ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 320 (1999) 70–81

CARBOHYDRATE RESEARCH

Separation and structural analysis of saponins in a bark extract from *Quillaja saponaria* Molina

Lars I. Nord, Lennart Kenne *

Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7015, SE-750 07 Uppsala, Sweden

Received 26 February 1999; accepted 24 May 1999

Abstract

Six major saponins were isolated from a bark extract from *Quillaja saponaria* Molina. Solid-phase extraction, followed by a two-step reversed-phase HPLC separation procedure with phosphate and ammonium acetate buffers of different pH values, was used. The compounds were characterised using NMR spectroscopy, mass spectrometry and chemical methods. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Quillaja saponaria* Molina; Saponins; Quillaic acid

1. Introduction

The bark of the *Quillaja saponaria* Molina tree contains at least 60 different saponins detected by mass spectrometry [1,2], of which several have been isolated and characterised [3–6]. Among them, the most common triterpenoid base structure reported is quillaic acid substituted with a trisaccharide at C-3. In addition, C-28 of this acid is substituted with an oligosaccharide through a fucosyl residue, to which are also linked two coupled C-9 aliphatic acids and an L-arabinofuranosyl group (Fig. 1).

The structures of saccharides linked at C-3 of the quillaic acid of *Quillaja* bark saponins have recently been reported [7]. They corresponded to the previously described trisaccharide β-D-Gal*p*-(1→2)-[β-D-Xyl*p*-(1→3)]-β-D-Glc*p*A [3–5], but also to the novel trisaccharide β-D-Gal*p*-(1→2)-[α-L-Rha*p*-(1→3)]-β-D-Glc*p*A and to minor amounts of the disaccharide β-D-Gal*p*-(1→2)-β-D-Glc*p*A. In that study, the structures of the oligosaccharides were elucidated after alkaline hydrolysis of intact saponins in order to remove the C-28 substituent from the quillaic acid.

Saponin components from *Quillaja* bark extracts are commonly separated by reversed-phase HPLC [1,2,5,6]. We are at present studying the structures of several *Quillaja* saponins with different mobility in reversed-phase systems. The aim of the present study is to show the structures of the later-eluting components and whether or not the mentioned oligosaccharides at C-3 of the quillaic acid are present.

The bark extract from *Q. saponaria* Molina was found to contain the saponins **S1** to **S6** given in Fig. 1, using reversed-phase HPLC analysis. Also present are the regio-isomers (**S1a**–**S6a**, structures not shown), where the fucosyl residue is substituted with the aliphatic acid group at C-3 instead of C-4. Two of the compounds found (**S4** and **S6**) are identical to

* Corresponding author. Tel.: +46-18-671573; fax: +46-18-673477.

E-mail address: lennart.kenne@kemi.slu.se (L. Kenne)

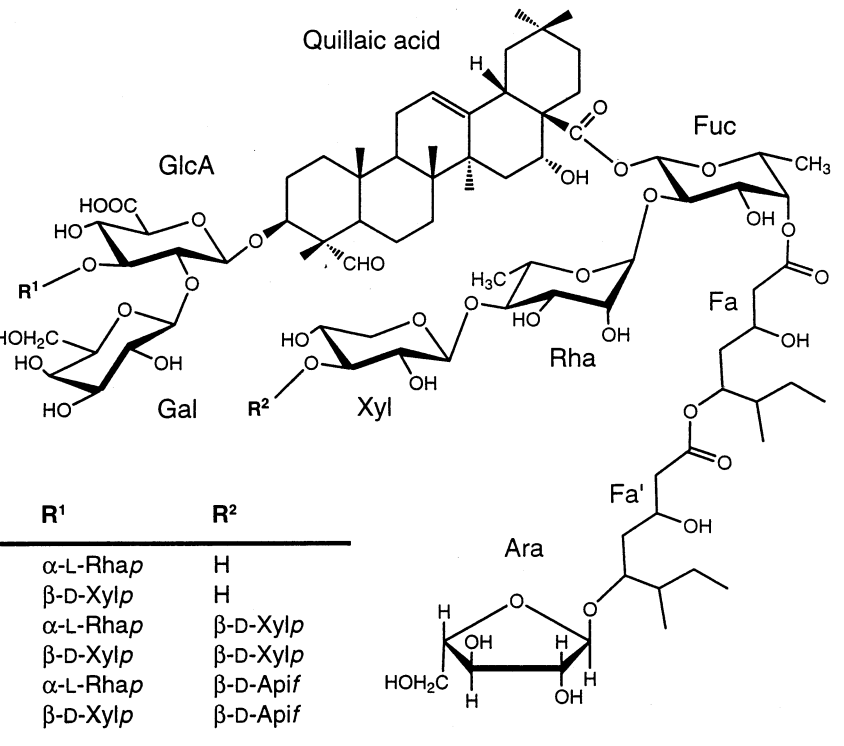
PII: S0008-6215(99)00134-2

to those in the previously described saponin mixture, QS-21 [5]. Compound **S2** is a novel structure, having only a disaccharide instead of a trisaccharide at the fucose C-2. Compounds **S1**, **S3** and **S5** are also novel structures, with the previously found [7] trisaccharide β-D-Gal*p*-(1→2)-[α-L-Rha*p*-(1→3)]-β-D-Glc*p*A at the quillaic acid C-3.

2. Results and discussion

HPLC.—An 80% methanol (aqueous) eluate from a solid-phase extraction (SPE) of bark extract from *Q. saponaria* Molina gave two main groups of peaks in the chromatogram (retention times 20–30 and 35–43 min, respectively) when analysed on reversed-phase HPLC using an ammonium acetate buffer at pH 6.4 (Fig. 2, vertical graph). The compounds in the first group (20–30 min) correspond to glucose-containing saponin structures that have been studied [8]. The 80% methanol eluate was fractionated using preparative HPLC and three fractions (Fractions I–III) were collected (Fig. 2, vertical graph). The fractions were characterised by analytical HPLC, using the same buffer system and by MALDI-TOF mass spectrometry. Samples, analysed immediately after collection, showed only a single peak in the analytical HPLC chromatogram as expected. However, if the isolated Fractions I–III were kept in a pH 6.4 buffer for 12 days at 7 °C, new peaks appeared in the 20–24 min region of the chromatogram. By employing MALDI-TOF mass spectrometry and NMR spectroscopy, it was shown that the compounds in the two groups (20–24 and 35–43 min, respectively) are regio-isomers. This is in agreement with the observation of Jacobsen et al. [5], who showed that migration of the aliphatic acid substituent occurs between O-3 and O-4 of the fucosyl residue.

All three fractions showed MALDI-TOF mass spectra with two $[M + Na]^+$ ions separated by 14 mass units, suggesting that they contained at least two components each. This was confirmed by ^{1}H NMR spectroscopy, which showed two small signals in the anomeric region with integral values that added up to the equivalent of one proton. The



	R¹	R²
S1	α-L-Rha*p*	H
S2	β-D-Xyl*p*	H
S3	α-L-Rha*p*	β-D-Xyl*p*
S4	β-D-Xyl*p*	β-D-Xyl*p*
S5	α-L-Rha*p*	β-D-Api*f*
S6	β-D-Xyl*p*	β-D-Api*f*

Fig. 1. Structures of the saponins **S1–S6**.

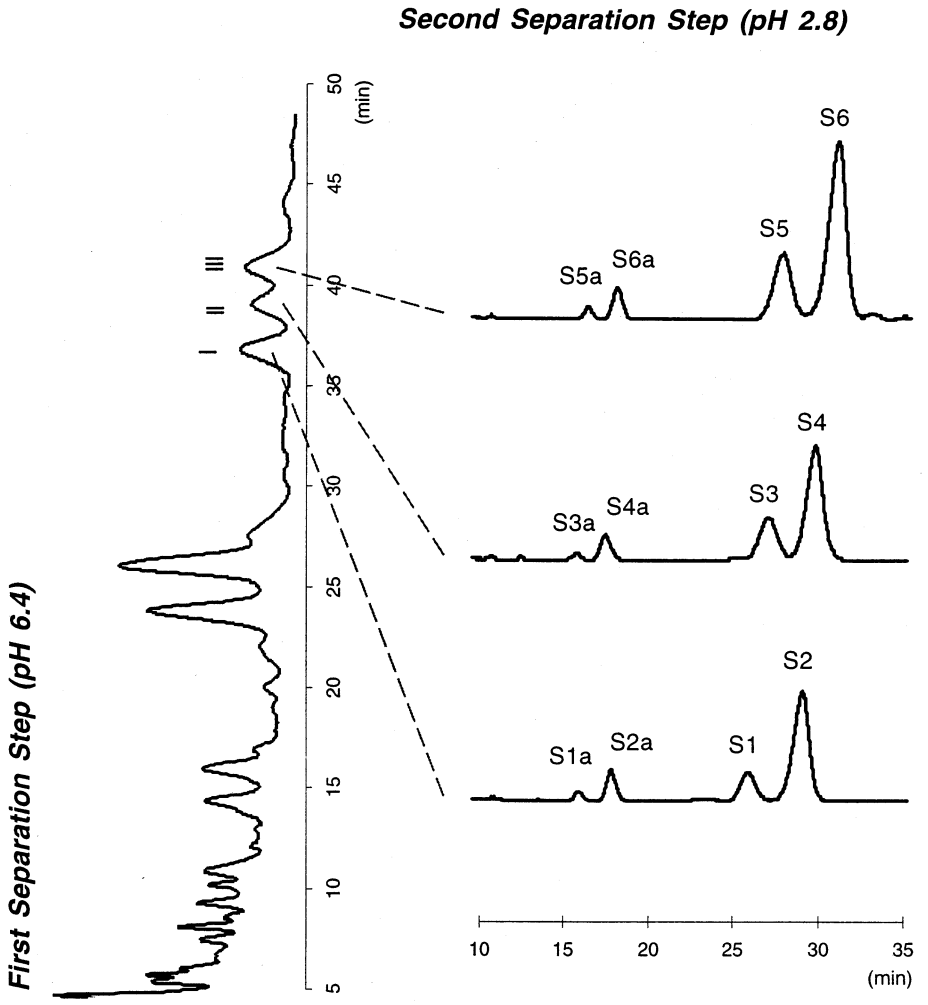


Fig. 2. HPLC chromatograms from the two separation steps of the saponin bark extract. At the first separation step (vertical graph), a buffer of pH 6.4 was used as mobile phase (see Section 3 for details). At the second separation (horizontal graphs), a buffer of pH 2.8 was used, which separated Rha and Xyl containing quillaic acid C-3 trisaccharides.

chemical shifts of these signals were consistent with those of the different trisaccharides at the quillaic acid C-3 found by Guo et al. [7].

A second separation step using a phosphate buffer of pH 2.8 gave four well-separated peaks for all fractions (I–III) (Fig. 2, horizontal graphs). The components in the two late-eluting peaks (**S1**–**S6** from Fractions I–III) were found to be substituted at the fucose C-4 by the fatty acyl group. Since the fractions from the first separation were allowed to equilibrate in a buffer at pH 6.4 for 12 days, the minor fucose 3-*O*-acyl isomers were also present (**S1a**–**S6a**).

Structural analysis.—The isolated compounds **S1**–**S6** and **S1a**–**S6a** were characterised by sugar analysis, MALDI-TOF MS and NMR spectroscopy in order to determine their structures. The neutral sugars released during acid hydrolysis of **S1**–**S6** and **S1a**–**S6a** were analysed by GC as their alditol acetates and their absolute configurations were assumed to be the same as those previously identified in similar *Quillaja* [3–5,7] and other saponins. The relative proportions of L-Ara, D-Xyl, D-Api, L-Rha, D-Fuc and D-Gal detected are given in Table 1. The molecular masses of all compounds were determined by MALDI-TOF MS and are given in Table 1. Further structural information was obtained by ^{1}H and ^{13}C NMR spectroscopy.

Assignments of NMR signals.—The NMR signals from the different sugar residues and the quillaic acid moiety were assigned by different 2D experiments for compounds **S1**–**S6**. ^{1}H NMR spectra are given in Fig. 3 for

S1–**S6** and **S2a**. Most of the proton spin systems were determined using ^{1}H,^{1}H-COSY and TOCSY experiments. The chemical shifts for ^{1}H and ^{13}C signals were extracted from the HSQC–DEPT (heteronuclear single quantum coherence–distortionless enhancement by polarisation transfer) spectra except for those of the quaternary and carbonyl carbons, which were observed and assigned by HMBC (heteronuclear multiple bond correlation). The signals in the triterpenoid structure were assigned from the spin systems observed and by comparison with data from quillaic acid without a C-28 substituent [7]. It was noted that the largest deviations (^{1}H: $\Delta\delta$ −0.15 ppm for H-15, ^{13}C: $\Delta\delta$ −4.0 ppm for C-28) were found for signals of atoms around C-28. This supports a C-28 substituted quillaic acid and was confirmed by comparison with data of a quillaic acid substituted in both C-3 and C-28 [3,5,8]. From the comparison of the chemical shifts and the pattern of the cross-peaks with those of corresponding monosaccharides [9,10], each sugar and its anomeric configuration could be identified. The anomeric configurations were also supported by the $^{3}J_{\mathrm{H\text{-}1,H\text{-}2}}$ values of the anomeric proton signals, and the $^{1}J_{\mathrm{CH}}$ values for the one-bond coupling between the anomeric atoms [11]. The linkage positions were indicated by the relatively high chemical shifts for the signals of the substituted carbons (4–9 ppm higher chemical shift relative to that of the unsubstituted monosaccharide).

The ^{1}H and ^{13}C NMR data for the quillaic acid moiety and the oligosaccharide at C-3 of saponins **S1**–**S6** are given in Table 2. From the data it is evident that the 3-substituent in compounds **S1**, **S3** and **S5** is the trisaccharide β-D-Gal*p*-(1→2)-[α-L-Rha*p*-(1→3)]-β-D-Glc*p*A and in compounds **S2**, **S4** and **S6** 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]. Fig. 4 shows the anomeric region in the HSQC–DEPT spectra of **S1** and **S2**, i.e., saponins with the two different trisaccharides at the quillaic acid C-3. The results are also in agreement with the results from the sugar analysis (Table 1).

All ^{1}H and ^{13}C signals for the oligosaccharides at C-28 were assigned and the NMR data for this part, including the C-9 fatty acyl part (Fa, Fa′ and Ara), of saponins **S1**–**S6** are given in Table 3. In the HSQC–DEPT spectra of these oligosaccharides of **S1**–**S6**, four common signals were observed in the region 4.4–5.5 ppm. Two of them originated from the fucose H-1 and H-4 (5.35–5.36/94.6–94.9 and

Table 1
Results from MALDI-TOF mass spectrometry [a] (second column) and sugar analysis [b] of the neutral sugars (columns 3 and forward)

Compound	MW	Rha		Fuc		Ara		Xyl		Gal		Api [c]	
S1a	1872	2.0	**2**	1.0	**1**	0.9	**1**	1.0	**1**	1.0	**1**		**0**
S2a	1859	1.0	**1**	1.0	**1**	1.0	**1**	1.8	**2**	1.0	**1**		**0**
S1	1873	2.0	**2**	1.0	**1**	1.0	**1**	1.0	**1**	1.0	**1**		**0**
S2	1859	1.0	**1**	1.0	**1**	1.0	**1**	1.9	**2**	1.0	**1**		**0**
S3a	2005	2.0	**2**	1.0	**1**	1.0	**1**	1.7	**2**	1.0	**1**		**0**
S4a	1990	1.0	**1**	1.0	**1**	1.0	**1**	2.2	**3**	1.0	**1**		**0**
S3	2005	1.9	**2**	1.0	**1**	0.9	**1**	1.6	**2**	1.0	**1**		**0**
S4	1989	1.0	**1**	1.0	**1**	0.9	**1**	2.2	**3**	1.0	**1**		**0**
S5a	2003	1.7	**2**	1.0	**1**	1.0	**1**	1.3	**1**	1.1	**1**	0.4	**1**
S6a	1990	1.0	**1**	1.0	**1**	0.9	**1**	1.6	**2**	1.0	**1**	0.5	**1**
S5	2004	1.6	**2**	1.0	**1**	0.9	**1**	1.1	**1**	1.0	**1**	0.4	**1**
S6	1989	1.0	**1**	1.0	**1**	0.9	**1**	1.8	**2**	1.0	**1**	0.6	**1**

[a] Molecular masses (MW) are corrected for Na by subtracting 23 and the uncertainty is ±1 mass unit.

[b] Presented as areas from GC chromatograms normalised against the fucitol peak, using a flame ionisation detector. The **bold-face** values are the assigned number of sugars for the compounds.

[c] The relative yield of Api was lower due to the hydrolytic conditions used in the analysis. However, ^{1}H NMR spectra showed equimolar amounts of apiose.

Table 2
NMR assignments for the quillaic acid and the oligosaccharide at C-3

Residue	S1		S2		S3		S4		S5		S6	
	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
Qa1	1.11, 1.71	39.1	1.12, 1.72	39.0	1.11, 1.71	39.3	1.12, 1.70	38.7	1.10, 1.72	39.2	1.11, 1.73	39.1
Qa2	1.78, 1.99	25.8	1.78, 1.98	25.7	1.78, 2.00	25.5	1.78, 1.98	25.5	1.79, 2.00	25.6	1.78, 1.98	25.5
Qa3	3.86	86.1	3.87	86.3	3.86	86.3	3.87	86.1	3.86	86.0	3.87	86.1
Qa4		56.3		56.3		56.5		56.0		56.1		56.1
Qa5	1.31	49.3	1.31	49.1	1.33	49.1	1.33	49.2	1.33	49.2	1.34	49.0
Qa6	0.93, 1.46	21.5	0.95, 1.46	21.4	0.94, 1.48	21.7	0.95, 1.47	20.9	0.95, 1.47	21.4	0.95, 1.48	21.2
Qa7	1.34, 1.50	33.7	1.33, 1.51	33.5	1.35, 1.50	33.6	1.36, 1.49	33.5	1.35, 1.50	33.5	1.37, 1.50	33.4
Qa8		41.1		41.1		41.0		40.9		41.0		41.0
Qa9	1.75	47.9	1.75	47.8	1.77	48.0	1.75	47.9	1.75	47.9	1.75	47.8
Qa10		37.0		37.3		37.0		36.9		37.1		36.9
Qa11	1.93, 1.93	24.4	1.93, 1.93	24.4	1.93, 1.93	24.4	1.93, 1.93	24.2	1.93, 1.93	24.3	1.93, 1.93	24.4
Qa12	5.33	123.1	5.33	123.1	5.33	123.3	5.32	123.1	5.33	123.1	5.33	122.9
Qa13		144.7		144.8		144.6		144.3		144.4		144.5
Qa14		42.6		42.7		43.0		42.4		42.7		42.4
Qa15	1.43, 1.68	36.4	1.43, 1.67	36.4	1.45, 1.62	36.3	1.47, 1.60	36.6	1.45, 1.61	36.5	1.45, 1.62	36.2
Qa16	4.48	74.5	4.48	74.5	4.48	74.4	4.48	74.3	4.48	74.4	4.48	74.2
Qa17		49.9		50.1		[b]		49.5		49.9		49.7
Qa18	2.95	[a]	2.95	42.1	2.94	42.3	2.95	42.3	2.95	42.2	2.95	42.1
Qa19	1.06, 2.31	48.0	1.06, 2.32	47.9	1.05, 2.33	48.0	1.06, 2.29	47.7	1.05, 2.32	48.0	1.06, 2.32	47.8
Qa20		31.2		31.3		31.1		30.9		31.2		31.1
Qa21	1.18, 1.96	36.6	1.17, 1.95	36.4	1.17, 1.97	36.8	1.17, 1.97	36.5	1.17, 1.95	36.5	1.16, 1.96	36.3
Qa22	[a]	[a]	1.77, 1.94	32.0	1.72, 1.96	32.1	1.73, 1.95	32.3	1.73, 1.95	32.0	1.73, 1.96	32.0
Qa23	9.45	210.9	9.45	210.9	9.46	211.2	9.46	210.7	9.46	211.0	9.46	211.4
Qa24	1.17	10.9	1.17	10.9	1.17	11.0	1.16	11.0	1.17	10.9	1.17	10.9
Qa25	1.01	16.4	1.01	16.4	1.02	16.5	1.01	16.6	1.02	16.4	1.00	16.4
Qa26	0.77	17.9	0.77	17.9	0.76	17.8	0.76	17.6	0.77	17.8	0.75	17.4
Qa27	1.40	27.1	1.40	27.1	1.40	26.9	1.40	27.1	1.40	27.0	1.40	27.1
Qa28		177.1		177.1		176.8		176.7		177.0		176.8
Qa29	0.89	33.3	0.89	33.2	0.89	33.2	0.89	33.3	0.89	33.2	0.89	33.1
Qa30	0.96	24.9	0.96	24.8	0.95	24.6	0.95	24.7	0.95	24.8	0.95	24.6
GlcA1	4.45	104.1	4.45	104.4	4.45	104.3	4.45	104.4	4.47	104.1	4.45	104.1
GlcA2	3.63	78.1	3.66	78.0	3.63	77.9	3.66	77.9	3.64	78.0	3.65	77.8
GlcA3	3.64	85.8	3.69	86.5	3.64	85.7	3.69	86.4	3.65	85.7	3.69	86.3
GlcA4	3.60	71.9	3.56	71.3	3.61	71.9	3.56	71.0	3.60	71.8	3.56	71.2
GlcA5	3.76	76.7	3.78	76.5	3.80	76.6	3.79	76.4	3.79	76.6	3.78	76.4
GlcA6		173.3		172.5		173.3		172.5		173.0		172.4
Gal1	4.46	104.2	4.80	103.7	4.46	104.1	4.81	103.6	4.46	104.1	4.81	103.3
Gal2	3.49	73.0	3.46	73.5	3.50	72.9	3.47	73.4	3.50	72.8	3.47	73.3
Gal3	3.48	74.9	3.43	75.3	3.48	74.9	3.43	75.1	3.49	74.8	3.44	75.1

Table 2 (continued)

Residue	S1		S2		S3		S4		S5		S6	
	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
Gal4	3.82	70.6	3.82	70.6	3.83	70.6	3.83	70.5	3.83	70.5	3.82	70.4
Gal5	3.47	76.9	3.49	76.6	3.47	76.7	3.49	76.4	3.48	76.9	3.49	76.4
Gal6	3.74, 3.79	62.2	3.74, 3.78	62.1	3.68, 3.79	62.1	3.68, 3.75	62.0	3.73, 3.80	62.2	3.73, 3.78	62.0
Xyl1			4.59	104.8			4.59	104.7			4.59	104.6
Xyl2			3.23	75.1			3.23	75.1			3.23	75.0
Xyl3			3.30	78.1			3.30	78.1			3.30	78.0
Xyl4			3.51	70.8			3.51	70.8			3.50	70.7
Xyl5			3.24, 3.90	67.1			3.23, 3.90	66.9			3.22, 3.90	66.9
Rha1	5.03	103.2			5.03	103.0			5.04	103.1		
Rha2	4.02	72.0			4.02	71.9			4.02	72.0		
Rha3	3.66	72.0			3.65	72.1			3.66	72.1		
Rha4	3.41	73.7			3.40	73.8			3.41	73.7		
Rha5	3.94	70.4			3.94	70.4			3.94	70.4		
Rha6	1.23	17.8			1.24	18.3			1.24	17.7		

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

5.10–5.11/75.1–75.3 ppm, respectively), suggesting that the fucose has the same substitution pattern in **S1**–**S6**. The other two signals were derived from the common fucose 4-substituent in *Quillaja* saponins, two 3,5-dihydroxy-C-9 acids with a terminal arabinofuranosyl group. At 5.18–5.19/75.1–75.2 ppm a characteristically split signal (double-triplet) was assigned to the Fa H-5 as there were three cross-peaks to signals between 1.6 and 1.9 ppm in the ^{1}H,^{1}H-COSY spectra [5]. The signal from the anomeric proton/carbon of the terminal Ara appeared at 4.98–4.99/108.3–108.6 ppm.

The sequence of the sugar residues in the C-28 oligosaccharide was obtained by the $^3J_{CH}$ connectivities over the glycosidic bonds, observed as cross-peaks in the HMBC spectra (Table 4). These connectivities give information on each disaccharide element in the oligosaccharide. For compounds **S1**–**S6**, a cross-peak at 3.73–3.76/101.2–101.5 ppm was found which connects Fuc H-2 to Rha C-1. For some compounds a weaker signal for Rha H-1 to Fuc C-2 was also observed. The relatively high chemical shift of the Rha C-4 (84.3–85.1 ppm) indicated substitution at that carbon. In the HMBC spectra, peaks at 3.52–3.53/106.3–107.3 ppm showed a coupling between Rha H-4 and Xyl C-1. The Xyl H-1 to Rha C-4 peak occurred at 4.46–4.51/84.3–85.6 ppm. The ^{13}C chemical shifts for the signals from this Xyl in **S1** and **S2** suggested that it was a terminal sugar. In **S3** and **S4**, however, the chemical shifts for the C-3 signal (87.2 and 87.3 ppm) of this Xyl residue indicated substitution and the cross-peak in the HMBC spectrum (4.52/87.0 and 87.4 ppm) showed a connectivity to H-1 of an additional β-D-Xyl. The extra Xyl is also supported by the sugar analysis (Table 1). For the C-3 signal (85.4 and 85.6 ppm) of the Xyl residue in **S5** and **S6** the cross-peak in the HMBC spectrum (5.25/85.4 and 85.7 ppm) showed a connectivity to H-1 of a β-D-Api*f* group. This apiose is supported by the sugar analysis (Table 1). In common for **S1**–**S6** is an HMBC connectivity from Fuc H-1 (5.35–5.36 ppm) to a carbon signal at 176.7–177.2 ppm and the latter was assigned to C-28 of the quillaic acid.

Also in common is a Fuc H-4 (5.10–5.11 ppm) connectivity to a carbon at 172.4–173.1 ppm, which is the carbonyl carbon of the first C-9 fatty acyl part (Fa, C-1). From H-5 of this C-9 fatty acyl part, an HMBC connectivity to another carbonyl carbon is observed which corresponds to C-1 of a second C-9 fatty acyl part (Fa′). Fa′ is linked via C-5 to H-1 of an arabinofuranosyl group which is confirmed by an observed HMBC connectivity between Ara H-1 and Fa′ C-5.

The NMR signals of one of the fucose 3-substituted regio-isomers (**S2a**) were assigned by comparison with those of the fucose 4-substituted analogue (**S2**). Table 5 lists the ^{1}H and ^{13}C signals for **S2a** for which the chemical shifts differ by more than 0.06 ppm for ^{1}H signals or 0.4 ppm for ^{13}C signals compared to those of **S2**. As expected, the signals from the fucose residue show the largest deviations but signals from the rhamnose residue at C-2 of the fucose are also affected.

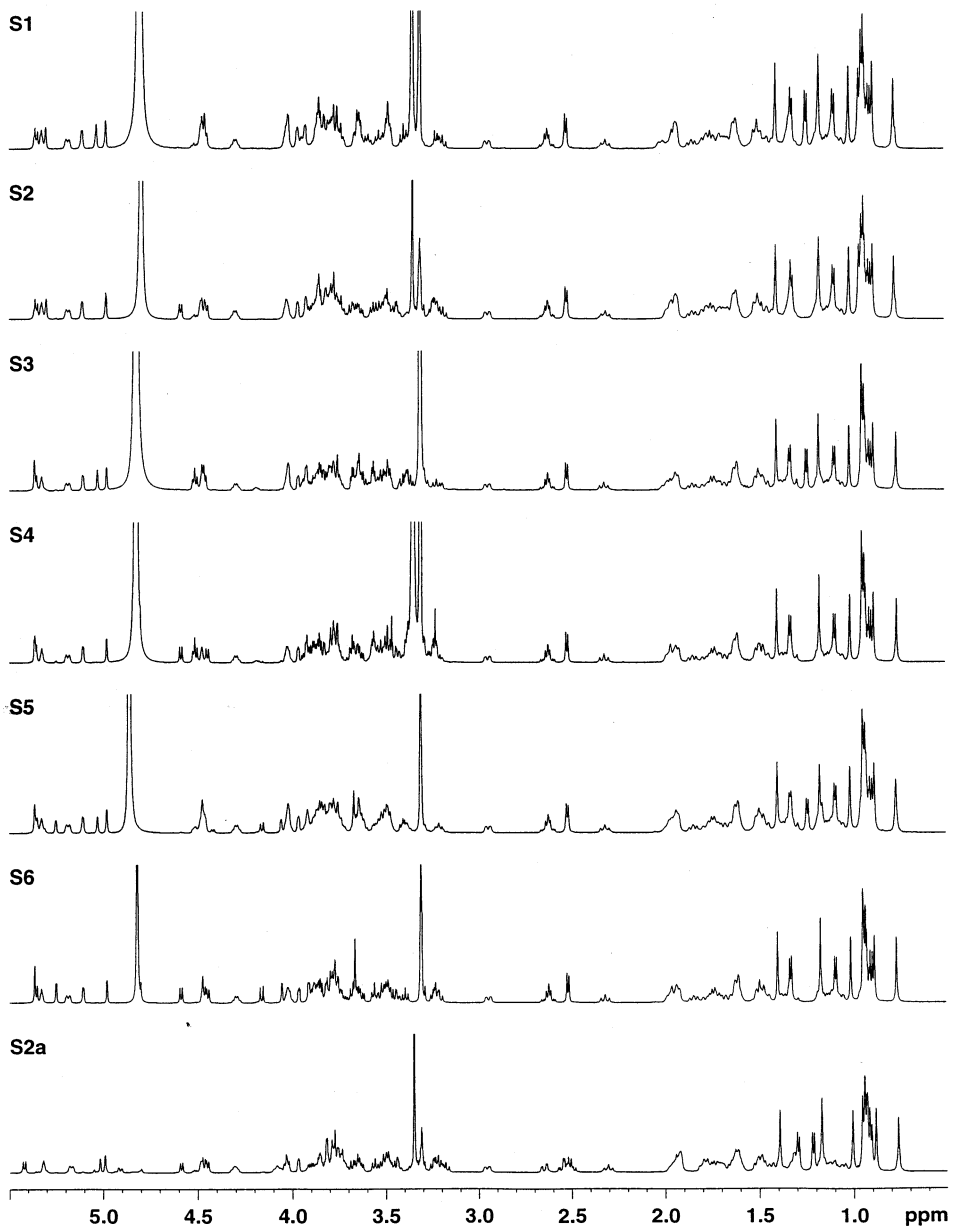


Fig. 3. Part of the ^{1}H NMR spectra of compounds **S1**–**S6** and **S2a**.

Table 3
NMR assignments for the C-28 oligosaccharide and the C-9 aliphatic acid parts

Residue	**S1**		**S2**		**S3**		**S4**		**S5**		**S6**	
	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
Fuc1	5.35	94.8	5.35	94.8	5.36	94.9	5.36	94.6	5.36	94.7	5.36	94.6
Fuc2	3.74	75.2	3.73	75.3	3.75	74.9	3.75	74.8	3.76	74.9	3.76	74.6
Fuc3	3.87	74.7	3.87	74.6	3.88	74.9	3.87	74.8	3.88	74.7	3.88	74.7
Fuc4	5.11	75.3	5.11	75.2	5.10	75.2	5.11	75.3	5.11	75.3	5.11	75.1
Fuc5	3.85	71.0	3.85	71.0	3.83	70.6	3.84	71.0	3.85	71.1	3.84	70.9
Fuc6	1.09	16.5	1.09	16.5	1.09	16.6	1.01	16.2	1.09	16.5	1.09	16.6
Rha1	5.30	101.6	5.30	101.5	5.37	101.2	5.36	101.2	5.36	101.3	5.37	101.1
Rha2	3.93	71.7	3.93	71.8	3.92	71.8	3.93	71.6	3.92	71.7	3.92	71.6
Rha3	3.80	72.0	3.81	72.1	3.79	72.2	3.79	72.2	3.79	72.2	3.79	72.1
Rha4	3.53	84.3	3.53	84.3	3.53	85.1	3.52	85.1	3.53	85.0	3.52	84.9
Rha5	3.79	68.8	3.78	68.8	3.77	68.6	3.77	68.5	3.77	68.7	3.78	68.6
Rha6	1.32	18.5	1.32	18.4	1.33	18.6	1.33	18.3	1.33	18.4	1.33	18.3
Xyl(1)1	4.47	107.0	4.47	107.0	4.51	107.1	4.52	107.0	4.47	107.2	4.47	107.1
Xyl(1)2	3.21	76.1	3.21	76.0	3.37	75.2	3.36	75.2	3.31	75.5	3.30	75.3
Xyl(1)3	3.32	78.1	3.31	78.1	3.49	87.2	3.49	87.3	3.40	85.6	3.40	85.4
Xyl(1)4	3.49	70.8	3.49	70.8	3.54	69.3	3.54	69.3	3.50	69.7	3.50	69.5
Xyl(1)5	3.19, 3.85	67.3	3.18, 3.84	67.2	3.22, 3.90	66.8	3.22, 3.89	66.9	3.21, 3.89	66.9	3.22, 3.90	66.9
Api1									5.25	110.9	5.25	110.8
Api2									4.06	77.7	4.06	77.5
Api3										80.6		80.2
Api4									3.82, 4.16	74.9	3.81, 4.17	74.7
Api5									3.67	65.3	3.67	65.1
Xyl(2)1					4.52	105.5	4.53	105.3				
Xyl(2)2					3.38	75.2	3.38	74.7				
Xyl(2)3					3.39	77.7	3.38	77.6				
Xyl(2)4					3.57	70.8	3.57	71.0				
Xyl(2)5					3.29, 3.94	67.1	3.29, 3.94	67.1				
Fa1		172.8		173.0		173.0		172.4		173.0		172.9
Fa2	2.62, 2.62	43.6	2.62, 2.62	43.5	2.62, 2.62	43.6	2.62, 2.62	43.4	2.62, 2.62	43.6	2.62, 2.62	43.4
Fa3	4.03	66.2	4.02	66.1	4.01	66.1	4.02	65.9	4.02	66.1	4.02	66.0
Fa4	1.68, 1.85	40.0	1.68, 1.85	39.6	1.68, 1.85	39.7	1.67, 1.85	39.7	1.67, 1.84	39.7	1.67, 1.84	39.6
Fa5	5.19	75.2	5.18	75.2	5.19	75.1	5.19	75.1	5.18	75.2	5.19	75.1
Fa6	1.61	40.1	1.61	40.0	1.60	40.0	1.62	40.1	1.61	40.1	1.61	40.0
Fa7	1.14, 1.51	26.4	1.17, 1.49	26.6	1.15, 1.51	26.5	1.16, 1.51	26.5	1.15, 1.49	26.4	1.16, 1.50	26.5
Fa8	0.94	12.2	0.94	12.2	0.95	12.4	0.93	12.1	0.93	12.2	0.93	12.1
Fa9	0.94	14.7	0.94	14.7	0.95	14.9	0.93	14.6	0.94	14.7	0.93	14.9
Fa′1		173.5		173.6		[a]		[a]		173.5		173.4
Fa′2	2.52, 2.52	43.9	2.52, 2.52	43.8	2.52, 2.52	44.0	2.52, 2.52	43.9	2.52, 2.52	43.9	2.52, 2.52	43.9
Fa′3	4.30	66.2	4.30	66.1	4.30	66.0	4.30	66.0	4.29	66.1	4.30	66.0

Table 3 (continued)

Residue	S1		S2		S3		S4		S5		S6	
	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
Fa′4	1.50, 1.63	39.3	1.50, 1.63	39.4	1.50, 1.65	39.2	1.49, 1.62	39.4	1.49, 1.64	39.5	1.50, 1.62	39.2
Fa′5	3.81	79.0	3.80	79.0	3.80	79.0	3.80	79.0	3.80	79.1	3.80	79.0
Fa′6	1.61	40.1	1.61	40.0	1.60	40.0	1.62	40.1	1.61	40.1	1.61	40.0
Fa′7	1.10, 1.59	25.2	1.10, 1.61	25.4	1.10, 1.61	25.7	1.12, 1.61	25.6	1.10, 1.59	25.4	1.12, 1.61	25.4
Fa′8	0.94	12.2	0.94	12.2	0.95	12.4	0.93	12.1	0.93	12.2	0.93	12.1
Fa′9	0.91	15.2	0.91	15.2	0.92	15.3	0.88	15.0	0.91	15.1	0.88	14.9
Ara1	4.98	108.6	4.98	108.6	4.99	108.5	4.98	108.4	4.99	108.5	4.98	108.3
Ara2	3.97	83.4	3.97	83.3	3.97	83.5	3.97	83.4	3.97	83.4	3.97	83.2
Ara3	3.85	78.2	3.86	78.2	3.85	78.1	3.85	78.1	3.86	78.3	3.85	78.0
Ara4	4.02	85.2	4.03	85.2	4.02	85.1	4.03	85.3	4.03	85.3	4.03	85.1
Ara5	3.64, 3.77	62.9	3.63, 3.77	62.9	3.64, 3.77	62.8	3.63, 3.76	62.8	3.64, 3.77	62.9	3.63, 3.76	62.8

[a] Signal not observed in the HMBC spectrum.

Structures.—The saponin structures **S1**–**S6** found in the *Q. saponaria* Molina bark extract are given in Fig. 1. The regio-isomers **S1a**–**S6a** are also present in the extract, where the aliphatic acyl substituents have migrated from the fucose O-4 to O-3. In addition, minor components with the same structure of the C-28 oligosaccharide as in **S1**/**S2** and **S5**/**S6**, respectively, but with a disaccharide at C-3 of the quillaic acid [7] were found. These structures were identified by NMR spectroscopy.

3. Experimental

Materials.—The *Quillaja* saponin bark extract was obtained from Berghausen (Cincinnati, OH, USA).

Course separation of bark extract.—*Quillaja* saponin bark extract (200 mg portions) was dissolved in 1 mL 10% v/v MeOH (aq) and the solution applied on an SPE column (C18, Isolute™, 10 g, International Sorbent Technology Ltd., UK). The column was eluted with a stepwise gradient of 10–80% aq MeOH, 10 mL portions (2 mL/min). The eluate from 80% MeOH (10 mL) was collected and concentrated to dryness and this material was used for further separation.

HPLC separation.—For separation of milligram amounts of the components, ~5 mg portions of the material from the course separation were repeatedly injected on a 20 × 150 mm C-18 column (Kromasil 100-5C18, HiCHROM, UK) connected to a semi-preparative HPLC instrument. The column was eluted with a mixture of MeCN and aq 0.03 M NH_4OAc buffer, pH 6.4 (34.2:65.8) at a flow rate of 10 mL/min and the eluate monitored by UV at 214 nm. Fractions containing saponins were collected and kept at 7 °C for 12 days, whereupon the MeCN concentration was lowered by evaporation and the solutions were applied on an SPE column (C18, Isolute™, 10 g) to concentrate and desalt. Methanol was used to elute the compounds from the SPE column and samples were evaporated to dryness.

A second separation step was carried out with a mixture of MeCN and aq 0.03 M

Table 4
Observed inter-residue $^3J_{CH}$ connectivities (HMBC) obtained for the C-28 oligosaccharides

Compound H no.	Observed ^{1}H (ppm)		C no.	Observed ^{13}C (ppm)
S1				
Fuc1	5.36	↔	Qa28	177.1
Fuc2	3.74	↔	Rha1	101.4
Fuc4	5.11	↔	Fa1	172.8
Rha4	3.53	↔	Xyl1	107.1
Xyl1	4.47	↔	Rha4	84.3
S2				
Fuc1	5.36	↔	Qa28	177.1
Fuc2	3.73	↔	Rha1	101.5
Fuc4	5.11	↔	Fa1	173.0
Rha1	5.30	↔	Fuc2	75.4
Rha4	3.53	↔	Xyl1	107.1
Xyl1	4.47	↔	Rha4	84.5
S3				
Fuc1	5.35	↔	Qa28	176.9
Fuc2	3.76	↔	Rha1	101.5
Fuc4	5.10	↔	Fa1	173.0
Rha1	5.36	↔	Fuc2	74.7
Rha4	3.53	↔	Xyl(1)1	106.3
Xyl(1)1	4.51	↔	Rha4	85.0
Xyl(1)3	3.49	↔	Xyl(2)1	105.6
Xyl(2)1	4.52	↔	Xyl(1)3	87.4
S4				
Fuc1	5.36	↔	Qa28	176.7
Fuc2	3.76	↔	Rha1	101.2
Fuc4	5.11	↔	Fa1	172.4
Rha1	5.36	↔	Fuc2	74.5
Xyl(1)1	4.51	↔	Rha4	84.8
Xyl(1)3	3.49	↔	Xyl(2)1	105.5
Xyl(2)1	4.52	↔	Xyl(1)3	87.0
S5				
Fuc1	5.36	↔	Qa28	177.2
Fuc2	3.75	↔	Rha1	101.3
Fuc4	5.11	↔	Fa1	173.1
Rha1	5.36	↔	Fuc2	74.7
Rha4	3.52	↔	Xyl1	107.3
Xyl1	4.46	↔	Rha4	85.6
Xyl3	3.40	↔	Api1	110.9
Api1	5.25	↔	Xyl3	85.7
S6				
Fuc1	5.36	↔	Qa28	176.8
Fuc2	3.76	↔	Rha1	101.3
Fuc4	5.11	↔	Fa1	172.9
Rha4	3.52	↔	Xyl1	107.3
Xyl1	4.47	↔	Rha4	84.9
Xyl3	3.41	↔	Api1	110.8
Api1	5.25	↔	Xyl3	85.4

phosphate buffer, pH 2.8 (40.5:59.5). The same instrument and settings as in the first separation step was used. Portions (1 mg) of the fractions from the first separation was injected repeatedly and the collected fractions were treated as in the first separation step.

Table 5
NMR signals from the Fuc 3-substituted regioisomer **S2a**

Residue	**S2a**	
	^{1}H (ppm) [a]	^{13}C (ppm) [a]
Fuc1	**5.42**	94.8
Fuc2	**4.04**	**72.7**
Fuc3	**4.91**	**78.5**
Fuc4	**3.82**	**70.4**
Fuc5	**3.77**	**72.2**
Fuc6	**1.22**	16.3
Rha1	**5.02**	101.5
Rha2	**3.79**	71.7
Rha3	**3.75**	72.0
Rha4	3.52	**83.7**
Fa1		**171.9**
Fa2	**2.52**, 2.65	43.6
Fa3	**4.08**	65.9

[a] Signals that differ significantly in chemical shifts from those of the corresponding Fuc 4-substituted regioisomer **S2**. A **bold-face** value indicates that there is a difference greater than or equal to $\Delta\delta$ 0.06 for ^{1}H and $\Delta\delta$ 0.4 for ^{13}C.

Analysis of fractions.—Each fraction from the semi-preparative HPLC was dissolved in MeOH and analysed by analytical HPLC on a 4.6×150 mm C-18 column (Kromasil 100-5C18, HiCHROM, UK). The mobile phases used were MeCN–aq 0.03 M NH_4OAc buffer, pH 6.4 (34:66) and MeCN–aq 0.03 M phosphate buffer, pH 2.8 (38:62). MALDI-TOF mass spectrometry on each fraction was carried out on a Linear LDI-1700XS spectrometer using a 337 nm nitrogen laser and 2,5-dihydroxybenzoic acid as the matrix.

Sugar analysis.—The isolated saponins (0.5 mg) were hydrolysed in 2 M CF_3COOH (0.4 mL) at 120 °C for 1 h, whereupon the solvent was evaporated by flushing with N_2. Then 1 M NH_4OH (0.1 mL) was added and the product was reduced with $NaBD_4$ (3 mg) in 1 M NH_4OH (0.2 mL) for 40 min at 40 °C. Excess $NaBD_4$ was quenched with 0.3 mL HOAc (concd) and the boric acid formed was removed by co-distillation with

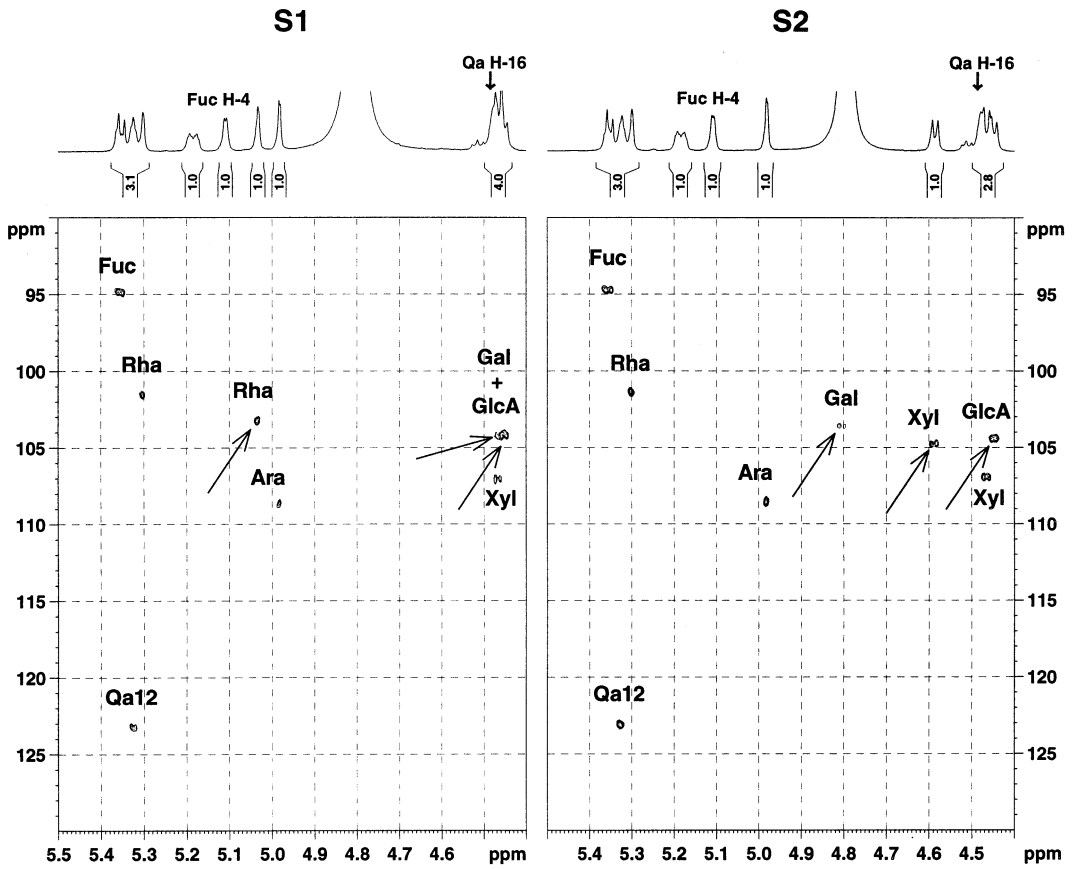


Fig. 4. Part of the HSQC–DEPT spectra of compounds **S1** and **S2** showing the difference for signals in the anomeric region between the two different trisaccharides (marked with arrows) at C-3 of the quillaic acid.

10% HOAc in MeOH (3 × 0.5 mL) followed by MeOH (3 × 0.5 mL). The resulting alditols were acetylated with Ac_2O–pyridine (1:1, 0.3 mL) at 120 °C for 20 min and analysed by GC (flame ionisation detection) using authentic samples as standards. Two alditol derivatives were formed from the apiose, one with a tertiary hydroxyl group and one with this group acetylated.

NMR spectroscopy.—NMR spectra were recorded on a Bruker DRX-600 spectrometer (600 MHz proton frequency) equipped with a 2.5 mm microprobe or a 5 mm triple-resonance inverse probe. Compounds in their protonated form were dissolved in CD_3OD (3–10 mg/mL) and all spectra were acquired at 30 °C without spinning. Chemical shifts are reported in ppm using the solvent peak as a reference (^1H 3.31 ppm and ^{13}C 49.15 ppm, respectively).

Acknowledgements

This work was supported by grants from the Swedish Natural Science Research Council and the Swedish Council for Forestry and Agricultural Research.

References

[1] D.C. van Setten, G. van de Werken, G. Zomer, G.F.A. Kersten, *Rapid Commun. Mass Spectrom.*, 9 (1995) 660–666.
[2] 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.
[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] N.E. Jacobsen, W.J. Fairbrother, C.R. Kensil, A. Lim, D.A. Wheeler, M.F. Powell, *Carbohydr. Res.*, 280 (1996) 1–14.

[6] C.R. Kensil, U. Patel, M. Lennick, D. Marciani, *J. Immunol*., 146 (1991) 431–437.
[7] S. Guo, L. Kenne, L.N. Lundgren, B. Rönnberg, B.G. Sundquist, *Phytochemistry*, 48 (1998) 175–180.
[8] N.T. Nyberg, L. Kenne, B. Rönnberg, B.G. Sundquist, *Carbohydr. Res*. (1999) accepted for publication.
[9] P.-E. Jansson, L. Kenne, G. Widmalm, *Carbohydr. Res*., 188 (1989) 169–191.
[10] P.K. Agrawal, *Phytochemistry*, 31 (1992) 3307–3330.
[11] K. Bock, C. Pedersen, *J. Chem. Soc*., *Perkin Trans. 2*, (1974) 293–297.