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Characterization of some *O*-acetylated saponins from *Quillaja saponaria* Molina

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Abstract

Sixteen saponins were identified from a bark extract of *Quillaja saponaria* Molina. The compounds were characterized, using NMR spectroscopy, mass spectrometry and monosaccharide analysis, as quillaic acid substituted at C-3 with oligosaccharides consisting of a disaccharide, β-D-Gal*p*-(1 → 2)-β-D-Glc*p*A substituted with either D-xylose or L-rhamnose and at C-28 with complex oligosaccharide structures consisting of a disaccharide, α-L-Rha*p*-(1 → 2)-4-*O*-acetyl-β-D-Fuc*p*, substituted with various amount of D-xylose, D-glucose, D-apiose, and L-rhamnose.

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

1. Introduction

The bark of the *Quillaja saponaria* Molina tree is a rich source of saponins and at least 60 different saponins in bark extract were partly characterized using HPLC in combination with mass spectrometry (van Setten et al., 1995, 1998). Partly purified bark extracts, such as Quil A, have found wide-spread use as an adjuvant in veterinary vaccines (Kensil et al., 1998; Campbell and Peerbaye, 1992; Claassen and Osterhaus, 1992). More than 30 components have been completely characterized and these have in common the triterpene quillaic acid with di- and tri-saccharides attached at C-3 and several oligosaccharides attached at C-28. The latter oligosaccharide is substituted with either two C_9 aliphatic acids (Higuchi et al., 1987, 1988; Jacobsen et al., 1996; Nord and Kenne, 1999; Nyberg et al., 2000) or an *O*-acetyl group (Guo et al., 2000). The structures of several *Quillaja* saponins were previously indicated by multiple stage mass spectrometry on an ion-trap instrument (van Setten et al., 1998).

In this study we present the separation and characterization of 16 novel quillaic acid saponins from a bark extract of *Q. saponaria* Molina which have less affinity to a C-18 column than the major part of the *Quillaja* saponins. In the isolated saponins, the quillaic acid was substituted at C-3 with two different trisaccharides and at C-28 with several oligosaccharides containing one or two *O*-acetyl groups (Fig. 1).

2. Results and discussion

A commerical bark extract was first fractionated by SPE on a C-18 column. The saponins collected from the 60% MeOH eluate (the first fraction containing saponins) were further fractionated by column chromatography on silica gel and the eluate was monitored by TLC, ^{1}H MNR and MALDI-TOF mass spectrometry. The isolation and identification of the early eluting components (**1**–**10**) have been described for a similar fraction of a bark extract in previous work (Guo et al., 2000, 1998). Further elution gave two fractions and the MALDI-TOF mass spectra of these

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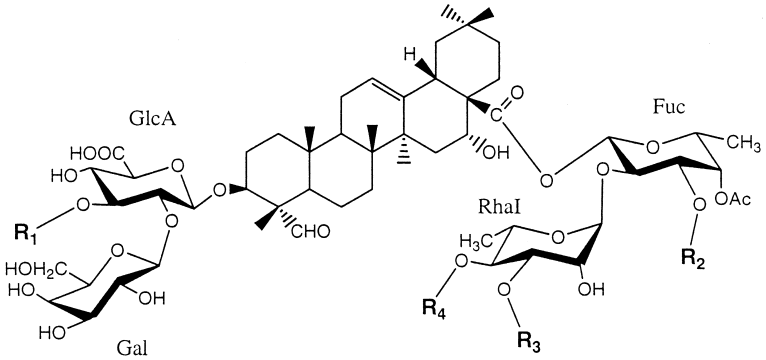
showed major peaks at *m/z* 1621.6/1607.7, 1651.8/1637.7, 1753.7/1739.7, 1664.0/1650.1, 1826.0/1812.1 for the first fraction and at *m/z* 1783.9/1770.1, 1767.7/1753.7, 1899.2/1885.2 for the second. These peaks were assigned to $[M + Na]^+$ ions and for some of the compounds various intensities of $[M + H]^+$, $[M + K]^+$ and $[M - H + 2Na]^+$ ions were also observed. The two fractions were further separated by reverse phase HPLC using an ammonium acetate buffer of pH 6.8. The separation obtained by this procedure is mainly due to the different structures of the oligosaccharide at C-28 of the quillaic acid (Guo et al., 2000; Nord and Kenne, 1999; Nyberg et al., 2000). These conditions gave eight fractions and MALDI-TOF MS of each fraction showed two $[M + Na]^+$ ions with a molecular mass difference of 14 Da, suggesting that each fraction contained at least two components. This was confirmed by ^{1}H NMR spectroscopy, which showed two anomeric signals (a terminal rhamnose at δ 5.03–5.05 ppm and a terminal xylose at δ 4.61–4.63 ppm) with integral values that added up to the equivalent of one proton. Among these 16 saponins, only one pair of components **14a**,**b** were further separated by a second separation step, using a phosphate buffer of pH 2.8. Separation of the components is obtained due to the different structures of the oligosaccharide at C-3 of the quillaic acid (Guo et al., 2000; Nord and Kenne, 1999; Nyberg et al., 2000), resulting in two pure compounds, **14a** and **14b**, according to ^{1}H NMR spectroscopy and MALDI-TOF MS. The other compounds, **11a**,**b**–**18a**,**b**, were analyzed as pairs of components with either a terminal Rha or Xyl group in the C-3 trisaccharide.

The components were analyzed by monosaccharide analysis and methylation analysis (only **14a** and **14b**), MALDI-TOF MS (Table 1), and NMR spectroscopy in order to determine their complete structures. The neutral sugars released during acid hydrolysis of **11a**,**b**–**18a**,**b** were analyzed by GC-MS as their alditol acetates (Sawardeker et al., 1965) and their absolute configurations were assumed to be the same as those in previously identified saponins (van Setten and van



	R_1	R_2	R_3	R_4
11a,b	Rha/Xyl	GlcI	H	Xyl
12a,b	Rha/Xyl	GlcI	GlcII	H
13a,b	Rha/Xyl	H	GlcII	Api-Xyl
14a	Rha	GlcI	GlcII	Xyl
14b	Xyl	GlcI	GlcII	Xyl
15a,b	Rha/Xyl	GlcI6OAc	H	Xyl
16a,b	Rha/Xyl	GlcI6OAc	GlcII	Xyl
17a,b	Rha/Xyl	RhaII	GlcII	Xyl
18a,b	Rha/Xyl	RhaII	GlcII	Api-Xyl

Rha = α-L-Rha*p*
Xyl = β-D-Xyl*p*
Glc = β-D-Glc*p*
Glc6OAc = 6-*O*-acetyl-β-D-Glc*p*
Api-Xyl = β-D-Api*f*-(1→3)-β-D-Xyl*p*

Fig. 1. Structures of saponins **11a**,**b**–**18a**,**b**.

de Werken, 1996; Guo et al., 1998, 2000). D-Gal, D-Glc, D-Xyl, L-Rha, D-Fuc and D-Api were the sugars detected in the relative proportions given in Table 1.

Hydrolysis of permethylated **14a** and **14b** followed by reduction and acetylation afforded partially methylated alditol acetates which were analyzed by GC-MS (Hakomori, 1964; Jansson et al., 1976) indicating one equivalent of 2,3-disubstituted Fuc, 3,4-disubstituted Rha, terminal Gal, terminal Xyl and two equivalents of terminal Glc in both components. In addition to these monosaccharides, a terminal Rha was obtained for **14a** and a terminal Xyl for **14b**.

^{1}H and ^{13}C NMR spectroscopy provided further structural information (Tables 2 and 3). First, all proton spin-systems were determined using different ^{1}H, ^{1}H-COSY, TOCSY and NOESY experiments. Starting with the signal from the anomeric proton, the H-2 to H-6 signals were identified from the COSY, TOCSY and NOESY spectra. The ^{13}C signals could be assigned by the one- and three-bond heteronuclear connectivities observed in the HSQC and HMBC spectra. The cross-peaks observed in NOESY and HMBC spectra could be used to assign the sequence of sugar residues and the overlapped signals in complex saponins. From the comparison of the chemical shifts and the pattern of the cross-peaks with those of corresponding monosaccharides (Jansson et al., 1989; Agrawal, 1992) each sugar and its anomeric configuration could be identified. The anomeric configurations were also supported by the $^{3}J_{\text{H-1, H-2}}$-values observed for the anomeric protons in the ^{1}H NMR spectra and $^{1}J_{\text{C-1, H-1}}$-values observed for the anomeric atoms in non-decoupled HSQC spectra (Bock and Pedersen, 1974). For compounds **18a** and **18b**, the $^{1}J_{\text{C-1, H-1}}$-values were 170, 172 and 173 Hz for the α-linked rhamnoses and 160, 160, 160, 162, and 167 Hz for the β-linked Gal, GlcA, Glc, Xyl and Fuc, respectively, and 175 Hz for the β-furanosidic Api. The relative high chemical shift for the signal of the substituted carbon relative to that of the unsubstituted monosaccharide and connectivities observed in NOESY and HMBC spectra supported the substitution positions determined for **14a** and **14b** in the methylation analysis.

The signals from the triterpene moiety and the trisaccharide at C-3 were assigned from the spin-systems observed and by comparison of the data with those of previously identified saponins (Guo et al., 1998, 2000; Nord and Kenne 1999; Nyberg et al., 2000). The data from different 2D NMR experiments demonstrated that the aglycone is quillaic acid, substituted at both C-3 and C-28, for all 16 saponins since almost identical chemical shifts and coupling patterns with those of saponins found in our previous studies were observed. The structure of the trisaccharide at C-3 in compound **14a** is β-D-Gal*p*-(1 → 2)-[α-L-Rha*p*-(1 → 3)]-β-D-Glc*p*A and in **14b** 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 trisaccharide substituents in previously identified saponins (Guo et al., 2000; Nord and Kenne, 1999; Nyberg et al., 2000; Guo et al., 1998). These results are also in agreement with the results from the sugar and methylation analyses of **14a** and **14b**.

For compounds **11**–**13** and **15**–**18**, the ^{1}H NMR spectra of each compound contain both anomeric signals in the region δ 5.03–5.05 and at δ 4.61–4.63, besides all other anomeric signals, and these belong to

Table 1
Molecular masses and number of neutral monosaccharide residues[a] in saponins **11a**,**b**–**18a**,**b** of *Quillaja saponaria* Molina

Compound	M_w[b]	M_w[c]	D-Gal	D-Glc	D-Xyl	D-Fuc	L-Rha	D-Api
11a	1598.6	1598.6	1	1	1	1	2	–
11b	1584.7	1584.7	1	1	2	1	1	–
12a	1628.8	1628.7	1	2	–	1	2	–
12b	1614.7	1614.7	1	2	1	1	1	–
13a	1730.7	1730.7	1	1	1	1	2	1
13b	1716.7	1716.7	1	1	2	1	1	1
14a	1760.9	1760.8	1	2	1	1	2	–
14b	1747.1	1746.7	1	2	2	1	1	–
15a	1641.0	1640.7	1	1	1	1	2	–
15b	1627.1	1626.7	1	1	2	1	1	–
16a	1803.0	1802.8	1	2	1	1	2	–
16b	1789.1	1788.8	1	2	2	1	1	–
17a	1744.7	1744.8	1	1	1	1	3	–
17b	1730.7	1730.7	1	1	2	1	2	–
18a	1876.2	1876.8	1	1	1	1	3	1
18b	1862.2	1862.8	1	1	2	1	2	1

[a] The number of neutral monosaccharide residues was estimated from the results of the monosaccharide analyses and the integrals of the signals for anomeric protons in the ^{1}H NMR spectra.
[b] Molecular mass determined by MALDI-TOF mass spectrometry.
[c] Molecular mass of the assigned structures.

Table 2
The ^{1}H and ^{13}C NMR chemical shifts (ppm) for the oligosaccharide in the 28-position of saponins **11–18**

Atoms no.	**11**		**12**		**13**		**14a**		**14b**		**15**		**16**		**17**		**18**	
	^{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	^{1}H	^{13}C
Fuc1	5.40	94.8	5.44	94.9	5.34	94.9	5.41	94.9	5.40	94.9	5.41	94.9	5.41	94.9	5.44	94.9	5.44	94.9
Fuc2	3.90	73.5	3.92	74.8	3.68	75.6	3.91	74.6	3.91	74.6	3.91	73.4	3.91	74.4	3.85	76.4	3.86	76.2
Fuc3	4.03	83.0	4.03	82.4	3.85	73.9	4.03	82.4	4.02	82.3	3.98	82.8	3.97	82.3	3.97	81.7	3.96	82.3
Fuc4	5.37	74.8	5.36	74.8	5.08	74.8	5.36	74.7	5.36	74.7	5.31	74.4	5.30	74.7	5.18	74.0	5.18	74.3
Fuc5	3.86	71.0	3.86	70.8	3.82	70.7	3.86	70.8	3.86	70.8	3.83	71.0	3.83	70.7	3.87	71.3	3.87	71.1
Fuc6	1.06	16.2	1.06	16.3	1.07	16.3	1.06	16.3	1.06	16.4	1.06	16.2	1.06	16.5	1.06	16.6	1.05	16.4
RhaI1	5.41	101.1	5.33	101.6	5.21	101.6	5.33	102.1	5.33	102.1	5.39	101.3	5.32	101.1	5.08	101.7	5.09	101.8
RhaI2	3.95	71.6	4.22	70.9	4.25	71.0	4.24	70.9	4.24	70.91	3.96	71.5	4.24	70.9	4.10	71.3	4.11	71.3
RhaI3	3.80	71.9	3.67	83.0	3.92	82.8	3.89	82.8	3.90	82.8	3.81	71.9	3.90	82.7	3.86	83.1	3.86	83.2
RhaI4	3.55	83.9	3.55	72.2	3.67	78.7	3.68	78.7	3.68	78.8	3.55	83.9	3.68	78.6	3.67	79.0	3.66	78.9
RhaI5	3.80	68.9	3.80	69.8	3.80	68.8	3.84	68.8	3.84	68.7	3.80	68.8	3.85	68.8	3.81	69.3	3.79	69.4
RhaI6	1.33	18.4	1.29	18.4	1.29	18.1	1.28	18.4	1.28	18.4	1.33	18.0	1.29	18.6	1.27	18.8	1.29	18.7
RhaII1															4.93	104.6	4.92	104.8
RhaII2															3.87	72.3	3.87	72.1
RhaII3															3.55	72.2	3.55	72.2
RhaII4															3.36	73.8	3.37	73.8
RhaII5															3.59	70.9	3.58	71.1
RhaII6															1.17	18.4	1.20	17.9
Xyl1	4.48	106.9			4.70	104.9	4.67	105.1	4.67	105.1	4.49	107.0	4.68	104.9	4.67	105.2	4.69	105.2
Xyl2	3.22	75.9			3.21	75.1	3.10	75.4	3.10	75.4	3.22	75.9	3.12	75.3	3.09	75.4	3.19	75.3
Xyl3	3.32	78.0			3.35	85.8	3.27	78.5	3.27	78.5	3.33	78.0	3.28	78.4	3.26	78.4	3.34	86.0
Xyl4	3.48	70.9			3.49	73.1	3.47	71.2	3.47	71.2	3.49	70.8	3.47	71.1	3.46	71.1	3.48	70.7
Xyl5	3.18	67.0			3.18	66.5	3.16	66.8	3.16	66.8	3.21	67.0	3.17	66.8	3.16	66.8	3.17	66.9
Xyl5′	3.85				3.88		3.84		3.84		3.86		3.84		3.83		3.87	
Api1					5.28	111.1											5.29	111.3
Api2					4.03	77.7											4.03	78.0
Api3					–	80.3											–	80.2
Api4					3.80	74.9											3.80	75.0
Api4′					4.14												4.14	
Api5					3.64	65.2											3.64	65.6
GlcI1	4.49	105.2	4.48	105.5			4.48	105.5	4.48	105.4	4.49	104.9	4.48	105.0				
GlcI2	3.15	75.2	3.16	75.2			3.15	75.1	3.15	75.1	3.18	75.1	3.18	75.1				
GlcI3	3.33	78.0	3.36	77.8			3.35	77.9	3.35	77.9	3.35	77.9	3.36	77.6				
GlcI4	3.23	71.1	3.22	71.2			3.22	71.1	3.22	71.1	3.33	70.9	3.28	70.9				
GlcI5	3.28	78.0	3.28	78.0			3.29	77.8	3.28	77.9	3.43	75.0	3.45	74.9				
GlcI6	3.61	62.7	3.62	62.7			3.61	62.7	3.61	62.6	4.43	63.9	4.43	63.9				
GlcI6′	3.85		3.86				3.85		3.86		4.14		4.14					
GlcII1			4.51	105.4	4.51	105.2	4.55	104.9	4.55	104.8			4.56	104.7	4.57	105.1	4.55	105.1
GlcII2			3.29	75.2	3.30	75.1	3.29	75.1	3.29	75.1			3.31	75.0	3.29	75.4	3.29	75.4
GlcII3			3.38	77.6	3.26	77.7	3.35	77.8	3.36	77.8			3.33	77.8	3.36	77.9	3.35	77.7
GlcII4			3.33	71.1	3.34	70.9	3.34	71.0	3.34	71.0			3.36	70.9	3.34	71.2	3.34	71.2
GlcII5			3.34	78.0	3.33	78.0	3.35	78.0	3.35	77.9			3.29	78.4	3.37	77.8	3.36	77.9
GlcII6			3.70	62.3	3.69	62.1	3.70	62.2	3.71	62.1			3.71	62.0	3.71	62.0	3.71	62.2
GlcII6′			3.85		3.84		3.85		3.85				3.86		3.85		3.85	

Table 3
Observed inter-residue NOE and $^{3}J_{H,C}$ connectivities from the anomeric proton of the sugar residues in the 28-*O*-oligosaccharide of saponin **11**–**18**[a]

Compound	Residue	Anomeric proton ^{1}H (ppm)	Inter-residual connectivities			Atom
			δ_C	δ_H	Residue	
11a,**b**	β-D-Xyl-(1 →	4.48	83.9		→ 4)-α-L-Rha-(1 →	C-4
		4.48		*3.55*	→ *4*)-α-*L*-*Rha*-(*1* →	*H-4*
	→ 4)-α-L-Rha-(1 →	5.41	73.5		→ 2,3)-β-D-Fuc-(1 →	C-2
	(RhaI)	*5.41*		*3.90*	→ *2,3*)-β-*D*-*Fuc*-(1 →	*H-2*
	D-Glc-(1 →	4.49	83.0		→ 2,3)-β-D-Fuc-(1 →	C-3
	(GlcI)	*4.49*		*4.03*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-3*
	→ 2,3)-β-D-Fuc-(1 →	5.40	177.1		Quillaic acid	C-28
12a,**b**	β-D-Glc-(1 →	4.51	83.0		→ 3)-α-L-Rha-(1 →	C-3
	(GlcII)	*4.51*		*3.67*	→ *3*)-α-*L*-*Rha*-(*1* →	*H-3*
	→ 3)-α-L-Rha-(1 →	5.33	74.8		→ 2,3)-β-D-Fuc-(1 →	C-2
	(RhaI)	*5.33*		*3.92*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-2*
	β-D-Glc-(1 →	4.48	82.4		→ 2,3)-β-D-Fuc-(1 →	C-3
	(GlcI)	*4.48*		*4.03*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-3*
	→ 2,3)-β-D-Fuc-(1 →	5.44	176.8		Quillaic acid	C-28
13a,**b**	β-D-Api-(1 →	5.28	85.8		→ 3)-β-D-Xyl-(1 →	C-3
		5.28		*3.35*	→ *3*)-β-*D*-*Xyl*-(*1* →	*H-3*
	→ 3)-β-D-Xyl-(1 →	4.70	78.7		→ 3,4)-α-L-Rha-(1 →	C-4
		4.70		*3.67*	→ *3,4*)-α-*L*-*Rha*-(*1* →	*H-4*
	β-D-Glc-(1 →	4.51	82.8		→ 3,4)-α-L-Rha-(1 →	C-3
	(GlcII)	*4.51*		*3.92*	→ *3,4*)-α-*L*-*Rha*-(*1* →	*H-3*
	→ 3,4)-α-L-Rha-(1 →	5.21	75.6		→ 2)-β-D-Fuc-(1 →	C-2
	(RhaI)	*5.21*		*3.68*	→ *2*)-β-*D*-*Fuc*-(*1* →	*H-2*
	→ 2-β-D-Fuc-(1 →	5.34	177.3		Quillaic acid	C-28
14a,**b**	β-D-Xyl-(1 →	4.67	78.8		→ 3,4)-α-L-Rha-(1 →	C-4
		4.67		*3.68*	→ *3,4*)-α-*L*-*Rha*-(*1* →	*H-4*
	β-D-Glc-(1 →	4.55	82.8		→ 3,4)-α-L-Rha-(1 →	C-3
	(GlcII)	*4.55*		*3.89*	→ *3,4*)-α-*L*-*Rha*-(*1* →	*H-3*
	→ 3,4)-α-L-Rha-(1 →	5.33	74.6		→ 2,3)-β-D-Fuc-(1 →	C-2
	(RhaI)	*5.33*		*3.91*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-2*
	β-D-Glc-(1 →	4.48	82.4		→ 2,3)-β-D-Fuc-(1 →	C-3
	(GlcI)	*4.48*		*4.03*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-3*
	→ 2,3)-β-D-Fuc-(1 →	5.41	177.1		Quillaic acid	C-28
15a,**b**	β-D-Xyl-(1 →	4.49	83.9		→ 4)-α-*L*-Rha-(1 →	C-4
		4.67		*3.68*	→ *4*)-α-*L*-*Rha*-(*1* →	*H-4*
	→ 4)-α-L-Rha-(1 →	5.39	73.4		→ 2,3)-β-D-Fuc-(1 →	C-2
	(RhaI)	*5.39*		*3.91*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-2*
	β-D-6-*O*-acetyl-Glc-(1 →	4.49	82.8		→ 2,3)-β-D-Fuc-(1 →	C-3
	(GlcI)	*4.49*		*3.98*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-3*
	→ 2,3)-β-D-Fuc-(1 →	5.41	177.0		Quillaic acid	C-28
16a,**b**	β-D-Xyl-(1 →	4.68	78.6		→ 3,4)-α-*L*-Rha-(1 →	C-4
		4.68		*3.68*	→ *3,4*)-α-*L*Rha-(*1* →	*H-4*
	β-D-Glc-(1 →	4.56	82.7		→ 3,4)-α-L-Rha-(1 →	C-3
	(GlcII)	*4.56*		*3.90*	→ *3,4*)-α-*L*Rha-(*1* →	*H-3*
	→ 3,4)-α-L-Rha-(1 →	5.32	74.4		→ 2,3)-β-D-Fuc-(1 →	C-2
	(RhaI)	*5.32*		*3.91*	→ *2,3*)-β-*D*-*Fuc*-(1 →	*H-2*
	β-D-6-*O*-acetyl-Glc-(1 →	4.48	82.3		→ 2,3)-β-D-Fuc-(1 →	C-3
	(GlcI)	*4.48*		*3.97*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-3*
	→ 2,3)-β-D-Fuc-(1 →	5.41	177.3		Quillaic acid	C-28
17a,**b**	β-D-Xyl-(1 →	4.67	79.0		→ 3,4)-α-L-Rha-(1 →	C-4
		4.67		*3.67*	→ *3,4*)-α-*L*-*Rha*-(*1* →	*H-4*
	β-D-Glc-(1 →	4.57	83.1		→ 3,4)-α-L-Rha-(1 →	C-3
	(GlcII)	*4.57*		*3.86*	→ *3,4*)-α-*L*-*Rha*-(*1* →	*H-3*
	α-L-Rha-(1 →	4.93	81.7		→ 2,3)-β-D-Fuc-(1 →	*C-3*
	(RhaII)	*4.93*		*3.97*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-3*
	→ 3,4)-α-L-Rha-(1 →	5.08	76.4		→ 2,3)-β-D-Fuc-(1 →	C-2
	(RhaI)	*5.08*		*3.85*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-2*
	→ 2,3)-β-D-Fuc-(1 →	5.41	177.0		Quillaic acid	C-28

(continued on next page)

Table 3 (*continued*)

Compound	Residue	Anomeric proton ^{1}H (ppm)	Inter-residual connectivities			Atom
			δ_C	δ_H	Residue	
18a,**b**	β-D-Api-(1 →	5.29	86.0		→ 3)-β-D-Xyl-(1 →	C-3
		5.29		*3.34*	→ *3*)-β-*D*-*Xyl*-(*1* →	*H-3*
	→ 3)-β-D-Xyl-(1 →	4.69	78.9		→ 3,4)-α-L-Rha-(1 →	C-4
		4.69		*3.66*	→ *3,4*)-α-*L*-*Rha*-(*1* →	*H-4*
	β-D-Glc-(1 →	4.55	83.2		→ 3,4)-α-L-Rha-(1 →	C-3
	(GlcII)	*4.55*		*3.86*	→ *3,4*)-α-*L*-*Rha*-(*1* →	*H-3*
	α-L-Rha-(1 →	4.92	82.3		→ 2,3)-β-D-Fuc-(1 →	C-3
	(RhaII)	*4.92*		*3.96*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-3*
	→ 3,4)-α-L-Rha-(1 →	5.09	76.2		→ 2,3)-β-D-Fuc-(1 →	C-2
	(RhaI)	*5.09*		*3.86*	→ *2,3*)-β-*D*-*Fuc*-(*1* →	*H-2*
	→ 2,3)-β-D-Fuc-(1 →	5.41	176.9		Quillaic acid	C-28

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

the terminal Rha and Xyl of the trisaccharide at C-3 of quillaic acid. It is thus evident from the NMR data and monosaccharide analysis that each fraction of **11**–**13** and **15**–**18** contains pairs of components with either the terminal Rha or terminal Xyl in the C-3 trisaccharide. A relative proportion of the two forms could be obtained from the intensities of the signals for the anomeric protons of the terminal Rha and Xyl group in the ^{1}H NMR spectra. The results showed that **11**–**13** contained approximately the same proportion of the two forms, whereas **15**–**18** contained a high amount of the rhamnose form (~70%). From the 2D NMR spectra, the structure of the two compounds in each pair could be completely elucidated, and these were designated **11a**–**18a** for the Rha form and **11b**–**18b** for the Xyl form.

In all compounds, the oligosaccharide at C-28 of the quillaic acid is linked via the β-D-Fuc*p* residue through an ester linkage, which is evident from the chemical shifts of the anomeric atoms of Fuc ($\delta\sim$ 5.4/95 ppm) and heteronuclear connectivity between the anomeric proton and C-28 observed in HMBC spectra. The characterization of the glycosyl moiety at C-28 started from **14a** and **14b** since they are the components with only one form of trisaccharide obtained from two separation steps. All ^{1}H and ^{13}C signals of the oligosaccharide at C-28 were assigned and the NMR data are given in Table 2. The ^{1}H NMR spectrum of **14a** contains 11 signals in the anomeric region δ 4.4–5.5. However, in the HSQC spectrum, only eight signals could arise from anomeric protons as for signals at δ 5.36, 5.34 and 4.44, the ^{13}C chemical shifts were not in the region for anomeric carbon signals (δ 94–112). The latter signals were assigned to H-4 of the 4-*O*-acetyl substituted Fuc residue and from H-12 and H-16 of the quillaic acid, respectively (Guo et al., 2000). Three out of the eight signals for anomeric protons will result from the trisaccharide at C-3 indicating that the oligosaccharide at C-28 consists of a pentasaccharide. According to the ^{1}H and ^{13}C chemical shifts (Table 2) and the results of monosaccharide (Table 1) and methylation analyses, the pentasaccharide consists of a terminal Xyl, two terminal Glc, a 3,4-disubstituted Rha and a 2,3-disubstituted Fuc. In order to determine the sequence of the sugar residues in the pentasaccharide, NOESY and HMBC experiments were performed and the inter-residual NOES and three-bond connectivities observed are given in Table 3. It is thus obvious that Fuc, which is linked to C-28 of the quillaic acid, is substituted at C-3 with the terminal β-D-Glc group (GlcI, δ 4.48/82.4) and at C-2 with the 3,4-disubstituted α-L-Rha (RhaI, δ 5.33/74.6). The latter residue is substituted at C-3 with another terminal β-D-Glc residue (GlcII, δ 4.55/82.8) and at C-4 with terminal β-D-Xyl (δ 4.67/78.7). Two more connectivities were observed for Fuc, between H-4 and the carbonyl carbon of the acetyl group (δ 5.36/173.6) and between H-1 and the carbonyl carbon, C-28, of the quillaic acid (δ 5.41/177.1). These results support that Fuc is 4-*O*-substituted by an acetyl group and it is linked to C-28 of the quillaic acid. Similar structures were found in the previously studied saponins (Guo et al., 2000).

From the NMR data (Tables 2 and 3), the results of sugar and methylation analyses, and the molecular masses (Table 1) of **14a** and **14b**, it is obvious that they have the same pentasaccharide, β-D-Xyl*p*-(1 → 4)-[β-D-Glc*p*-(1 → 3)]-α-L-Rha*p*-(1 → 2)-[β-D-Glc*p*-(1 → 3)]-4-*O*-acetyl-β-D-Fuc*p*, at C-28 but different trisaccharides at C-3.

Fractions **11** and **12** contain almost equal amounts of Rha (**11a** and **12a**) and Xyl form (**11b** and **12b**) of the trisaccharide at C-3 according to the intensities of the signals from the anomeric protons. NMR data of **11a**,**b** (Tables 2 and 3), together with the results of monosaccharide analysis and MALDI-TOF MS (Table 1), showed that the only difference in the structure of the oligosaccharide at C-28 between **11a**,**b** and

14a,**b** is that compounds **11a**,**b** lack the Glc group at C-3 of RhaI. Thus, the structure of the oligosaccharide at C-28 for **11a** and **11b** could be identified as 28-*O*-{β-D-Xyl*p*-(1 → 4)-α-L-Rha*p*-(1 → 2)-[β-D-Glc*p*-(1 → 3)]-4-*O*-acetyl-β-D-Fuc*p*}. The same analyses were applied for compounds **12a**,**b**, resulting in no Xyl group at C-4 of RhaI in **12a**,**b** compared with **14a**,**b**. Thus, the structure of the oligosaccharide at C-28 for **12a** and **12b** is 28-*O*-{β-D-Glc*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-[β-D-Glc*p*-(1 → 3)]-4-*O*-acetyl-β-D-Fuc*p*}.

The ^{1}H and ^{13}C NMR spectra of compound **13**, with almost equal amounts of Rha (**13a**) and Xyl form (**13b**), showed signals for five anomeric protons (in addition to those of the trisaccharide at C-3), which demonstrated a pentasaccharide at C-28. Comparison of NMR data of compound **13** with those of **14a** and **b** showed that **13** lacks a β-D-Glc (GlcI) at C-3 of Fuc, but instead has a sugar residue, β-D-apiose, with the signals for anomeric atoms at δ 5.28, $J_{H\text{-}1,\ H\text{-}2}$ = 2.9 Hz and δ 111.1 ppm, at C-3 of the Xyl residue. The chemical shifts of the signals from the sugars in the pentasaccharide part showed only significant differences for the C-3 signal of the Fuc residue, −8.5 ppm, which indicates a non-substituted Fuc C-3 in **13**, and for the signals of the Xyl residue with a major shift for the C-3 signal of +7.3 ppm. The relative high chemical shift of the C-3 signal of Xyl, the three bond-connectivity between Xyl H-3 and Api C-1 observed in the HMBC spectrum, and comparison with NMR data of previously identified saponins (Guo et al., 2000) demonstrated that the Api residue is linked to C-3 of Xyl (Fig. 1). Thus, the structure of the pentasaccharide of saponins **13a**,**b** is β-D-Api*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-[β-D-Glc*p*-(1 → 3)]-α-L-Rha*p*-(1 → 2)-4-*O*-acetyl-β-D-Fuc*p*. The compounds with a 2-substituted 4-*O*-acetyl-β-D-Fuc residue in fraction **13** have the same basic structures as those previously identified saponins **B1** and **B2** (Nyberg et al., 2000). However, in the latter saponins, the 4-*O*-acyl group on the Fuc residue was the dimeric C_9 acyl group terminated with an arabinofuranosyl group instead of the *O*-acetyl group in **13a** and **b**. When the 3 position of Fuc residue was unsubstituted, the 3-*O*-acyl substituted isomers of the β-D-Fuc residue were formed by migration of the *O*-acyl substituent (Jacobsen et al., 1996; Nord and Kenne, 1999; Nyberg et al., 2000). By comparison of the 1D and 2D NMR spectra of fraction **13** with those of the 3-*O*-acyl isomers **B1a** and **B2a** (Nyberg et al., 2000), signals from a 3-*O*-acetylated Fuc residue (δ 5.43/94.7, 4.08/73.0, 4.91/78.8, 3.79/70.4, and 1.22/16.2) were also observed, demonstrating the occurrence of these isomers in fraction **13**. The amount of the 3-*O*-acetylated isomers was estimated to ∼25% according to the relative intensity of the signals.

The oligosaccharide at C-28 of the quillaic acid in compound **15** contains D-Fuc, L-Rha, D-Xyl and D-Glc, according to the sugar analysis and the NMR spectra which showed almost identical chemical shifts and coupling pattern as those of compound **11**. An additional singlet at δ 2.08 corresponding to 3 H and a two bond-connectivity between these protons and a carbonyl carbon at δ 173.1 in the spectra of **15** were observed. The relative high chemical shifts for the signals from H-6 and H-6′ (δ 4.43 and 4.14) and from C-6 (δ 63.9) of GlcI and three-bond connectivities between the two protons and the same carbonyl carbon at δ 173.1 observed in HMBC spectra, suggesting the presence of an *O*-acetyl group attached to the 6-position of GlcI (Jansson et al., 1987). The difference in molecular mass between **15** and **11** measured by MALDI-TOF mass spectrometry is 42 Da, consistent with the mass of one acetyl group. Thus, the oligosaccharide structure of **15** is the same as that of **11** but with an *O*-acetyl group on GlcI in the former (Fig. 1).

The comparison of NMR data (Tables 2 and 3), results of the monosaccharide analysis and molecular masses (Table 1) of **15** and **16** demonstrated that the only difference between these compounds is an additional terminal Glc (GlcII) in **16**, with signals for anomeric atoms at δ 4.56/104.7 ppm. The HMBC and NOESY spectra showed that GlcII is linked to C-3 of RhaI, an element also found in **12**–**14**. The 6-position of GlcI in **16** is also *O*-acetylated which was confirmed by the similar chemical shifts and the connectivities found for GlcI in NMR spectra of **15** and **16**. Thus, the structure of the pentasaccharide of **16a**,**b** is β-D-Xyl*p*-(1 → 4)-[β-D-Glc*p*-(1 → 3)]-α-L-Rha*p*-(1 → 2)-[6-*O*-acetyl-β-D-Glc*p*-(1 → 3)]-4-*O*-acetyl-β-D-Fuc*p*.

In fraction **17**, the presence of five sugar residues in the oligosaccharide at C-28 of the quillaic acid was deduced from the observation of five cross-peaks in the HSQC spectrum for anomeric atoms at *δ* 5.44/94.9, 5.08/101.7, 4.93/104.6, 4.57/105.1 and 4.67/105.2 (in addition to those of the trisaccharide at C-3), which were identified as D-Fuc, D-Xyl, D-Glc and two L-Rha. Comparison of the NMR spectra of **17** with those of **14a** and **14b** showed that the spin-system of a terminal Glc (GlcI) in **14a** and **b** was replaced by a spin-system of a terminal Rha (RhaII) in compound **17**. The connectivities observed in the HMBC and NOESY spectra of **17** displayed that RhaII is linked to C-3 of the Fuc residue, the position of which is substituted with GlcI in **14a** and **14b**. Thus, the structure of the pentasaccharide of **17a**,**b** is β-D-Xyl*p*-(1 → 4)-[β-D-Glc*p*-(1 → 3)]-α-L-Rha*p*-(1 → 2)-[α-L-Rha*p*-(1 → 3)]-4-*O*-acetyl-β-D-Fuc*p*.

The NMR spectra of fraction **18** demonstrated almost identical chemical shifts and coupling patterns of the ^{1}H and ^{13}C signals for the pentasaccharide element (Tables 2 and 3) as those observed for the corresponding moiety in **17**. Only the signals from the Xyl residue deviated from those of **17**, indicating the same

pentasaccharide element in both **17** and **18**, but an additional β-D-Api, with the anomeric atoms at δ 5.29, $J_{H\text{-}1,\ H\text{-}2} = 2.9$ Hz and δ 111.3, in **18**. The position of β-D-Api was shown by the NMR data (Tables 2 and 3) of the disaccharide element β-D-Api-(1 → 3)-β-D-Xyl which were identical to the corresponding signals of compound **13** and previously identified saponins (Guo et al., 2000). Thus, the structure of the hexasaccharide of **18a**,**b** was identified as β-D-Api*f*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-[β-D-Glc*p*-(1 → 3)]-α-L-Rha*p*-(1 → 2)-[α-L-Rha*p*-(1 → 3)]-4-*O*-acetyl-β-D-Fuc*p*.

The structures of compounds **11**–**18** (Fig. 1) are different from the structures previously reported for saponins isolated from bark extract of *Q. saponaria* Molina (Guo et al., 2000; Higuchi et al., 1987, 1988; Jacobsen et al., 1996; Nord and Kenne, 1999; Nyberg et al., 2000). With exception of the saponins in fraction **13**, the fucosyl residue, linked to the carboxyl group of the quillaic acid, is fully substituted with an *O*-acetyl group at C-4, a mono- or di-substituted α-L-Rha (RhaI) at C-2 and a terminal β-D-Glc (GlcI) or a terminal α-L-Rha (RhaII) at C-3. In compounds **15** and **16**, the 6-position of GlcI was substituted by an additional *O*-acetyl group. This *O*-acetyl group makes these compounds more lipophilic which is indicated by the higher affinity of **15** and **16** to a C18 column than that of **11** and **14**. The compounds analyzed in this study are either pure Rha or Xyl form of the trisaccharide at C-3 of the quillaic acid isolated by a two-step separation procedure or mixtures of the two forms isolated by a one-step separation procedure.

3. Experimental

3.1. Materials

The bark extract from *Quillaja saponaria* Molina was obtained from Berghausen (Cincinnati, OH, USA). This material (200 mg portions) was dissolved in 1 ml aq. 10% MeOH and the solution applied to an Isolute SPE column [C-18 (EC), 10 g]. The column was eluated with a stepwise gradient of aq. 10–80% MeOH (10 ml each step). The eluate from 60% MeOH was collected and used for further separation.

3.2. Isolation of compounds *11–18*

A part of the material obtained from the course separation was fractionated on a column (5 × 45 cm) of silica gel 60 (0.04–0.063 mm, Merck) using a mixture of $CHCl_3$, MeOH, H_2O and HOAc (24:17.5:3:0.1) as solvent. The elution of saponins were monitored by TLC, ^{1}H NMR and MALDI-TOF MS. A relative slow-moving fraction with components of molecular mass between 1600 and 1900 Da was pooled into two fractions and these were concentrated to dryness yielding 106 and 85 mg, respectively. The two fractions were then separately redissolved in a mixture of MeCN and aq. 0.02 M ammonium acetate buffer in 20% MeCN, pH 6.8 (8.5:91.5) and loaded on a semi-preparative HPLC column (Kromasil 100-5C18, 2 × 15 cm). The column was eluated with the same solvent at a flow rate of 9 ml min^{-1} and the eluate monitored by UV at 205 nm. By this procedure fractions containing saponin components were obtained and these were first evaporated to remove the MeCN, then diluted with H_2O and the solutions applied on Isolute SPE columns [C-18 (EC), 10 g]. The columns were washed with H_2O to remove salts and the compounds were eluted with MeOH (~20 ml) and individually evaporated to dryness yielding saponins **11**–**18** which were analyzed by MALDI-TOF MS, NMR spectroscopy and monosaccharide analysis.

For saponin **14**, a second HPLC separation was carried out with the same system as described above but with a mixture of MeCN and aq. 0.01 M phosphate buffer, pH 2.8 (30:70) as mobile phase. The pure saponins, **14a** and **14b**, were isolated in the same way as described for the first separation.

3.3. Monosaccharide and methylation analyses

Monosaccharide and methylation analyses were performed as described previously (Guo et al., 1998, 2000).

3.4. Mass spectrometry

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

3.5. 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. 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 (spin-lock time 80 ms), NOESY (mixing time 300 ms), HMQC, HSQC and HMBC (delay times of 50 or 70 ms) experiments were performed according to standard pulse sequences.

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