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TRITERPENOID SAPONINS FROM *QUILLAJA SAPONARIA*

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Key Word Index—*Quillaja saponaria* Molina; Rosaceae; saponins; quillaic acid.

Abstract—Three new saponins were isolated from a commercial bark extract of *Quillaja saponaria* Molina. These compounds were also obtained as degradation products from larger saponins in this extract when treated with strong alkali. The compounds were characterized, using mainly NMR spectroscopy, mass spectrometry and chemical methods, as quillaic acid 3-*O*-{β-D-galactopyranosyl-(1 → 2)-β-D-glucopyranosiduronic acid}, 3-*O*-{α-L-rhamnopyranosyl-(1 → 3)-[β-D-galactopyranosyl-(1 → 2)]-β-D-glucopyranosiduronic acid} and 3-*O*-{β-D-xylopyranosyl-(1 → 3)-[β-D-galactopyranosyl-(1 → 2)]-β-D-glucopyranosiduronic acid}, respectively.

INTRODUCTION

The structures of several saponins from the bark of the tree *Quillaja saponaria* Molina have been reported [1–3] and fifty have been partially characterized [4]. These structures have in common the triterpene quillaic acid with a unique trisaccharide attached to C-3 and different oligosaccharides attached to C-28. Several of these saponins are known to have immunoenhancing activity and are, together with antigen, cholesterol and phospholipids, able to form immunostimulating complexes [5–7]. These complexes are energetically stable constructs which offer the advantages of reducing the required dose of both antigen and adjuvant, potent immunogenicity for both humoral and cell-mediated responses and minimal side-effects [6, 7]. A large-scale procedure has been developed to isolate two *Quillaja* saponin fractions, designated QH-A and QH-C [8], which in certain proportions are used for immunostimulating complex formation (ISCOPREP™703; Iscotec AB, Uppsala, Sweden).

In this study we present the structural elucidation of three quillaic acid saponins, isolated from QH-A, lacking the oligosaccharide group at C-28. These components were also identified as products formed during alkali treatment [1] of C-28-substituted saponins in QH-A and QH-C.

RESULTS AND DISCUSSION

The saponin fraction QH-A was analysed by MALDI-TOF mass spectrometry (Fig. 1) which showed several components of molecular masses corresponding to saponins of similar structures to those previously identified from *Q. saponaria* [1–3]. The spectrum also contained $[M+Na]^+$ ions of *m/z* 847, 979 and 993 indicating the occurrence of disaccharide- and trisaccharide-substituted quillaic acid, respectively. The material was fractionated by column chromatography on silica gel and the eluate was checked by TLC and MALDI-TOF mass spectrometry. A fast moving component on TLC (R_f 0.43) had the molecular mass 824, as indicated by the $[M+Na]^+$ ion *m/z* 847. Two other fast moving components (R_f 0.40) with molecular masses of 956 and 970 showed one spot on TLC and these were collected into one fraction. The components were further fractionated by reverse phase HPLC. By these procedures three different saponins, **1**, **2** and **3**, were isolated. These compounds had the elemental compositions $C_{42}H_{64}O_{16}$, $C_{48}H_{74}O_{20}$ and $C_{47}H_{72}O_{20}$, respectively, as indicated by FAB-MS, which gave the $[M-H]^-$ ions at *m/z* 823.42, 969.52 and 955.47. The masses correspond to a hexose and a hexuronic acid in addition to quillaic acid for compound **1** and an additional deoxyhexose or pentose for **2** and **3**, respectively.

Analyses of the neutral sugars released during acid hydrolysis of the saponins showed that compound **1** contained D-galactose, compound **2** contained L-rhamnose and D-galactose and compound **3** contained D-xylose and D-galactose in equimolar proportions. The sugars were analysed as their alditol acetates by GC-MS [9] and the absolute configurations determined by GC of the trimethylsilylated (+)-2-butyl glycosides [10]. The linkages, by which the sugars are connected, were determined by methylation analysis [11, 12], yielding 2,3,4,6-tetra-*O*-methyl-D-galactose

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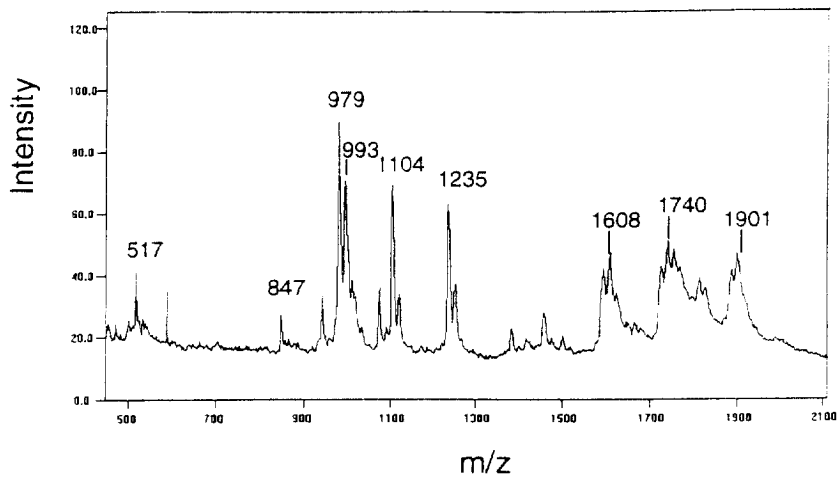
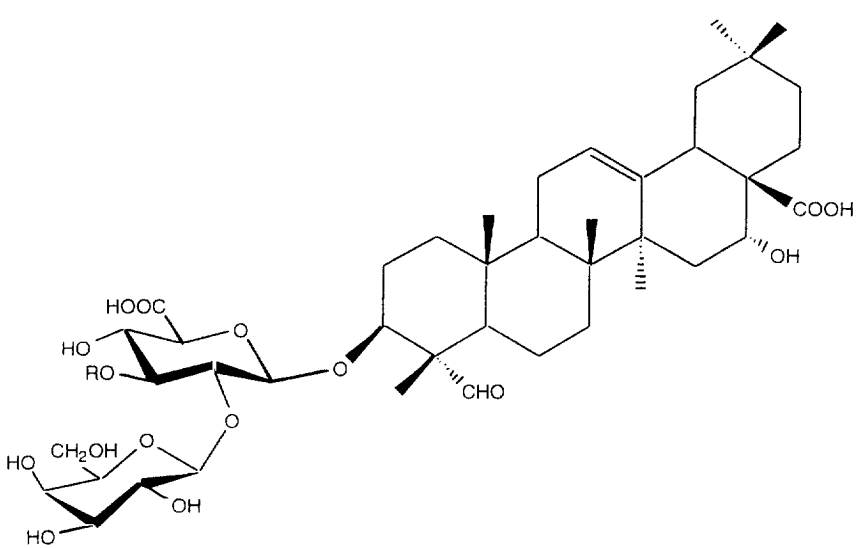


Fig. 1. MALDI-TOF mass spectrum of QH-A. The ions formed from each component are $[M+Na]^+$ and $[M+K]^+$ and their masses are indicated as nominal values.

from compound **1**, 2,3,4-tri-*O*-methyl-L-rhamnose and 2,3,4,6-tetra-*O*-methyl-D-galactose from compound **2** and 2,3,4-tri-*O*-methyl-D-xylose and 2,3,4,6-tetra-*O*-methyl-D-galactose from compound **3**, indicating that all these sugars occupy terminal positions. If the components were first methylated and subsequently reduced with "Superdeuteride" [13], a derivative of 3,4-di-*O*-methyl-D-glucose-6-d_2 was also present in the analysis of compound **1**, and a derivative of 4-*O*-methyl-D-glucose-6-d_2 in the analyses of compounds **2** and **3**. These results indicated that the β-D-galactopyranosyl group is linked to the 2-position of the β-D-glucuronic acid residue in **1** and the two terminal sugars in compound **2** and **3** are linked to the 2- and 3-positions of the β-D-glucuronic acid residue.

Further structural information was obtained by ^1H and ^{13}C NMR spectroscopy. Using different 1D and 2D experiments the ^1H and ^{13}C signals could be assigned (Table 1) and the $^3J_{H,H}$-values for the coupling between vicinal protons estimated. The anomeric configurations of the sugar residues were indicated by the chemical shifts, the $^3J_{H\text{-}1,H\text{-}2}$-values of the anomeric proton signals, and the $^1J_{C,H}$-values for the one-bond coupling between the anomeric atoms [14]. As the ^1H



Compound	R
1	H
2	α-L-Rha*p*
3	β-D-Xyl*p*

Table 1. Chemical shift assignments for compounds **1**, **2** and **3**

Atoms	1		2		3	
no	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
1	1.11, 1.71	39.3	1.11, 1.71	39.2	1.11, 1.71	39.2
2	1.99, 1.78	25.5	1.98, 1.78	25.6	1.97, 1.76	25.7
3	3.88	85.2	3.85	86.1	3.89	86.2
4	—	—[a]	—	56.2	—	56.2
5	1.34	49.2	1.33	49.1	1.33	49.0
6	0.92, 1.51	21.3	0.93, 1.50	21.3	0.90, 1.50	21.3
7	1.56, 1.24	33.6	1.53, 1.24	33.5	1.55, 1.23	33.5
8	—	—[a]	—	40.9	—	40.9
9	1.76	48.2	1.75	47.7	1.75	47.7
10	—	—[a]	—	37.1	—	37.1
11	1.93, 1.93	24.5	1.92, 1.92	24.4	1.92, 1.92	24.5
12	5.30	123.4	5.30	123.2	5.30	123.2
13	—	—[a]	—	145.1	—	145.2
14	—	—[a]	—	42.7	—	42.8
15	1.84, 1.33	36.3	1.83, 1.32	36.6	1.82, 1.32	36.0
16	4.45	75.3	4.45	75.0	4.45	75.3
17	—	—[a]	—	49.6	—	49.7
18	3.00	42.0	3.02	42.1	3.02	42.1
19	2.29, 1.02	47.9	2.26, 1.02	47.7	2.29, 1.03	47.3
20	—	—[a]	—	30.8	—	30.8
21	1.94, 1.15	36.7	1.92, 1.14	36.2	1.93, 1.15	36.2
22	1.91, 1.76	32.9	1.90, 1.73	32.8	1.90, 1.76	32.7
23	9.46	—[a]	9.44	210.8	9.44	210.7
24	1.14	10.7	1.15	10.8	1.15	10.8
25	1.00	16.2	1.00	16.2	1.00	16.3
26	0.79	17.7	0.79	17.7	0.79	17.8
27	1.40	27.3	1.39	27.2	1.39	27.3
28	—	—[a]	—	181.1	—	181.1
29	0.88	33.5	0.88	33.4	0.88	33.5
30	0.96	24.9	0.97	24.8	0.97	24.8
GlcA1	4.40	103.7	4.47	104.2	4.47	104.5
GlcA2	3.48	81.3	3.63	78.1	3.65	78.2
GlcA3	3.57	78.0	3.63	85.7	3.67	86.2
GlcA4	3.49	72.9	3.47	71.8	3.55	70.8
GlcA5	3.73	76.6	3.80	77.0	3.80	76.1
GlcA6	—	—[a]	—	—[a]	—	172.3
Gal1	4.49	105.1	4.45	104.3	4.80	103.8
Gal2	3.53	74.0	3.48	73.0	3.44	73.5
Gal3	3.46	75.0	3.48	75.2	3.44	75.3
Gal4	3.83	70.8	3.79	70.6	3.80	70.8
Gal5	3.52	77.1	3.48	77.5	3.49	76.8
Gal6	3.82, 3.74	62.5	3.79, 3.71	62.3	3.76, 3.69	62.3
Rha1			5.02	103.3		
Rha2			4.01	72.2		
Rha3			3.63	72.1		
Rha4			3.40	73.0		
Rha5			3.91	70.7		
Rha6			1.24	17.8		
Xyl1					4.58	105.0
Xyl2					3.23	74.4
Xyl3					3.31	78.2
Xyl4					3.52	70.5
Xyl5					3.24, 3.90	67.2

[a] Signal not detected; too weak or not observed in the HMQC spectrum.

NMR spectra were complex, the assignment of the signals was made by different COSY experiments. Using these techniques, the spin-systems starting with the anomeric proton signals and those belonging to the triterpene could be determined. Thereafter the ^{13}C signals were assigned by the C-H-connectivities

Table 2. The significant NOEs for the anomeric protons of the sugar residues of compound **2**, observed as cross-peaks in the NOESY spectrum

	Anomeric proton		Observed NOE	
Residue	(δ)	(δ)	Residue	Atom
α-L-Rha	5.02	4.01	α-L-Rha	H-2
		3.63	β-D-GlcA	H-3
β-D-GlcA	4.49	3.63	β-D-GlcA	H-3
		3.85	Quillaic acid	H-3
β-D-Gal	4.47	3.48	β-D-Gal	H-3 and 5
		3.63	β-D-GlcA	H-2

observed as cross-peaks in the HMQC spectra. The chemical shifts of the signals from each sugar residue (Table 1) were compared with those reported for the corresponding monosaccharides [15]. Comparison of the signals from the triterpene moiety in the ^{1}H NMR (Table 1) and ^{13}C NMR spectra (Table 1) with those from quillaic acid in the saponin QS-21 [3] showed that the aglycone in all three compounds was quillaic acid substituted in the C-3 position.

For compound **1** signals for two anomeric protons appeared at δ 4.49 ($J_{1,2}$ = 7.7 Hz) and δ 4.40 ($J_{1,2}$ = 7.5 Hz), indicating two β-pyranosides. The two spin-systems corresponded to a β-D-Gal*p*-(1 → and a → 2)-β-D-Glc*p*A-(1 → residue. The D-glucuronic acid was 2-substituted as indicated by the high chemical shift of the C-2 signal relative to that of the monosaccharide [15]. Compound **1** was thus composed of a disaccharide [β-D-Gal*p*-(1 → 2)-β-D-Glc*p*A-(1 →)] substituted to the 3-position of quillaic acid as evident from the molecular mass, the sugar and methylation analyses and the ^{1}H and ^{13}C NMR chemical shifts (Table 1).

For compound **2** the anomeric proton signals appeared at δ 5.02 ($J_{1,2}$ = 1.6 and $^1J_{C,H}$ = 173 Hz), δ 4.49 ($J_{1,2}$ = 7.1 and $^1J_{C,H}$ = 158 Hz) and δ 4.42 ($J_{1,2}$ = 7.2 and $^1J_{C,H}$ = 158 Hz), respectively, indicating one α- and two β-pyranosides. The results showed that the L-rhamnose had the α-configuration and the other sugars the β-configuration. The substitution positions of the D-glucuronic acid residue were indicated by the high chemical shift of the substituted carbon signals relative to those of the monosaccharide [15] and those of the (→ 2)-β-D-Glc*p*A-(1 →) residue in compound **1**. As evident from the molecular mass, the sugar and methylation analyses and the ^{1}H and ^{13}C NMR chemical shifts compound **2** was composed of a branched trisaccharide (α-L-Rha*p*-(1 → 3)-[β-D-Gal*p*-(1 → 2)]-β-D-Glc*p*A-(1 →) substituted to the 3-position of quillaic acid. The substitution positions of the α-L-rhamnopyranosyl and β-D-galactopyranosyl groups were determined by a NOESY experiment (Table 2) in which the inter-residue NOE connectivities involving the anomeric protons were observed as cross-peaks. An NOE between H-1 of the β-D-galactopyranosyl group and H-2 of the β-D-glucopyranosyluronic acid residue and an NOE between H-1 of the α-L-rhamnopyranosyl group and H-3 of the β-D-glucopyranosyluronic acid residue showed that these terminal sugars were linked to the 2- and 3-position of the β-D-glucopyranosyluronic acid residue, respectively.

The structure of compound **3** was evident from the molecular mass, the sugar and methylation analyses, and the similarities of the ^{1}H and ^{13}C NMR signals (Table 1) with those of corresponding components in the saponin QS-21 [3]. When the NMR spectra of **3** were obtained in the same solvent mixture (D_2O and CD_3CN) as that used for analysis of QS-21 almost identical chemical shifts were observed for all signals except for those of the atoms close to the C-28 substituent in QS-21. These results show that **3** is composed of a branched trisaccharide substituted to the 3-position of quillaic acid, identical to the trisaccharide-substituted prosapogenin previously obtained after alkaline hydrolysis of some *Quillaja* saponins [1].

Compounds **1**–**3** are minor components found in QH-A, which is a chromatographic fraction of a bark extract of *Q. saponaria* Molina. The three saponins were also components of the larger saponins in both QH-A and QH-C and could be isolated from these after alkaline cleavage of the ester-linked oligosaccharide at C-28. The proportions of the three oligosaccharide components in the saponin mixtures could be determined by HPLC. Compound **3** has previously been obtained after alkaline treatment of saponins from *Q. saponaria* Molina [1]. However, the present study shows that the oligosaccharide in the 3-position of quillaic acid consists of different structures and thus that both the C-3 and C-28 oligosaccharide substituents vary in structure. A previous mass spectral study [4] of fifty *Quillaja* saponins indicated that two different trisaccharides occupy the 3-position of the quillaic acid, although no complete structure was given.

Saponins containing the disaccharide of **1** could be a product from a selective acid hydrolysis of the pentose or 6-deoxyhexose of the two trisaccharides during the extraction procedure (treatment with hot water).

To test if this occurs a mixture of **2** and **3** in a water solution of pH 3.5 was heated at 95°C for 3 hours and the products were analysed by MALDI-TOF mass spectrometry and HPLC. Only a minor amount (≈3%) of compound **1** was detected by MALDI-TOF whereas no peak for **1** could be observed by HPLC. This amount is less than that found after alkaline hydrolysis of QH-A and QH-C indicating that the disaccharide is a minor component of the natural saponins. However, when the C-28 substituted saponins were treated using the same conditions several degradation products occurred, among these also compounds **1–3**. Thus different bark extracts were tested for the content of compounds **1–3** by MALDI-TOF mass spectrometry and minor amounts of these were observed by MALDI-TOF and HPLC after removal of the major components using silica gel chromatography. These results indicate that compounds **1–3** are new saponins found as minor components in the bark of *Quillaja saponaria* Molina.

EXPERIMENTAL

General. Solns were concd under red. pres. at temps. not exceeding 40°. A GC instrument fitted with an FID was used. Sepn of the alditol acetates and the partially methylated alditol acetates was performed on an HP-5 fused-silica capillary column, using a temp. program, from 140° (1 min) to 230° at 3° min^{-1}. Electron impact ionization (EI, 70 eV) GC-MS was performed using the column and conditions mentioned above.

FAB-MS spectra were recorded in both positive and negative mode on a JEOL JMS-SX/SX-102A tandem mass spectrometer by bombardment of the samples (dissolved in a glycerol matrix) with Xe atoms of average translational energy of 6 keV. For the determination of the molecular masses the mass spectrometer was operated at an accelerating voltage of 10 kV, PEG-1000 was mixed with the sample for reference peaks and a resolution of 3000 was used. MALDI-TOF mass spectra were recorded on a Linear LDI-1700XS spectrometer using a 339 nm nitrogen laser and 2,5-dihydroxybenzoic acid as matrix.

^1H (400 and 600 MHz) and ^{13}C (100 MHz) NMR spectra were obtained for CD_3OD solutions or a mixture of acidified D_2O (brought to pH 4 by addition of HOAc-d_4) and MeCN-d_3 (7:3) at 30°. Chemical shifts are reported in ppm, using TMS (δ_H 0.00) and Me_2CO (δ_C 31.00) as internal references. 2D (COSY, relay COSY, NOESY and HMQC) experiments were performed according to standard pulse sequences. A 90° pulse was used in the correlation experiments, and in the NOESY experiment mixing times of 0.2 and 0.5 sec.

TLC was run on silica gel (Merck) using $CHCl_3$-MeOH-H_2O-HOAc (240:175:30:1) as solvent and HPLC on a NOVA Pak C-18 RAD PAK cartridge (8 × 100 mm), using a UV detector at 205 nm. For analysis of the components 31% MeCN in aq. 0.01 M phosphate buffer, pH 2.8, was used at the flow rate 1.0 ml · min^{-1}. For the preparative experiments each injection was 15 μl containing 0.5–0.6 mg saponin material using a flow rate of 1.0 ml · min^{-1}.

Materials. Quillaja saponin fractions QH-A and QH-C were obtained from Iscotec AB (Uppsala, Sweden).

Isolation of compounds **1–3**. Dried QH-A (1.7 g) was fractionated on a column (5.0 × 45 cm) of silica gel 60 (Merck) using $CHCl_3$-MeOH-H_2O-HOAc (240:175:30:1) as solvent. TLC, HPLC and MALDI-TOF mass spectrometry were used to monitor the eluate. Fractions containing a faster moving component (**1**) were pooled, yielding 27 mg, and fractions containing compounds **2** and **3** were pooled yielding 100 mg. Compound **1** was further purified by reverse phase HPLC using MeCN and aq. 0.01 M phosphate buffer, pH 2.8 (2:3) as eluent and pure **1** (5 mg) was obtained. The component was analysed by MALDI-TOF mass spectrometry and ^1H NMR spectroscopy. The fractions containing compounds **2** and **3** were further separated by HPLC using MeCN and aq. 0.01 M phosphate buffer, pH 2.8 (31:69) as eluent. Compound **2** (12 mg) was eluted between 16.6 and 18 min and compound **3** (38 mg) between 19 and 20.5 min.

Alkaline hydrolysis of QH-A and QH-C. QH fractions (40 mg) were treated with aq. 1 M NaOH (8 ml) for 2 hr at 95° whereafter the reactions were stopped by the addition of HOAc. The resulting saponins, devoid of the oligosaccharides linked to C-28, were first isolated as a mixture by reverse phase chromatography on a Sep-Pak C_{18} cartridge and then separated on HPLC using a gradient of MeCN and aq. 0.01 M phosphate buffer, pH 2.8 (33% MeCN in 11 min, decreased to 27% and then from 27 to 30% MeCN in 8 min). Fractions containing pure compound **2**, **1** and **3**, respectively, were obtained.

Sugar analysis. Samples (1 mg) were hydrolysed with 2 M TFA (0.2 ml) at 120° for 1 hr whereafter the solvent was evapd with a stream of N_2. The product was reduced with $NaBD_4$ (2 mg) in 0.5 M NH_4OH (0.4 ml) for 40 min at room temp. Excess $NaBD_4$ was quenched with a few drops of HOAc and formed boric acid removed by co-distillation with 10% HOAc in MeOH (3 × 0.5 ml) and MeOH (3 × 0.5 ml). The resulting alditols were acetylated with Ac_2O-pyridine (1:1, 0.15 ml) at 120° for 30 min and analysed by GC-MS [9] using authentic samples as standards.

Methylation analysis. Dry samples (1 mg) in sealed vials were dissolved in dry DMSO (0.25 ml), Na methylsulfinyl methanide in DMSO (2 M, 0.25 ml) was added and the soln stirred for 5 hr at room temp. It was then frozen and MeI (0.35 ml) was added followed by stirring for 1 hr. The excess MeI was removed by flushing with N_2 for 1 hr and the soln was then diluted with H_2O. The methylated products were isolated using a Sep-Pak C_{18} cartridge [16] and treated with "Superdeuteride" ($LiB(C_2H_5)_3D$) according to Bhat *et al.* [13]. The products were hydrolysed with 2 M aq. TFA at 120° for 2 hr. The partially methylated

sugars in the hydrolysate were then reduced, acetylated as described for the sugar analysis above and analysed as described [12].

Analysis of bark extracts. Bark extract (1 g) was dissolved in $CHCl_3$-MeOH-H_2O-HOAc (240:175:30:1). Some insoluble material was removed by centrifugation and the soln applied to a silica gel column. The fractions containing components with the same mobility as compounds **1**–**3** were concd and the material analysed by MALDI-TOF mass spectrometry and NMR spectroscopy.

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REFERENCES

1. Higuchi, R., Tokimitsu, Y., Fujioka, T., Komori, T., Kawasaki, T. and Oakenful, D. G., *Phytochemistry*, 1987, **26**, 229.
2. Higuchi, R., Tokimitsu, Y. and Komori, T., *Phytochemistry*, 1988, **27**, 1165.
3. Jacobsen, N. E., Fairbrother, W. J., Kensil, C. R., Lim, A., Wheeler, D. A. and Powell, M. F., *Carbohydr. Res.*, 1996, **280**, 1.
4. van Setten, D. C., van de Werken, G., Zomer, G. and Kersten, G. F. A., *Rapid Commun. Mass Spectrom.*, 1995, **9**, 660.
5. Morein, B., Sundquist, B., Höglund, S., Dalsgaard, K. and Osterhaus, A., *Nature*, 1984, **308**, 457.
6. Morein, B., Villacres-Eriksson, M., Åkerblom, L., Rönnberg, B., Lövgren, K. and Sjölander, A., *Aids Res. Human Retroviruses*, 1994, **10**, S109.
7. Morein, B., Lövgren, K., Rönnberg, B., Sjölander, A. and Villacres-Eriksson, M., *Clin. Immunother.*, 1995, **3**, 461.
8. Rönnberg, B., Fekadu, M. and Morein, B., *Vaccine*, 1995, **13**, 1375.
9. Sawardeker, J. S., Sloneker, J. H. and Jeanes, A. R., *Anal. Chem.*, 1965, **37**, 1602.
10. Gerwig, G. J., Kamerling, J. P. and Vliegenthart, J. F. G., *Carbohydr. Res.*, 1978, **62**, 349.
11. Hakomori, S., *J. Biochem. (Tokyo)*, 1964, **55**, 205.
12. Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B. and Lönngren, J., *Chem. Commun. (Stockholm Univ.)*, 1976, **8**, 1.
13. Bhat, U. R., Krishnaiah, B. S. and Carlsson, R. W., *Carbohydr. Res.*, 1991, **220**, 219.
14. Bock, K. and Pedersen, C., *J. Chem. Soc. Perkin Trans.* 2, 1974, 283.
15. Jansson, P. E., Kenne, L. and Widmalm, G., *Carbohydr. Res.*, 1989, **188**, 169.
16. Waeghe, T. J., Darvill, A. G., McNeil, M. and Albersheim, P., *Carbohydr. Res.*, 1983, **123**, 281.