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Steroidal saponins from the roots of *Asparagus racemosus*

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Abstract

Five steroidal saponins, shatavarins VI–X, together with five known saponins, shatavarin I (or asparoside B), shatavarin IV (or asparinin B), shatavarin V, immunoside and schidigerasaponin D5 (or asparanin A), have been isolated from the roots of *Asparagus racemosus* by RP-HPLC and characterized by spectroscopic (1D and 2D NMR experiments) and spectrometric (LCMS) methods.

Keywords: Saponins; Shatavarins; *Asparagus racemosus*; Asparagaceae; NMR

1. Introduction

Asparagus racemosus, commonly known as Shatavari, is found in forests throughout India, especially in the North of India and is cultivated for both medicinal and ornamental purposes. The tuberous root of *A. racemosus* is used in traditional Indian medicine as a galactologue and for the treatment of diverse ailments including dysentery, tumors, inflammations, neuropathy, nervous disorders, bronchitis, hyperacidity, certain infectious diseases (Goyal et al., 2003), conjunctivitis (Sharma and Singh, 2002), spasm, chronic fevers, and rheumatism (Capasso et al., 2003). Pharmacological studies with animals have manifested the potency of *A. racemosus* extract as an antioxidant (Parihar and Hemnani, 2004; Kamat et al., 2000), antianaphylactic (Padmalatha et al., 2002), adaptogen (Rege et al., 1999; Bhattacharya et al., 2000), antistress (Bhattacharya et al., 2002; Muruganandam et al., 2002), antiulcer (De et al., 1997; Goel and Sairam, 2002; Datta et al., 2002; Sairam et al., 2003), antidiarrhoeal (Venkatesan et al., 2005), antibacterial (Mandal et al., 2000a), antitussive (Mandal et al., 2000b), molluscicide (Chifundera et al., 1993) and radioprotective agent (Arora et al., 2005), and as a substrate for inulinase production (Singh et al., 2006), with the biggest focus being on its ability in modulating the immune system (Thatte et al., 1987; Thatte and Dahanukar, 1988; Rege et al., 1989; Rege and Dahanukar, 1993; Dhuley, 1997; Diwanay et al., 2004; Gautam et al., 2004). One human trial confirmed the herb's potency in treating dyspepsia (Dalvi et al., 1990).

A limited number of steroidal saponins have been reported previously from the roots of this plant, with the major one being shatavarins I and IV (Ravikumar et al., 1987; Joshi and Sukh, 1988; Jadhav and Bhutani, 2006), immunoside (Handa et al., 2003) and shatavarin V (Hayes et al., 2006b). Recently, the racemosides (Mandal et al., 2006) were described from the only study of the fruits of *A. racemosus*.

Our preliminary work on the saponin content of *A. racemosus* roots revised the structures of the two *major* saponins of this plant, shatavarins I, **10** and IV, **7** (Hayes et al., 2006a). We unambiguously demonstrated (based on extensive 1D and 2D NMR experiments) that the previous reports wrongly assigned the glycosidic linkages in both shatavarin I and IV. Further confirmation was provided by the isolation of a new *minor* steroidal saponin from the *A. racemosus* roots (Hayes et al., 2006b), shatavarin

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V, **6** and the demonstration that this saponin in fact possessed the structure previously incorrectly assigned to shatavarin IV (Jadhav and Bhutani, 2006; Ravikumar et al., 1987). The present paper describes in detail the isolation and structure elucidation of the steroidal saponins isolated from the root of this plant.

2. Results and discussion

Semi preparative RP-HPLC (Varian HPLC column, Dynamax HPLC column, 250 × 10 mm, OmniSpher 5 C18) of the crude extract of *A. racemosus* roots furnished ten pure saponins **1**–**10**. Five new steroidal saponins, shatavarin VI–X, **1**–**5** and five known steroidal saponins, shatavarin I **10** and shatavarin IV **7** (Hayes et al., 2006a), asparinin A **8** (Zhang et al., 2004), shatavarin V, **6** (fully characterized in our previous communication, Hayes et al., 2006b) and immunoside **9** (Huang and Kong, 2006). The structures of the new saponins were elucidated by 1D and 2D NMR studies in combination with MS studies (Fig. 1).

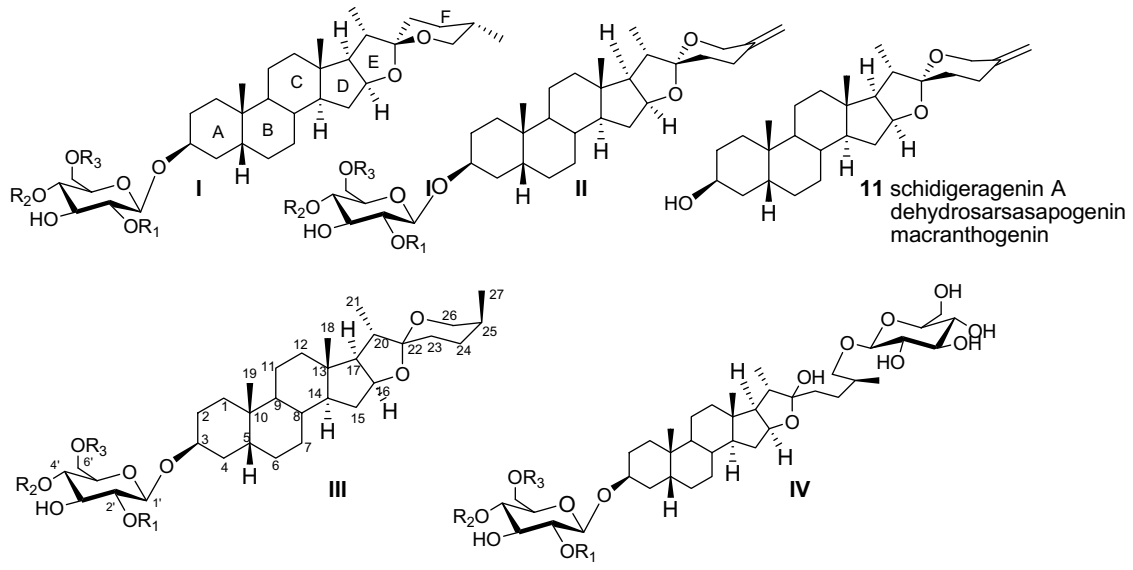
The absolute configuration of the sugar components was determined by enantioselective GC (König et al., 1981), after acid hydrolysis of the crude saponin mixture and comparison with authentic standards. The mixture from saponin hydrolysis was subjected to methanolysis followed by trifluoroacetylation, and then analysed by gas chromatography using a Chirasil-L-Val column as the stationary phase. The sugar residues were determined to be D-glucose, L-arabinose and L-rhamnose.

Compound **1** was isolated as an amorphous solid, and exhibited a molecular ion (positive-ion ESI-HRMS) at 909.4837 ([M+Na$^+$]) corresponding to the same molecular formula ($C_{45}H_{74}O_{17}$) as that of shatavarin IV **7** and shatavarin V **6**. Comparable ^{13}C and ^{1}H chemical shifts for the A–D rings were observed for **1**, **6** and **7**, but with a noticeable difference for the carbons of rings E and F, indicating that a different stereostructure may characterize these rings in the case of **1**. The proton signal for H-27 moved upfield from 1.07 ppm to 0.68 ppm, and the carbon signal for C-27 downfield from 16.3 ppm to 17.4 ppm, suggestive of an equatorially (*alpha*) oriented methyl group in **1** (Agrawal, 2003), instead of axial (*beta*) as in **6** and **7**. Additionally, the downfield shift for H-20, from 1.91 to 2.32 ppm, as well as ROESY correlations between H-16 and H-21/H-23*eq* is consistent with a *cis* relationship between H-20 and the oxygen of the F-ring (Tobari et al., 2000) indicating an equatorial position for the oxygen of the F-ring and the (*S*) configuration of C-22. Additional 2D ROESY correlations between H-14, H-16 and H-17, confirm the *cis* junction of the D and E rings (see Fig. 2).

This rare 22-S configuration has been observed before in a saponin isolated from the tubers of *Dichelostemma multiflorum* (Inoue et al., 1995) and the ^{13}C shifts reported for the E/F rings are in excellent agreement with these reported here for **1**. In fact, the aglycone of the saponin from *D. multiflorum* differs from **1** only in the configuration of C-5, which results in significantly different chemical shifts for the carbons of the A/B rings in these two compounds. Both **1** and **7** could presumably form from a common intermediate which undergoes cyclisation to give an F ring with differing stereochemistry at C-22. Maintenance of the anomeric effect then results in the differing disposition of the C-27 methyl group; *axial (beta)* in **7** and *equatorial* (*alpha*) in **1**.

The ^{1}H NMR spectrum of **1** displayed signal for three anomeric protons at δ_H 4.85 (*d*, $J = 8.2$ Hz), 5.43 (*d*, $J = 7.6$ Hz) and 5.90 (brs) which correlated, in the HSQC spectrum, with ^{13}C signals at δ_c 102.5, 105.7 and 101.9, respectively. The vicinal coupling constants (for the sugars) were determined via 1D-TOCSY experiments, which indicated the presence of two β-D-glucopyranoses and one α-rhamnopyranose (see Table 3). This conclusion is in agreement with the sugar distribution obtained by GCMS analysis after acid hydrolysis of the saponin, which provided rhamnose and glucose in a 1:2 ratio. In the HMBC spectrum, a cross peak between the ^{1}H NMR signal at δ 4.85 (H-1′, 2,4-disubstituted glucose) and the carbon signal at δ 75.4 (C-3, aglycone) indicated glycosylation of the aglycone at C-3. Similarly, anomeric protons at δ 5.43 (H-1, terminal glucose) and δ 5.90 (H-1, rhamnose) showed cross-peaks with the carbon signals at δ 82.9 (C-2 of the 2,4-disubstituted glucose), and at δ 77.4 (C-4 of the 2,4-disubstituted glucose), respectively. Compound **1** was therefore established as 3-*O*-{[β-D-glucopyranosyl(1 → 2)]- [α-L-rhamnopyranosyl(1 → 4)]-β-D-glucopyranosyl}-(22S,25S)-5β-spirostan-3β-ol (Shatavarin VI).

Compound **2** was isolated as an amorphous solid, and positive-ion ESI-HRMS provided an ion at 907.4664 ([M+Na$^+$]) corresponding to a molecular formula of $C_{45}H_{72}O_{17}$. The ^{1}H NMR spectrum (in d_5-pyridine) revealed the presence of two methyl groups (δ 0.84 and 0.97 ppm) attached to quaternary carbons, corresponding to the angular methyl groups of a steroidal saponin, and only one methyl group [δ 1.08 (J 7.0 Hz)] on a secondary carbon, suggesting a different type of aglycone. This hypothesis was reinforced by the presence of two carbon signals (not observed in the case of sarsasapogenin) at δ 144.6 (quaternary) and δ 109.1 (CH_2) in the ^{13}C NMR spectrum with HSQC correlations to two proton signals (both as broad singlets) at δ 4.80 and δ 4.82 ppm, respectively. The ^{13}C chemical shifts were in good agreement with those of sarsasapogenin for the rings A–E, with noticeable differences only for the F ring. Further comparison with the ^{13}C chemical shifts reported for schidigeragenin A, **11** from *Yucca schidigera* (Miyakoshi et al., 2000), known also as dehydrosarsasapogenin (Petricic, 1974) or macranthogenin (Petricic et al., 1969) from *Helleborus macranthus*, led to the conclusion that **2** possesses a dehydrosarsasapogenin aglycone, reported here for the first time in the Asparagaceae family. Acid hydrolysis yielded rhamnose and glucose in a 1:2 ratio. This was in agreement with the NMR which contained signals for a secondary methyl group at δ 1.66



	Type	R_1	R_2	R_3	
1	I	β-D-Glc	α-L-Rha	H	shatavarin VI
2	II	β-D-Glc	α-L-Rha	H	shatavarin VII
3	III	β-D-Glc	α-L-Ara	β-D-Glc	shatavarin VIII
4	III	β-D-Glc	β-D-Glc	H	shatavarin IX
5	III	α-L-Rha	β-D-Glc (6-OAc)	H	shatavarin X
6	III	α-L-Rha	β-D-Glc	H	shatavarin V
7	III	β-D-Glc	α-L-Rha	H	shatavarin IV
8	III	β-D-Glc	H	H	asparinin A
9	III	α-L-Rha	α-L-Rha	H	immunoside
10	IV	β-D-Glc	α-L-Rha	H	shatavarin I

Fig. 1. Structures of furostanol and spirostanol glycosides 1–10 isolated from *A. racemosus* roots, divided in four major structural types (I–IV).

(*d*, *J* 6.2 Hz) and three anomeric protons [4.87 (*d*, *J* 7.2 Hz), δ 5.45 (*d*,*J* 7.8 Hz), δ 5.82 (brs)], suggesting the presence of three monosaccharides including one deoxyhexose. Comparison of the ^{1}H NMR spectrum for **1** and **2** indicated identical chemical shifts for the oligosaccharide chain for both compounds, confirmed by similar correlations in the HMBC spectrum. The structure of compound **2** was therefore elucidated as 3-*O*-{[β-D-glucopyranosyl(1 → 2)][α-L-rhamnopyranosyl(1 → 4)]-β-D-glucopyranosyl}-5β-spirost-25(27)-en-3β-ol (Shatavarin VII).

Compound **3** was isolated as an amorphous solid, and positive-ion ESI-HRMS provided an ion at 1057.5203 ([M+Na$^+$]), corresponding to a molecular formula of $C_{50}H_{82}O_{22}$. Analysis of the ^{1}H and ^{13}C NMR spectra indicated the presence of four sugar units with four anomeric protons [δ 4.75 (*d*, *J* 8.2 Hz), δ 5.27 (*d*, *J* 8.0 Hz), δ 5.29 (*d*, *J* 8.1 Hz), δ 5.36 (*d*, *J* 8.0 Hz)] with correlation with these carbon signals [δ 101.3, 105.1, 105.2 and 105.2 ppm, respectively] in the HSQC spectrum. The ^{13}C NMR spectrum suggested a spirostanol-type of steroid aglycone for **3**, and was then found to be sarsasapogenin by comparison with literature data (Agrawal et al., 1997; Miyakoshi et al., 2000). Acid hydrolysis yielded arabinose and glucose in a 1:3 ratio. The vicinal coupling constants (for the sugars of the saponin) were determined through 1D-TOCSY experiments, which indicated the presence of three β-D-glucopyranoses and one α-arabinose (see Table 3). In the HMBC spectrum, a cross peak between the ^{1}H NMR signal at δ 4.75 (glucose) and the carbon signal at δ 75.1 (C-3, aglycone) indicated glycosylation of the aglycone at C-3. The carbon chemical shifts C-2, C-4 and C-6 of this glucose moiety were downfield (by comparison with a non-substituted glucose unit) by 5.4 ppm (from 75.2 to 80.6 ppm), 9.7 ppm (from 69.9 to 79.6 ppm) and 5.5 ppm (from 62.8 to 68.3 ppm) indicating that this glucose was 2,4,6-trisubstituted. This was corroborated by the detection of cross-peaks between the anomeric protons at δ

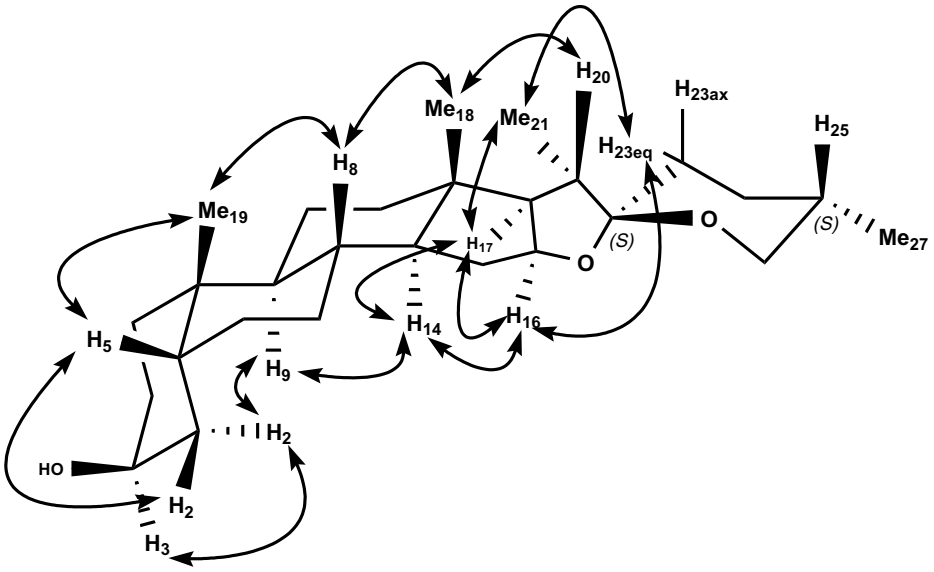


Fig. 2. ROESY-correlation for aglycone moiety of shatavarin VI 1.

5.27 (H-1 6'-*O*-β-D-glucose), 5.29 (H-1, 4′-*O*-α-arabinose) and δ 5.36 (H-1 2'-*O*-β-D-glucose) with the carbon signals at δ 68.3 (C-6 of the 2,4,6-trisubstituted glucose), δ 76.9 (C-4 of the 2,4,6-trisubstituted glucose) and δ 80.6 (C-2 of the 2,4,6-trisubstituted glucose), respectively. Compound **3** was determined as 3-*O*-{[β-D-glucopyranosyl(1 → 2)][α-L-arabinopyranosyl(1 → 4)][β-D-glucopyranosyl (1 → 6)]-β-D-glucopyranosyl}-(25S)-5β-spirostan-3β-ol (Shatavarin VIII).

Compound **4** was isolated as an amorphous solid, and positive-ion ESI-HRMS provided an ion at 925.4775 ([M+Na$^+$]) corresponding to a molecular formula of $C_{45}H_{74}O_{18}$. Acid hydrolysis provided glucose only. The spectral data for **4** exhibited close similarity with the aglycone part of compounds, **3**, **6** and **7**, indicating a sarsasapogenin aglycone. The HMBC spectrum again indicated the connectivity of the three sugars. The anomeric proton signals at δ 4.84 (H-1, 2,4-substituted glucose), δ 5.40 (H-1, second glucose) and δ 5.14 (H-1, third glucose), correlated with the carbon signals at δ 75.1 (C-3 of the aglycone), δ 81.2 (C-2 of the 2,4-disubstituted glucose) and δ 80.8 (C-4 of the 2,4-disubstituted glucose), respectively. The structure of compound **4** was therefore elucidated as 3-*O*-{[β-D-glucopyranosyl(1 → 2)][β-D-glucopyranosyl (1 → 4)]-β-D-glucopyranosyl}-(25S)-5β-spirostan-3β-ol (Shatavarin IX).

Compound **5** was isolated as an amorphous solid, and exhibited a molecular ion (positive-ion ESI-HRMS) at 951.4937 ([M+Na$^+$]) corresponding to a molecular formula of $C_{47}H_{76}O_{18}$. Examination of the ^{1}H and ^{13}C NMR spectra suggested a spirostanol-type steroid aglycone for **5**, and this was found to be sarsasapogenin by comparison with literature data (Agrawal et al., 1997; Miyakoshi et al., 2000). Acid hydrolysis furnished rhamnose and glucose in a 1:2 ratio. The FABMS fragment ions of **5** at *m/z* 927 [M−H] and 885 [M−H-43] in negative ion mode, and 929 [M+H], 783 [M+H-Rha], 725 [M+H-Glc-43], 579 [783-Glc-43 or 725-Rha] and 417 [579-Glc] in positive ion mode, suggested that the sugar moiety of **5** was branched, with one inner glucose, and most likely a terminal mono-acetylated glucose. This explains the lost of [43 + glucose] in some of the fragmentations.

The occurrence of an acetyl group was confirmed by the presence of a quaternary signal at 171.6 ppm and an extra methyl signal at 20.8 ppm in the ^{13}C spectrum, and a methyl singlet at 2.07 ppm in the ^{1}H NMR spectrum. In the HMBC spectrum, a cross peak between the ^{1}H NMR signal at δ 4.82 (H-1, inner glucose) and the carbon signal at δ 76.0 (C-3, aglycone) indicated glycosylation of the aglycone at C-3 with a glucose unit. The downfield shift of the C-4 signal of this inner glucose by more than 13 ppm indicated the substitution at this position by the second glucose unit. This was confirmed by the presence of a cross peak between this carbon (C-4) and the anomeric proton at δ 5.06 ppm in the HMBC spectrum. An upfield shift of 3.3 ppm (from 78.5 to 75.2 ppm) and a downfield shift by 2.7 ppm (from 62.1 to 64.8 ppm) for the C-5 and C-6 carbons of the terminal glucose unit would be consistent with acetylation at C-6 of the terminal glucose. HMBC correlations between the carbon at δ 171.1 ppm (carbonyl of the acetyl group) and the proton signals at δ 4.90 and 4.57 ppm (H-6, terminal glucose) definitely established C-6 acetylation of this glucose. The rhamnose unit was found to be linked at the C-2 position of the inner glucose with correlations between the signal at δ 76.8 ppm (C-2 inner glucose) and δ 6.32 ppm (H-1 rhamnose) in the HMBC spectrum. Therefore, Compound **5** was established as (3-*O*-{[α-L-rhamnopyranosyl(1 → 2)][β-D-6-acetyl-glucopyranosyl(1 → 4)]-β-D-glucopyranosyl}-(25*S*)-5β-spirostan-3-β-ol) (Shatavarin X). The fact that this acetylated saponin was observed only from a minor component, shatavarin V, **6** and not from the major shatavarin IV, **7** indicates that **5**

is unlikely to be an artifact formed during the extraction/isolation process.

Our investigation of the roots of *A. racemosus* led to the isolation of ten steroidal saponins, with a majority containing a sarsasapogenin aglycone commonly found in the Asparagaceae family. While the major ones, shatavarin I and IV have been previously isolated from different species of asparagus, some of the novel, minor ones were found to possess rare (22S configuration, or dehydrosarsasapogenin) aglycones, reported for only two members of the Liliaceae family (*D. multiflorum* and *Y. schidigera*) and one member of the Ranunculaceae family (*Helloborus macranthus*) but never reported for the Asparagaceae family. (Some classifications place the *Asparagus* genus within the Liliaceae family.)

3. Experimental

3.1. General

Optical rotations were measured at 20 °C on a Perkin–Elmer 241 MC polarimeter. ^{1}H NMR spectra were recorded on Bruker AV500 or AV750 MHz spectrometers with the residual protonated signal in the d_5-pyridine solvent (δ 8.71 ppm) as internal standard. ^{13}C NMR spectra were recorded at 125 or 188 MHz with the broad singlet of pyridine (δ 149.9 ppm) as internal standard. J values are reported in Hz. Mass spectra were recorded on a Shimadzu LCMS-QP8000 alpha equipped with a SPD-M10Avp diode array detector and an Adsorbosphere HS C18 7 micron column (150 mm × 4.6 mm ID, Alltech). High resolution mass spectra were recorded on a Kratos MS-25RFA spectrometer. Compounds **1**–**10** were purified by semi-preparative RP-HPLC with an LC-10AT Shimadzu Liquid Chromatograph (acetonitrile/water gradient from 8% to 100% of acetonitrile in 50 min) using an ELSD-LT Shimadzu detector (52 °C, P 200 KPa) and a Varian HPLC column (Dynamax HPLC column, 250 × 10 mm, OmniSpher 5 C18).

3.2. Plant material

The roots of *A. racemosus* were harvested in September 2005 in Bangalore; South India, and were identified by Dr.

Table 1
^{1}H spectral data (δ in ppm) for the algycone part of compounds **1**–**5** in pyridine-d_5

Carbon no.	**1**	**2**	**3**	**4**	**5**
1a	1.43 *m*[a]	1.42 *m*[a]	1.46 *m*[a]	1.45 *m*[a]	1.50 *m*[a]
1b	1.78 *m*[a]	1.78 *m*[a]	1.83 *m*[a]	1.81 *m*[a]	1.84 *m*[a]
2a	1.21 *m*[a]	1.14 *m*[a]	1.47 *m*[a]	1.48 *m*[a]	1.51 *m*[a]
2b	1.82 *m*[a]	1.75 *m*[a]	1.82 *m*[a]	1.85 *m*[a]	1.85 *m*[a]
3	4.23 *m*[a]	4.28 *m*[a]	4.22 *m*[a]	4.22 *m*[a]	4.22 *m*[a]
4a	1.73 *m*[a]	1.77 *m*[a]	1.75 *m*[a]	1.77 *m*[a]	1.82 *m*[a]
4b	1.79 *m*[a]	1.77 *m*[a]	1.75 *m*[a]	1.77 *m*[a]	1.825 *m*[a]
5	2.18 *m*[a]	2.16 *brs*	2.22 *m*[a]	2.17 *m*[a]	2.16 *m*[a]
6a	1.47 *m*[a]	1.46 *m*[a]	1.18 *m*[a]	1.15 *m*[a]	1.35 *m*[a]
6b	1.83 *m*[a]	1.84 *m*[a]	1.84 *m*[a]	1.78 *m*[a]	1.98 *m*[a]
7a	0.9 5*m*[a]	0.91 *m*[a]	0.92 *m*[a]	0.92 *m*[a]	0.98 *m*[a]
7b	1.22 *m*[a]	1.20 *m*[a]	1.23 *m*[a]	1.22 *m*[a]	1.28 *m*[a]
8	1.45 *m*[a]	1.48 *m*[a]	1.47 *m*[a]	1.47 *m*[a]	1.54 *m*[a]
9	1.27 *m*[a]	1.25 *m*[a]	1.25 *m*[a]	1.25 *m*[a]	1.31 *m*[a]
11a	1.19 *m*[a]	1.16 *m*[a]	1.18 *m*[a]	1.18 *m*[a]	1.22 *m*[a]
11b	1.31 *m*[a]	1.28 *m*[a]	1.31 *m*[a]	1.31 *m*[a]	1.32 *m*[a]
12a	1.05 *m*[a]	1.05 *m*[a]	1.08 *m*[a]	1.06 *m*[a]	1.08 *m*[a]
12b	1.63 *m*[a]	1.66 *m*[a]	1.66 *m*[a]	1.67 *m*[a]	1.68 *m*[a]
14	0.96 *m*[a]	1.08 *m*[a]	1.05 *m*[a]	1.03 *m*[a]	1.08 *m*[a]
15a	1.44 *m*[a]	1.43 *m*[a]	1.40 *m*[a]	1.40 *m*[a]	1.38 *m*[a]
15b	2.02 *m*[a]	2.03 *m*[a]	2.09 *m*[a]	2.00 *m*[a]	2.01 *m*[a]
16	4.37 *m*[a]	4.63 *dt* (6.8, 7.6)	4.58 *m*[a]	4.57 *m*[a]	4.56 *m*[a]
17	1.63 *dd* (6.5, 8.7)	1.86 *dd* (6.8, 7.6)	1.81 *m*[a]	1.82 *m*[a]	1.81 *m*[a]
18	0.94 *s*	0.84 *s*	0.81 *s*	0.81 *s*	0.81 *s*
19	0.94 *s*	0.97 *s*	0.98 *s*	0.96 *s*	1.06 *s*
20	2.32 *dq* (6.5, 6.5)	1.99 *dq* (6.8, 7.0)	1.91 *m*[a]	1.91 *m*[a]	1.90 *m*[a]
21	1.00 *d* (7.2)	1.08 *d* (7.0)	1.12 *d* (7.0)	1.14 *d* (7.0)	1.14 *d* (7.0)
23a	1.46 *m*[a]	1.82 *m*[a]	1.45 *m*[a]	1.44 *m*[a]	1.42 *m*[a]
23b	1.67 *dbrt* (2.8, 12.0)	1.82 *m*[a]	1.90 *m*[a]	1.90 *m*[a]	1.88 *m*[a]
24a	1.55 *m*[a]	2.24 *brd* (12.0)	1.35 *m*[a]	1.35 *m*[a]	1.34 *m*[a]
24b	1.55 *m*[a]	2.70 *td* (5.4, 12.0)	2.12 *m*[a]	2.13 *m*[a]	2.13 *m*[a]
25	1.63 *m*[a]		1.59 *m*[a]	1.57 *m*[a]	1.56 *m*[a]
26a	3.56 *dd* (10.5, 11.0)	4.06 *d* (12.0)	3.36 *brd* (11.0)	3.36 *brd* (11.6)	3.36 *brd* (11.6)
26b	3.70*rdd* (5.6, 11.0)	4.49 *d* (12.0)	4.07 *brdd* (2.2, 11.0)	4.07 *dd* (2.9, 11.6)	4.06 *brdd* (2.6, 11.6)
27a	0.68 *d* (6.4)	4.80 *brs*	1.07 *d* (6.7)	1.07 *d* (7.2)	1.06 *d* (7.2)
27b		4.82 *brs*			

[a] Indicates overlapping signals.

M. Madhukar Reddy, Taxonomist from Heritage Bio-Natural Systems Pvt. Ltd. A voucher specimen (Ref: NCM-D-06-043) was deposited at the Medicinal Plant Herbarium of the Southern Cross University, Lismore, Australia.

3.3. Extraction and isolation

Powdered roots (11.3 g) of *A. racemosus* were extracted at room temperature (90% acetonitrile/water, 200 mL), assisted by sonication (2 × 20 min), and then left to stir at room temperature for 12 h. After filtration and removal of the solvent under vacuum, the residue was diluted in 5 mL (90% methanol/water) and filtered to remove the insoluble material. This crude solution was then purified by semi-preparative RP-HPLC performed on a Shimadzu LC-10AT Liquid Chromatograph equipped with a Shimadzu ELSD-LT detector (Flow rate: 2 mL/min, 52 °C, 200 KPa) and a Varian HPLC column (Dynamax HPLC column, 250 × 10 mm, OmniSpher 5 C18). An acetonitrile/water gradient from 8% to 100% of acetonitrile in 50 min was used and 100 μL were injected each time. Under these conditions, ten different pure saponins were collected in order of elution (decreasing order of polarity): shatavarin I, **10** (R_t: 19.5 min, 8.2 mg) (Hayes et al., 2006a), shatavarin VIII, **3** (R_t: 29.3 min, 1.6 mg), shatavarin VI, **1** (R_t: 32.0 min, 3.2 mg), shatavarin VII, **2** (R_t: 32.7 min, 0.5 mg), shatavarin IX, **4** (R_t: 33.7 min, 1.1 mg), shatavarin IV, **7** (R_t: 34.8 min, 19.4 mg) (Hayes et al., 2006a), asparanin A, **8** (R_t: 37.6 min, 0.61 mg) (Zhang et al., 2004), shatavarin V, **6** (R_t: 38.8 min, 1.3 mg) (Hayes et al., 2006b) immunoside, **9** (R_t: 40.5 min, 1.8 mg) (Huang and Kong, 2006), and shatavarin X, **5** (R_t: 43.9 min, 0.3 mg).

3.4. Acid hydrolysis

3.4.1. Aglycone analysis

Acid hydrolysis of the powdered roots by heating under reflux for 15 h in 2 N HCl in 70:30 isopropanol: water (Sauvaire and Baccou, 1978a, 1978b) yielded sarsasapogenin (0.37%) as the main aglycone, which had identical spectroscopic (^{1}H and ^{13}C NMR) properties to literature reports (Agrawal et al., 1997; Miyakoshi et al., 2000).

3.4.2. Sugar analysis

The saponins (0.5–1 mg) were heated with 90% formic acid (0.4 mL) for 1 hr at 100 °C, cooled and concentrated. The residue was then heated with 2M TFA (0.3 mL) for 2 h at 120 °C, and after cooling the solution was evaporated to dryness and rinsed twice with methanol (2 × 0.5 mL). The resulting product was reduced at room temperature for 30 min using a solution of 0.25 M $NaBH_4$ in NH_4OH (0.3 mL) and the reaction was quenched with a solution of 10% of acetic acid in MeOH (4 × 0.5 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 the extract analyzed by GCMS (Cyclo-β 0.25 μm, 25 m × 0.22 mm, Alltech) and retention times were compared with those of authentic standards.

3.4.3. Determination of the sugar absolute configurations

The crude saponin mixture was filtrated through a C_{18} SPE cartridge and then evaporated to dryness. A portion of the saponin mixture (approximately 1 mg) was heated under reflux for 2 hours at 100 °C in 1N HCl in methanol (0.5 mL). After cooling, the solution was concentrated under a stream of nitrogen and then partitioned between water (500 μL) and $CHCl_3$ (500 μL). The aqueous layer was separated and concentrated under a stream of nitrogen. The methanolysis product was dissolved in a mixture of trifluoroacetic anhydride (TFAA)/CH_2Cl_2 (1/1 200 μL) and heated at 100 °C for 10 min, cooled to room temperature and then concentrated under a stream of nitrogen. The residue was redissolved in CH_2Cl_2 (1.5 mL) and then analyzed by enantioselective GC (König et al., 1981), using a Chirasil-L-Val capillary column (25 m × 0.32 mm × 0.20 μm), FID detection, carrier gas: Helium, injector: 200 °C, detector: 200 °C, column flow: 1.32 mL/min, split ratio: 56.0, pressure: 40 KPa. Temperature program: initial temperature 50 °C for 6 min, raised at 4 °C per min to a final temperature of 160 °C for 5 min. The standards were prepared following the same procedure. Under these conditions, the retention times for the standards were: L-Glc (24.88 and 27.95 min), D-Glc (24.95 and 28.11 min), L-Rha (16.86 and 22.26 min), D-Rha (17.11 and 22.53 min), L-Ara (18.81, 19.96 and 22.49 min) and D-Ara (18.81, 19.85 and 22.67 min). For the

Table 2
^{13}C spectral data (δ in ppm) for the algycone part of compounds **1**–**5** in pyridine-d_5

Carbon no.	1	2	3	4	5
1	30.9	31.1	30.9	31.2	31.1
2	27.0	27.2	27.1	27.0	26.9
3	75.4	75.6	75.1	75.6	76.0
4	30.8	30.8	30.5	31.0	31.1
5	36.8	36.8	36.5	36.8	37.3
6	26.8	26.9	26.9	27.1	26.8
7	26.9	27.0	26.8	26.9	26.9
8	35.3	35.8	35.6	35.7	35.7
9	40.4	40.4	40.4	40.3	40.4
10	35.3	35.6	35.3	35.4	35.4
11	21.2	21.4	21.2	21.4	21.2
12	40.8	40.5	40.3	40.5	40.4
13	41.5	41.3	40.9	41.1	41.0
14	55.7	56.7	56.5	56.7	56.7
15	33.2	32.4	32.2	32.3	32.2
16	81.0	81.9	81.4	81.5	81.4
17	62.8	63.2	63.0	63.1	63.0
18	17.0	16.9	16.6	16.7	16.9
19	24.0	24.2	24.1	24.1	23.9
20	42.2	42.2	42.5	42.5	42.4
21	16.8	15.2	14.9	15.0	14.9
22	110.6	109.8	109.8	109.9	109.8
23	28.3	33.5	26.5	26.6	26.5
24	28.2	29.2	26.2	26.3	26.2
25	30.8	144.6	27.6	27.7	27.7
26	69.6	65.3	65.2	65.2	65.2
27	17.4	109.1	16.3	16.3	16.3

A. racemosus hydrolysate, peaks were observed at 16.91 (L-Rha), 18.81 (L-Ara), 19.84 (L-Ara), 22.37 (L-Rha), 22.49 (L-Ara), 25.14 (D-Glc) and 28.33 min (D-Glc). Due to the retention time variations, co-injection studies of the crude extract with the different standards were conducted and confirmed the presence of D-glucose, L-rhamnose and L-arabinose only in the extract.

3.5. Shatavarin VI (**1**)

Amorphous solid, $[\alpha]_D$ −46.7 (*c*, 0.12, pyridine). HRMS: Calcd for $C_{45}H_{74}NaO_{17}$ (M+Na): 909.4824. Found: 909.4837. Negative ion ESI *m/z*: 885 [M−1]. Positive ESI-MS *m/z*: 887 [M+1], 725 [M-Glc], 579 [M-Glc-Rha], 417 [M-Glc-Rha-Glc], 399. ^{1}H (d_5-pyridine, 500 MHz) and ^{13}C NMR (d_5-pyridine, 125 MHz), see Tables 1–4.

3.6. Shatavarin VII (**2**)

Amorphous solid, $[\alpha]_D$ −42.1 (*c*, 0.04, pyridine). HRMS: Calcd for $C_{45}H_{72}NaO_{17}$ (M+Na): 907.4667. Found: 907.4664. Negative ion ESI *m/z*: 883 [M−1]. Positive ESI-MS *m/z*: 885 [M+1], 723 [M-Glc], 577 [M-Glc-Rha], 415 [M-Glc-Rha-Glc], 397. ^{1}H (d_5-pyridine, 750 MHz) and ^{13}C NMR (d_5-pyridine, 188 MHz), see Tables 1–4.

3.7. Shatavarin VIII (**3**)

Amorphous solid, $[\alpha]_D$ −40.6 (*c*, 0.16, pyridine). HRMS: Calcd for $C_{50}H_{82}NaO_{22}$ (M+Na): 1057.5195. Found: 1057.5203. Negative ion ESI *m/z*: 1033 [M−1]. Positive ESI-MS *m/z*: 1035 [M+1], 873 [M-Glc], 741 [M-Glc-Xyl], 711[M-Glc-Glc], 579 [M-Glc-Glc-Xyl], 579 [M-Glc-Glc-Xyl-Glc], 399. ^{1}H (d_5-pyridine, 750 MHz) and ^{13}C NMR (d_5-pyridine, 188 MHz), see Tables 1–4.

3.8. Shatavarin IX (**4**)

Amorphous solid, $[\alpha]_D$ −42.2 (*c*, 0.04, pyridine). HRMS: Calcd for $C_{45}H_{74}NaO_{18}$ (M+Na): 925.4772. Found: 925.4775. Negative ion ESI *m/z*: 901 [M−1]. Positive ESI-MS *m/z*: 903 [M+1], 741 [M-Glc], 579 [M-Glc-Glc], 417 [M-Glc-Glc-Glc], 399. ^{1}H (d_5-pyridine, 500 MHz) and ^{13}C NMR (d_5-pyridine, 125 MHz), see Tables 1–4.

3.9. Shatavarin X (**5**)

Amorphous solid, $[\alpha]_D$ −50.0 (*c*, 0.02, pyridine). HRMS: Calcd for $C_{47}H_{76}NaO_{18}$ (M+Na): 951.4929. Found: 951.4937. Negative ion ESI *m/z*: 927 [M−1]. Positive ESI-MS *m/z*: 929 [M+1], 783 [M-Rha], 725 [M-Glc-43], 579

Table 3
^{1}H spectral data (δ in ppm) for the sugar part of compounds **1**–**5** in pyridine-d_5

Proton	**1**	**2**	**3**	**4**	5
	3-*O*-β-D-Glucose	3-*O*-β-D-Glucose	3-*O*-β-D-Glucose	3-*O*-β-D-Glucose	3-*O*-β-D-Glucose
H-1	4.85 *d* (8.2)	4.88 *d* (8.0)	4.75 *d* (8.2)	4.84 *d* (8.0)	4.82 *d* (7.8)
H-2	4.20 *dd* (8.8, 9.0)	4.27 *dd* (9.2, 8.0)	4.11 *dd* (8.2, 9.2)	4.26 *dd* (9.2, 8.0)	4.24 *m*[a]
H-3	4.23 *dd* (9.1, 9.1)	4.28 *dd* (9.2, 9.2)	4.16 *dd* (9.2, 9.2)	4.29 *dd* (9.2, 9.2)	4.22 *m*[a]
H-4	4.45 *dd* (9.1, 9.1)	4.38 *dd* (9.1, 9.1)	4.34 *dd* (9.2, 9.2)	4.23 *dd* (9.1, 9.1)	4.15 *dd* (9.4, 9.4)
H-5	3.60 *brd* (9.2)	3.62 *brd* (9.0)	3.75 *brd* (9.2)	3.80 *brd* (9.0)	3.84 *ddd* (3.3, 3.3, 9.4)
H-6a	4.04 *dd* (5.7, 12.0)	4.07 *dd* (3.6, 12.0)	4.60 *dd* (1.3, 12.0)	4.42-4-51 *m*	4.47 *m*[a]
H-6b	4.73 *brd* (12.0)	4.23 *brd* (12.0)	4.73 *dd* (2.8, 12.0)		
	2′-*O*-β-D-Glucose	2′-*O*-β-D-Glucose	2′-*O*-β-D-Glucose	2′-*O*-β-D-Glucose	4′-*O*-β-D-Glucose
H-1	5.43 *d* (7.6)	5.45 *d* (7.8)	5.36 *d* (8.0)	5.40 *d* (8.0)	5.06 *d* (7.9)
H-2	4.07 *dd* (7.6, 8.6)	4.07 *dd* (7.8, 8.6)	3.98 *dd* (8.2, 9.0)	4.02 *dd* (8.0, 8.8)	4.00 *dd* (8.0, 9.3)
H-3	4.24 *dd* (8.8, 8.6)	4.25 *dd* (8.9, 8.9)	4.23 *dd* (9.1, 9.1)	4.24 *dd* (8.8, 9.0)	4.17 *dd* (9.3, 9.3)
H-4	4.31 *dd* (8.8, 8.8)	4.27 *dd* (8.8, 8.8)	4.22 *dd* (9.2, 9.0)	4.29 *dd* (9.0, 9.0)	3.93 *dd* (9.3, 9.3)
H-5	3.94 *ddd* (3.4, 4.9, 9.2)	3.97 *ddd* (2.7, 5.1, 9.2)	3.95 *ddd* (3.1, 4.9, 9.2)	3.95 *ddd* (3.3, 5.0, 9.0)	4.10 *ddd* (2.2, 8.0, 9.3)
H-6a	4.48 *dd* (4.9, 12.0)	4.45 *dd* (5.2, 12.0)	4.44 *dd* (5.0, 12.0)	4.47 *brd* (12.0)	4.57 *dd* (8.0, 12.0)
H-6b	4.56 *brd* (12.0)	4.58 *dd* (2.7, 12.0)	4.57 *dd* (3.1, 12.0)	4.56 *brd* (12.0)	4.90 *dd* (2.2, 12.0)
6-0-Ac					171.6 (Me)
	4’-*O*-α-L-rhamnose	4’-*O*-α-L-rhamnose	6’-*O*-β-D-Glucose	4’-*O*-β-D-Glucose	2’-*O*-α-L-rhamnose
H-1	5.90 *brs*	5.82 *brs*	5.27 *d* (8.0)	5.14 *d* (8.3)	6.32 *brd* (1.1)
H-2	4.66 *brs*	4.67 *brs*	4.04 *dd* (8.0, 8.7)	4.05 *dd* (8.3, 8.7)	4.72 *brs*
H-3	4.53 *dd* (2.9, 9.2)	4.55 *dd* (3.8, 9.1)	4.22 *dd* (8.8, 8.8)	4.19 *m*	4.52 *dd* (3.2, 9.6)
H-4	4.31 *dd* (9.2, 9.2)	4.35 *dd* (9.2, 9.2)	4.24 *dd* (9.8, 9.6)	4.21 *m*	4.30 *dd* (9.6, 9.6)
H-5	4.97 *dq* (5.8, 9.2)	4.93 *dq* (6.2, 9.2)	3.94 *ddd* (2.8, 5.2, 8.6)	3.98 *m*	4.78 *dq* (6.3, 9.6)
H-6a	1.66 *d* (5.8)	1.66 *d* (6.2)	4.35 *dd* (5.2, 12.0)	4.28 *m*	1.73 *d* (6.3)
H-6b			4.49 *dd* (2.8, 12.0)	4.49 *brd* (12.0)	
			4’-*O*-α-L-Arabinose		
H-1			5.29 *d* (8.1)		
H-2			4.43 *dd* (8.2, 8.3)		
H-3			4.22 *m*[a]		
H-4			4.22 *m*[a]		
H-5			4.22 *m*[a]		

[a] Indicates overlapping signals.

Table 4
^{13}C spectral data (δ in ppm) for the sugar moiety of compounds 1-5 in pyridine d_5

Carbon no.	1	2	3	4	5
Sugar moiety	3-*O*-β-D-Glucose	3-*O*-β-D-Glucose	3-*O*-β-D-Glucose	3-*O*-β-D-Glucose	3-*O*-β-D-Glucose
C-1	102.5	101.7	101.3	101.7	102.2
C-2	82.9	82.1	80.6	81.4	76.8
C-3	76.5	76.6	76.2	76.6	78.1
C-4	77.4	77.6	79.6	81.4	83.6
C-5	77.1	77.0	75.0	76.3	76.1
C-6	61.4	61.3	68.3	62.0	62.2
	2'-*O*-β-D-Glucose	2'-*O*-β-D-Glucose	2'-*O*-β-D-Glucose	2'-*O*-β-D-Glucose	4'-*O*-β-D-Glucose
C-1	105.7	105.4	105.2	1055	105.7
C-2	77.1	76.6	79.6	77.1	75.0
C-3	78.0	77.7	78.4	77.9	78.1
C-4	71.9	71.7	71.7	72.1	72.1
C-5	78.6	78.5	78.7	78.6	75.2
C-6	63.0	62.9	63.3	63.0	64.8
6-0-Ac					171.6 (C=O)
					20.8(Me)
	4'-*O*-α-L-rhamnose	4'-*O*-α-L-rhamnose	6'-*O*-β-D-Glucose	4'-*O*-β-D-Glucose	2'-*O*-α-L-rhamnose
C-1	101.9	102.5	105.1	105.0	101.9
C-2	72.6	72.4	75.3	75.1	72.4
C-3	72.8	72.6	78.0	78.5	74.2
C-4	74.0	73.9	69.9	71.6	74.2
C-5	70.3	70.3	78.5	78.5	69.7
C-6	18.5	18.5	62.8	62.3	18.9
			4'-*O*-α-L-Arabinose		
C-1			105.2		
C-2			72.6		
C-3			74.7		
C-4			72.3		
C-5			67.8		

[M-Rha-Glc-43], 417 [M-Glc-Rha-Glc-43], 399. ^1H (d_5-pyridine, 500 MHz) and ^{13}C NMR (d_5-pyridine, 125 MHz), see Tables 1–4.

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