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Triterpenoid saponins containing an acetylated branched D-fucosyl residue from *Quillaja saponaria* Molina

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Abstract

Seven novel saponins were isolated from a bark extract of *Quillaja saponaria* Molina. the compounds were characterized, using mainly NMR spectroscopy, mass spectrometry and chemical methods, as quillaic acid substituted at C-3 with oligosaccharides consisting of various compositions of D-glucuronic acid D-galactose, D-xylose, and L-rhamnose and at C-28 with complex oligosaccharide structures consisting of various compositions of D-xylose, L-rhamnose, D-apiose and a branched 4-*O*-acetyl-D-fucose residue.

Keywords: *Quillaja saponaria* Molina; Structural analysis; Saponins; Quillaic acid; Acetyl group

1. Introduction

The structures of several saponins from the bark of the tree *Quillaja saponaria* Molina have been reported (Higuchi, Tokimitsu, Fujioka, Komori, Kawasaki & Oakenful, 1987; Higuchi, Tokimitsu & Komori, 1988; Jacobsen, Fairbrother, Kensil, Lim, Wheeler, & Powell, 1996) and other saponins have been partially characterised using mass spectrometry and monomer mapping (van Setten, van de Werken, Zomer & Kersten, 1995). In another study (van Setten, ten Hove, Wiertz, Kamerling & van de Werken, 1998) 60 saponins were partly characterised using HPLC in combination with ion-trap mass spectrometry. The completely characterised structures have in common the triterpene quillaic acid with a unique trisaccharide attached to C-3 and several oligosaccharides, substituted with two C_9 aliphatic acids, attached to C-28. Recently, we reported the occurrence of three new quillaic acid saponins in a bark extract lacking the oligosaccharide at C-28 but with variation in the structure of the oligosaccharide attached to C-3 (Guo, Kenne, Lundgren, Rönnberg & Sundquist, 1998). These oligosaccharides consisted of the disaccharide β-D-galactopyranosyl-(1 → 2)-β-D-glucopyranosiduronic acid and the two trisaccharides β-D-galactopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 3)]-β-D-glucopyranosiduronic acid and β-D-galactopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosiduronic acid.

Several of the *Quillaja* saponins are known to have immuno-enhancing activity and are, together with antigen, cholesterol and phospholipids, able to form immunostimulating complexes (Morein, Sundquist, Höglund, Dalsgaard & Osterhaus, 1984; Morein, Villacres-Eriksson, Åkerblom, Rönnberg, Lövgren & Sjölander, 1994; Morein, Lövgren, Rönnberg, Sjölander & Villacres-Eriksson, 1995). A large-scale procedure has been developed to isolate *Quillaja* saponin fractions, called QH-A and QH-C (Rönnberg, Fekadu & Morein, 1995), which in certain proportions are used for immunostimulating complex formation (ISCOPREP™703; Iscotec AB, Uppsala, Sweden).

In this study, we present the separation and charac-

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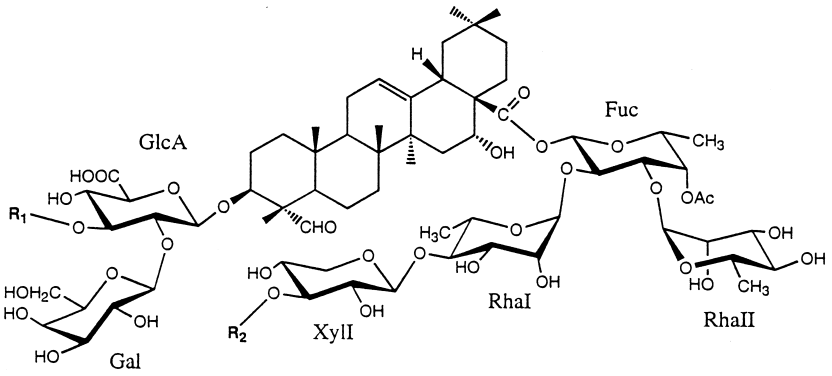


		R_1	R_2
4	=	H	H
5	=	α-L-Rha	H
6	=	β-D-Xyl	H
7	=	α-L-Rha	β-D-Api
8	=	β-D-Xyl	β-D-Api
9	=	α-L-Rha	β-D-Xyl (XylII)
10	=	β-D-Xyl	β-D-Xyl (XylII)

Fig. 1. Structures of saponins **4**–**10**.

terization of seven novel quillaic acid saponins from the fraction QH-A with a different basic structure of the oligosaccharide group at C-28 (Fig. 1). This novel structure consists of a branched β-D-fucopyranosyl residue substituted with an *O*-acetyl group instead of a 2-substituted β-D-fucopyranosyl residue with an additional acyl substituent of two C_9 aliphatic acids as reported for previously identified structures (Higuchi et al., 1987, 1988; Jacobsen et al., 1996).

2. Results and discussion

The saponin fraction QH-A (Rönnberg et al., 1995), obtained from a bark extract, was further fractionated by column chromatography on silica gel and the eluate monitored by TLC and MALDI-TOF mass spectrometry. The first eluted components, designated **1**, **2** and **3**, from the column were described in our previous work (Guo et al., 1998). Further elution of the column yielded a fraction containing mainly a new saponin, **4**, that according to the $[M + Na]^+$ ion at 1461 in the MALDI–TOF mass spectrum had a molecular mass of 1438. The next fraction, partly overlapping with the first fraction, contained several main components which in the MALDI–TOF MS showed $[M + Na]^+$ ions of 1461, 1593, 1607, 1725, and 1739. The components were further purified by reverse phase HPLC using two different solvents. When an ammonium acetate buffer of pH 6.8 was used the saponins separated mainly due to the different structures of the 28-*O*-oligosaccharide. These conditions gave fractions containing pairs of components with a molecular mass difference of 14 Da, according to MALDI–TOF mass spectra. Using a phosphate buffer of pH 2.8 the pairs separated due to the difference in the structures of the oligosaccharide in the 3-position, and seven saponins, designated **4**–**10**, were obtained. The isolated compounds were analysed by sugar and methylation analyses and by MALDI–TOF MS and NMR spectroscopy in order to determine their structures.

The neutral sugars released during acid hydrolysis of **4**–**10** were analysed by GC/MS as their alditol acetates (Sawardeker, Sloneker & Jeanes, 1965) and their absolute configurations determined by GC of the trimethylsilylated (+)-2-butyl glycosides (Gerwig, Kamerling &

Table 1
Neutral sugar content in saponins **4**–**10** of *Quillaja saponaria* Molina

	Residue/mol						
	4	**5**	**6**	**7**	**8**	**9**	**10**
D-Galactose	1	1	1	1	1	1	1
D-Xylose	1	1	2	1	2	2	3
D-Fucose	1	1	1	1	1	1	1
L-Rhamnose	2	3	2	3	2	3	2
D-Apiose	–	–	–	1	1	–	–

Vliegenthart, 1978). D-Galactose, D-xylose, L-rhamnose, D-fucose and D-apiose were the sugars detected in the relative proportions given in Table 1.

The linkages, by which the sugars are connected, were determined by methylation analysis. The samples were methylated according to the Hakomori procedure (Hakomori, 1964), hydrolysed by acid, and the released partially methylated monosaccharides analysed as their alditol acetates by GC/MS (Jansson, Kenne, Liedgren, Lindberg & Lönngren, 1976). The different methylated sugars obtained are listed in Table 2. The results demonstrate the presence of one 2,3-disubstituted fucose in all seven components. This substitution pattern is different from that of the fucose residue in the *Quillaja* saponins previously identified (Higuchi et al., 1987, 1988; Jacobsen et al., 1996). When **4** was first methylated and subsequently reduced with 'Superdeuteride' (Bhat, Krishnaiah & Carlsson, 1991), a derivative of 3,4-di-*O*-methyl-D-glucose-6-d_2 was also present in the analysis. This result demonstrates that the oligosaccharide attached to C-3 of the quillaic acid in **4** consists of the previously found disaccharide β-D-galactopyranosyl-(1 → 2)-β-D-glucopyranosyluronic acid (Guo et al., 1998).

Further structural information was obtained by ^{1}H- and ^{13}C-NMR spectroscopy. All NMR signals from the different sugar residues and the quillaic acid moiety were assigned by different 2D experiments and the chemical shifts are given in Tables 3 and 4. First, all proton spin-systems were determined using different ^{1}H, ^{1}H-COSY, TOCSY and NOESY experiments and then the ^{13}C signals could be assigned by the one- and three-bond H,C-connectivities observed in the HMQC and HMBC spectra. The ^{1}H- and ^{13}C-signals from the triterpene moiety could be identified and when compared to data for previous identified saponins (Jacobsen et al., 1996; Guo et al., 1998) the data supported a quillaic acid substituted at both C-3 and C-28.

Table 2
Methylation analysis of saponins **4**–**10** of *Quillaja saponaria* Molina

Methylated sugar[a]	Residue/mol						
	4	**5**	**6**	**7**	**8**	**9**	**10**
2,3,4-Rha	1	2	1	2	1	2	1
2,3,4-Xyl	1	1	2	–	1	1	2
2,3-Rha	1	1	1	1	1	1	1
2,4-Xyl	–	–	–	1	1	1	1
2,3,4,6-Gal	1	1	1	1	1	1	1
4-Fuc	1	1	1	1	1	1	1
2,3,3′-Apiose[b]	–	–	–	1	1	–	–

[a] 2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, etc.
[b] 2,3,3′-Apiose = 2,3-di-*O*-methyl-3-C-(methoxymethyl)-D-*glycero*-tetrose.

From the comparison of the chemical shifts and the pattern of the cross-peaks with those of corresponding monosaccharides (Jansson, Kenne & Widmalm, 1989; Agrawal, 1992) each sugar and its anomeric configuration could be identified. The anomeric configurations were also supported by the observed $^1J_{C,H}$-values observed for the anomeric atoms (Bock & Pedersen, 1974). The relative high chemical shift for the signal of the substituted carbon (4–9 ppm relative to that of the unsubstituted monosaccharide) confirmed the substitution position determined in the methylation analysis.

The ^{1}H- and ^{13}C-NMR data for the quillaic acid moiety and the oligosaccharide in its 3-position of saponins **4**–**10** are given in Table 3. From the data it is evident that the 3-substituent in compound **4** is the disaccharide β-D-Gal*p*-(1 → 2)-β-D-Glc*p*A, in compounds **5**, **7** and **9** the trisaccharide β-D-Gal*p*-(1 → 2)-[α-L-Rha*p*-(1 → 3)]-β-D-Glc*p*A and in compounds **6**, **8** and **10** 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 di- and trisaccharide 3-substituents in previously identified saponins (Guo et al., 1998). These results are also in agreement with the results from the sugar and methylation analyses.

All ^{1}H and ^{13}C signals for the 28-oligosaccharides were assigned and the NMR data for this part of saponins **4**–**10** are given in Table 4. The ^{1}H-NMR spectrum of **4** contains 9 signals in the region δ 4.4–5.5, where signals from anomeric protons resonate. However, in the HSQC spectrum only six of the signals could derive from anomeric protons as for three signals, δ 5.18, 5.34 and 4.43, the ^{13}C chemical shifts were not in the region for anomeric carbons (δ 94–112). These three unanomeric signals could be derived from H-4 of the acetyl-substituted fucosyl residue and from H-12 and H-16 of the quillaic acid, respectively. Thus, the oligosaccharide in the 28-position consists of a tetrasaccharide and according to the ^{1}H and ^{13}C chemical shifts (Table 4) and the sugar and methylation analyses (Tables 1 and 2) it is build of terminal xylosyl and rhamnosyl groups and 4-substituted rhamnosyl and 2,3-di-substituted fucosyl residues. In order to determine the sequence of the sugar residues in the tetrasaccharide NOESY and HMBC experiments were performed and the inter-residual NOE- and $^3J_{H,C}$-connectivities observed are given in Table 5 and Fig. 2. It is thus obvious that the fucosyl residue, which is linked to C-28 of the quillaic acid, is substituted at C-2 with the 4-substituted α-L-Rha residue (RhaI) and at C-3 with the terminal α-L-Rha group (RhaII). RhaI is then substituted at C-4 with the terminal β-D-Xyl group (XylI). Two more $^3J_{H,C}$-connectivities were observed for the fucosyl residue, between H-4 and the carbonyl carbon of the acetyl group (δ 5.18/171.9) and H-1 and the carbonyl carbon, C-28, of the quillaic acid (δ 5.44/177.1). These results confirm that the fucose residue is

Table 3
The ^{1}H- and ^{13}C-NMR chemical shifts (ppm) for the quillaic acid moiety and the oligosaccharide in its 3-position of saponins **4**–**10**

Atoms no.	**4**		**5**		**6**		**7**		**8**		**9**		**10**	
	^{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	1.11, 1.72	39.0	1.10, 1.71	39.1	1.11, 1.72	39.1	1.11, 1.72	39.2	1.11, 1.73	39.2	1.11, 1.72	39.1	1.10, 1.73	39.2
2	2.00, 1.78	25.3	2.00, 1.77	25.2	1.97, 1.79	25.4	2.09, 1.78	25.4	1.97, 1.77	25.4	2.05, 1.79	25.4	2.04, 1.79	25.1
3	3.89	84.9	3.86	85.9	3.87	86.1	3.87	85.4	3.87	85.8	3.86	85.7	3.86	85.7
4		56.3		56.1		56.2		56.4		56.4		56.2		56.2
5	1.33	49.0	1.32	49.0	1.32	49.0	1.35	49.0	1.34	49.1	1.34	49.0	1.34	48.9
6	0.94, 1.48	21.1	0.93, 1.51	21.0	0.96, 1.50	21.2	0.94, 1.48	21.2	0.93, 1.52	21.1	0.93, 1.50	21.1	0.94, 1.48	21.1
7	1.53, 1.31	33.4	1.55, 1.31	33.4	1.50, 1.31	33.5	1.52, 1.37	33.5	1.57, 1.36	33.4	1.53, 1.37	33.3	1.52, 1.36	33.4
8		41.0		41.0		41.2		41.2		41.2		40.9		40.9
9	1.74	47.8	1.75	47.6	1.75	47.7	1.75	47.8	1.75	47.8	1.75	47.7	1.75	477
10		37.1		36.9		37.1		37.1		37.1		37.1		37.1
11	1.93, 1.93	24.2	1.90, 1.93	24.0	1.93, 1.92	24.2	1.94, 1.93	24.3	1.94,1.94	24.1	1.94, 1.94	24.2	1.93, 1.93	24.2
12	5.34	122.9	5.34	123.0	5.34	122.8	5.34	122.9	5.34	122.9	5.34	123.2	5.34	123.0
13		144.8		144.7		144.6		144.8		144.8		144.7		145.2
14		42.7		42.8		42.8		42.8		42.8		42.7		42.8
15	1.69, 1.42	36.2	1.68, 1.42	36.2	1.70, 1.43	36.1	1.62, 1.45	36.4	1.64, 1.45	36.3	1.63, 1.45	36.2	1.63, 1.45	36.2
16	4.43	74.5	4.44	74.5	4.44	74.4	4.45	74.2	4.45	74.2	4.44	74.2	4.44	74.2
17		49.6		49.7		50.4		49.9		50.1		49.6		49.7
18	2.94	42.1	2.99	41.9	2.94	42.1	2.95	42.1	2.93	42.1	2.95	42.0	2.95	42.0
19	2.30, 1.06	47.9	2.30, 1.06	47.8	2.30, 1.06	47.9	2.31, 1.07	47.9	2.31, 1.07	47.9	2.31, 1.07	47.8	2.30, 1.06	47.9
20		30.8		31.0		30.8		31.3		31.3		30.8		30.8
21	1.92, 1.18	36.4	1.92, 1.17	36.3	1.93, 1.18	36.2	1.93, 1.17	36.3	1.94, 1.18	36.4	1.94, 1.18	36.3	1.92, 1.17	36.4
22	1.91, 1.83	31.5	1.89, 1.82	31.4	1.91, 1.85	31.5	1.94, 1.77	31.7	1.93, 1.90	31.5	1.94, 1.78	31.5	1.93, 1.78	31.5
23	9.46	210.8	9.45	210.8	9.45	210.7	9.47	–[a]	9.47	–[a]	9.44	–[a]	9.44	–[a]
24	1.16	10.5	1.17	10.3	1.17	10.6	1.16	10.8	1.18	10.6	1.17	10.5	1.15	10.6
25	1.01	16.2	1.00	16.1	1.00	16.5	1.01	16.4	1.01	16.2	1.02	16.1	1.01	16.2
26	0.80	17.8	0.79	17.6	0.80	17.8	0.79	17.4	0.79	17.6	0.79	17.5	0.79	17.6
27	1.40	26.8	1.40	26.9	1.40	27.0	1.40	26.9	1.40	26.8	1.41	26.9	1.40	26.8
28		177.1		177.3		177.3		176.9		177.1		176.9		177.1
29	0.88	33.2	0.88	33.1	0.88	33.2	0.88	33.0	0.88	33.1	0.88	33.0	0.88	33.0
30	0.96	24.9	0.96	24.8	0.96	27.9	0.95	24.7	0.95	24.7	0.95	24.6	0.95	24.6
GlcA1	4.40	103.7	4.45	104.0	4.45	104.2	4.39	103.8	4.45	104.2	4.41	103.9	4.40	104.2
GlcA2	3.47	80.9	3.63	78.2	3.66	78.0	3.62	78.2	3.65	77.9	3.62	78.0	3.65	78.1
GlcA3	3.54	77.8	3.64	85.9	3.69	86.5	3.63	85.9	3.68	86.4	3.63	85.6	3.68	86.4
GlcA4	3.49	72.8	3.48	72.9	3.56	73.3	3.55	72.0	3.55	71.7	3.55	71.9	3.53	71.7
GlcA5	3.72	76.2	3.77	76.7	3.79	76.5	3.67	78.3	3.72	77.0	3.69	76.8	3.60	77.7
GlcA6		173.6		172.6		172.7		-[a]		172.7		174.3		174.8
Gal1	4.50	105.0	4.46	104.1	4.80	103.5	4.47	103.9	4.80	103.3	4.46	104.0	4.80	103.4
Gal2	3.55	73.3	3.56	73.4	3.45	73.3	3.51	72.7	3.47	73.2	3.51	72.7	3.48	73.3
Gal3	3.46	74.7	3.48	74.9	3.43	75.2	3.49	74.7	3.44	75.1	3.48	74.8	3.45	75.1
Gal4	3.83	70.3	3.82	70.4	3.82	70.5	3.83	70.4	3.83	70.4	3.83	70.4	3.84	70.4
Gal5	3.52	76.7	3.47	76.9	3.49	76.5	3.47	76.6	3.49	76.5	3.47	76.6	3.49	76.3
Gal6	3.74, 3.80		3.73, 3.79	61.9	3.73, 3.78	61.9	3.73, 3.78	61.9	3.74, 3.77	62.0	3.47, 3.78	61.9	3.74, 3.77	61.8
Xyl1					4.58	104.7			4.58	104.6			4.62	104.5
Xyl2					3.24	75.0			3.23	75.0			3.23	75.1
Xyl3					3.30	78.0			3.31	77.9			3.31	78.0
Xyl4					3.50	70.8			3.51	70.7			3.51	70.7
Xyl5					3.24, 3.91	66.8			3.25, 3.92	66.7			3.24, 3.91	66.9
Rha1			5.04	103.1			5.06	102.8			5.04	103.0		
Rha2			4.02	71.9			4.01	71.9			4.01	71.9		
Rha3			3.65	72.1			3.67	72.1			3.66	72.0		
Rha4			3.40	73.7			3.38	73.7			3.39	73.6		
Rha5			3.95	70.3			4.03	70.0			3.98	70.1		
Rha6			1.25	17.4			1.24	17.6			1.23	17.5		

[a] Signal not detected.

Table 4
The ^{1}H- and ^{13}C-NMR chemical shifts (ppm) for the oligosaccharide in the 28-position of saponins **4**–**10**

Atoms no.	**4**		**5**		**6**		**7**		**8**		**9**		**10**	
	^{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
Fuc1	5.43	94.9	5.44	94.9	5.44	94.8	5.44	94.5	5.44	94.6	5.44	94.6	5.44	94.5
Fuc2	3.85	75.2	3.85	75.4	3.85	75.2	3.88	74.3	3.88	74.3	3.88	74.3	3.88	74.4
Fuc3	3.95	81.8	3.95	81.9	3.96	81.7	3.97	82.4	3.95	82.3	3.96	82.4	3.96	82.4
Fuc4	5.18	74.1	5.18	74.1	5.18	74.1	5.19	74.1	5.18	74.1	5.18	74.2	5.18	74.1
Fuc5	3.87	70.9	3.87	70.9	3.87	71.0	3.86	71.0	3.87	71.0	3.87	71.0	3.87	71.0
Fuc6	1.05	16.3	1.06	16.3	1.06	16.5	1.04	16.4	1.06	16.6	1.05	16.3	1.05	16.4
RhaI1	5.08	101.9	5.08	101.7	5.08	101.6	5.12	101.3	5.11	101.4	5.12	101.5	5.12	101.4
RhaI2	3.84	71.7	3.83	71.7	3.83	71.7	3.84	71.7	3.84	71.6	3.84	71.6	3.84	71.6
RhaI3	3.78	72.0	3.78	72.1	3.79	72.0	3.76	72.1	3.76	72.1	3.76	72.1	3.76	72.1
RhaI4	3.55	83.7	3.55	83.9	3.38	83.7	3.54	84.5	3.54	84.4	3.54	84.4	3.54	84.4
RhaI5	3.77	69.1	3.77	68.9	3.77	69.0	3.77	68.7	3.77	68.7	3.76	68.7	3.76	68.6
RhaI6	1.30	18.1	1.30	18.0	1.31	18.4	1.33	18.3	1.33	18.2	1.32	18.2	1.32	18.2
RhaII1	4.89	104.8	4.89	104.6	4.89	104.5	4.89	104.4	4.88	104.5	4.89	104.9	4.89	104.6
RhaII2	3.87	72.0	3.87	71.9	3.87	71.9	3.87	71.9	3.86	72.0	3.86	71.9	3.87	72.0
RhaII3	3.55	71.9	3.56	71.9	3.56	72.0	3.55	72.0	3.56	71.9	3.56	71.9	3.55	71.9
RhaII4	3.37	73.4	3.38	73.4	3.37	73.3	3.37	73.4	3.38	73.2	3.37	73.4	3.38	73.2
RhaII5	3.58	70.7	3.58	70.6	3.57	70.6	3.57	70.6	3.57	70.7	3.57	70.6	3.57	70.6
RhaII6	1.22	17.7	1.23	17.4	1.23	17.7	1.24	17.6	1.22	17.7	1.21	17.7	1 .22	17.7
XylI1	4.48	106.8	4.48	107.0	4.48	106.8	4.48	106.9	4.48	106.8	4.53	106.5	4.53	106.5
XylI2	3.20	75.9	3.20	75.9	3.20	75.8	3.30	75.3	3.30	75.2	3.35	74.9	3.35	74.9
XylI3	3.31	78.0	3.31	78.2	3.32	78.0	3.39	85.5	3.39	85.5	3.48	87.0	3.49	87.1
XylI4	3.46	70.9	3.47	70.9	3.47	70.9	3.49	69.5	3.49	69.3	3.52	69.2	3.53	69.3
XylI5	3.18, 3.85	67.0	3.19, 3.85	67.0	3.19, 3.85	67.0	3.19, 3.85	67.0	3.21, 3.89	66.8	3.21, 3.89	66.6	3.22, 3.89	66.7
Api1							5.25	110.7	5.25	110.8				
Api2							4.07	77.6	4.06	77.5				
Api3							–	80.3	–	80.3				
Api4							3.81, 4.16	74.8	3.81, 4.15	74.7				
Api5							3.67	65.1	3.67	65.0				
XylII1											4.52	105.3	4.52	105.3
XylII2											3.38	74.8	3.38	74.8
XylII3											3.39	77.5	3.39	77.6
XylII4											3.56	70.7	3.50	70.6
XylII5											3.28, 3.94	67.0	3.29, 3.94	66.9

4-*O*-substituted by an acetyl group and that it is linked to C-28 of the quillaic acid.

Both compounds **5** and **6** have the same tetrasaccharide at C-28 of the quillaic acid as compound **4** according to almost identical chemical shifts and coupling patterns of the ^{1}H and ^{13}C signals for the tetrasaccharide (Table 4) as those observed for the corresponding moiety in **4**. The only difference between these three saponins is in the structure of the oligosaccharide at C-3 of the quillaic acid (Fig. 1). Compounds **5** and **6** are substituted with a trisaccharide instead of the disaccharide in **4**. The molecular masses and the results of the sugar and methylation analyses (Tables 1 and 2) were consistent with this conclusion.

Saponins **7** and **8** have the same trisaccharide at C-3 of quillaic acid as **5** and **6**, respectively, but contain an additional sugar residue, β-D-apiose, according to the molecular mass, the sugar and methylation analyses (Tables 1 and 2) and the appearance of an additional spin-system with the signals for anomeric atoms at δ 5.25, $J_{1,2} = 2.9$ Hz and δ 110.7 in the ^{1}H- and ^{13}C-NMR spectrum, respectively. The similar chemical shifts of both carbon and proton signals also confirmed the same configuration at C-3 as those previously reported for *Quillaja* saponins (Jacobsen et al., 1996). The chemical shifts of the signals from the sugars in the tetrasaccharide part, occurring in **5** and **6**, showed only significant changes for signals from XylI with a major shift for the C-3 signal of +7.3 ppm. This result and the occurrence of a 3-substituted xylosyl residue in the methylation analysis indicated that the β-D-apiosyl group is linked to this position in saponin **7** and **8**. This structure of the pentasaccharide (Fig. 1) was corroborated by the $^{3}J_{H,C}$-connectivities (Fig. 2) observed in the HMBC spectra between the anomeric atoms of the β-D-apiosyl group and the linkage atoms of XylI.

Table 5
Observed NOE and $^3J_{H,C}$ connectivities for the anomeric atoms of the sugar residues in the 28-*O*-oligosaccharide of saponin **4**[a]

Residue	Anomeric atom		Connectivities to		Intra-residue	Inter-residue	
	δ_H	δ_C	δ_C	δ_H	Atom	Residue	Atom
D-Xyl-(1 →	*4.48*			*3.55*		→ 4)-α-L-Rha-(1 →	*H-4*
(XylI)	*4.48*		83.7			→ 4)-α-L-Rha-(1 →	C-4
		106.8		3.55		→ 4)-α-L-Rha-(1 →	H-4
		106.8		3.18, 3.85	H-5		
		106.8		3.53	H-4		
	4.48			*3.31*	*H-3*		
	4.48		78.0		C-3		
	4.48		67.0		C-5		
→ 4)-α-L-Rha-(1 →	*5.08*			*3.85*		→ 2,3)β-D-Fuc-(1 →	*H-2*
(RhaI)	5.08		75.2			→ 2,3)-β-D-Fuc-(1 →	C-2
		101.9		3.85		→ 2,3)-β-D-Fuc-(1 →	H-2
	5.08			*3.84*	*H-2*		
	5.08		72.0		C-3		
	5.08		69.1		C-5		
α-L-Rha-(1 →	*4.89*			*3.95*		→ 2,3)-β-D-Fuc-(1 →	*H-3*
(RhaII)		104.8		3.95		→ 2,3)-β-D-Fuc-(1 →	H-3
	4.89		81.8			→ 2,3)-β-D-Fuc-(1 →	C-3
	4.89			*3.87*	*H-2*		
	4.89		70.7		C-5		
		104.8		3.87	H-2		
→ 2,3-β-D-Fuc-(1 →	*5.44*			*3.85*	*H-2*		
		94.9		3.85	H-2		
	5.44			*3.95*	*H-3*		
	5.44		177.1			Quillaic acid	C-28

[a] The connectivities were observed as cross-peaks in NOESY and HMBC spectra (NOE connectivities are shown in italics).

Compounds **9** and **10** had the same molecular mass as **7** and **8** but the sugar and methylation analyses (Tables 1 and 2) identified an additional xylose instead of the apiose. Similar chemical shift changes for the tetrasaccharide part as those observed for **7** and **8** and the additional spin-system, with the signals for anomeric atoms at δ 4.52, $J_{1,2} = 7.5$ Hz and δ 105.3, in the NMR spectra (Table 4) identified a β-D-xylosyl group (XylII) linked to the 3-position of XylI. This structure of the pentasaccharide (Fig. 1) was corroborated by the $^3J_{H,C}$-connectivities (Fig. 2) observed in the HMBC spectra between the anomeric atoms of the β-D-xylosyl group and the linkage atoms H-3/C-3 of XylI.

The structures of the saponins **4**–**10** (Fig. 1) are different from the structures previously identified for saponins isolated from bark extract of *Quillaja saponaria* Molina (Higuchi et al., 1987, 1988; Jacobsen et al., 1996). In **4**–**10** the fucosyl residue linked to the carboxyl group of the quillaic acid is branched and substituted with an *O*-acetyl group instead of the C_9 aliphatic acid. In this study, we have completely characterised the basic structure of these *Quillaja* saponins which was previously indicated by mass spectrometry using MS-MS on a ion-trap instrument (van Setten et al., 1998) and suggested for QS-7 from FAB-MS and carbohydrate linkage analysis (Kensil, Wu, Anderson, Wheeler & Amsden, 1998).

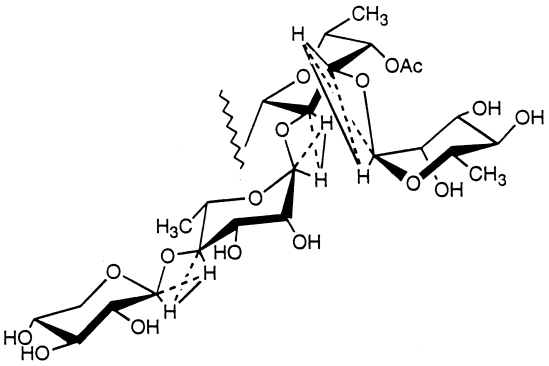
3. Experimental

3.1. Materials

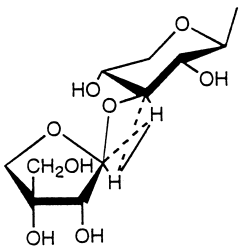
The saponin fraction from a bark extract of *Quillaja saponaria* Molina, called QH-A, was obtained from Iscotec AB (Uppsala, Sweden).

3.2. Isolation of compounds *4*–*10*

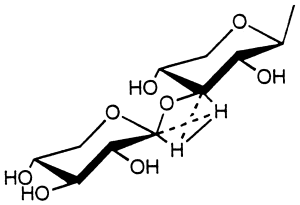
A sample of QH-A (1.7 g) was fractionated on a column (5 × 45 cm) of silica gel 60 (0.04–0.063 μm, Merck) using a mixture of $CHCl_3$, MeOH, H_2O and HOAc (24 : 17.5 : 3 : 0.1) as solvent. On the basis of TLC and MALDI–TOF MS, a fraction was obtained which contained mainly **4** (55 mg). Further elution of the column gave a fraction which after concentration to dryness gave a mixture of **4**–**10** (200 mg). The two fractions were further separated by reverse phase



4-6 (4-10)



7, 8



9, 10

Fig. 2. Inter-residual NOE- (solid lines) and $^{3}J_{H,C}$- (dashed lines) -connectivities as observed in NOESY and HMBC spectra, respectively, for the oligosaccharide at C-28 of compounds **4**–**10**.

HPLC on a preparative system using a Kromasil 100-5C18 (20 × 150 mm) column (Hichrom, UK) and the eluate was monitored using a UV detector at 205 nm. For compound **4** elution was carried out with MeCN and aq. 0.01 M phosphate buffer, pH 2.8 (31 : 69) at the flow rate 7 ml min^{-1}. The fraction containing a mixture of **4**–**10** was separated on the same system using MeCN and aq. 0.02 M ammonium acetate buffer in 20% MeCN, pH 6.8 (9 : 91) at the flow rate 9 ml min^{-1} and the fractions obtained were checked by MALDI–TOF MS. The latter fractions, containing pairs of compounds separated by 14 Da, were further separated on the same system using MeCN and aq. 0.01 M phosphate buffer, pH 2.8 (31 : 69) at the flow rate 8.5 ml min^{-1}. The fractions containing pure saponins were first evaporated to remove the MeCN, then diluted with H_2O and the solutions passed through Isolute SPE columns (C-18, 10 g). The columns were washed with H_2O to remove salts, the saponins eluted with MeOH (~30 ml) and the solutions obtained concentrated to dryness.

Sugar and methylation analyses were performed as previously described (Guo et al., 1998). For analysis of apiose the same conditions were used but the acidic hydrolysis was performed at 90° for 30 min. The absolute configurations of the sugars were determined by GC of the trimethylsilylated (+)-2-butyl glycosides (Gerwig et al., 1978).

3.3. Mass spectrometry

The MALDI-TOF mass spectra were recorded on a Linear LDI-1700XS spectrometer using a 337 nm nitrogen laser and 2,5-dihydroxybenzoic acid as matrix.

3.4. NMR spectroscopy

NMR spectra were recorded for samples in CD_3OD on a Bruker DRX-600 spectrometer with a proton frequency of 600 MHz equipped with a 5 mm triple-resonance inverse probe or a 2.5 mm microprobe. All spectra were acquired at 30° without spinning the sample. Chemical shifts are reported in ppm using the solvent peak as a reference (δ_H 3.31 and δ_C 49.0). DQF–COSY, TOCSY, NOESY (mixing times of 200 ms or 400 ms), HMQC, HSQC and HMBC (delay times of 50 or 70 ms) experiments were performed according to standard pulse sequences.

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