(1→4)-[β-D-Glc*p*-(1→3)]-α-L-Rha*p*-(1→2)-β-D-Fuc*p*. These compounds were further substituted with an acyl group either at O-3 or O-4 of the fucose residue, which is the sugar linked to C-28 of the quillaic acid.

Keywords: *Quillaja saponaria* Molina; Saponins; Quillaic acid; *O*-Acyl migration

1. Introduction

Saponins from the bark of the South American tree *Quillaja saponaria* Molina are known to have immunostimulating properties and function as adjuvants in vaccine formulations. Together with antigen, cholesterol and phospholipids, they form immunostimulating complexes, ISCOMs, which have proved to be an effective way of presenting antigens to the immune system [1,2].

An extract of the *Quillaja* bark is, however, a heterogeneous mixture of saponins and only a few of the major components have been fully structurally elucidated and reported [3–8]. High-performance liquid chromatography (HPLC), in combination with FABMS and monomer mapping, showed up to 50 different saponins differing mainly in the carbohydrate composition [9]. Ion-trap MS analysis of HPLC fractions showed the substitution pattern of 60 components, which appeared to be variants of one basic structure [10]. The common building block is the triterpenoid quillaic acid with different oligosaccharide substituents at C-3 and C-28. Additionally, a dimeric C_9 acyl group, terminated by one or two sugar residues [4–6,8,11], has been found on the first sugar of the C-28 oligosaccharide. Other acyl groups in this position have also previously been described [10,12].

One of the emerging commercial products, the ISCOPREP™ 703, is a 7:3 mixture of two

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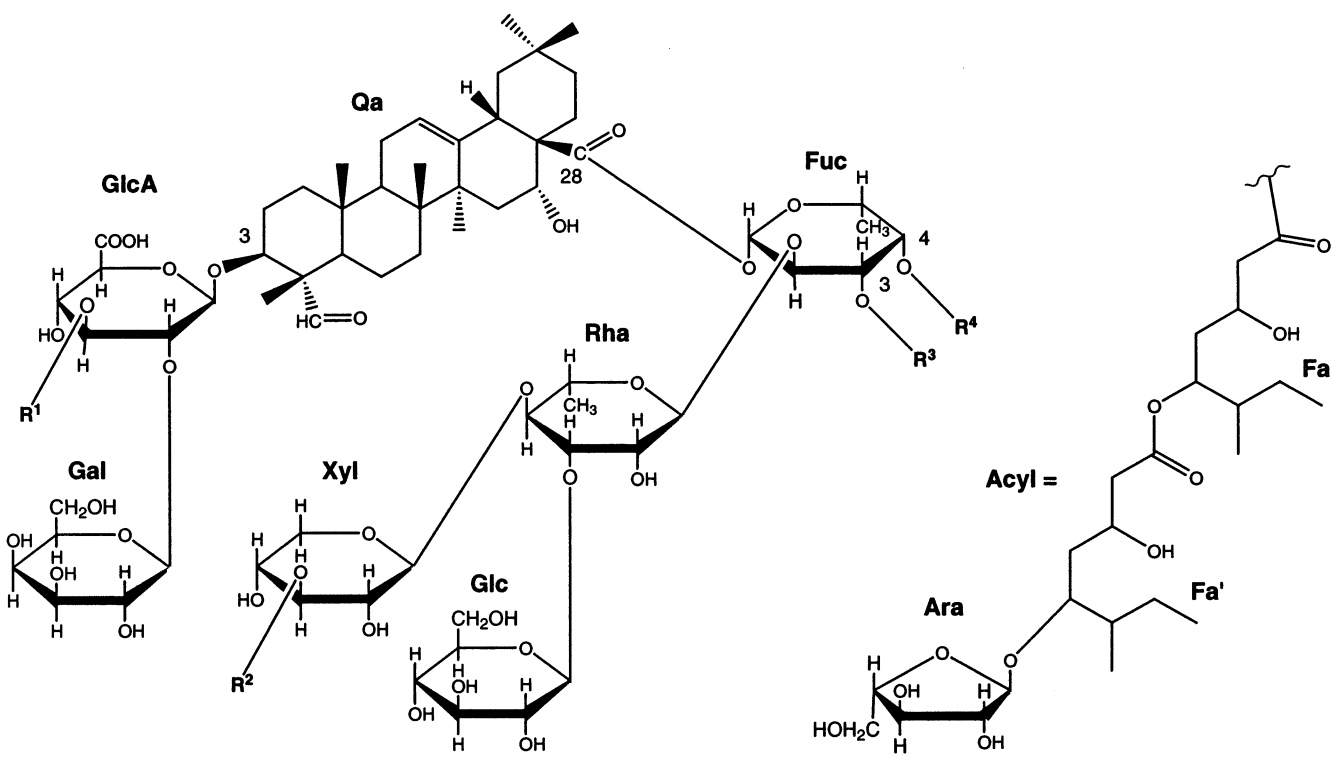
different fractions of *Quillaja* saponins, QH-A and QH-C [13]. The zero in the middle denotes a removed fraction, QH-B, which also has a strong adjuvant activity, but is more toxic than the other fractions. Doses in the range 50–200 μg are toxic to mice, while QH-A and QH-C seem to be virtually non-toxic in these doses [14,15]. The fraction QH-B from *Q. saponaria* Molina was found to contain, as major components, the saponins **B1** to **B4**, together with the respective regioisomers **B1a** to **B4a**, given in Fig. 1. In this work we present the isolation and characterisation of these major components.

2. Results and discussion

HPLC of QH-B.—The saponin fraction QH-B [13], obtained from a bark extract, was further fractionated by reverse-phase HPLC in three steps. In the first step, a coarse preparative separation using a C8 column and a gradient system, most of the major components were collected. Hence, both earlier- and later-eluting minor components, and a fraction containing several compounds normally occurring in fraction QH-C, were removed. This coarse separation step made the following fractionations more efficient. The main fraction containing the major components was analysed



	R¹	R²	R³	R⁴
B1	α-L-Rha*p*	H	H	Acyl
B2	β-D-Xyl*p*	H	H	Acyl
B3	α-L-Rha*p*	β-D-Api*f*	H	Acyl
B4	β-D-Xyl*p*	β-D-Api*f*	H	Acyl
B1a	α-L-Rha*p*	H	Acyl	H
B2a	β-D-Xyl*p*	H	Acyl	H
B3a	α-L-Rha*p*	β-D-Api*f*	Acyl	H
B4a	β-D-Xyl*p*	β-D-Api*f*	Acyl	H

Fig. 1. Structures of the *Quillaja saponaria* saponins isolated from the QH-B fraction.

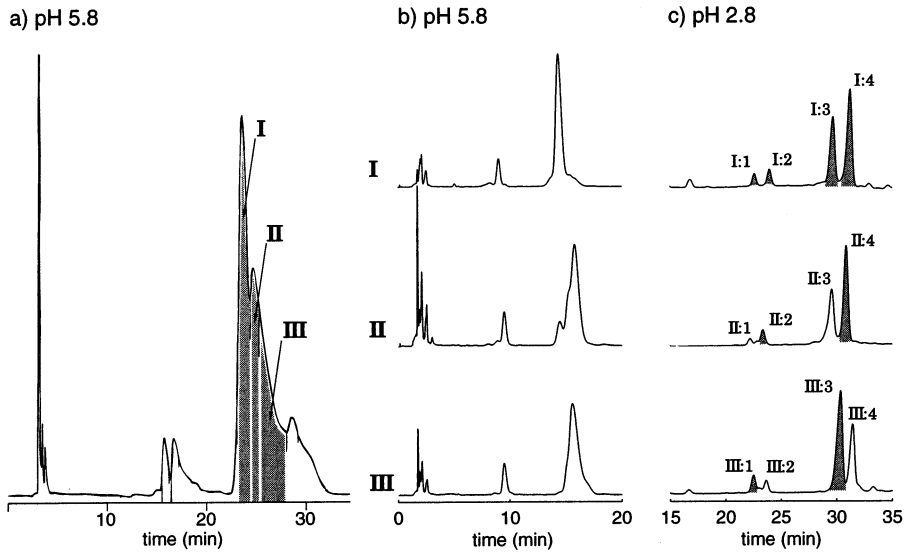


Fig. 2. (a) The second HPLC step of the main components using isocratic elution with 35% MeCN in 20 mM NH_4OAc buffer (pH 5.8) on a C18-column and monitored at 205 nm. The three obtained subfractions, **I**, **II** and **III**, were analysed with two different HPLC systems; (b) 20 mM NH_4OAc, pH 5.8, 35% MeCN and (c) 20 mM phosphate buffer, pH 2.8, 35–45% MeCN in 60 min. The shaded fractions were further analysed.

by MALDI-TOF MS. The main peaks in the mass spectrum revealed that the molecular masses of these components, as represented by their $[M + Na]^+$ peaks, were 2019.9, 2033.9, 2152.5 and 2166.4 Da. In the negative mode $[M - H]^-$ ions with 24 amu smaller values were observed. The differences between these masses suggested pairwise additive terms of 14 and 132 amu, assigned as the mass differences between a pentose and a deoxyhexose as well as an extra pentose. This assignment was also in accordance with results from previous studies of *Quillaja* saponins [3,4,7,9].

The fraction, from the first separation step, containing the main components was further fractionated by a second HPLC system. Using an ammonium acetate buffer of pH 5.8 and a C18 column three fractions, **I**–**III**, were collected (Fig. 2(a)). MALDI-TOF MS showed that these conditions separated the components with the mass difference of 132 Da, with the components containing the additional pentose eluting later (Fig. 3).

Quillaja saponins have previously been shown to form an equilibrium of two isomers, where the acyl group migrates between two positions [6,8]. The same feature applies to the saponins in this study. The analytical HPLC analysis of fractions **I**–**III** showed, in addition to the main components, earlier-eluting components (Fig. 2(b) and (c)). When the isolated early eluting components were reinjected after a few days in solution, the later eluting components had formed.

To further separate the components in fractions **I**–**III**, a third HPLC system with a phosphate buffer of pH 2.8 was used. By lowering the pH of the mobile phase, the components with the mass difference of 14 Da were sepa-

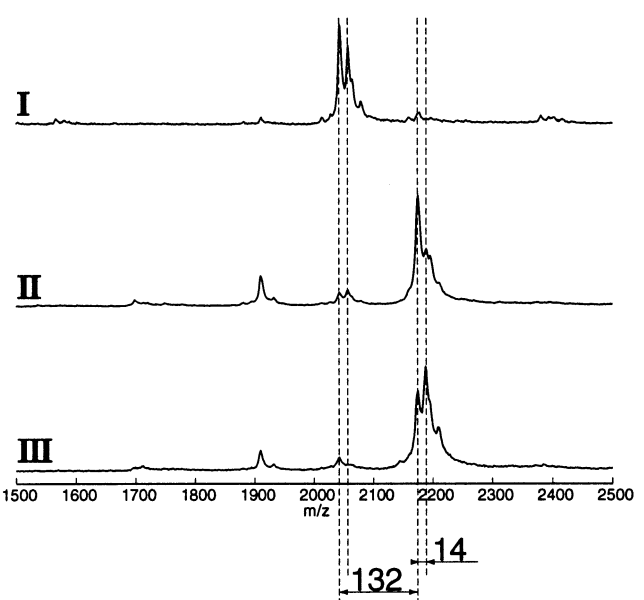


Fig. 3. MALDI-TOF mass spectrum, acquired in the positive mode, of fractions **I**–**III**. The mass differences of 132 and 14 amu are indicated.

Table 1
Molecular masses and the number of neutral monosaccharides as determined by MALDI-TOF MS and sugar analysis, respectively [a]

	M_w [b]	M_w [c]	Api [d]	Rha	Fuc	Ara	Xyl	Glc	Gal
B1	2034.2	2033.9	— (0)	1.8 (2)	1.0 (1)	0.8 (1)	0.6 (1)	1.0 (1)	1.0 (1)
B2	2020.2	2019.9	— (0)	0.9 (1)	1.0 (1)	0.8 (1)	1.0 (2)	1.2 (1)	1.0 (1)
B3	2166.3	2166.4	0.2 (1)	1.6 (2)	1.0 (1)	0.8 (1)	0.6 (1)	1.1 (1)	1.0 (1)
B4	2152.3	2152.5	0.2 (1)	1.0 (1)	1.0 (1)	0.9 (1)	1.0 (2)	1.1 (1)	1.0 (1)

[a] The assigned number of monosaccharide residues is given in parentheses.
[b] Molecular mass of the assigned structures.
[c] Molecular mass as determined by MALDI-TOF mass spectrometry in the positive mode.
[d] The relative yields were lower due to the hydrolytic conditions used in the analysis.

rated as indicated by MALDI-TOF analysis. Prior to this separation step the fractions were kept in solution at room temperature for at least 1 week to allow the chemical equilibrium between the isomers to establish. Using this procedure, the less-preferred isomers were formed, albeit in small amounts, and they could be isolated in the same run as the main components.

Twelve subfractions were collected according to Fig. 2(c). Since fractions **II** and **III**, obtained in the second HPLC step, were overlapping some components occurred in both. As a result some subfractions contained the same components, as evident from the identical ^{1}H NMR spectra.

The isolated compounds obtained from the different fractions were named **B1**–**B4** for the major components and **B1a**–**B4a** for the corresponding isomers. Compounds from the first main fraction (**I**) were **B1a**, the component found in fraction **I**:**1**, **B2a** in **I**:**2**, **B1** in **I**:**3** and **B2** in **I**:**4**. Subfractions **II**:**2** and **II**:**4** contained compounds **B4a** and **B4**, respectively. Compounds **B3a** and **B3** were components in subfractions **III**:**1** and **III**:**3**, respectively.

Structural analysis.—The compounds were further analysed by sugar analysis, MALDI-TOF MS and NMR spectroscopy in order to elucidate the structures. The four major components, **B1**–**B4**, were subjected to sugar analysis whereby the neutral monosaccharides released during acid hydrolysis were determined as the corresponding alditol acetates by GC. The results from the sugar analysis and the determined molecular masses by MALDI-TOF MS are given in Table 1.

NMR spectroscopy, assignments of signals.—As the NMR spectra were complex, for all eight compounds, the major signals and spin systems were assigned by COSY, TOCSY with different mixing times, and HSQC experiments. Starting with the signal from the anomeric proton, H-1, the COSY spectrum identified the H-2 and the TOCSY spectra the H-3 to H-6. From the assigned ^{1}H signals and the one-bond C–H connectivities, the carbon signals were assigned in HSQC-DEPT experiments. The quartenary and carbonyl carbons were assigned by HMBC experiments when sufficient material was available. Overlapping signals within the same spin system were assigned by HSQC-DEPT and the intramolecular connectivities observed in the HMBC spectra. The ^{1}H and ^{13}C chemical shifts are given in Tables 2 and 3.

The designations used for the different moieties are given in Fig. 1. The common aglycone for all eight compounds was determined as quillaic acid. Comparison of obtained proton and carbon chemical shifts with data given for the quillaic acid moiety with oligosaccharides at C-3 [7], C-3 and C-28 [6,8] demonstrated a quillaic acid substituted at C-3 and C-28.

By comparison of the ^{1}H and ^{13}C chemical shifts (Tables 2 and 3) with published NMR data for the corresponding monosaccharides and methyl glycosides [16,17] and previously characterised saponins [6–8] and, as observed in the 2D spectra, the $^3J_{H,H}$ values coupling between ring protons, each monosaccharide and its anomeric configuration was identified. The anomeric configurations were also supported by the $^3J_{H\text{-}1,H\text{-}2}$ values obtained from

the 1D spectra. The absolute configurations of the sugars were expected to be the same as those determined from *Quillaja* saponins [3,7,11] and those generally occurring in saponins — L-Ara, D-Xyl, D-Api, L-Rha, D-Fuc, D-Gal, D-GlcA and D-Glc. The linkage positions were indicated by a 4 to 9 ppm higher chemical shift of the signal from the substituted carbon, relative to those of the unsubstituted monosaccharides. The sequence of the monosaccharide residues was determined by the $^3J_{CH}$ connectivities over the glycosidic bonds, observed as cross-peaks in the HMBC spectra. Hence, each disaccharide element in the oligosaccharides could be identified. Since the acquired HMBC spectra for the acyl migrated products, **B1a**–**B4a**, were of low signal to noise ratio, the monosaccharide sequences are based on those determined for compounds **B1**–**B4**. The fatty acyl group was evaluated in a similar way to the carbohydrates. The starting point in the determination of the spin systems was the signal from the proton at the ether-bound carbon (Fa′-5), which has a lower ^{1}H chemical shift than the signal from the corresponding proton at the ester-bound carbon (Fa-5).

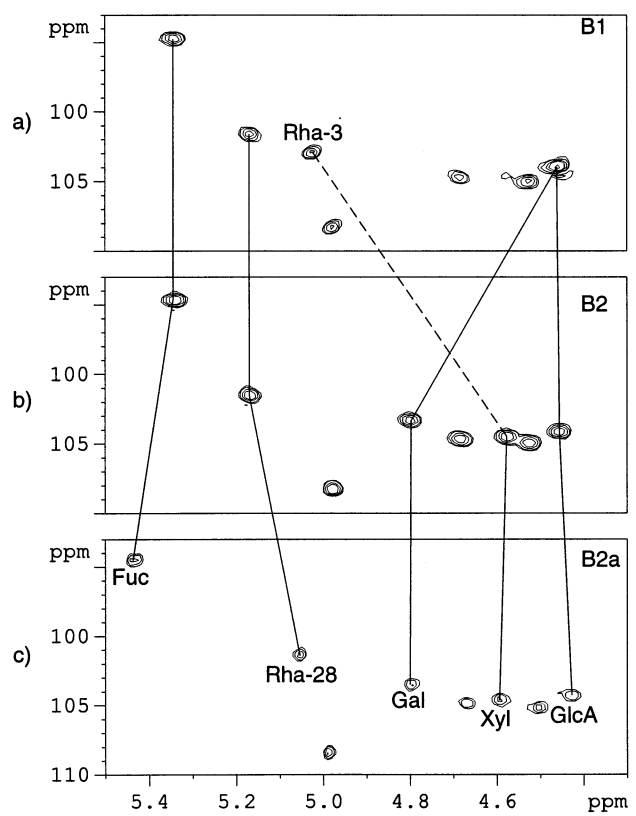


Fig. 4. Anomeric signals in HSQC-DEPT spectra of compounds **B1**, **B2** and **B2a** indicating (a, b) the differences between the two different trisaccharides at C-3 and (c) the differences between these and the acyl migrated variant of the saponin with xylose in the trisaccharide at C-3. Solid lines mark corresponding saccharides and dotted lines interchanged monosaccharides.

The assigned chemical shifts of the quillaic acid moiety and the oligosaccharide at C-3 are given in Table 2. From the data it is evident that the 3-substituent in compounds **B1**, **B1a**, **B3** and **B3a** is β-D-Gal*p*-(1→2)-[α-L-Rha*p*-(1→3)]-β-D-Glc*p*A and in **B2**, **B2a**, **B4** and **B4a** the trisaccharide β-D-Gal*p*-(1→2)-[β-D-Xyl*p*-(1→3)]-β-D-Glc*p*A, as all ^{1}H and ^{13}C chemical shifts are similar to those of the trisaccharides occurring as 3-substituents in previously identified saponins [7,8]. The β-configuration of GlcA, Gal and Xyl was confirmed by the $^3J_{H\text{-}1,H\text{-}2}$ values (7.2, 7.2 and 7.7 Hz, respectively). The α anomeric configuration for Rha ($^3J_{H\text{-}1,H\text{-}2}$ 1.8 Hz) is the same as in the previously described trisaccharide [7] as the ^{1}H and ^{13}C chemical shifts are similar. Fig. 4(a) and (b) demonstrates the differences in the anomeric region of the HSQC-DEPT spectra of saponins containing the two trisaccharides. In addition to the characteristic signals at ~5.03 and ~4.59 ppm for H-1 of the rhamnosyl and xylosyl group, respectively, the most prominent change is the ^{1}H chemical shift for the Gal H-1 signal, which shifts from ~4.45 to ~4.79 ppm.

All signals from the oligosaccharides at C-28 were assigned (Table 3). The β anomeric configuration of Fuc, Glc, and Xyl was based on the chemical shifts and the $^3J_{H\text{-}1,H\text{-}2}$ values — 7.9, 7.2 and 7.9 Hz, respectively. The chemical shifts of Api C-1 and H-1 at 110.7–110.9 and 5.28 ppm, respectively, showed that this monosaccharide is linked in β configuration [3,6]. Rha was assigned α configuration by comparison of ^{1}H- and ^{13}C-chemical shifts with these for α-Rha [16] and for previously characterised saponins [6]. The substitution positions of the monosaccharides were shown by the high chemical shifts of the substituted carbon relative to that of the unsubstituted monosaccharide. The sequence of the monosaccharides was shown by the inter-glycosidic $^3J_{C,H}$ connectivities, which identified each disaccharide element. An HMBC connectivity between Glc H-1 and Rha C-3 estab-

Table 2
NMR assignments for the quillaic acid and the oligosaccharide at C-3. Chemical shifts (at 27 °C) in ppm are referenced against the solvent signal (methanol) at 3.31/49.0 ppm

Residue	**B1**		**B2**		**B3**		**B4**		**B1a**		**B2a**		**B3a**		**B4a**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
Qa-1	1.11, 1.72	38.9	1.12, 1.72	39.1	1.10, 1.71	38.9	1.09, 1.71	39.0	1.11, 1.70	38.9	1.10, 1.70	39.1	1.10, 1.71	39.1	1.08, 1.71	39.3
Qa-2	1.79, 1.98	25.4	1.79 1.97	25.5	1.78, 2.01	25.3	1.78, 1.98	25.4	1.79, 1.98	25.4	1.78, 1.98	25.5	1.79, 2.01	25.0	1.78, 1.98	25.4
Qa-3	3.86	85.7	3.87	86.1	3.86	85.6	3.87	85.8	3.86	85.7	3.86	86.2	3.86	85.4	3.88	85.3
Qa-4		56.0		55.5		56.0		56.0		56.0		56.2		56.1		56.0
Qa-5	1.33	48.9	1.33	48.6	1.34	48.8	1.32	48.8	1.33	48.9	1.31	49.0	1.33	48.8	1.32	48.8
Qa-6	0.95, 1.47	21.0	0.94, 1.47	21.0	0.95, 1.47	21.1	0.95, 1.47	21.1	0.95, 1.47	21.0	0.96, 1.48	21.3	0.95, 1.47	21.1	0.94, 1.46	21.2
Qa-7	1.29, 1.54	33.3	1.29, 1.54	33.4	1.31, 1.55	33.3	1.31, 1.55	33.3	1.29, 1.54	33.3	1.30, 1.52	33.4	1.31, 1.55	33.3	1.31, 1.54	33.5
Qa-8		40.8		40.9		40.8		41.7		40.9		40.9		41.3		42.1
Qa-9	1.75	47.6	1.76	47.8	1.74	47.7	1.74	47.7	1.74	47.6	1.74	47.8	1.73	47.5	1.74	47.6
Qa-10		37.0		36.6		37.0		37.0		36.8		37.2		38.0		36.7
Qa-11	1.93, 1.93	24.3	1.94, 1.94	24.3	1.93, 1.93	24.3	1.93, 1.93	24.3	1.93, 1.93	24.3	1.92, 1.92	24.3	1.92, 1.92	24.3	1.92, 1.92	24.3
Qa-12	5.33	122.8	5.33	122.8	5.33	122.9	5.33	122.9	5.33	122.9	5.32	123.1	5.32	122.9	5.33	122.8
Qa-13		144.6		144.4		144.6		144.6		144.4		144.4		143.7		144.6
Qa-14		42.4		42.5		42.4		41.9		42.0		42.6		41.5		42.5
Qa-15	1.41, 1.74	36.1	1.42, 1.74	36.2	1.41, 1.70	36.1	1.41, 1.70	36.1	1.41, 1.74	36.1	1.40, 1.74	36.3	1.41, 1.70	35.9	1.41, 1.70	36.2
Qa-16	4.45	74.6	4.46	74.7	4.45	74.4	4.45	74.4	4.45	74.4	4.47	74.4	4.45	73.9	4.46	74.3
Qa-17		[a]		48.5		48.5		48.6		[a]		49.9		48.6		48.9
Qa-18	2.93	41.9	2.95	42.0	2.93	41.9	2.93	41.9	2.93	41.9	2.95	42.1	2.94	41.9	2.94	42.1
Qa-19	1.05, 2.30	47.8	1.07, 2.31	47.9	1.06, 2.30	47.7	1.05, 2.30	47.8	1.05, 2.30	47.8	1.06, 2.31	47.8	1.06, 2.30	47.7	1.06, 2.31	47.8
Qa-20		31.0		31.0		31.0		31.0		31.0		31.0		30.8		31.5
Qa-21	1.19, 1.96	36.1	1.17, 1.96	36.2	1.17, 1.94	36.2	1.17, 1.94	36.2	1.19, 1.96	36.1	1.18, 1.95	36.2	1.17, 1.94	36.0	1.16, 1.95	36.4
Qa-22	1.84, 1.83	31.6	1.82, 1.93	31.6	1.80, 1.94	31.5	1.81, 1.92	31.5	1.83, 1.91	31.6	1.83, 1.92	31.6	1.79, 1.92	31.5	1.80, 1.93	31.5
Qa-23	9.44	210.8	9.45	210.6	9.44	210.8	9.45	210.8	9.44	210.8	9.45	211.0	9.44	210.8	9.44	211.1
Qa-24	1.17	10.6	1.17	10.7	1.16	10.6	1.16	10.6	1.17	10.6	1.18	10.7	1.16	10.6	1.16	10.8
Qa-25	1.02	16.3	1.01	16.4	1.01	16.3	0.99	16.3	1.00	16.3	1.01	16.2	1.00	16.0	1.01	16.2
Qa-26	0.81	17.6	0.80	17.7	0.80	17.5	0.79	17.3	0.78	17.6	0.77	17.7	0.77	17.3	0.79	17.7
Qa-27	1.40	26.9	1.40	27.0	1.40	26.9	1.40	26.9	1.40	26.9	1.39	27.0	1.39	26.8	1.40	26.9
Qa-28		177.0		176.6		177.0		176.6		[a]		176.9		[a]		[a]
Qa-29	0.89	33.0	0.89	33.1	0.89	33.0	0.88	32.8	0.89	33.0	0.89	33.1	0.88	32.7	0.89	33.1
Qa-30	0.97	24.7	0.97	24.8	0.96	24.7	0.96	24.7	0.97	24.7	0.96	24.7	0.95	24.4	0.96	24.7
GlcA-1	4.46	103.9	4.46	104.2	4.45	103.8	4.44	104.0	4.46	103.9	4.43	104.3	4.43	103.6	4.36	103.9
GlcA-2	3.63	77.7	3.65	77.8	3.63	77.8	3.67	78.3	3.63	77.7	3.66	78.0	3.62	77.6	3.66	78.1
GlcA-3	3.64	85.4	3.68	86.2	3.64	85.4	3.69	86.1	3.64	85.4	3.69	86.5	3.63	85.2	3.67	86.2
GlcA-4	3.60	71.5	3.56	71.0	3.58	71.6	3.56	71.0	3.60	[a]	3.55	71.4	3.57	71.5	3.54	71.6
GlcA-5	3.80	76.2	3.80	76.1	3.76	76.4	3.80	76.1	3.80	[a]	3.75	[a]	3.72	76.2	3.79	[a]
GlcA-6		172.5		171.7		172.5		171.7		[b]		[b]		173.6		[b]
Gal-1	4.46	103.9	4.80	103.5	4.45	103.8	4.80	103.3	4.45	103.9	4.80	103.5	4.46	103.6	4.80	103.3
Gal-2	3.48	72.7	3.45	73.3	3.50	72.6	3.47	73.3	3.50	72.7	3.46	73.4	3.50	72.4	3.49	73.3
Gal-3	3.47	74.6	3.42	75.1	3.47	74.6	3.44	75.0	3.47	74.6	3.43	75.1	3.48	74.5	3.45	75.1
Gal-4	3.82	70.3	3.81	70.5	3.82	70.3	3.82	70.3	3.82	70.3	3.81	70.3	3.82	70.1	3.83	70.4
Gal-5	3.47	76.6	3.49	76.4	3.47	76.5	3.49	76.3	3.47	76.6	3.49	76.4	3.47	76.6	3.49	76.1
Gal-6	3.73, 3.79	61.9	3.72, 3.77	61.9	3.72, 3.79	62.0	3.73, 3.77	61.8	3.73. 3.79	61.9	3.66, 3.73	61.9	3.72, 3.78	62.0	3.72, 3.77	61.8
Xyl-1			4.58	104.5			4.59	104.3			4.59	104.6			4.63	104.4
Xyl-2			3.23	74.9			3.22	74.8			3.23	74.9			3.23	74.8
Xyl-3			3.30	77.9			3.31	77.7			3.30	78.0			3.31	77.7
Xyl-4			3.50	70.6			3.50	70.3			3.51	70.8			3.50	70.7
Xyl-5			3.23, 3.90	66.9			3.24, 3.89	66.7			3.24, 3.91	67.0			3.22, 3.89	67.0
Rha-1	5.02	102.9			5.03	102.9			5.04	102.5			5.03	102.7		
Rha-2	4.01	71.7			4.01	71.8			4.01	71.7			4.01	71.5		
Rha-3	3.65	71.8			3.65	71.8			3.66	71.9			3.65	71.6		
Rha-4	3.40	73.5			3.40	73.5			3.39	73.6			3.39	73.3		
Rha-5	3.92	70.2			3.95	70.2			3.92	[a]			3.95	69.9		
Rha-6	1.22	17.4			1.23	17.3			1.22	16.5			1.23	17.2		

[a] Signal not observed in HSQC-DEPT spectrum.
[b] Signal not observed in HMBC spectrum.

Table 3
NMR assignments for the oligosaccharide at C-28 and the acyl group. Chemical shifts (at 27 °C) in ppm are referenced against the solvent signal (methanol) at 3.31/49.0 ppm.

Residue	**B1**		**B2**		**B3**		**B4**		**B1a**		**B2a**		**B3a**		**B4a**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
Fuc-1	5.34	94.9	5.34	94.8	5.34	94.8	5.35	94.7	5.42	94.3	5.43	94.5	5.42	94.2	5.43	94.1
Fuc-2	3.67	75.9	3.66	76.0	3.68	75.8	3.68	75.7	4.06	72.9	4.08	73.0	4.06	72.8	4.07	73.1
Fuc-3	3.85	73.5	3.85	73.7	3.85	73.7	3.85	73.5	4.93	78.6	4.93	78.8	4.94	78.5	4.93	78.9
Fuc-4	5.12	74.8	5.12	74.9	5.12	74.9	5.12	74.9	3.80	70.1	3.82	70.3	3.80	69.8	3.81	70.3
Fuc-5	3.84	70.8	3.84	70.9	3.84	70.8	3.84	70.8	3.76	71.9	3.77	72.1	3.77	71.7	3.77	71.9
Fuc-6	1.09	16.3	1.10	16.5	1.09	16.3	1.08	16.3	1.21	16.3	1.22	16.2	1.21	16.0	1.21	16.8
Rha-1	5.17	101.6	5.17	101.6	5.19	101.6	5.18	101.4	5.04	101.1	5.05	101.4	5.04	100.9	5.05	101.5
Rha-2	4.25	71.0	4.23	71.0	4.24	71.0	4.25	70.9	4.00	71.6	4.01	71.6	4.01	71.5	4.02	71.6
Rha-3	3.93	82.5	3.93	82.6	3.91	82.6	3.92	82.4	3.79	82.6	3.79	82.6	3.76	82.6	3.77	82.5
Rha-4	3.67	78.5	3.67	78.6	3.66	78.6	3.67	78.3	3.64	78.2	3.64	78.7	3.64	78.2	3.65	78.6
Rha-5	3.82	68.9	3.81	69.0	3.80	68.8	3.80	68.7	3.77	68.8	3.78	68.8	3.75	68.2	3.75	68.6
Rha-6	1.27	18.4	1.27	18.4	1.28	18.4	1.27	18.4	1.28	18.5	1.29	18.5	1.29	18.5	1.29	18.4
Glc-1	4.53	105.0	4.53	105.0	4.52	105.0	4.52	104.9	4.49	105.2	4.51	105.2	4.49	104.8	4.51	104.9
Glc-2	3.29	74.9	3.29	75.0	3.30	75.0	3.30	74.9	3.28	74.9	3.29	74.9	3.29	74.9	3.30	75.0
Glc-3	3.26	77.5	3.26	77.5	3.27	77.5	3.26	77.5	3.24	77.4	3.25	77.4	3.24	77.1	3.26	77.7
Glc-4	3.31	70.9	3.31	70.9	3.33	70.8	3.33	70.6	3.39	70.5	3.40	70.5	3.39	70.3	3.40	70.6
Glc-5	3.31	77.9	3.31	77.9	3.32	77.9	3.31	77.7	3.30	78.0	3.32	78.0	3.32	77.6	3.32	77.8
Glc-6	3.68, 3.84	62.1	3.68, 3.84	62.1	3.69, 3.83	62.0	3.69, 3.84	62.1	3.72, 3.82	61.9	3.75, 3.82	61.7	3.74, 3.82	61.6	3.74, 3.63	61.9
Xyl-1	4.69	104.7	4.69	104.7	4.71	104.7	4.71	104.6	4.66	104.7	4.67	104.9	4.68	104.5	4.70	104.6
Xyl-2	3.10	75.4	3.10	75.5	3.20	74.9	3.20	74.9	3.08	75.4	3.09	75.5	3.17	74.7	3.21	75.1
Xyl-3	3.26	78.3	3.26	78.4	3.34	85.6	3.35	85.5	3.26	78.2	3.26	78.5	3.34	85.7	3.34	85.7
Xyl-4	3.47	71.1	3.47	71.1	3.49	69.8	3.49	69.8	3.46	71.1	3.47	71.1	3.48	69.7	3.49	70.2
Xyl-5	3.16, 3.82	66.7	3.15, 3.82	66.8	3.18, 3.87	66.3	3.17, 3.85	66.5	3.15, 3.81	66.7	3.16, 3.82	66.8	3.17, 3.85	66.3	3.18, 3.87	66.6
Api-1					5.27	110.9	5.28	110.7					5.28	110.8	5.28	110.8
Api-2					4.01	77.4	4.02	77.3					4.03	77.4	4.03	77.6
Api-3						80.0		80.0						80.3		80.2
Api-4					3.79, 4.14	74.7	3.79, 4.14	74.7					3.79, 4.14	74.6	3.80, 4.15	74.7
Api-5					3.63	64.9	3.63	64.9					3.64	64.9	3.65	65.1
Fa-1		172.7		172.6		172.7		172.7		172.2		172.2		172.2		173.0
Fa-2	2.62, 2.62	43.2	2.63, 2.63	43.3	2.62, 2.62	43.2	2.62, 2.62	43.2	2.51, 2.63	43.6	2.51, 2.64	43.6	2.50, 2.64	43.5	2.51, 2.63	43.4
Fa-3	4.04	65.8	4.03	65.8	4.02	65.8	4.02	65.7	4.07	65.6	4.08	65.6	4.08	65.4	4.08	65.7
Fa-4	1.67, 1.83	39.4	1.67, 1.84	39.6	1.67, 1.83	39.4	1.67, 1.83	39.4	1.62, 1.77	39.4	1.64, 1.77	39.4	1.62, 1.77	39.4	1.64, 1.77	39.4
Fa-5	5.18	74.9	5.19	75.0	5.18	74.9	5.18	74.9	5.16	74.9	5.18	74.9	5.17	74.8	5.18	75.0
Fa-6	1.61	39.3	1.62	39.4	1.61	39.3	1.61	39.3	1.60	39.8	1.60	39.8	1.60	39.8	1.60	40.0
Fa-7	1.16, 1.50	26.3	1.16, 1.49	26.3	1.16, 1.50	26.3	1.14, 1.50	26.3	1.17, 1.50	26.3	1.17, 1.50	26.3	1.16, 1.49	26.3	1.15, 1.48	26.4
Fa-8	0.93	12.1	0.93	11.9	0.93	12.1	0.93	12.0	0.93	12.1	0.93	12.1	0.93	11.9	0.93	12.1
Fa-9	0.93	14.9	0.94	14.7	0.93	14.9	0.93	14.6	0.94	14.4	0.94	14.4	0.93	14.3	0.94	14.4
Fa′-1		173.3		171.8		173.3		173.3		173.1		173.5		173.1		173.3
Fa′-2	2.52, 2.52	43.7	2.52, 2.52	43.7	2.52, 2.52	43.7	2.52, 2.52	43.7	2.51, 2.52	43.7	2.52, 2.52	43.7	2.51, 2.52	43.5	2.51, 2.53	43.5
Fa′-3	4.29	65.9	4.30	65.9	4.29	65.9	4.30	65.8	4.30	66.1	4.31	66.1	4.31	65.8	4.30	66.0
Fa′-4	1.51, 1.61	39.0	1.50. 1.62	39.1	1.49, 1.60	39.1	1.48, 1.60	38.9	1.50, 1.62	39.1	1.50, 1.62	39.1	1.50, 1.62	38.9	1.50 1.62	38.9
Fa′-5	3.80	78.7	3.80	78.9	3.80	78.7	3.80	78.6	3.79	79.1	3.81	79.1	3.80	78.8	3.80	78.8
Fa′-6	1.62	39.9	1.62	40.0	1.61	39.9	1.61	39.9	1.62	40.0	1.62	40.0	1.60	39.7	1.60	39.9
Fa′-7	1.10, 1.62	25.2	1.11, 1.61	25.2	1.10, 1.62	25.2	1.10, 1.60	25.2	1.12, 1.62	25.2	1.12, 1.62	25.2	1.12, 1.61	25.2	1.10, 1.60	25.3
Fa′-8	0.93	12.1	0.93	11.9	0.93	12.1	0.93	12.0	0.93	12.1	0.93	12.1	0.93	11.9	0.93	12.1
Fa′-9	0.88	14.9	0.91	14.7	0.88	14.9	0.88	14.7	0.91	14.9	0.91	14.9	0.90	14.5	0.91	14.9
Ara-1	4.98	108.3	4.98	108.3	4.98	108.3	4.98	108.1	4.98	108.3	4.99	108.5	4.98	108.1	4.99	108.3
Ara-2	3.96	83.1	3.96	83.3	3.96	83.1	3.97	83.0	3.96	83.1	3.97	83.3	3.96	82.9	3.97	83.2
Ara-3	3.85	78.0	3.85	78.1	3.85	78.0	3.85	77.9	3.84	78.0	3.86	78.1	3.85	77.8	3.85	77.9
Ara-4	4.03	84.9	4.02	85.0	4.02	84.9	4.03	84.9	4.03	84.9	4.03	85.2	4.03	84.8	4.03	85.0
Ara-5	3.63, 3.76	62.7	3.63, 3.75	62.8	3.63, 3.76	62.6	363, 3.76	62.5	3.63, 3.77	62.7	3.64, 3.77	62.8	3.63, 3.76	62.4	3.63, 3.76	62.0

lished the linkage position of the glucosyl group to the rhamnosyl residue. Glucose-containing saponins from *Q. saponaria* were previously shown, by linkage analysis, to have a 3,4-disubstituted rhamnose with a β-D-Glc*p* at C-3 [3,5,12]. The branched rhamnosyl residue is then linked to the 2-position of the fucosyl residue. This structural element was shown for **B1**–**B4**, where cross-peaks in the HMBC spectra at 3.66–3.68/101.4–101.6 ppm demonstrated the $^{3}J_{CH}$ connectivity between Rha H-1 and Fuc C-2. For **B1** and **B2**, there were also cross-peaks between Fuc H-2 and Rha C-1 at 5.17/75.9–76.0 ppm. A Xyl is linked to C-4 of Rha as shown by cross-peaks at 4.67–4.71/78.3–78.6 ppm. The Xyl is terminal in compounds **B1**, **B1a**, **B2** and **B2a**, but substituted at C-3 in **B3**, **B3a**, **B4** and **B4a** as revealed by the downfield shift for the signals from Xyl H-3 and Xyl C-3. These signals shift from 3.26/78.2–78.5 ppm for the terminal xylose group to 3.34–3.35/85.5–85.7 ppm for Xyl substituted with Api. The anomeric atoms of the terminal Api also gave a distinct peak at 5.27–5.28/110.7–110.8 in the HSQC DEPT spectra. Cross-peaks in the HMBC spectra of **B3**, **B3a**, **B4** and **B4a** between Xyl H-3 and Api C-1 at 3.34–3.35 and 110.7–110.8 ppm support the linkage. The fucose, the central saccharide residue of these molecules, is connected via an ester linkage to C-28 of the quillaic acid, as evident from the cross-peak in the HMBC spectra between Fuc H-1 and a carbonyl carbon at 176.6–177.0 ppm.

Two variants, both with a Glc attached to the branched Rha, were found. The first was a tetrasaccharide, β-D-Xyl*p*-(1→4)-[β-D-Glc*p*-(1→3)]-α-L-Rha*p*-(1→2)-β-D-Fuc*p*, with a terminal Xyl and the second a pentasaccharide with a terminal Api, β-D-Api*f*-(1→3)-β-D-

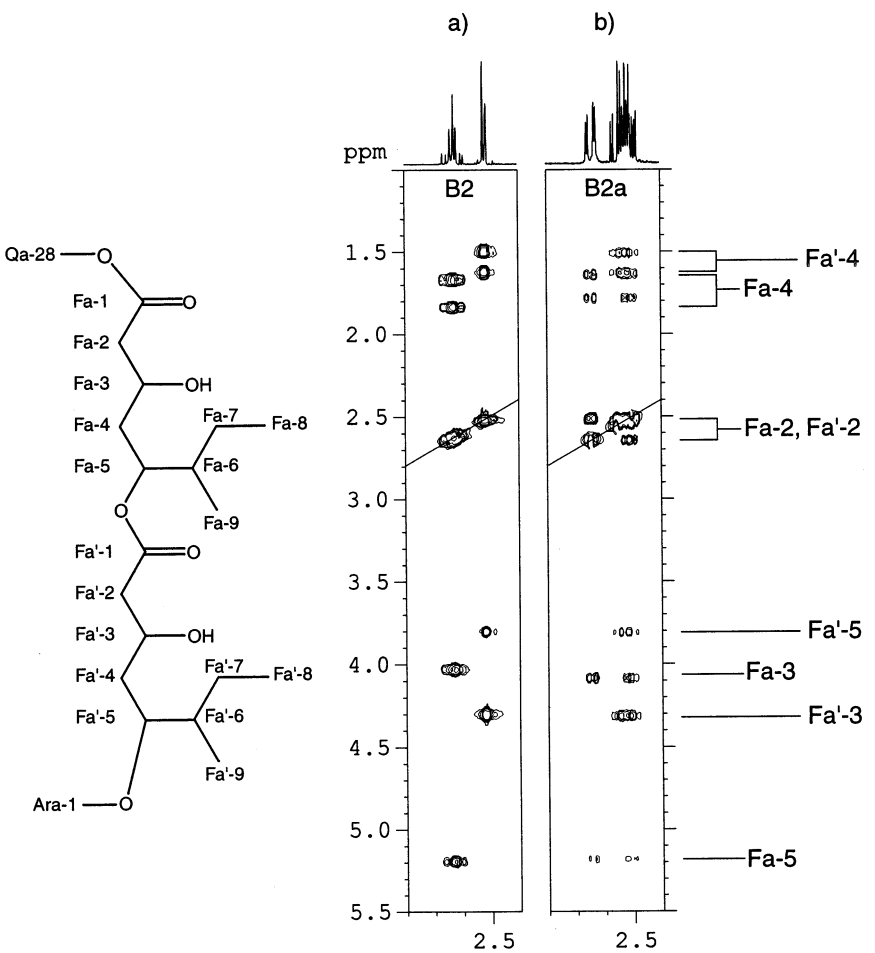


Fig. 5. TOCSY spectra (80 ms mixing time) from (a) **B2** and (b) **B2a** showing the differences in ^{1}H cross-peaks from Fa-2 and Fa′-2 between the 4-*O*- and 3-*O*-acyl-substituted D-fucose.

Xyl*p*-(1→4)-[β-D-Glc*p*-(1→3)]-α-L-Rha*p*-(1→2)-β-D-Fuc*p*.

The acyl group with the terminal arabinosyl (named Acyl in Fig. 1) is a common component in previously identified structures [4–6,8]. All signals from this group were assigned (Table 3). Fig. 5 shows the TOCSY spectrum with the connectivities from Fa-5 to Fa-2 and Fa′-5 to Fa′-2. The signals from the two monomers of the dimeric 3,5-dihydroxy acids were distinguished by the different chemical shifts of the ether-bound Fa′-5, at ~3.80 ppm, compared with the ester-bound Fa-5 at ~5.17 ppm. The signal from the anomeric atoms of the arabinofuranosyl group was identified by the distinct peak at ~4.99/~108.3 ppm and a $^{3}J_{H\text{-}1,H\text{-}2}$ value of 2.2 Hz. The connectivity to Fa′-5 was established by cross-peaks in HMBC spectra at the positions for the ^{1}H and ^{13}C chemical shifts for Fa′-5 and Ara-1. This connectivity could be discerned in all HMBC spectra except for **B1a** and **B2a**, where only small amounts of sample were available.

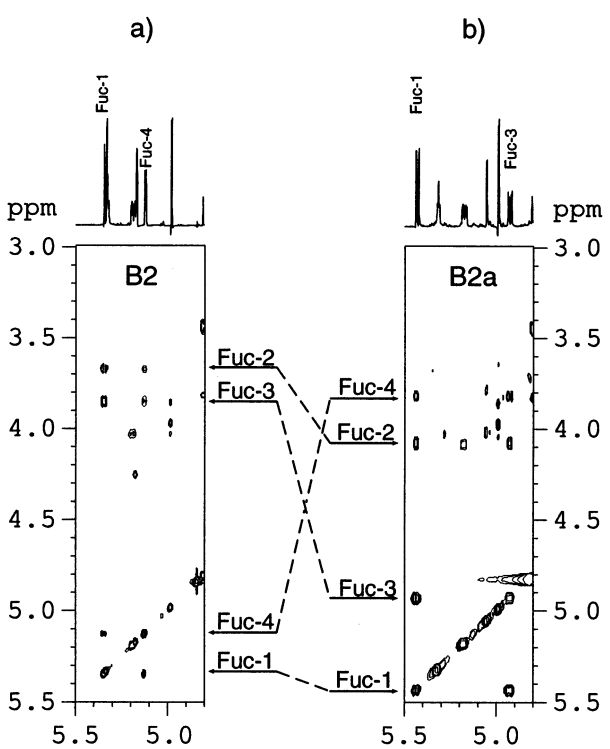


Fig. 6. TOCSY spectra (80 ms mixing time) from (a) **B2** and (b) **B2a** showing the differences in the pattern of ^{1}H cross-peaks in the spin system of fucose for two acyl migrated isomers.

The acyl group is attached either to Fuc-3 or Fuc-4. These isomers interconvert in solution but they are stable enough to be analysed by NMR spectroscopy when dissolved in MeOH and kept at 4 °C between experiments. The equilibrium, however, favours the isomer substituted at Fuc-4, so the collected amounts of the Fuc-3 isomer are considerably lower. These compounds also have a notably shorter retention time in the RP-HPLC systems used (Fig. 2). The differences in the chemical shifts between the isomers are largest in the proximity of the substitution position in the monosaccharide. The signal from the proton at the acylated carbon (Fuc-3 or Fuc-4) is clearly shifted downfield compared with that from the free hydroxyl group (Fig. 6). The signal for the anomeric proton of the rhamnose is also affected. The chemical shift is decreased from 5.19–5.17 to 5.04–5.05 ppm (Fig. 4(b) and (c)). Another change in the appearance of the spectra is the larger dispersion of chemical shifts for the two methylene protons at Fa-2. This can be seen in the spin systems displayed by the TOCSY spectra (Fig. 5). This effect might be induced by hindered movement of the inner part of the dimeric fatty acyl group in the Fuc-3-substituted isomers relative to those substituted at Fuc-4. The expected cross-peak in the HMBC spectra was only weak for compounds **B1**–**B4** and not observed in the Fuc-3-substituted isomers.

Structures.—The structures of the major saponins **B1**–**B4** found in the chromatographic fraction QH-B are given in Fig. 1. Also present in the fraction are the regioisomers **B1a**–**B4a**, where the acyl group has migrated from fucose O-4 to O-3. The structure of compound **B4** match that of QS-18 [5,10].

3. Experimental

Materials.—*Quillaja* saponin fraction QH-B was obtained from Iscotec AB (Uppsala, Sweden). The MeCN (LiChrosolv®) was obtained from E. Merck and the Milli-Q-water was produced in house.

Isolation of compounds.—The separation of QH-B was performed in three steps on a

semi-preparative HPLC instrument with UV detection at 205 nm.

(i) Gradient elution on a 5 μm, 250 × 20 mm Hichrom RP-C8 column (Chromtech, Sweden). The mobile phases were 20 mM NH_4OAc (pH 5.8) premixed with MeCN to 35% and pure MeCN. The concentration of MeCN was held at 35% for the first 10 min, raised to 41.5% during the next 10 min and kept at this level for a total of 30 min. The flow rate was 10 mL/min. Approximately 875 mg QH-B was separated in 50 mg portions injected in each run. The fraction containing the major components was collected. Most of the MeCN was removed by careful evaporation and then the residual material was lyophilised yielding 350 mg.

(ii) Isocratic separation with 35% MeCN–20 mM NH_4OAc (pH 5.8) using a C18-column, 5 μm, 100 × 25 mm (Hichrom, Chromtech, Sweden) and a flow rate of 7.5 mL/min. The fraction obtained above was separated in 26 runs, each 10–15 mg. Three main fractions, **I** (46 mg), **II** (32 mg) and **III** (60 mg), were collected.

(iii) Isocratic separation of fractions **I**–**III** with 35% MeCN/20 mM phosphate buffer (pH 2.8) on a C18-column, 5 μm, 100 × 25 mm (Hichrom, Chromtech, Sweden) using a flow rate of 7.5 mL/min. Each of the three fractions was separated (between 0.7 and 1.5 mg per injection) and several subfractions were collected. The last number in the final fraction name refers to this subfractionation. The amount of material collected in the last step was for **I**:**1** 1.1 mg, **I**:**2** 2.0 mg, **I**:**3** 11.0 mg, **I**:**4** 19.3 mg, **II**:**1** 0.3 mg, **II**:**2** 2.5 mg, **II**:**3** 4.6 mg, **II**:**4** 17.2 mg, **III**:**1** 2.3 mg, **III**:**2** 4.2 mg, **III**:**3** 21.0 mg and **III**:**4** 2.9 mg.

General work-up after fractionation.—Due to the strong foaming properties of the compounds, the concentration after each separation step was performed by solid-phase extraction. Most of the MeCN was evaporated and the remains were diluted to approximately twice the volume with water containing 4% CH_3OH. The solutions were slowly sucked through endcapped C18 Sep-Pak cartridges (Isolute™, Sorbent AB, Sweden). The size of the cartridges was either 10 g or 500 mg, depending on the amount of material. Possible loss (to the water phase) was controlled by checking for formation of foam. The saponins were collected by elution with CH_3OH, evaporation, dilution with water and lyophilised.

Analytical HPLC system.—To monitor the separation, an analytical HPLC system consisting of a 150 × 4.6 mm Hichrom RP-C8 column and a UV detector at 205 nm was used. The compounds were eluted either with 35% MeCN in 20 mM NH_4OAc (pH 5.8) or with 20 mM phosphate buffer at pH 2.8 and a gradient running from 35 to 45% MeCN in 60 min, using a flow rate of 0.8 mL/min.

Mass spectrometry.—Mass spectra were acquired, in the positive and negative modes, on an LDI-1700XS time-of-flight instrument with 2,5-dihydroxybenzoic acid (Aldrich, Steimheim, Germany) as matrix. A similar saponin, S3 [8], with molecular weight of 2004.3 Da, was used as internal reference.

Sugar analysis.—Samples were hydrolysed with 2 M CF_3COOH at 120 °C for 1 h. After evaporation of solvent, the products were reduced with $NaBH_4$. The alditols were acetylated with Ac_2O–pyridine at 120 °C for 45 min and analysed by GC [18].

GC analysis.—A Hewlett–Packard 6890 instrument with an FID and a splitless injector was used. The column was a HP-5 (Hewlett–Packard, 25 m × 0.32 mm, 0.17 μm phase). The temperature program used was 140 °C (1 min) → 230 °C (3 °C/min). The retention times were compared with those of authentic standards.

NMR spectroscopy.—Prior to NMR analysis, the saponins were exchanged with D_2O (99.5% D, Cambridge Isotope Laboratories) and freeze-dried. The spectra were recorded on a Bruker DRX-600 MHz spectrometer at 27 °C with CD_3OD as solvent (min 99.8% D, Cambridge Isotope Laboratories). Chemical shifts are reported in ppm referenced against the solvent peak (δ_H 3.31 and δ_C 49.0).

For samples with more than 3 mg material available for NMR analysis a 5 mm triple resonance inverse probe was used. These samples were dissolved in approximately 700 μL solvent in an ordinary NMR tube. For smaller amounts a 2.5 mm microprobe was used. Samples down to 1.6 mg were dissolved in 120–150 μL and analysed in closed capillaries with 2.5

mm O.D. For samples <1 mg capillaries with 1.9 or 1.7 mm O.D. were used. One-dimensional, both with and without presaturation of the water signal, COSY, TOCSY (80 ms mixing time), HSQC-DEPT and HMBC (with a delay time of 65 ms) experiments were performed with Bruker standard pulse sequences.

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References

[1] B. Morein, B. Sundquist, S. Höglund, K. Dalsgaard, A. Osterhaus, *Nature*, 308 (1984) 457–460.
[2] I.G. Barr, G.F. Mitchell, *Immunol. Cell Biol.*, 74 (1996) 8–25.
[3] R. Higuchi, Y. Tokimitsu, T. Fujioka, T. Komori, T. Kawasaki, D.G. Oakenful, *Phytochemistry*, 26 (1987) 229–235.
[4] R. Higuchi, Y. Tokimitsu, T. Komori, *Phytochemistry*, 27 (1988) 1165–1168.
[5] C.R. Kensil, S. Soltysik, U. Patel, D.J. Marciani, in F. Brown (Ed.), *Vaccines*, Cold Spring Harbor Laboratory Press, 1992, pp. 35–40.
[6] N.E. Jacobsen, W.J. Fairbrother, C.R. Kensil, A. Lim, D.A. Wheeler, M.F. Powell, *Carbohydr. Res.*, 280 (1996) 1–14.
[7] S.J. Guo, L. Kenne, L.N. Lundgren, B. Rönnberg, B.G. Sundquist, *Phytochemistry*, 48 (1998) 175–180.
[8] L.I. Nord, L. Kenne, *Carbohydr. Res.*, 320 (1999) 70–81.
[9] D.C. van Setten, G. van de Werken, G. Zomer, G.F.A. Kersten, *Rapid Commun. Mass Spectrom.*, 9 (1995) 660–666.
[10] D.C. van Setten, G.J. ten Hove, E.J.H.J. Wiertz, J.P. Kamerling, G. van de Werken, *Anal. Chem.*, 70 (1998) 4401–4409.
[11] D.C. van Setten, G. van de Werken, in *Advances in Experimental Medicine and Biology*, Vol. 404, Plenum Press, New York, 1996, pp. 185–193.
[12] C.R. Kensil, J.Y. Wu, C.A. Anderson, D.A. Wheeler, J. Amsden, *Dev. Biol. Stand.*, 92 (1998) 41–47.
[13] B. Rönnberg, M. Fekadu, B. Morein, *Vaccine*, 13 (1995) 1375–1382.
[14] B. Morein, K. Lövgren, B. Rönnberg, A. Sjölander, M. Villacrés-Eriksson, *Clin. Immunother.*, 3 (1995) 461–475.
[15] B. Rönnberg, M. Fekadu, S. Behboudi, L. Kenne, B. Morein, *Vaccine*, 15 (1997) 1820–1826.
[16] P.-E. Jansson, L. Kenne, G. Widmalm, *Carbohydr. Res.*, 188 (1989) 169–191.
[17] P.K. Agrawal, *Phytochemistry*, 31 (1992) 3307–3330.
[18] J.S. Sawardeker, J.H. Sloneker, A.R. Jeanes, *Anal. Chem.*, 37 (1965) 1602–1604.