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Steroidal saponins from the fruits of *Asparagus racemosus*

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Abstract

Three steroidal saponins, racemosides A (**1**), B (**2**) and C (**3**), were isolated from the methanolic extract of the fruits of *Asparagus racemosus*, and characterized as (25*S*)-5β-spirostan-3β-ol-3-*O*-{β-D- glucopyranosyl (1 → 6)-[α-L-rhamnopyranosyl (1 → 6)-β-D-glucopyranosyl (1 → 4)]-β-D-glucopyranoside}, (25*S*)-5β-spirostan-3β-ol-3-*O*-α-L-rhamnopyranosyl (1 → 6)-β-D-glucopyranosyl (1 → 6)-β-D-glucopyranoside and (25*S*)-5β-spirostan-3β-ol-3-*O*-{α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl (1 → 4)]-β-D-glucopyranoside}, respectively, by spectrometric analysis and some chemical strategies.

Keywords: *Asparagus racemosus*; Liliaceae; Steroidal saponins; Sarsasapogenin; Racemosides

1. Introduction

Asparagus racemosus Willd. (Liliaceae), commonly known as "Shatavari", is a much-branched, spinous under shrub found growing wild in tropical and sub-tropical parts of India. The plant enjoys considerable reputation in Indian medicine as an antispasmodic, aphrodisiac, demulcent, diuretic, galactogogue and refrigerant. It is also used in the treatment of diarrhoea, rheumatism, diabetes and brain complaints (Chadha, 2003). Earlier phytochemical investigators reported the isolation of isoflavones (Saxena and Chourasia, 2001), steroidal glycosides (Joshi and Dev, 1988; Handa et al., 2003), polycyclic alkaloids and a dihydrophenanthrene derivative (Sekine et al., 1995, 1997) from the roots of the plant but there has been no report on the chemistry of the constituents of its fruits. In continuation of our work on chemical studies on naturally occurring bioactive glycosides (Mandal et al., 2006), we become interested in the saponin content of the fruits of the plant. The present paper reports the isolation and structure elucidation of the compounds isolated from the methanol extract of the fruits.

2. Results and discussion

The *n*-BuOH soluble fraction of the methanol extract of the defatted fruits of *A. racemosus* on repeated chromatographic purification over Diaion HP-20, silica gel column and preparative thin layer chromatography (TLC) furnished three major compounds designated as racemosides A, B and C according to decreasing order of polarity. All the three compounds gave positive Liebermann–Burchard test for steroids and Molisch test for sugar. Structure determinations of the compounds were mainly accomplished by extensive analysis of 1D and 2D NMR results.

Racemoside A (**1**), colourless crystalline needles, displayed a quasimolecular ion peak at *m*/*z* 1071 attributed to $[M+Na]^+$ in the ESI-TOF mass spectrum indicating the molecular weight to be 1048. This information coupled with the data from elemental analysis and ^{13}C NMR DEPT

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spectrum suggested the molecular formula to be $C_{51}H_{84}O_{22}$. Anomeric $^{1}H/^{13}C$ signals of four sugar units were observed in the NMR spectra of **1** [δ_H 5.59 (*brs*), 5.47 (*d*, $J = 7.8$ Hz), 4.82 (*d*, $J = 7.2$ Hz), 4.78 (*d*, $J = 7.8$ Hz) and δ_C 102.5 (*d*), 106.0 (*d*), 102.2 (*d*), 106.0 (*d*)] (Tables 1 and 2). The aglycone of **1** was determined to be a spirostanol but not a furostanol by comparison of its NMR data (Table 1) with those of known spirostane and furostane type steroidal glycosides (Li et al., 2005; Zhang et al., 2004). Hydrolysis of the saponin yielded a sapogenin whose NMR data was identical to those reported in the literature for sarsasapogenin (Agrawal et al., 1997). The monosaccharide constituents were identified as glucose and rhamnose by PC analysis of the acid hydrolysate and its GLC analysis after derivatization. The chemical shifts of all the individual protons of the monosaccharides were ascertained from a combination of TOCSY, DQF-COSY, HMQC and HMBC spectral analysis. Due to the highly overlapping nature of sugar proton signals within a 2-ppm region in the ^{1}H NMR spectrum, the assignment of all the sugar protons for a molecule like **1** was not an easy task. Information from COSY and 2D-TOCSY furnished most of the assignments. On the basis of assigned protons, the ^{13}C chemical shifts (Table 1) of each sugar unit were assigned by HSQC and further confirmed by HMBC experiment. An unambiguous determination of the sequence and sites of linkage was made from the HMBC and NOESY correlations. Thus the HMBC spectrum displayed cross peaks between the ^{1}H NMR signal at δ 4.82 (*d*, $J = 7.2$ Hz, Glc H-1′) and the ^{13}C NMR signal at δ 76.2 (Agly C-3) while the NOESY spectrum showed correlation between δ 4.82 (Glc H-1′) and δ 4.28 (*m*, Agly H-3) signals proving that a glucose unit was linked at C-3 of the aglycone. In addition, ^{13}C NMR spectral comparison of **1** with its aglycone revealed expected glycosylation shifts in resonance positions of C-2 (−1.9 ppm), C-3 (+10.1 ppm) and C-4 (−3.4 ppm) of the aglycone moiety (Tori et al., 1978). The linkages of other sugar moieties were deduced from the following HMBC correlations: anomeric proton signal (δ 4.78) of glucose II with C-6 chemical shift (δ 68.1) of glucose I; anomeric proton signal (δ 5.47) of glucose III with C-4 signal (δ 81.7) of glucose I; and anomeric proton signal (δ 5.59) of rhamnose with C-6 resonance position (δ 67.1) of glucose III. The ^{13}C glycosylation shifts of these carbons also indicated that these were the linkage sites. The same conclusion was also drawn from the NOESY experiment.

All the monosaccharides were in the pyranose forms as determined from their ^{13}C NMR data. The β anomeric configurations ($^{4}C_1$ conformation) for the glucose units were based on their large $^{3}J_{H1,H2}$ coupling constants (7–8 Hz) and small $J_{C1,H1}$ (159–160 Hz) values. The non-splitting pattern of the anomeric proton signal, the ^{13}C chemical shift of the anomeric carbon, and the large

Table 1
^{13}C NMR spectral data (δ) of racemosides **1**–**3** (150 MHz) in pyridine-d_5[a]

Carbon no.	1	2	3	4		1	2	3
1	31.3	31.2	31.2	30.6	Sugar moiety	glc I	glc I	glc
2	26.7	26.7	26.8	28.6	C-1	102.2	103.5	103.6
3	76.2	75.6	75.4	66.1	C-2	76.4	75.0	75.8
4	31.0	32.5	30.4	34.4	C-3	80.5	76.9	77.1
5	37.1	37.4	37.4	37.0	C-4	81.7	74.5	79.8
6	27.3	27.3	27.3	27.1	C-5	75.2	75.5	75.9
7	27.1	27.1	27.1	26.9	C-6	68.1	68.2	67.5
8	35.6	35.6	35.9	35.6		glc II	glc II	rha I
9	40.6	40.6	40.7	40.4	C-1	106.0	106.2	102.4
10	35.9	35.9	35.6	35.6	C-2	72.6	72.6	72.7
11	21.5	21.5	21.5	21.2	C-3	74.9	75.2	73.1
12	40.6	40.7	40.6	40.9	C-4	69.9	69.9	74.4
13	41.2	41.2	41.2	40.1	C-5	81.7	81.2	70.2
14	56.8	56.8	56.8	56.6	C-6	63.3	67.6	19.1
15	32.5	32.5	32.5	32.1		glc III	rha	rha II
16	81.9	81.7	81.7	81.3	C-1	106.0	102.7	103.3
17	63.3	63.3	63.3	63.0	C-2	77.4	72.8	73.0
18	16.9	16.9	17.0	16.6	C-3	78.3	73.2	73.1
19	24.3	24.2	24.2	24.2	C-4	72.1	75.5	74.2
20	42.8	42.8	42.8	42.5	C-5	79.2	70.2	70.9
21	16.6	16.6	16.7	16.3	C-6	67.1	19.1	18.9
22	110.0	110.1	110.1	109.7		rha		
23	27.0	27.1	27.2	26.2	C-1	102.5		
24	26.5	26.5	26.6	26.4	C-2	72.8		
25	27.9	27.9	27.9	27.5	C-3	73.1		
26	65.4	65.4	65.4	65.1	C-4	74.4		
27	15.3	15.2	15.3	14.9	C-5	70.2		
					C-6	19.1		

[a] Assignments based on ^{13}C, DEPT, HMQC, HMBC experiments; glc, glucose; rha, rhamnose.

Table 2
^{1}H NMR data of **1**–**3** (600 MHz, pyridine-d_5, J in Hz)

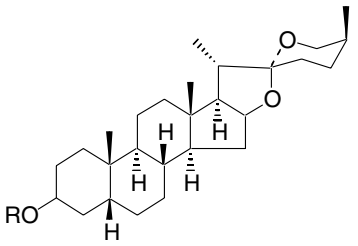
Aglycone of **1**		3-*O*-sugar	**1**	**2**	**3**
H-1a	1.78 *dd* (3.6, 11.4)	Glc-I-1	4.82 *d* (7.2)	4.85 *d* (7.8)	4.82 (7.8)
H-1b	1.46[b]	2	4.26[a]	4.07 *t* (9)	4.0[b]
H-2a	1.92[b]	3	4.08 *t* (9)	4.23[b]	4.20[b]
H-2b	1.46[b]	4	4.35[b]	4.25[b]	4.15[b]
H-3	4.28[a]	5	3.85 *dd* (5.4, 8.4)	3.99 *dd* (4.8,8.4)	3.87 *dd* (3.6, 7.2)
H-4a	1.75[b]	6	4.23 *dd* (1.8, 12)	4.27[b]	4.36[b]
H-4b	1.85[b]		3.64 *d* (11.4)	3.66 *d* (10.8)	3.98 *dd* (4.8, 11.4)
H-5	2.15[b]				
		Glc-II-1	4.78 *d* (7.8)	4.84 *d* (7.8)	
H-6a,	1.77[b]	2	4.43 *t* (8.4)	4.49[b]	
H-6b	1.14[b]	3	4.03[b]	4.07 *t* (9)	
H-7a	1.19[b]	4	4.18[b]	4.18 *t* (8.4)	
H-7b	0.91 *brd* (10.8)	5	4.59[b]	4.13[b]	
H-8	1.46[b]	6	4.62 *dd* (2.4,11.4)	4.75 *d* (11.4)	
H-9	1.24[b]		4.54[b]	4.35 *dd* (4.2, 11.4)	
H-11a	1.31[b]	Glc-III-1	5.47 *d* (7.8)		
H-11b	1.14[b]	2	4.11[b]		
H-12a	1.66[b]	3	4.33[b]		
H-12b	1.04[b]	4	4.36[b]		
H-14	1.04[b]	5	4.05[b]		
H-15a	2.01 *ddd* (4.8, 7.2, 11.4)	6	4.33[b]		
H-15b	1.46[b]		4.65 *dd* (2.4,11.4)		
H-16	4.35[b]	Rha-I	5.59 *brs*	5.63 *brs*	5.40 *brs*
H-17	1.80 *dd* (6.6, 8.4)	2	4.67 *brs*	4.69 *brs*	4.64 *d* (3)
H-18	0.82 *s*	3	4.56[b]	4.57 *t* (8.4)	4.52 *dd* (3, 9)
H-19	0.96 *s*	4	4.30 *t* (10.2)	4.29 *t* (9)	4.26[b]
H-20	1.83[b]	5	4.41 *t* (3.6)	4.41[a]	4.36[b]
H-21	1.17 *d* (6.6)	6	1.68 *d* (6.6)	1.69[a]	1.68 *d* (6. 4)
H-23a	1.89 *brd* (9.6)	Rha-II-1			5.67 *brs*
H-23b	1.24[b]	2			4.68 *d* (2.4)
H-24a	2.15[b]	3			4.57 *dd* (3, 9)
H-24b	1.37[a]	4			4.36[b]
H-25	1.66[b]	5			4.95 *dq* (6.0, 8.4)
H-26a	4.10[b]	6			1.72 *d* (6.6)
H-26b	3.38 *d* (10.8)				
H-27	1.09 *d* (6.6)				

[a] Unresolved.
[b] Overlapped with other signals.

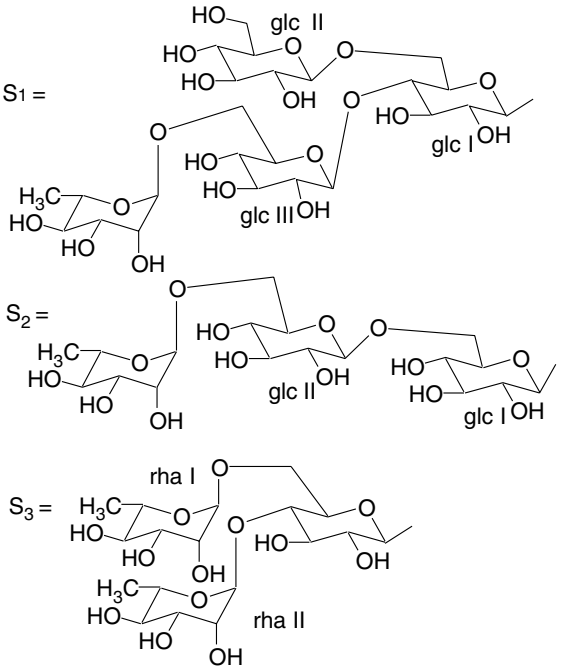
$J_{C1,H1}$ coupling constant (170 Hz) of the rhamnose indicated α-configuration in $^1C_4$ conformation (Sahu et al., 1995). The absolute configurations of these monosaccharides were chosen in keeping with those mostly encountered among plant glycosides. From the foregoing evidences, the structure of racemoside A (**1**) was elucidated to be (25*S*)-5β-spirostan-3β-ol-3-*O*-{β-D-glucopyranosyl (1 → 6)-[α-L-rhamnopyranosyl (1 → 6)-β-D-glucopyranosyl (1 → 4)]-β-D-glucopyranoside}.

Racemoside B (**2**) crystallized from methanol and showed a sodiated molecular ion at *m*/*z* 909 in its ESI-TOF mass spectrum suggesting a molecular ion of 886, 162 mass units less than that of **1**. Combined with the results of an elemental analysis, ^{13}C NMR and DEPT spectra, the molecular formula of **2** was deduced to be $C_{45}H_{74}O_{17}$. Its ^{1}H and ^{13}C NMR spectra indicated that **2** had the same aglycone as that of **1** but differed in the oligosaccharide chain (Table 1). On acid hydrolysis, **2** afforded the same aglycone, sarsasapogenin (**4**), and the monosaccharides were identified to be glucose and rhamnose in the ratio 2:1 from GLC analysis. Moreover, the difference of 162 mass units compared to **1** suggested that **2** has one glucose unit less than that of **1**. The overall structure assignment was accomplished using the same protocol as with **1**. The presence of three monosaccharides was indicated by the three anomeric proton signals at δ 4.85 (*d*, $J = 7.2$ Hz), 4.84 (*d*, $J = 7.2$ Hz) and 5.63 (*brs*), and carbon signals at δ 106.5, 103.5 and 102.7. The exact linkage position of the trisaccharide unit was established using the following HMBC correlations: H-1 (δ 5.63) of the rhamnose with the C-6 (δ 67.6) of glucose II and H-I (δ 4.84) of glucose II with C-6 (68.2) of glucose I. The attachment of the trisaccharide chain to C-3 of the aglycone was based on HMBC correlation between H-1 (δ 4.85) of glucose I and the C-3 (δ 75.6) of the aglycone. The same conclusion has been also achieved from the NOESY experiment. Thus

the structure of **2** was elucidated as (25*S*)-5β-spirostan-3β-ol-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside.



1. Racemoside A: R = S1
2. Racemoside B: R = S2
3. Racemoside C: R = S3
4. Sarsasapogenin: R = H



Racemoside C (**3**) was obtained as white crystalline needles. The molecular formula $C_{45}H_{74}O_{16}$ was deduced from the ^{13}C NMR data, sodiated molecular ion location in the ESI-TOF mass spectrum (positive) at *m*/*z* 893.14 $[M+Na]^+$ and elemental analysis. Comparison of the ^{13}C NMR spectrum of **3** with those of **1** and **2** indicated that it had the same aglycone. Acid hydrolysis of **3** furnished sarsasapogenin as the aglycone together with glucose and rhamnose as sugar components in the ratio 1:2, respectively, by GLC analysis. Three anomeric carbon signals of **3** were observed at δ 103.6 (C-1 of glucose), 102.4 (C-1′ of rhamnose I) and 103.3 (C-1″ of rhamnose II), which corresponded to three anomeric proton signals at δ 4.82 (*d*, *J* = 7.8 Hz, H-1 of glucose), 5.41 (*brs*, H-1′ of rhamnose I) and 5.67 (*brs*, H-1″ of rhamnose II). In the HMBC spectrum, the correlations between H-1′ (δ 5.41) of rhamnose I/C-6 (δ 67.5) and H″ (δ 5.67) of rhamnose II/C-4 (δ 79.8) indicated that rhamnoses were linked at C-6 and C-4 of glucose. The sugar-aglycone linkage was deduced from the correlation between H-1 (δ 4.82) of glucose and C-3 (δ 75.44) of the aglycone. Thus **3** was characterized as (25*S*)-5β-spirostan-3β-ol-3-*O*-{α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside}.

It may be mentioned that the methonolic extract of the defatted fruits of *A. racemosus* was subjected to TLC examination; the spots of the compounds (**1**–**3**) were clearly visible, and were negative to Ehrlich's reagent (Farid et al., 2002), but positive to Liebermann–Burchard reagent, indicating that the compounds were spirostanol glycosides but not furostanol glycoside.

3. Experimental

3.1. General procedures

All melting points were measured on a Yanagimoto micromelting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively, using a Bruker AVANCE 600 spectrometer in C_5D_5N with TMS as internal standard. ESI-TOF mass was performed on a Q-TOF-Micromass spectrometer. IR spectra were recorded as KBr pellets using a JASCO 7300FTIR spectrometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. Diaion HP-20 (Mitsubishi Chemicals) and silica gel (silica gel 60, Merck) were used for column chromatography. TLC was carried out on silica gel 60 F_{254}, and spots were visualized by spraying with 10% H_2SO_4 solution followed by heating. Preparative TLC was carried out on precoated silica gel 60 plates, (thickness: 0.5 mm, E. Merck, Germany). Paper chromatography was performed on Whatman paper No. 1 (46 × 57 cm) with solvent system *n*-BuOH-C_5H_5N–H_2O (6:4:3); a saturated solution of aniline oxalate in water was used as staining agent, and the spots were visualized after heating at 100 °C. GLC was performed on a Hewlett-Packard model 5730 A instrument using 2% SE-30 on Chromosorb W (60–80 mesh), 3 mm i.d., 1.5 m, 150 °C column temperature, nitrogen as carrier gas with a flow rate of 15 ml/min.

3.2. Plant material

The plant material was collected from the suburbs of Kolkata, India and identified at Indian Botanic Garden, Botanical Survey of India, Howrah, West Bengal, India. A voucher specimen (No. 345) was deposited at the Steroids and Terpenpoids Chemistry Department, Indian Institute of Chemical Biology, Kolkata.

3.3. Extraction and isolation

The air-dried powdered fruits of *A. racemosus* (2 kg) was first defatted at room temperature with petroleum

ether (60–80 °C) and then successively extracted with MeOH (3 × 4 l) at ambient temperature. The combined MeOH extract was concentrated under reduced pressure. The concentrated extract was partitioned between *n*-BuOH and H_2O. The organic layer was further washed with water for complete removal of inorganic impurities and free sugars and the solvent removed under reduced pressure to yield a dark-brown residue (42 g). The residue was applied to a column of Diaion HP-20 (500 g) and, washed with water followed by 30%, 50%, 80% and 100% of MeOH. Fraction eluted with 50% MeOH (4.5 g) was chromatographed over silica gel (100 g). Graded elution was carried out with chloroform followed by various mixtures of $CHCl_3$–MeOH (19:1, 9:1, 4:1 and 3:1). A total of 35 fractions (each 75 ml) were collected and those giving similar spots on TLC were combined. The more polar fractions eluted with $CHCl_3$–MeOH (4:1) were combined and rechromatographed over silica gel. Earlier fractions eluted with $CHCl_3$–MeOH (17:3) was found to be a mixture of racemosides B and C (120 mg), while later fractions furnished racemoside A (75 mg). Racemosides B and C were successfully separated by preparative TLC (mobile phase: $CHCl_3$:MeOH:H_2O; 60:23:3) to give racemoside B (39 mg) and racemoside C (28 mg).

3.4. Racemoside A (*1*)

Colourless needles from MeOH, m.p. 244–246 °C; $[\alpha]_D^{26}$ − 34.9° (*c* 0.90, H_2O); IR (KBr): ν_{max} cm^{-1} ; 3415, 1435, 1367, 1150, 1060, 986, 918, 900 and 851 (absorption 918 > 900); ESI-TOFMS (positive): *m*/*z* 1071.55 $[M+Na]^+$; ^1H NMR and ^{13}C NMR (Tables 1 and 2); (Found: C, 58.31; H, 8.12; $C_{51}H_{84}O_{22}$ requires: C, 58.38; H, 8.07%).

3.5. Acid hydrolysis of *1*

Racemoside A (**1**) (15 mg) was heated with 1 mol/L HCl in aqueous dioxane (1:1, v/v, 10 ml) at 80 °C for 3 h, cooled, and 5 ml of water was added. Dioxane was removed under reduced pressure, and the solution was extracted with EtOAc (5 ml × 3). The organic layer was washed with water until free from acid and dried to give a white powder. Purification of the product over a silica gel column and subsequent crystallization from chloroform–methanol mixture afforded the aglycone in fine needles, identified as sarsasapogenin by TLC comparison with an authentic sample and ^{13}C NMR chemical shifts (Agrawal et al., 1997). The aqueous part of the acid hydrolysate was neutralized with silver carbonate and filtered. The filtrate containing sugar mixture was evaporated to dryness in vacuo. This was then dissolved in water (10 ml) and divided into two parts. The first part was examined for sugars by paper chromatography, leading to the identification of glucose and rhamnose using authentic specimens. The second part was reduced with $NaBH_4$ (200 mg) for 2 h. The reduced product was treated with Dowex 50 (H^+) and the solution concentrated. Boric acid was removed by co-distillation with MeOH and the product acetylated with acetic anhydride–pyridine (1:1) at 100 °C for 1 h, diluted with water and extracted with $CHCl_3$. The alditol acetates of the monosaccharides obtained after removal of the solvent were identified to be glucose and rhamnose in the ratio 3:1 from GLC analysis.

3.6. Racemoside B (*2*)

Colourless crystals from MeOH, m.p. 240–242 °C, $[\alpha]_D^{26}$ − 41.1° (*c* 0.81, MeOH); IR (KBr): ν_{max} cm^{-1}; 3410, 1440, 1360, 1150, 1050, 978, 920 and 901 (absorption 920 > 901); ESI-TOFMS (positive): *m*/*z* 909.34 $[M+Na]^+$; ^1H NMR (C_5D_5N): 0.81 (s, H_3-18), 0.85 (s, H_3-19), 1.17 (*d*, *J* = 6.6 Hz, H_3-21), 1.08 (*d*, *J* = 6.6 Hz, H_3-27), 3.38 (*d*, *J* = 11.4 Hz, H-26), 4.07 (unresolved, H-26), 4.31 (unresolved, H-3), 4.57 (unresolved, H-16) and sugar moiety (Table 2); ^{13}C NMR (Table 1); (Found: C, 60.87; H, 8.46; $C_{45}H_{74}O_{17}$ requires: C, 60.93; H, 8.41%).

3.7. Racemoside C (*3*)

Colourless needles from MeOH, m.p. 236–238 °C, $[\alpha]_D^{26}$ − 55.4° (*c* 0.56, MeOH); IR (KBr): ν_{max} cm^{-1}; 3430, 1445, 1365, 1155, 1050, 980, 920 and 900 (absorption 920 > 900); ESI-TOFMS (positive): *m*/*z* 893.14 $[M+Na]^+$; ^1H NMR (C_5D_5N): 0.82 (s, H_3-19), 0.83 (s, H_3-18), 1.17 (*d*, *J* = 7.2 Hz, H_3-21), 1.09 (*d*, *J* = 7.2 Hz, H_3-27), 3.40 (*d*, *J* = 11.4 Hz, H-26), 4.09 (*d*, *J* = 9.0 Hz, H-26), 4.32 (H-3), 4.61 (*t*-like, *J* = 7.2 Hz H-16) and sugar moiety (Table 2); ^{13}C NMR (Table 1); (Found: C, 61.97; H, 8.41; $C_{45}H_{74}O_{16}$ requires C, 62.05; H, 8.56%).

3.8. Acid hydrolysis of *2* and *3*

A solution of **2** and **3** (8 mg each) was subjected to acid hydrolysis by the same procedure as described for **1**. Usual work up followed by chromatographic purification on a silica gel column furnished sarsasapogenin as aglycone for both **2** and **3**. The monosaccharides were identified as glucose and rhamnose in the ratios 2:1 and 1:2 for **2** and **3**, respectively, by GLC analysis on comparison with authentic samples.

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