ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 329 (2000) 817–829

CARBOHYDRATE RESEARCH

Novel acetylated triterpenoid saponins in a chromatographic fraction 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 8 May 2000; accepted 8 August 2000

Abstract

Six novel fucose 3-O-acetylated saponins, with a quillaic acid aglycone, were isolated from a bark extract from the *Quillaja saponaria* Molina tree. In addition, a saponin with a novel aglycone (phytolaccagenic acid) and a novel fatty acyl group [(*S*)-2-methylbutanoyl] for *Quillaja* saponins was found. The compounds were characterised using NMR spectroscopy, mass spectrometry and chemical methods. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Quillaja saponaria* Molina; Saponins; Quillaic acid; Acetyl group; Phytolaccagenic acid; (*S*)-2-Methylbutanoyl group

1. Introduction

Saponins from the bark of the *Quillaja saponaria* Molina tree are used as adjuvants with vaccines [1,2]. The bark extracts from *Q. saponaria* Molina contain a complex mixture of saponins that are difficult to separate into individual components. A large number of the saponin components have been isolated and characterised [3–11]. Further studies using HPLC combined with mass spectrometry and monomer mapping partly characterised 60 different saponins in a bark extract [12,13], and showed the existence of at least two additional aglycone structures. Four different acyl groups, as constituents of the saponins, were detected using multiple-stage tandem mass spectrometry [13].

The most common basic structure reported for *Quillaja* saponins is quillaic acid substituted at C-3 with a trisaccharide, and at C-28 with an oligosaccharide through a fucose residue. This fucose residue is usually substituted by an acyl group consisting of two 3,5-dihydroxy-6-methyloctanoic acid groups (C-9) terminated by an L-arabinofuranosyl group (Fig. 1). The acyl group can migrate between O-3 and O-4 of the fucose residue [6,8,9,14]. However, the present study reports new *Quillaja* saponin structures with an acetyl group at O-3 of the fucose residue in addition to the acyl group at O-4. The acetyl group at O-3 prevents the acyl group from migrating between O-3 and O-4. The stereochemistry of the C-9 fatty acyl group commonly found in *Quillaja* saponins was recently elucidated by enantioselective synthesis [15] and X-ray crystallography [16] giving (3*S*,5*S*,6*S*)-3,5-dihydroxy-6-methyloctanoic acid.

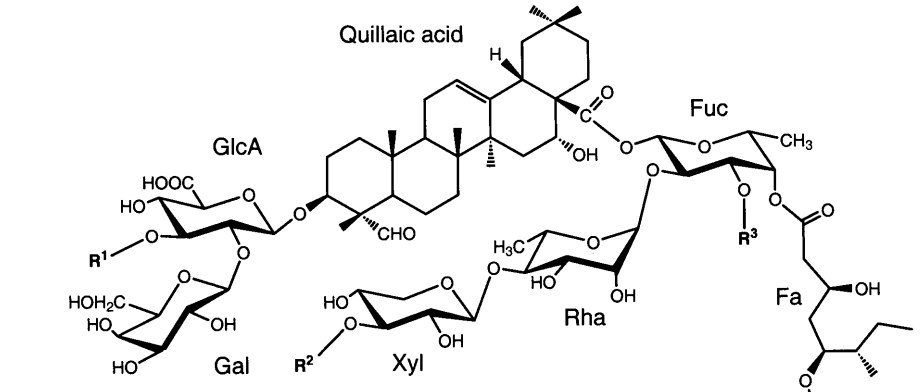
Recently, we reported six major saponin structures (**S1**–**S6**, Fig. 1) from a bark extract

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

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

PII: S0008-6215(00)00248-2

of the *Q. saponaria* Molina tree [8]. Here we report the same structures as **S1**–**S6** with the only difference being that O-3 of the fucose is acetylated in the new structures (**S7**–**S12**, Fig. 1). In addition, a saponin structure (**S13**, Fig. 2) with a different aglycone and two C-5 aliphatic acyl groups was found. The aglycone in the novel compound is the triterpene phytolaccagenic acid substituted with a 23-*O*-acetyl group.



	R¹	R²	R³
S1	α-L-Rha*p*	H	H
S2	β-D-Xyl*p*	H	H
S3	α-L-Rha*p*	β-D-Xyl*p*	H
S4	β-D-Xyl*p*	β-D-Xyl*p*	H
S5	α-L-Rha*p*	β-D-Api*f*	H
S6	β-D-Xyl*p*	β-D-Api*f*	H
S7	α-L-Rha*p*	H	Acetyl
S8	β-D-Xyl*p*	H	Acetyl
S9	α-L-Rha*p*	β-D-Xyl*p*	Acetyl
S10	β-D-Xyl*p*	β-D-Xyl*p*	Acetyl
S11	α-L-Rha*p*	β-D-Api*f*	Acetyl
S12	β-D-Xyl*p*	β-D-Api*f*	Acetyl

Fig. 1. Structures of the saponins **S1**–**S6** and the novel **S7**–**S12**.

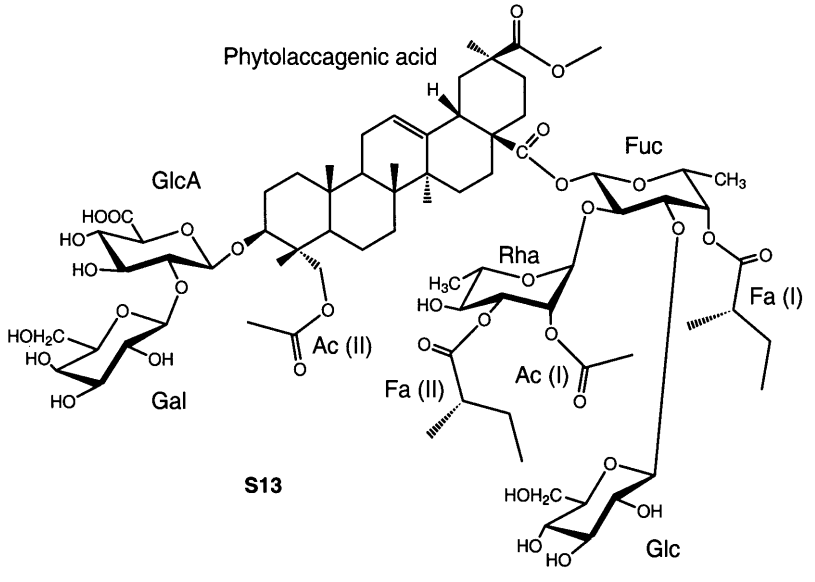


Fig. 2. Structure of the saponin **S13**.

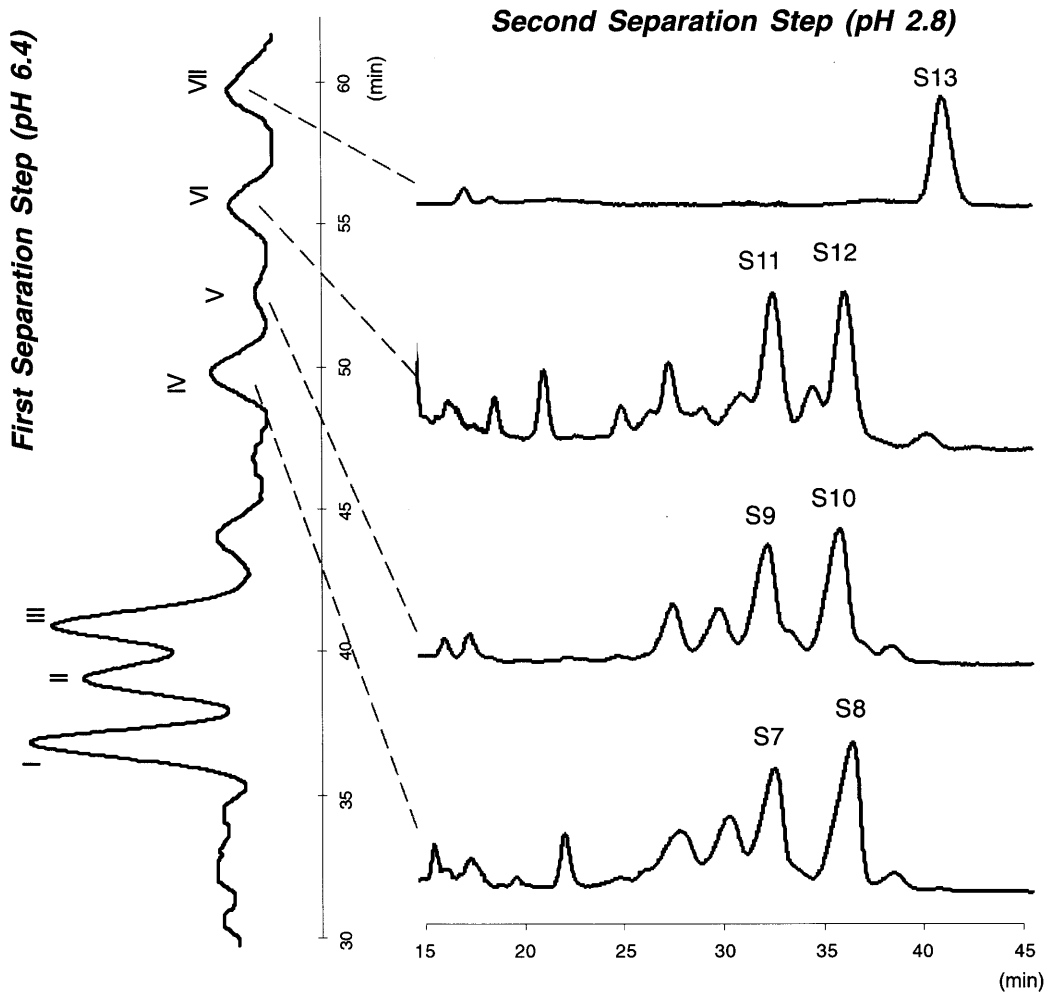


Fig. 3. HPLC chromatograms from the two separation steps of the saponin fraction. At the first separation step (vertical graph) a buffer at pH 6.4 was used as the mobile phase. At the second separation (horizontal graphs) a buffer at pH 2.8 was used, which separated compounds with Rha and Xyl containing trisaccharides at C-3 of quillaic acid, present in fractions IV–VI. No additional separation was achieved for fraction VII indicating the absence of trisaccharide pairs, verified by MALDI-TOF MS and NMR.

2. Results and discussion

HPLC.—Bark extract from *Q. saponaria* Molina was subjected to the crude separation step using solid-phase extraction (SPE) as previously described [8]. Collected 80–90% methanol (aqueous) eluates showed several peaks in the reversed-phase HPLC chromatogram (Fig. 3, vertical graph) using ammonium acetate buffer at pH 6.4. The compounds in fractions I–III have already been investigated [8] but fractions IV–VII contained more lipophilic novel structures and these were collected. Analytical HPLC with the same buffer system was used to analyse the fractions. Samples, analysed immediately after collection, showed only one main peak in the analytical HPLC chromatogram as expected. Even if fractions IV–VII were allowed to stand in the pH 6.4 buffered eluate for 2 weeks at 7 °C no emerging regioisomer could be detected by HPLC. This is in contrast to the case for fractions I–III (**S1**–**S6**) [8] and similar *Quillaja* saponins [6,9], where the acyl group at fucose O-4 could migrate to fucose O-3, thus giving a new peak in the chromatogram. The absence of regioisomers supports a structure with a 3,4-substituted fucose since it would prevent migration of the larger acyl group from fucose O-4 to O-3. A second separation step using a phosphate buffer at pH 2.8 was employed (Fig. 3, horizontal graphs) and seven compounds **S7**–**S13** were isolated and characterised.

Structural analysis.—Isolated compounds were characterised by monosaccharide analysis, MALDI-TOF MS and NMR spectroscopy in order to determine their structures. Neutral sugars, converted to alditol acetates, were analysed by GC and Table 1 gives the relative proportions of L-Rha, D-Fuc, L-Ara, D-Xyl, D-Gal, D-Glc and D-Api. NMR spectroscopy was used to identify the additional D-GlcA residues. The absolute configurations of the sugars were assumed to be the same as those previously identified in similar *Quillaja* saponins [3,7,17]. The molecular masses of all compounds were determined by MALDI-TOF MS and are given in Table 1. Further structural information was obtained by 1D and 2D NMR experiments.

Assignments of NMR signals.—The proton spin systems of the sugar residues were determined using COSY and TOCSY experiments for all compounds. Starting with the signal from H-1 (the anomeric proton) the COSY spectra identified the H-2 signal, and then the COSY together with TOCSY spectra were used to assign the rest of the protons in the spin system. Overlapping signals within the same spin systems were assigned by HSQC-DEPT, intramolecular connectivities observed in the HMBC spectra and by comparison with NMR data of similar elements in previously investigated saponins [6–10]. The chemical shifts for ^{1}H and ^{13}C signals were extracted from the HSQC-DEPT spectra. The signals from quaternary and carbonyl carbons in apiose and glucuronic acid, respectively, were assigned by HMBC spectra. The different sugars and their anomeric configurations could be identified by comparison of chemical shifts and the pattern of the cross-peaks with those of the corresponding monosaccharides and methyl glycosides [18,19] and previously characterised saponins [6–9]. Anomeric configurations were also confirmed by the $^{3}J_{H\text{-}1,H\text{-}2}$ values (β-D-Fuc: 8.1–8.2 Hz, α-L-Ara: 2.0–2.2 Hz, β-D-Xyl: 6.9–7.9 Hz, β-D-Gal: 6.4–7.7 Hz, β-D-Glc: 7.7 Hz, β-D-Api: 2.7–2.8 Hz, and β-D-GlcA: 7.6–7.7 Hz). The linkage positions for the sugar residues were indicated by the relatively high chemical shifts for the signals of the substituted carbons (4–9 ppm higher than corresponding unsubstituted carbon). The sequence of the sugar residues and the attachment of the oligosaccharides to the aglycone were obtained by the three-bond heteronuclear connectivities over the glycosidic bonds, observed as cross-peaks in the HMBC spectra.

The ^{1}H and ^{13}C signals from the quillaic acid (Qa) moiety were assigned from ^{1}H, HSQC-DEPT and HMBC spectra for compounds **S7**, **S8**, **S11**, and **S12** (Table 2). The chemical shifts were in good agreement ($|\Delta\delta| < 0.05$ ppm in ^{1}H and < 0.6 ppm in ^{13}C) with the corresponding values from the quillaic acid moiety in compounds **S1**–**S6** [8], and support substitution at C-3 and C-28 of the quillaic acid. The oligosaccharide at C-3 of the quillaic acid in compounds **S7** and **S11** was

Table 1
Molecular masses and results from monosaccharide analysis [a] of the neutral sugars

	M_w [b]	M_w [c]	Rha		Fuc		Ara		Xyl		Gal		Glc		Api [d]	
S7	1912.9	1912.9	1.9	**2**	1.0	**1**	0.9	**1**	1.0	**1**	1.0	**1**		0		**0**
S8	1898.9	1898.8	1.0	**1**	1.0	**1**	0.9	**1**	1.8	**2**	1.0	**1**		0		**0**
S9	2045.0	2045.0	1.7	**2**	1.0	**1**	0.8	**1**	1.8	**2**	1.1	**1**		0		**0**
S10	2030.9	2030.8	1.0	**1**	1.0	**1**	0.9	**1**	2.5	**3**	1.0	**1**		0		**0**
S11	2045.0	2044.8	1.9	**2**	1.0	**1**	0.9	**1**	0.9	**1**	1.0	**1**		0	0.5	**1**
S12	2030.9	2030.7	1.0	**1**	1.0	**1**	0.9	**1**	1.7	**2**	1.0	**1**		0	0.2	**1**
S13	1560.7	1560.6	1.3	**1**	1.0	**1**		**0**		**0**	1.1	**1**	0.9	1		**0**

[a] Presented as areas from GC chromatograms normalised against the fucitol peak. The bold-face values are the assigned number of sugars for the compounds.

[b] Monoisotopic molecular mass (Da) of the assigned structures.

[c] Monoisotopic molecular mass (Da) as determined by MALDI-TOF MS, corrected for Na by subtracting 23 mass units.

[d] 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 for compounds **S7**, **S8**, **S11**, and **S12**

Residue	**S7**		**S8**		**S11**		**S12**	
	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
Qa-1	1.10, 1.72	39.1	1.11, 1.72	39.2	1.11, 1.72	39.2	1.11, 1.73	39.3
Qa-2	1.78, 2.01	25.5	1.77, 1.98	25.5	1.78, 2.01	25.5	1.79, 1.99	25.4
Qa-3	3.86	86.0	3.87	86.2	3.86	85.8	3.88	86.2
Qa-4		56.1		55.9		56.2		55.9
Qa-5	1.31	49.1	1.31	48.9	1.33	49.1	1.32	49.2
Qa-6	0.93, 1.47	21.3	0.93, 1.46	21.3	0.94, 1.47	21.3	0.94, 1.46	21.2
Qa-7	1.31, 1.50	33.5	1.31, 1.52	33.3	1.34 1.51	33.5	1.33, 1.49	33.6
Qa-8		41.0		41.0		40.9		40.9
Qa-9	1.74	47.9	1.75	47.8	1.75	47.9	1.75	47.9
Qa-10		37.1		37.0		37.1		36.9
Qa-11	1.93, 1.93	24.4	1.94, 1.94	24.3	1.93, 1.93	24.3	1.94, 1.94	24.4
Qa-12	5.34	123.1	5.34	123.0	5.34	123.1	5.34	123.2
Qa-13		144.5		144.4		144.4		144.2
Qa-14		42.3		42.2		42.8		42.4
Qa-15	1.41, 1.68	36.4	1.42, 1.68	36.3	1.44, 1.64	36.4	1.45, 1.65	36.4
Qa-16	4.47	74.6	4.47	74.5	4.46	74.4	4.47	74.4
Qa-17		[a]		50.2		[a]		[a]
Qa-18	2.96	42.1	2.96	42.0	2.95	42.1	2.96	42.2
Qa-19	1.06, 2.31	47.9	1.06, 2.31	47.9	1.06, 2.31	48.0	1.06, 2.31	47.9
Qa-20		31.0		31.0		31.1		30.9
Qa-21	1.18, 1.94	36.4	1.17, 1.95	36.4	1.16, 1.95	36.4	1.18, 1.95	36.6
Qa-22	1.79, 1.94	31.8	1.80, 1.95	31.8	1.75, 1.95	32.0	1.78, 1.95	32.2
Qa-23	9.45	211.1	9.45	210.6	9.45	210.9	9.46	210.8
Qa-24	1.17	10.8	1.17	10.7	1.17	10.7	1.17	10.8
Qa-25	1.02	16.4	1.02	16.3	1.02	16.4	1.02	16.4
Qa-26	0.78	17.9	0.78	17.8	0.78	17.8	0.78	17.9
Qa-27	1.40	27.0	1.40	27.0	1.40	27.0	1.40	27.0
Qa-28		176.8		176.6		176.7		176.2
Qa-29	0.89	33.2	0.89	33.1	0.89	33.2	0.89	33.2
Qa-30	0.96	24.8	0.97	24.8	0.96	24.7	0.96	24.8
GlcA-1	4.46	104.1	4.44	104.3	4.46	103.9	4.44	104.2
GlcA-2	3.64	77.9	3.66	78.1	3.63	78.0	3.66	78.0
GlcA-3	3.64	85.7	3.68	86.4	3.64	85.7	3.69	86.4
GlcA-4	3.59	71.8	3.56	71.3	3.59	71.8	3.56	71.3
GlcA-5	3.76	76.6	3.76	76.6	3.76	76.6	3.76	76.8
GlcA-6		173.3		172.7		173.1		172.4
Gal-1	4.46	104.1	4.80	103.6	4.46	103.9	4.81	103.7
Gal-2	3.49	72.9	3.45	73.4	3.50	72.8	3.47	73.4
Gal-3	3.48	74.8	3.43	75.2	3.48	74.9	3.44	75.3
Gal-4	3.82	70.6	3.82	70.6	3.82	70.5	3.83	70.7
Gal-5	3.47	76.8	3.49	76.5	3.47	76.8	3.50	76.6
Gal-6	3.72, 3.79	62.1	3.73, 3.77	61.9	3.73, 3.79	62.1	3.74, 3.78	62.1
Xyl-1			4.59	104.6			4.60	104.8
Xyl-2			3.23	75.1			3.24	75.1
Xyl-3			3.30	78.0			3.31	78.1
Xyl-4			3.49	70.7			3.51	70.9
Xyl-5			3.24, 3.91	66.9			3.24, 3.91	67.1
Rha-1	5.03	103.0			5.04	103.0		
Rha-2	4.02	71.9			4.01	71.9		
Rha-3	3.66	72.1			3.65	72.1		
Rha-4	3.40	73.6			3.40	73.8		
Rha-5	3.95	70.4			3.94	70.4		
Rha-6	1.24	17.7			1.24	17.8		

[a] Signal not observed in the HMBC spectrum.

found to be β-D-Gal*p*-(1→2)-[α-L-Rha*p*-(1→3)]-β-D-Glc*p*A as the chemical shifts (Table 2) were similar to those of **S1**, **S3** and **S5** [8] and other *Quillaja* saponins [7,9]. Analogously, compounds **S8** and **S12** contained the trisaccharide β-D-Gal*p*-(1→2)-[β-D-Xyl*p*-(1→3)]-β-D-Glc*p*A at the quillaic acid C-3. Fig. 4 gives the ^{1}H NMR spectra for compounds **S7**, **S9**, **S10**, and **S13**. Spectra are given from both the protonated and deprotonated form of **S7** for comparison and, as can be seen, the GlcA H-1 signal is shifted upfield 0.10 ppm and the Rha H-1 signal is shifted downfield 0.02 ppm when the acidic group is deprotonated. The assignment of the shifted signals in the spectrum for the deprotonated form was verified by a TOCSY experiment. The sample amount of compounds **S9** and **S10** was not sufficient to give a desirable quality of the ^{1}H–^{13}C correlated spectra and thus no data are given in Table 2 for **S9** and **S10**. However, it was possible to identify signals in the ^{1}H, and ^{1}H–^{1}H correlated NMR spectra that together with the results from MALDI-TOF MS and monosaccharide analysis ascertain the identity of compounds **S9** and **S10**. Signals from protons in quillaic acid that were identified in the ^{1}H NMR spectra for **S9** and **S10** are; 0.78 ppm (3 H, s, H-26), 0.89 ppm (3 H, s, H-29), 1.01 ppm (3 H, s, H-25), 1.16 ppm (3 H, s, H-24), 1.40 ppm (3 H, s, H-27), 2.32 ppm (1 H, m, H-19), 2.96 ppm (1 H, dd, H-18), 5.34 ppm (1 H, m, H-12), 9.45 ppm (1 H, s, H-23), and overlapped signals at 4.45–4.47 ppm (H-16) and 0.96 ppm (H-30). Further assignments were made from the COSY spectra; 2.32/1.07 ppm (H-19,19′), 4.46/1.43 ppm (H-16,15), 4.46/1.65 ppm (H-16,15′), 5.34/1.94 ppm (H-12,11), and TOCSY spectra; 3.86/1.12 ppm (H-3,1), 3.86/1.71 ppm (H-3,1′), 3.86/1.79 ppm (H-3,2), 3.86/2.08 ppm (H-3,2′). Fig. 4 shows ^{1}H NMR spectra from the deprotonated forms of **S9** and **S10**, which expose the GlcA H-1 signal at 4.35–4.37 ppm. Compound **S9** was expected to contain the same trisaccharide at the quillaic acid C-3 as compounds **S7** and **S11** and this was confirmed by the following signals in the ^{1}H spectrum for **S9**; 1.23 ppm (3 H, d, Rha H-6), 5.05 ppm (1 H, d, Rha H-1) and the overlapped signal at 4.45–4.47 ppm (Gal H-1). Additional signals were identified in the COSY spectrum; 4.37/3.62 ppm (GlcA H-1,2), 4.46/3.50 ppm (Gal H-1,2), 5.05/3.99 ppm (Rha H-1,2), and 4.03/1.23 ppm (Rha

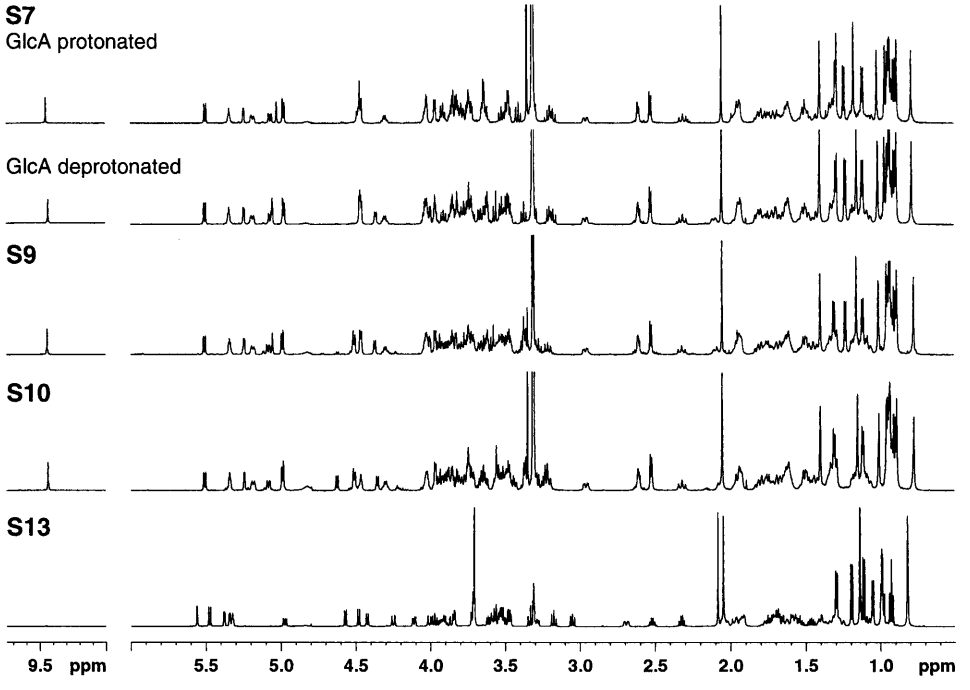


Fig. 4. Parts of the ^{1}H NMR spectra for compounds **S7**, **S9**, **S10**, and **S13**. Two spectra are given for compound **S7**, demonstrating the differences induced by a protonated and a deprotonated GlcA residue. Compounds **S9**, **S10** and **S13** were acquired in their deprotonated forms.

H-5,6). The COSY and TOCSY spectra showed similar patterns of cross-peaks from the spin systems of the triterpenoid moiety and the three sugars as for the previously studied **S1**, **S3** and **S5**, and compounds **S7** and **S11** (quillaic acid with a rhamnose containing trisaccharide at C-3). Analogously, compound **S10** was expected to contain the same trisaccharide as compounds **S8** and **S12**. This was confirmed by the signal at 4.62 ppm (1 H, d, Xyl H-1) in the ^{1}H NMR spectrum for **S10**. Also, a signal at 4.81 ppm (1 H, d, Gal H-1) was observed in a ^{1}H spectrum acquired at decreased temperature (24 °C instead of 30 °C) inducing a shift of the solvent –OH signal from 4.8 to 4.9 ppm. Additional signals in the COSY spectrum were identified; 4.35/3.65 ppm (GlcA H-1,2), 4.81/3.47 ppm (Gal H-1,2), 4.63/3.24 ppm (Xyl H-1,2). The COSY and TOCSY spectra showed similar patterns of cross-peaks from the spin systems of the triterpenoid moiety and the three sugars as for the previously studied **S2**, **S4** and **S6**, and compounds **S8** and **S12** (quillaic acid with a xylose containing trisaccharide at C-3).

NMR data for the C-28 oligosaccharides including the C-9 fatty acyl parts (Fa, Fa′ and Ara) are given in Table 3 for compounds **S7**, **S8**, **S11**, and **S12**. The structure of the C-28 oligosaccharide in compounds **S7** and **S8** was found to be identical to the corresponding oligosaccharide in structures **S1** and **S2** [8], except for an additional 3-*O*-acetyl group in the fucose residue in **S7** and **S8**. The position of the acetyl group was readily obtained by the three-bond heteronuclear connectivity between the acetyl carbonyl carbon and fucose H-3 observed as cross-peaks in the HMBC spectra (Fig. 5). The acetyl group induced a marked difference in the chemical shifts for most of the ^{1}H and ^{13}C signals of the fucose residue. The largest differences in ^{1}H and ^{13}C chemical shifts between saponin **S2** and the fucose 3-O-acetylated compound **S8** are given as $\Delta\delta$ values in Table 4. Apart from the fucose residue, the signals of the rhamnose residue are also affected by the acetyl group, e.g., the Rha H-1 signal was shifted upfield by 0.33 ppm. A similar behaviour was observed for the fucose 3-O-acylated *Quillaja* saponin **S2a** [8], which had an upfield shift of 0.28 ppm for the Rha H-1 signal as compared with that from **S2**. Analogously compounds **S11** and **S12** were found to contain the same C-28 oligosaccharide as **S5** and **S6**, except for the fucose 3-*O*-acetyl group. Compounds **S9** and **S10** were expected to contain the same C-28 oligosaccharide as **S3** and **S4**, substituted by a fucose 3-*O*-acetyl group, and the presence of the following signals in the ^{1}H NMR spectra verified this; 1.12 ppm (3 H, d, Fuc H-6), 1.31 ppm (3 H, d, Rha H-6), 2.05 ppm (3 H, s, Ac H-2), 4.50–4.51 ppm (2 H, overlapped, Xyl(1) + Xyl(2) H-1), 4.98 ppm (1 H, d, Rha H-1), 5.08 ppm (1 H, dd, Fuc H-3), 5.24 ppm (1 H, d, Fuc H-4), 5.51 ppm (1 H, d, Fuc H-1). Also, cross-peaks in the COSY spectra for **S9** and **S10** were identified; 5.51/3.94 ppm (Fuc H-1,2), 5.08/3.94 ppm (Fuc H-3,2), 5.24/5.08 ppm (Fuc H-4,3), 3.97/1.12 ppm (Fuc H-5,6), 4.99/3.74 ppm (Rha H-1,2), 3.72/1.31 ppm (Rha H-5,6), 4.51/3.36 ppm (overlapping signals from both Xyl H-1,2), 3.89/3.21 ppm (Xyl H-5,5′), and 3.93/3.27 ppm (terminal Xyl H-5,5′). The patterns of cross-peaks in the COSY and TOCSY spectra for **S9** and **S10** are similar to those for **S7**, **S8**, **S11**, and **S12** regarding the fucose residue, and similar to those for **S3** and **S4** regarding the two xylose residues in the C-28 oligosaccharide. All signals from the C-9 fatty acyl parts including the Ara were assigned for compounds **S7**, **S8**, **S11**, and **S12**. The same acyl group was found in compounds **S9** and **S10** and this was based on the following signals in the ^{1}H NMR spectra; 2.52 ppm (2 H, m, Fa′ H-2), 2.61 ppm (2 H, m, Fa H-2), 4.30 ppm (1 H, m, Fa′ H-3), 4.99 ppm (1 H, broad, Ara H-1), 5.18 ppm (1 H, m, Fa H-5). Cross-peaks in the COSY spectra gave additional evidence; 4.02/2.61 ppm (Fa H-3,2), 4.02/1.68 ppm (Fa H-3,4), 4.02/1.81 ppm (Fa H-3,4′), 5.18/1.68 ppm (Fa H-5,4), 5.18/1.81 ppm (Fa H-5,4′), 5.18/1.62 ppm (Fa H-5,6), 4.30/2.52 ppm (Fa′ H-3,2), 4.30/1.50 ppm (Fa′ H-3,4), 4.30/1.63 ppm (Fa′ H-3,4′), 3.80/1.50 ppm (Fa′ H-5,4), and 3.80/1.63 ppm (overlapped Fa′ H-5,4′ + Fa′ H-5,6).

Compound **S13** gave no signal from an aldehyde group in the ^{1}H NMR spectrum (Fig. 4) and hence the aglycone was not quillaic acid. Signals from five methyl groups on tertiary carbons at 0.82–1.14 ppm (3 H each, s) and one olefinic proton at 5.32 ppm (1 H,

Table 3
NMR assignments for the C-28 oligosaccharide and the C-9 aliphatic acid parts for compounds **S7**, **S8**, **S11**, and **S12**

Residue	**S7**		**S8**		**S11**		**S12**	
	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
Fuc-1	5.50	94.5	5.50	94.4	5.50	94.5	5.50	94.5
Fuc-2	3.91	73.6	3.91	73.6	3.93	73.0	3.93	73.0
Fuc-3	5.07	75.4	5.06	75.3	5.08	75.6	5.08	75.7
Fuc-4	5.25	71.8	5.24	71.6	5.24	71.7	5.25	71.9
Fuc-5	3.97	70.4	3.96	70.5	3.97	70.5	3.97	70.6
Fuc-6	1.12	16.2	1.11	16.2	1.11	16.2	1.12	16.3
Ac-1		171.5		171.2		171.4		171.3
Ac-2	2.06	20.8	2.06	20.8	2.05	20.8	2.05	20.9
Rha-1	4.97	101.8	4.97	101.7	4.99	101.5	4.99	101.5
Rha-2	3.75	71.6	3.74	71.7	3.74	71.7	3.74	71.7
Rha-3	3.73	72.0	3.72	71.9	3.71	72.1	3.72	72.1
Rha-4	3.52	83.7	3.52	83.8	3.52	84.5	3.52	84.5
Rha-5	3.72	69.1	3.72	69.1	3.72	68.9	3.73	69.0
Rha-6	1.29	18.4	1.29	18.3	1.31	18.3	1.31	18.4
Xyl-1	4.47	106.8	4.46	106.7	4.47	107.1	4.47	107.0
Xyl-2	3.20	75.9	3.20	76.0	3.29	75.5	3.30	75.4
Xyl-3	3.31	78.1	3.30	78.0	3.39	85.7	3.39	85.7
Xyl-4	3.49	70.7	3.49	70.7	3.50	69.5	3.50	69.6
Xyl-5	3.18, 3.84	67.1	3.18, 3.84	67.1	3.20, 3.88	66.8	3.21, 3.89	66.9
Api-1					5.24	110.8	5.25	110.9
Api-2					4.04	77.7	4.04	77.7
Api-3						80.1		80.0
Api-4					3.80, 4.16	74.8	3.81, 4.16	74.9
Api-5					3.65	65.2	3.66	65.2
Fa-1		172.4		172.3		172.4		172.3
Fa-2	2.61, 2.61	43.2	2.61, 2.61	43.1	2.61, 2.61	43.2	2.61, 2.61	43.3
Fa-3	4.02	65.7	4.02	65.7	4.02	65.7	4.03	65.7
Fa-4	1.67, 1.81	39.7	1.66, 1.81	39.7	1.67, 1.81	39.7	1.68, 1.82	39.9
Fa-5	5.18	75.2	5.18	75.1	5.18	75.2	5.18	75.2
Fa-6	1.62	40.0	1.62	40.1	1.61	40.0	1.61	40.0
Fa-7	1.16, 1.50	26.4	1.16, 1.50	26.5	1.15, 1.49	26.4	1.16, 1.50	26.6
Fa-8	0.93	12.2	0.94	12.1	0.93	12.1	0.93	12.2
Fa-9	0.95	14.7	0.95	14.6	0.94	14.7	0.95	14.8
Fa′-1		173.5		173.3		173.5		173.3
Fa′-2	2.53, 2.53	43.8	2.52, 2.52	43.7	2.53, 2.53	43.7	2.53, 2.53	43.8
Fa′-3	4.30	66.1	4.30	66.1	4.30	66.0	4.30	66.1
Fa′-4	1.50, 1.63	39.2	1.50, 1.65	39.4	1.49, 1.63	39.5	1.51, 1.64	39.5
Fa′-5	3.80	79.1	3.80	79.0	3.80	79.1	3.80	79.1
Fa′-6	1.62	40.0	1.62	40.1	1.61	40.0	1.61	40.0
Fa′-7	1.12, 1.60	25.4	1.11, 1.60	25.3	1.10, 1.60	25.3	1.11, 1.60	25.6
Fa′-8	0.93	12.2	0.94	12.1	0.93	12.1	0.93	12.2
Fa′-9	0.90	15.1	0.91	15.1	0.90	15.0	0.91	15.1
Ara-1	4.99	108.6	4.98	108.6	4.98	108.7	4.98	108.7
Ara-2	3.97	83.3	3.97	83.2	3.97	83.3	3.97	83.3
Ara-3	3.85	78.0	3.85	78.1	3.85	78.2	3.85	78.2
Ara-4	4.03	85.2	4.03	85.2	4.02	85.2	4.03	85.3
Ara-5	3.63, 3.77	62.9	3.63, 3.77	62.9	3.63, 3.76	62.9	3.63, 3.77	62.9

m) were observed. The proton spin systems were identified by use of COSY and TOCSY experiments and inter-connected by use of an HMBC experiment. The chemical shifts for all ^{1}H and ^{13}C signals (Table 5) were extracted from the HSQC-DEPT spectrum except for quaternary and carbonyl carbons, which were obtained from the HMBC spectrum. A three

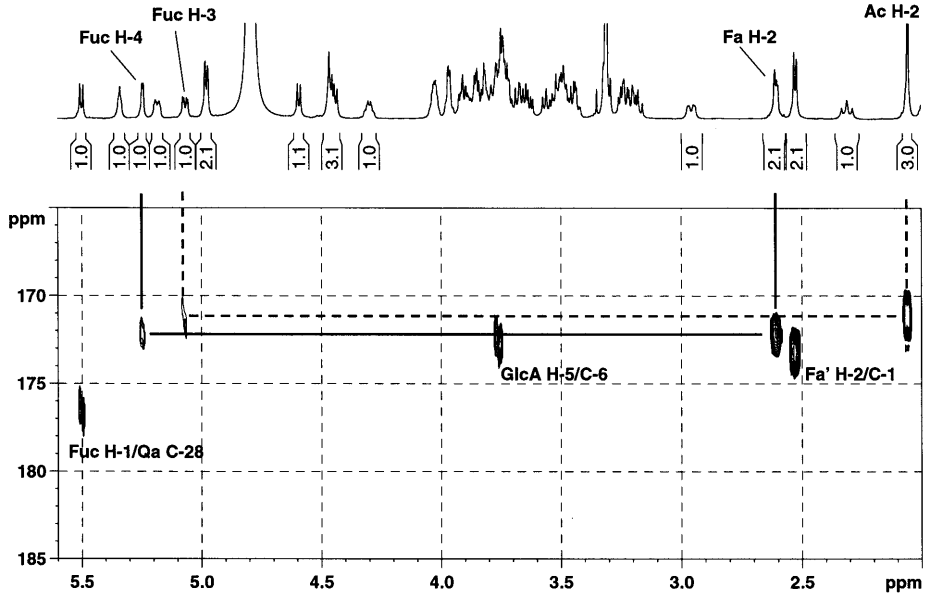


Fig. 5. Part of the HMBC spectrum for saponin **S8**. The solid lines indicate the three-bond heteronuclear connectivity between Fuc H-4/Fa C-1 and the two-bond connectivity between Fa H-2/Fa C-1. The dashed lines indicate the three-bond connectivity between Fuc H-3/Ac C-1 and the two-bond connectivity between Ac H-2/Ac C-1.

proton singlet at 3.71 ppm with a one-bond heteronuclear coupling to a carbon resonance at 52.3 ppm and a three-bond heteronuclear correlation to a carboxyl carbon at 178.6 ppm observed in the HSQC-DEPT and HMBC spectrum, respectively, indicated the presence of a methyl ester in the aglycone. A three-bond heteronuclear correlation from the protons of the methyl group at C-20 to the carbonyl carbon (1.14/178.6 ppm in the HMBC spectrum) showed that a methoxycarbonyl group is linked to C-20. A cross-peak at 3.71/2.69 ppm in a ROESY spectrum demonstrated that the *O*-methyl group of the ester and H-18 were positioned on the same side of ring E. Hence the methyl group at C-20 was in the α-position (C-29) and the methoxycarbonyl group was in the β-position (C-30). Thus it was possible to identify the aglycone as the C-30 methyl ester of 3β,23α-dihydroxy-12-oleanen-28,30-dioic acid. This triterpenoid aglycone was previously found in a saponin from *Phytolacca americana* [20] and other plants [21,22], and is called phytolaccagenic acid (Pa). The ^{1}H and ^{13}C chemical shifts for the aglycone in **S13** were similar to those previously found [20–22] except for signals from C-23 and adjacent atoms. This was found to be due to an acylation of the C-23 hydroxymethyl group, which induced a downfield shift of the methylene protons by ~0.6 ppm. Cross-peaks in the HMBC spectrum (Fig. 6) further demonstrated that there was an acetyl group ester linked to the hydroxyl at C-23. Both C-3 and C-28 of the aglycone in **S13** were found to be substituted by oligosaccharide chains. Recently Guo et al. isolated a saponin from *Quillaja* containing the non-acetylated phytolaccagenic acid aglycone [23].

Table 4
$\Delta\delta$ values induced by fucose 3-O-acetylation [a]

Position	$\Delta\delta$	
	^{1}H (ppm) [b]	^{13}C (ppm) [b]
Fuc-1	0.15	
Fuc-2	0.18	−1.7
Fuc-3	1.20	0.8
Fuc-4	0.14	−3.5
Fuc-5	0.11	
Rha-1	−0.33	
Rha-2	−0.18	
Rha-3	−0.08	
Rha-4		−0.6
Rha-5	−0.06	
Fa-1		−0.7

[a] NMR signals from compound **S2** [8] are subtracted from the corresponding values for compound **S8**.

[b] $|\Delta\delta| > 0.05$ for ^{1}H and $|\Delta\delta| > 0.5$ for ^{13}C are shown.

Table 5
NMR assignments for compound **S13**

Residue	**S13**				
	^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)
Pa-1	0.98, 1.64	39.4	Fuc-1	5.47	95.0
Pa-2	1.77, 1.97	26.4	Fuc-2	3.97	73.3
Pa-3	3.58	84.4	Fuc-3	4.11	82.6
Pa-4		43.2	Fuc-4	5.37	74.9
Pa-5	1.09	49.0	Fuc-5	3.90	71.0
Pa-6	1.30, 1.35	19.2	Fuc-6	1.05	16.4
Pa-7	1.31, 1.41	33.4	Rha-1	5.56	98.7
Pa-8		40.7	Rha-2	5.34	71.1
Pa-9	1.59	49.2	Rha-3	4.97	73.0
Pa-10		37.8	Rha-4	3.51	70.8
Pa-11	1.92, 1.92	24.5	Rha-5	3.93	70.1
Pa-12	5.32	124.0	Rha-6	1.29	17.8
Pa-13		144.2	Glc-1	4.48	105.3
Pa-14		42.6	Glc-2	3.05	75.2
Pa-15	1.25, 1.57	29.3	Glc-3	3.33	77.8
Pa-16	1.70, 2.06	23.9	Glc-4	3.17	71.3
Pa-17		47.6	Glc-5	3.29	77.8
Pa-18	2.69	44.2	Glc-6	3.61, 3.86	62.9
Pa-19	1.70, 1.95	43.2	Fa(I)-1		178.4
Pa-20		44.7	Fa(I)-2	2.52	42.5
Pa-21	1.39, 2.03	31.2	Fa(I)-3	1.54, 1.74	27.9
Pa-22	1.60, 1.69	34.2	Fa(I)-4	0.99	12.0
Pa-23	4.01, 4.25	66.8	Fa(I)-5	1.19	17.3
Pa-24	0.82	13.3	Fa(II)-1		177.4
Pa-25	0.99	16.4	Fa(II)-2	2.32	42.1
Pa-26	0.82	17.7	Fa(II)-3	1.47, 1.67	27.5
Pa-27	1.14	25.8	Fa(II)-4	0.93	12.0
Pa-28		177.3	Fa(II)-5	1.11	16.6
Pa-29	1.14	28.5	Ac(I)-1		171.9
Pa-30		178.6	Ac(I)-2	2.08	20.9
OMe	3.71	52.3	Ac(II)-1		172.8
GlcA-1	4.42	105.1	Ac(II)-2	2.05	20.8
GlcA-2	3.52	82.4			
GlcA-3	3.58	77.9			
GlcA-4	3.52	72.9			
GlcA-5	3.71	76.4			
GlcA-6		173.5			
Gal-1	4.57	105.9			
Gal-2	3.55	73.6			
Gal-3	3.47	74.8			
Gal-4	3.84	70.0			
Gal-5	3.47	76.8			
Gal-6	3.71, 3.71	61.9			

The saccharide part at C-3 of the aglycone was identified as the disaccharide β-D-Gal*p*-(1→2)-β-D-Glc*p*A. This disaccharide has been described as a C-3 substituent in a *Quillaja* saponin [7] and chemical shifts both ^{1}H and ^{13}C signals were similar ($|\Delta\delta| < 0.11$ ppm in ^{1}H and < 1.4 ppm in ^{13}C, with the largest deviations for GlcA C-1 and C-2 and Gal C-1 signals).

The C-28 substituent in **S13** was the branched trisaccharide α-L-Rha*p*-(1→2)-[β-D-Glc*p*-(1→3)]-β-D-Fuc*p* substituted with three acyl groups. The anomeric signals were found at 5.47/95.0 ppm ($^3J_{\text{H-1,H-2}} = 8.1$ Hz), 5.56/98.7

ppm ($^3J_{H\text{-}1,H\text{-}2} = 1.3$ Hz), and 4.48/105.3 ($^3J_{H\text{-}1,H\text{-}2} = 7.7$ Hz) for the Fuc, Rha and Glc residues, respectively. The identity of the sugars was verified by COSY and TOCSY experiments and the $^3J_{H,H}$ values. A three-bond heteronuclear connectivity, observed as a cross-peak at 5.47/177.3 ppm in the HMBC spectrum (Fig. 6), connects Fuc H-1 to C-28 of the aglycone. The position of the Rha residue was demonstrated by cross-peaks in the HMBC spectrum at 3.97/98.7 and 5.56/73.3 ppm between Fuc H-2/Rha C-1 and Rha H-1/Fuc C-2, respectively. The position of the Glc residue was demonstrated by cross-peaks at 4.11/105.3 and 4.48/82.6 ppm between Fuc H-3/Glc C-1 and Glc H-1/Fuc C-3, respectively. The substitution positions of the acyl groups were elucidated from the three-bond heteronuclear connectivities observed as cross-peaks in the HMBC spectrum (Fig. 6). A connectivity between Rha H-2 and a carbonyl carbon at 5.34/171.9 ppm was detected and a two-bond connectivity between a three proton singlet at 2.08 and the carbonyl carbon indicated the presence of an acetyl group at Rha O-2. A connectivity between Fuc H-4 and a carbonyl carbon at 5.37/178.4 ppm was observed. This carbon showed a second cross-peak assumed to be the two-bond heteronuclear connectivity to H-2 (1 H, m in the ^{1}H spectrum) of an acyl group. The COSY spectrum showed cross-peaks from this proton to a methyl group (3 H, d, Fa(I) H-5) and a methylene group (negative signals in HSQC-DEPT, Fa(I) H-3). The TOCSY spectrum revealed the presence of an additional methyl group (3 H, t, Fa(I) H-4) in the spin system. The carbonyl carbon showed cross-peaks in the HMBC spectrum associated with all protons in the spin system except for the last mentioned methyl group. A similar pattern of cross-peaks and signals was observed for the acyl group at Rha O-3. Thus, the Fuc O-4 and Rha O-3 substituents were both identified as 2-methylbutanoyl groups. The stereochemistry of this acyl group was assessed by GC–MS after hydrolysis and isolation of the resulting 2-methylbutanoic acid [16]. The hydrolysis product as well as (*S*)- and racemic 2-methylbutanoic acid, used as references, were analysed on a chiral capillary column of fused-silica coated with derivatised β-cyclodextrin. The mass spectra verified the identity of the 2-methylbutanoic acid in the sample and references (prominent peaks; *m*/*z* 41, 57, 74, and 87). The (*S*)-2-methylbutanoic acid gave one peak in the chromatogram, whereas the racemic acid gave two well separated ($\alpha =$

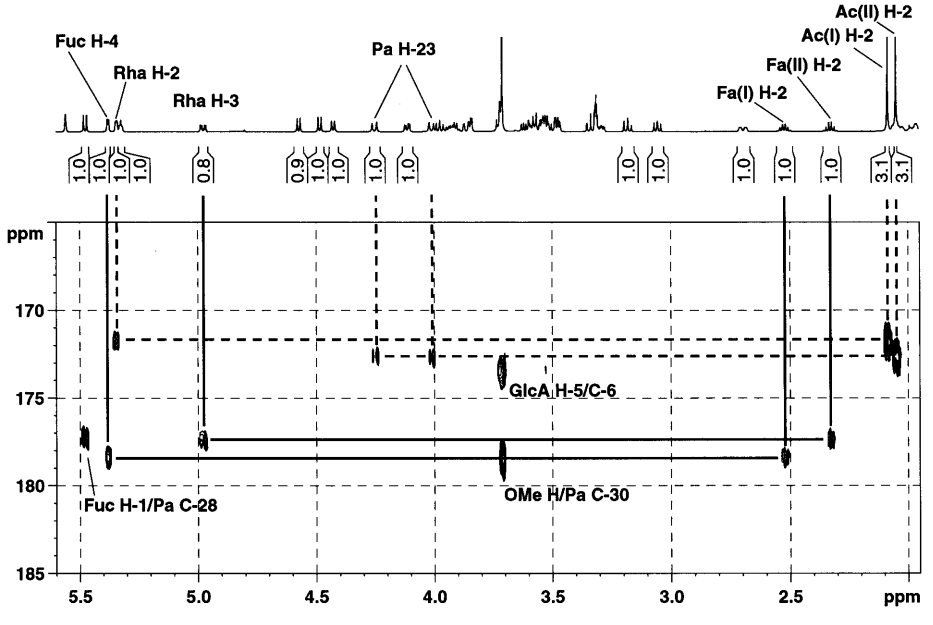


Fig. 6. Part of the HMBC spectrum for saponin **S13**. The solid lines indicate the three-bond heteronuclear connectivities between Fuc H-4/Fa(I) C-1 and Rha H-3/Fa(II) C-1, as well as the two-bond connectivities between Fa(I) H-2/Fa(I) C-1 and Fa(II) H-2/Fa(II) C-1. The dashed lines indicate the three-bond connectivity between Rha H-2/Ac(I) C-1 and Pa H-23/Ac(II) C-1, as well as the two-bond connectivities between Ac(I) H-2/Ac(I) C-1 and Ac(II) H-2/Ac(II) C-1.

Table 6
Results from GC–MS analysis of isolated 2-methylbutanoic acid and (*S*)- and racemic 2-methylbutanoic acid as references

Sample	t_R (*S*) [a]	t_R (*R*) [b]
(*S*)-2-Methylbutanoic acid (reference)	17.54	
Racemic 2-methylbutanoic acid (reference)	17.59	18.65
Isolated 2-methylbutanoic acid	17.67	

[a] Retention time (min) for (*S*)-2-methylbutanoic acid.
[b] Retention time (min) for (*R*)-2-methylbutanoic acid.

1.07) peaks. The retention times for the sample and the references are summarised in Table 6. The isolated C-5 acid was thus identified as (*S*)-2-methylbutanoic acid.

Structures.—The saponin structures **S7**–**S12**, identified in the *Q. saponaria* Molina bark extract, have the commonly found quillaic acid as the aglycone and are acetylated at the fucose O-3, as shown in Fig. 1. The aglycone in the saponin **S13** (Fig. 2) is the triterpene phytolaccagenic acid (acetylated at O-23). This aglycone is substituted by a disaccharide at C-3 and a trisaccharide at C-28. The trisaccharide is further substituted by one acetyl and two (*S*)-2-methylbutanoyl groups.

3. Experimental

Materials.—The *Quillaja* saponin bark extract was obtained from Berghausen (Cincinnati, OH, USA). Commercial racemic 2-methylbutanoic acid (Riedel-de Haën AG, Seelze, Germany) and (*S*)-2-methylbutanoic acid (Sigma-Aldrich, Steinheim, Germany) were used as references.

Course separation of bark extract.—*Quillaja* saponin bark extract (200 mg portions) was dissolved in aq 10% v/v MeOH (1 mL) and the solution applied on an SPE column (C18, Isolute™, 10 g, International Sorbent Technology Ltd., UK). The column was eluated with a stepwise gradient of aq 10–90% MeOH, (10 mL portions, 2 mL/min). The eluate from 80 and 90% MeOH (20 mL) was collected and concd to dryness and this material was used for further separation.

HPLC separation.—For separation of mg 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 eluated 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 14–20 days whereupon the MeCN concentration was lowered by evaporation and the solutions were applied on an SPE column (C18, Isolute™, 10 g). The samples were desalted with aq 10% v/v MeOH (10 mL) and MeOH (20–30 mL) was used to elute the compounds from the SPE column whereupon the samples were evaporated to dryness.

A second separation step was carried out with a mixture of MeCN and aq 0.03 M phosphate buffer, pH 2.8 (40.5:59.5). The same instrument and settings as in the first separation step were used. One mg of the fractions from the first separation was injected repeatedly and fractions collected were treated as in the first separation step.

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 Bruker Reflex III spectrometer using a 337 nm nitrogen laser and 2,5-dihydroxybenzoic acid as matrix.

Monosaccharide analysis.—The isolated saponins (0.2 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. NH_4OH (1 M, 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 AcOH (conc) and formed boric acid removed by co-distillation first with 10% AcOH in MeOH (3 × 0.5 mL) and then with MeOH

(3×0.5 mL). The resulting alditols were acetylated with 1:1 Ac_2O–pyridine (0.3 mL) at 120 °C for 20 min and analysed by GC (flame ionisation detection) using authentic samples as standards. The column was an HP-5 (30 m × 0.32 mm, Hewlett–Packard, USA) and the carrier gas was He at 30 cm/s. The oven temperature was held at 140 °C for 1 min and then increased to 220 °C at 3 °C/min. Two alditol derivatives were formed from apiose, one with a tertiary alcohol 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. Compounds 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). Bruker standard pulse sequences were used. A mixing time of 80 ms was used for TOCSY experiments and a delay time of 65 ms for HMBC experiments.

Isolation of 2-methylbutanoic acid.—Compound **S13** (0.5 mg) was subjected to alkaline hydrolysis (0.5 mL 60 mM aq NaOH, 30 min, 20 °C). The acid was extracted with $CHCl_3$ (0.5 mL) after acidification to pH 2 with 1 M HCl. The $CHCl_3$ phase was transferred into a vial and evaporated to dryness by a gentle flush of N_2. The sample was then redissolved in 20 μl $CHCl_3$.

Stereochemistry of 2-methylbutanoic acid.—The sample containing 2-methylbutanoic acid was analysed by GC–MS without derivatisation (injection volume 5 μl). Racemic 2-methylbutanoic acid and (*S*)-2-methylbutanoic acid references were prepared as 1 mg/mL solutions in $CHCl_3$ and analysed on the GC–MS (injection volume 1 μl). The sample was analysed before the references to avoid cross-contamination in the injector. The GC column was a chiral fused-silica (30 m × 0.25 mm) β-Dex 120, coated with 0.25 μm 20% permethylated β-cyclodextrin in 80% poly-(35% diphenyl–65% dimethyl)-siloxane (Supelco, Bellefonte, PA, USA). The carrier gas was He at 35 cm/s and the column was run at 90 °C and the injector temperature was 240 °C. EIMS (70 eV) was recorded on a JEOL JMS-SX/SX-102A instrument with the GC–MS interface at 225 °C.

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] C.R. Kensil, *Crit. Rev. Ther. Drug Carr. Syst.*, 13 (1996) 1–55.
[2] I.G. Barr, A. Sjölander, J.C. Cox, *Adv. Drug Deliv. Rev.*, 32 (1998) 247–271.
[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, U. Patel, M. Lennick, D. Marciani, *J. Immunol.*, 146 (1991) 431–437.
[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] N.T. Nyberg, L. Kenne, B. Rönnberg, B.G. Sundquist, *Carbohydr. Res.*, 323 (2000) 87–97.
[10] S.J. Guo, E. Falk, L. Kenne, B. Rönnberg, B.G. Sundquist, *Phytochemistry*, 53 (2000) 861–868.
[11] S.J. Guo, L. Kenne, *Phytochemistry*, 54 (2000) 615–623.
[12] D.C. van Setten, G. van de Werken, G. Zomer, G.F.A. Kersten, *Rapid Commun. Mass Spectrom.*, 9 (1995) 660–666.
[13] 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.
[14] J.L. Cleland, C.R. Kensil, A. Lim, N.E. Jacobsen, L. Basa, M. Spellman, D.A. Wheeler, J.Y. Wu, M.F. Powell, *J. Pharm. Sci.*, 85 (1996) 22–28.
[15] X.M. Zhu, B. Yu, Y.Z. Hui, R. Higuchi, T. Kusano, T. Miyamoto, *Tetrahedron Lett.*, 41 (2000) 717–719.
[16] S.J. Guo, O. Kristiansson, L.I. Nord, L. Kenne, manuscript in preparation.
[17] D.C. van Setten, G. van de Werken, in G.R. Waller, K. Yamasaki (Eds.), Molecular structures of saponins from *Quillaja saponaria* Molina, in *Saponins used in traditional and modem medicine*, vol. 404, *Advances in Experimental Medicine and Biology*, Plenum, New York, 1996, pp. 185–193.
[18] P.-E. Jansson, L. Kenne, G. Widmalm, *Carbohydr. Res.*, 188 (1989) 169–191.
[19] P.K. Agrawal, *Phytochemistry*, 31 (1992) 3307–3330.
[20] S.S. Kang, W.S. Woo, *Planta Med.*, 53 (1987) 338–340.
[21] B.M.R. Bandara, U.L.B. Jayasinghe, V. Karunaratne, G.P. Wannigama, M. Bokel, W. Kraus, S. Sotheeswaran, *Planta Med.*, 56 (1990) 290–292.
[22] S.M. Spengel, *Phytochemistry*, 43 (1996) 179–182.
[23] S.J. Guo, L. Kenne, *Phytochemistry*, 55 (2000) 419–428.