Steroidal Saponins from *Asparagus racemosus*[1)]

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Two new steroidal saponins, shatavaroside A (1) and shatavaroside B (2) together with a known saponin, filiasparoside C, were isolated from the roots of *Asparagus racemosus*. Filiasparoside C was first time isolated from this plant. Their structures were elucidated by 1D and 2D NMR experiments including correlation spectroscopy, distortionless enhancement by polarization transfer, heteronuclear multiple quantum correlation and heteronuclear multiple bond coherence spectroscopy as well as electrospray ionization-QTOF-MS/MS analysis.

Key words *Asparagus racemosus*; steroidal saponin; shatavaroside A; shatavaroside B

The genus *Asparagus* has more than 300 species growing widely in eastern Asia including India, China, Korea and Japan.[2)] *Asparagus racemosus* Wild. (Asparagaceae), commonly known as "Shatavari" is mainly distributed wild in tropical and sub-tropical parts of India. It was believed to possess therapeutic properties and mentioned as "Rasayana" in Ayurveda, an Indian traditional system of medicine.[3,4)] The plant enjoys considerable reputation in Indian system of medicine as an antispasmodic, aphrodisiac, diuretic, immunoadjuvant and refrigerant.[5)] It is recommended for threatened abortion, lactation in women (galactogogue) and to normalize the uterus and hormone changes that occur during pregnancy period.[6)] The root of this herb is reported to have number of biological activities like antitussive, adaptogenic, immunomodulatory, anti-inflammatory, anti-bronchitis and anti dyspepsia.[7—9)]

Earlier investigations on this plant have revealed the presence of both furostanol and spirostanol steroidal saponins such as shatavarins I—IV[10,11)] and shatavarin V[12)] from roots and three racemoside (A—C) from fruits[13)] of the plant. Shatavarins I and IV were reported to be the major steroidal saponins in the roots of *A. racemosus* in which 20—500 μg/ml of shatavarin I was found to produce a specific and competitive block of oxytocin-induced contraction of rats, *in vitro* and *in vivo*.[14)]

In continuation to our work on the isolation of novel molecules from the western Himalayan medicinal plants,[15—21)] here we report the characterization of two new spirostanol steroidal saponins from roots of *A. racemosus* together with a saponin earlier isolated from *Asparagus filicinus*.[22)]

Results and Discussion

Methanolic extract of the roots of *A. racemosus* was repeatedly chromatographed over Diaion HP-20 and silica gel (60—120 mesh) column which resulted to the isolation of two new steroidal saponins. The new saponins were positive to Libermann–Burchard's reagent and negative to Ehrlich's reagent for furosatnol saponins.

Shatavaroside A (**1**) was obtained as white amorphous powder. The HR-ESI-QTOF-MS displayed a pseudomolecular ion peak at *m/z* 857.4866, $[M+H]^+$ (Calcd 857.4899), corresponded to the molecular formula $C_{44}H_{72}O_{16}$. The IR spectrum exhibited absorption for hydroxyl groups (3433, 1049 cm^{-1}) and strong absorption bands at 919, 900 and 847 cm^{-1} characteristic of spirostane-type steroidal sapogenins. The stronger intensity of the band at 919 compared with that at 900 cm^{-1} showed that **1** belongs to the 25*S* series of spirostanes.[23)] The 25*S*-configuaration of spirostane aglycone was also confirmed by the two proton signals at δ 3.97 (1H, m) and 3.31 (1H, d, J=10.5 Hz) which corresponded to the H-26 in the ^{1}H-NMR and the higher field resonance of C-27 (δ 16.8) compared to the ^{13}C-NMR shift of 25*R*-spirostanes (δ 17.1—17.2).[24)] The aglycon of **1** was identified as sarsasapogenin by the comparison of NMR data (Table 1) with reported values.[25)] The ^{1}H-NMR spectrum of **1** showed four methyl proton signals at δ 0.77 (3H, s, Me-18), 0.98 (3H, s, Me-19), 1.12 (3H, d, J=6.7 Hz, Me-21) and 1.07 (3H, d, J=6.6 Hz, Me-27). The presence of another methyl signal at δ 1.69 (3H, d, J=6.9 Hz) and three anomeric proton signals at δ 4.21 (1H, d, J=7.6 Hz), 4.10 (1H, d, J=7.4 Hz), 4.59 (1H, br s) suggested the presence of three monosaccharides including one deoxyhexose. The oxygenated signals at δ 4.30 (1H, m), 3.31 (1H, d, J=10.5 Hz) and 3.97 (1H, m) were assigned to H-3, Ha-26 and Hb-26. The decoupled ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra of **1** showed five methyls at δ 15.3, 16.8, 17.0, 18.7 and 24.4 for C-21, C-27, C-18, C-6 of rhamnose and C-19, respectively. Three anomeric carbon signals, correlated with above ascribed anomeric proton signals in heteronuclear multiple quantum correlation (HMQC) spectrum, were observed at δ 101.9, 104.5 and 101.4. These ^{1}H- and ^{13}C-NMR anomeric signals indicated the identity of sugar moieties as glucose, arabinose and rhamnose, respectively which was further confirmed by acid hydrolysis of **1** and comparison with reference sugars on TLC plate and GC-MS analysis of alditol derivatives of sugar portion. The ^{1}H coupling constants ($^3J_{1,2}$>7 Hz) were consistent with a β-configuration for the glucose and an α-configuration for arabinose. An α-configuration of rhamnose was deduced on the basis of C-5 signal of rahmnose at δ 68.7. The chemical shift of C-3 at δ 75.5 and its cross heteronuclear multiple bond coherence (HMBC) correlation with H-1′ (δ 4.21, d, J=7.6 Hz) of glucopyranosyl sugar indicated the linkage of glucose sugar at C-3 of the aglycone. The ^{1}H-NMR spectrum showed overlapping signals for other protons of sugar residues at δ 3.75—4.50. From HMBC correlation and mass fragmentation pattern it was evident that all three sugar residues were linked with each other. The linkages between sugar residues were assigned on the basis of ^{13}C-NMR values and HMBC correlations. HMBC experiments established correlations between

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Table 1. ^{1}H-NMR (300.13 MHz) and ^{13}C-NMR (75.46 MHz) Data for Compounds **1** (DMSO-d_6) and **2** (Pyridine-d_5)

Position	**1**		**2**		Position	**1**		**2**	
	δ	δ	δ	δ		δ	δ_H	δ	δ
1	30.7	1.43, 1.82 (m)	30.9	1.23, 1.90 (m)		Glc		Glc I	
2	26.8	1.46, 1.90 (m)	26.9	1.44, 1.94 (m)	1′	101.9	4.21 (d, 7.6)	102.0	4.78 (d, 7.3)
3	75.5	4.30 (m)[a]	75.3	4.31 (m)	2′	81.2	4.19[a]	80.3	4.23[a]
4	30.6	1.58, 1.79 (m)	30.1	1.58, 1.78 (m)	3′	75.5	4.30[a]	74.9	4.38[a]
5	37.0	2.15 (m)	36.7	2.16 (m)	4′	71.4	4.36[a]	79.0	4.04[a]
6	26.7	1.26, 1.34 (m)	26.9	1.23, 1.39 (m)	5′	74.4	3.75[a]	74.3	3.84[a]
7	27.0	0.94, 1.30 (m)	27.1	1.07, 1.31 (m)	6′	67.8	4.00,[a]	68.0	3.82,[a]
							4.45 (br d, 11.1)		4.04[a]
8	35.7	1.60 (m)	35.4	1.67 (m)		Ara		Glc II	
9	40.2	1.24 (m)	40.4	1.23 (m)	1″	104.5	4.10 (d, 7.4)	105.7	4.76 (d, 7.3)
10	36.0	—	35.7	—	2″	72.8	4.09[a]	74.8	3.88[a]
11	21.3	1.30 (m)	21.3	1.35 (m)	3″	74.4	4.29[a]	78.3	4.23[a]
12	40.2	1.27 (m)	40.4	1.27 (m)	4″	69.1	4.23[a]	72.1	4.27[a]
13	40.5	—	41.0	—	5″	66.8	3.82,[a] 4.06[a]	77.3	4.09[a]
14	56.2	1.09[a]	56.6	0.95[a]	6″			63.2	4.36,[a] 4.52[a]
15	32.2	1.39, 1.94 (m)	32.4	1.38, 1.95 (m)		Rham		Rham	
16	81.3	4.52 (m)	81.6	4.31 (m)	1‴	101.4	4.59 (br s)	102.4	5.56 (br s)
17	62.7	1.85 (m)	63.2	1.83 (m)	2‴	71.3	4.55	72.5	4.52[a]
18	17.0	0.77 (s)	16.5	0.81 (s)	3‴	71.5	4.36	72.7	4.49[a]
19	24.4	0.98 (s)	24.2	0.95 (s)	4‴	73.5	4.62	73.2	4.60[a]
20	42.4	1.78 (m)	42.6	1.76 (m)	5‴	68.7	4.45	69.8	4.49[a]
21	15.3	1.12 (d, 6.7)	15.1	1.17 (d, 6.7)	6‴	18.7	1.69 (d, 6.9)	19.0	1.66 (d, 6.9)
22	109.7	—	110.0	—				Xyl	
23	26.4	1.36,[a] 1.88[a]	26.6	1.35,[a] 1.87[a]	1⁗			105.7	5.44 (d, 6.8)
24	26.2	1.47,[a] 2.11[a]	26.4	1.44,[a] 2.01[a]	2⁗			73.0	4.09[a]
25	27.3	1.56[a]	27.7	1.56[a]	3⁗			76.2	4.23[a]
26	65.1	3.31 (d, 10.5), 3.97 m	65.3	3.39 (d, 10.5), 4.09 m	4⁗			72.1	4.27[a]
27	16.8	1.07 (d, 6.6)	16.9	1.09 (d, 6.6)	5⁗			67.0	4.82[a]

a) Multiplicities could not be determined due to the overlapping signals.

H-1″ (δ 4.10, d, J=7.4 Hz) of arabinopyranosyl and C-2′ (δ 81.2) of glucose, H-1‴ (δ 4.59, br s) of rhamnopyranosyl and C-6′ (δ 67.8) of glucose suggested that arabinose and rhamnose sugars were linked to C-2′ and C-6′ of glucose, respectively. The proposed linkages were further supported by the observation of downfield resonances of C-2′ and C-6′ (α-effect) of internal glucose and upfield resonances of adjacent carbons (β-effect).

ESI-QTOF-MS/MS of **1** (*m/z* 857) displayed the fragments at *m/z* 725 $[M+H-132]^+$ and 711 $[M+H-146]^+$ represented glycosidic cleavage by simultaneous loss of a pentose and a deoxyhexose sugar units which suggested the terminal linkage of both sugars. Further fragments at *m/z* 579 $[M+H-132-146]^+$ and 417 $[M+H-132-146-162]^+$ were observed due to the loss of a pentose from *m/z* 711 and/or a deoxyhexose from *m/z* 725 and a hexose sugar residue from *m/z* 579. The product ion at *m/z* 272 $[M+H-132-146-162-144]^+$ represented the loss of spirostane ring (144 amu) form *m/z* 417 (aglycone). The mass ion at *m/z* 441 [pentose+hexose+deoxyhexose+H]$^+$ was due to the cleavage of polysaccharide portion from aglycone part. In conclusion, **1** was assigned the structure as 3-*O*-{[α-L-arabinopyranosyl-(1→2)]-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranosyl}-(25*S*)-5β-spirostan-3β-ol and was designated as shatavaroside A (Fig. 1).

Shatavaroside B (**2**) was obtained as a white amorphous powder. The molecular formula was established as $C_{50}H_{82}O_{21}$ by HR-ESI-QTOF-MS at *m/z* 1019.5410 $[M+H]^+$ (Calcd 1019.5427). The IR spectrum exhibited absorption for hydroxyl groups (3429, 1069 cm^{-1}) and strong absorption bands at 918, 900 and 847 cm^{-1} characteristic of spirostane-type steroidal sapogenins. The stronger intensity of the band at 918 compared with that at 900 cm^{-1} showed that **2** belongs to the 25*S* series of spirostanes.[23] Comparison of the ^{1}H- and ^{13}C-NMR spectroscopic data of **2** with **1** suggested that it contained the same spirostane aglycone with 25*S*-configuration. The ^{1}H-NMR spectrum displayed four anomeric protons at δ 4.76 (1H, d, J=7.3 Hz), 4.78 (1H, d, J=7.3 Hz), 5.44 (1H, d, J=6.8 Hz) and 5.56 (1H, br s) with corresponding carbon signals at δ 105.7, 102.0, 105.7, and 102.4, respectively, indicating the presence of four sugar moieties in **2**. The identity of sugars was established as β-D-glucopyranosyl, β-D-glucopyranosyl, β-D-xylopyranosyl and α-L-

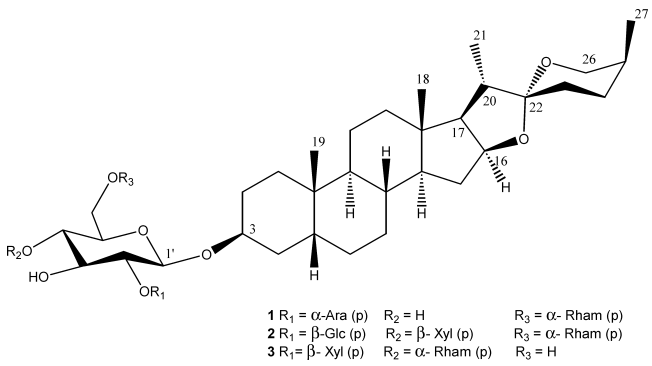

Fig. 1. Structures of Compounds **1**, **2** and **3**

rhamnopyranosyl by analyzing NMR values and coupling constants followed by acid hydrolysis of **2** and comparison with reference sugars on TLC plate. The sugars were further identified by GC-MS analysis of their alditol acetates with reference sugars. The linkages between sugar moieties were determined on the basis of HMBC spectrum, mass fragmentation pattern and shift values (α- and β-effects) in ^{13}C-NMR spectral data. The HMBC cross correlation between H-1′ (δ 4.78 d, J=7.3 Hz) of glc I and C-3 (δ 75.3) of aglycon suggested the direct linkage of glc I (inner sugar) to the aglycone part. Similarly, the correlations between H-1″ (δ 4.76, d, J=7.3 Hz) of glc II and C-2′ (δ 80.3) of glc I, H-1‴ (δ 5.56, br s) of rham and C-6′ (δ 68.0) of glc I and H-1⁗ (δ 5.44, d, J=6.8 Hz) of xyl and C-4′ (δ 79.0) of glc I indicated the direct attachment of glc II, rham and xyl to C-2′, C-6′ and C-4′ of glc I, respectively. The arrangement of sugar units was further supported by mass fragmentation pattern.

ESI-QTOF-MS/MS of **2** (*m/z* 1019) displayed the fragment at *m/z* 887 $[M+H-132]^+$, 873 $[M+H-146]^+$ and 857 $[M+H-162]^+$ represented glycosidic cleavage by simultaneous loss of a pentose, deoxyhexose and a hexose sugar unit from molecular ion peak, hence, suggested that the three sugar moieties were present in terminal positions. The loss of a deoxyhexose and/or pentose unit from *m/z* 887 and 873 revealed the fragment at *m/z* 741 $[M+H-132-146]^+$. Similarly the fragment at *m/z* 725 $[M+H-132-162]^+$ indicated the loss of a hexose and/or a pentose unit from *m/z* 887 and 857, respectively. The fragment at *m/z* 711 was observed due to the loss of a hexose and/or a deoxyhexose sugar unit from *m/z* 873 and 857, respectively. Other fragments at *m/z* 579 [aglycone+H+glc I]$^+$ and 417 [aglycone+H]$^+$ were observed due to the sequential loss of other remaining sugar units from *m/z* 741, 725 and 711. The mass ion at *m/z* 603 [pentose+2×hexose+deoxyhexose+H]$^+$ were due to the cleavage of polysaccharide portion from aglycone part which proved the presence of all sugar units in one group. The fragments at *m/z* 435 and 273 were observed due to the subtraction of ring F (spirostane, *m/z* 144) from *m/z* 579 and 417, respectively. Thus, on the basis of above evidences the structure of saponin **2** was assigned as 3-*O*-{[β-D-glucopyranosyl(1→2)]-[β-D-xylopyranosyl-(1→4)]-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranosyl}-(25*S*)-5β-spirostan-3β-ol and designated as shatavaroside B (Fig. 1).

Compound **3** was isolated as amorphous powder and was identified as filiasparoside C by comparing its spectroscopic data with reported values.[22)]

It is worth pointing out that the occurrence of steroidal saponins is characteristic of *Asparagus* genus and is considered as a chemotaxonomic marker of the genus.

Experimental

General Methods Melting points were determined on a Barnstead Electrothermal 9100 and were uncorrected. Optical rotation was determined on Horiba Sepa-300 Polarimeter and IR spectra were recorded on a NICOLET-6700 FT-IR spectrometer with KBr disc. Mass spectra were recorded on QTOF-Micro of Waters Micromass. NMR experiments were performed on Bruker Avance-300 spectrometer. Diaion HP-20 was procured from Kitten Enterprises Pvt. Ltd., Mumbai, India and Silica gel (60—120 mesh), TLC silica gel 60 F_{254} plates and all other chemicals used were purchased from Merck India Ltd. Standards glucose, xylose, rhamnose and arabinose were purchased from Life Technologies (India) Pvt. Ltd.

Plant Material The plant material was collected in August 2006 from Medicinal Plant Research Institute (ISM), Joginder Nagar, Himachal Pradesh, India. A voucher specimen (PLP-11100) has been deposited in the herbarium of IHBT, Palampur, India.

Extraction and Isolation Air dried powder of roots of *A. racemosus* (2.75 kg) was subsequently extracted with methanol (3×4 l) and water : methanol (7 : 3, 4×4 l) in a percolator at room temperature. Each combined percolation was dried under reduced pressure to yield 300 g and 700 g of methanol and water : methanol extract, respectively. Methanol extract (300 g) was subjected to a column chromatography over Diaion HP-20 (500 g) and eluted with deionised water followed by 10, 20, 30, 50 and 100% ethanol in water. Total 60 fractions (200 ml each) were collected. Fractions no. 51—60 eluted with 50 and 100% ethanol in water were combined and dried (15 g). The dried fraction (15 g) was further chromatographed over silica gel (60—120 mesh) using a gradient elution in $CHCl_3$ and mixture of $CHCl_3$: MeOH : H_2O (65 : 5 : 10, 65 : 10 : 10, 65 : 15 : 10, 65 : 20 : 10, 65 : 22 : 10, 65 : 25 : 10 and 65 : 30 : 10). Total 67 fractions (80 ml each) were collected. Fractions no. 24—30 eluted with $CHCl_3$: MeOH : H_2O (65 : 20 : 10) yielded **3** (85 mg) on crystallization with MeOH. The fractions no. 33—38 eluted in the same polarity as above afforded **2** (42 mg) after crystallization in methanol. The fractions no. 40—44 eluted with $CHCl_3$: MeOH : H_2O (65 : 22 : 10) yielded **1** (30 mg) after crystallization with methanol.

Shatavaroside A (**1**): White amorphous powder; mp 187—192 °C, $[\alpha]_D^{35}$ −31.4 (c=0.06, DMSO); IR (KBr) cm^{-1}: 3433 (OH), 2929 (CH), 1049, 919, 900, 847 cm^{-1}; HR-ESI-QTOF-MS (positive) *m/z*: 857.4866 $[M+H]^+$ (Calcd for $C_{44}H_{72}O_{16}$, 857.4899); MS/MS *m/z*: 725 $[M+H-132]^+$, 711 $[M+H-146]^+$, 579 $[M+H-132-146]^+$, 441 $[132+162+146+H]^+$, 417 $[M+H-132-146-162]^+$, 399 $[M+H-132-146-162-H_2O]^+$, 272 $[M+H-132-146-162-144]^+$; The ^{1}H- and ^{13}C-NMR data (DMSO-d_6): see Table 1.

Shatavaroside B (**2**): White amorphous powder; mp 217—219 °C; $[\alpha]_D^{19}$ −67.7 (c=0.53, py); IR (KBr) cm^{-1} 3429 (OH), 2932 (CH), 1069, 918, 900, 847 cm^{-1}; HR-ESI-QTOF-MS (positive) *m/z*: 1019.5410 $[M+H]^+$ (Calcd for $C_{50}H_{20}O_{21}$, 1019.5427); MS/MS *m/z*: 887 $[M+H-132]^+$, 873 [M+H−146], 857 $[M+H-162]^+$, 741 $[M+H-132-146]^+$, 725 $[M+H-132-162]^+$, 711 $[M+H-146-162]^+$, 603 $[132+162+146+162+H]^+$, 579 $[M+H-132-146-162]^+$, 435 $[M+H-132-146-162-144]^+$, 417 $[M+H-132-146-2\times162]^+$, 399 $[M+H-132-146-162-162-H_2O]^+$, 273 $[M+H-132-146-162-162-144]^+$; The ^{1}H- and ^{13}C-NMR data (C_5D_5N): see Table 1.

Acid Hydrolysis of 1 and 2 The saponins (**1**, **2**) (10 mg each) was hydrolyzed with 2 M HCl in 1,4-dioxane (1 : 1, 1.5 ml) and refluxed for 2 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 (5.7 mg from **1** and 4.9 mg from **2**) and identified as sarsasapogenin by comparing its ^{13}C-NMR data with earlier report.[25)] The aqueous part of the acid hydrolysate was neutralized with silver carbonate and filtered. The filtrate containing sugar mixture was evaporated to dryness in vacuum, dissolved in water (5 ml) and was examined by TLC [EtOAc–MeOH–HOAc–H_2O (6 : 2 : 1 : 1)] with reference sugars. Alditol acetates were prepared by reduction of sugar part and reference sugars at room temperature for 30 min using a solution of 0.25 M $NaBH_4$ in NH_4OH (1.5 ml) and then quenched by a solution of 10% of acetic acid in MeOH (20 ml). The reduced product was then acetylated with acetic anhydride–pyridine (1 : 1) at 100 °C for 1 h. This mixture was diluted with water and extracted with ethyl acetate and dried. The ethyl acetate extract was analyzed by GC-MS and sugars were identified by comparing their retention times with authentic sugars. GC-MS analyses were carried out on a Shimadzu QP 2010 GC-MS system equipped with a Carbowax BP20 capillary column (30 m×0.25 mm, 0.25 μm film thickness) from SGE, Australia; carrier gas, helium, at a flow rate of 1.1 ml/min; ionization energy, 70 eV; ion source temperature, 230 °C; injector temperature, 220 °C. Oven temperature was programmed as follows: initially at 70 °C for 4 min, rising at 4 °C/min to 220 °C and then held isothermal (4 min) at 220 °C. Peaks corresponding to L-rhamnose, L-arabinose, D-xylose and D-glucose appeared at 2.65, 3.31, 3.45, 5.86 min, respectively. The alditol acetates of sugars were identified as D-glucose, L-rhamnose and L-arabinose for saponin **1** and D-glucose, L-rahmnose and D-xylose for saponin **2**.

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