Antifungal Activity of the Saponin Fraction Obtained from *Asparagus officinalis* L. and Its Active Principle

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Antifungal activity was detected in the crude saponin fraction obtained from the bottom cut of *Asparagus officinalis* L. This activity was specific to certain fungi, for example *Candida, Cryptococcus, Trichophyton, Microsporum* and *Epidermophyton*. Attempts were made to isolate the active principles from this fraction; in this way a new saponin (AS-1) was isolated, and its structure was estimated to be 3-*O*-[{β-D-glucopyranosyl(1→2)}{β-D-xylopyranosyl(1→4)-β-D-glucopyranosyl]-(25*S*),5β-spirostan-3β-ol. The minimum inhibitory concentration (MIC) ranged from 0.5 μg/ml to more than 8 μg/ml depending upon the nature of the fungi. On the basis of the work carried out here, it is probable that asparagus will contain additional antifungal principles.

To date, many saponins have been isolated and characterized from asparagus.[1] These constituents may have a bitter taste,[2] and those parts which have a large amount of saponins in the 'bottom cut' must be discarded during processing. In India and other countries, asparagus has long been utilized for medicinal purposes, and saponins are considered to be the active principles possessing such physiological activities.[3] Antifungal activities have been reported for several kinds of saponins.[4–6] We have thus attempted to determine the antifungal activity of the waste products from asparagus processing and to isolate and characterize the active antifungal principle.

Materials and Methods

Material. The crude saponin fraction was prepared from the bottom cut of white asparagus (*Asparagus officinalis* L. cv. Merry Washington 500W), which was obtained from factory waste.

Preparation of the crude saponin fraction from asparagus. Dried bottom cut (3 kg) was extracted twice with 60% ethanol (50 l) at room temperature for 24 hr. The two extracts were combined, the solvent being removed under reduced pressure. The product was dispersed in 1-butanol–water (1 : 1, v/v), the mixture centrifuged, and the 1-butanol layer collected and concentrated under reduced pressure. The concentrated extract was dispersed in benzene–water (1 : 1, v/v), the water phase extracted again with 1-butanol, and the butanol extract dissolved in methanol (300 ml). Ether (8 l) was added dropwise, with stirring; the precipitate was collected as the crude saponin fraction (11.6 g).

Measurement of antifungal activity. Antifungal activity was determined by using the paper disc method.[7] The fungi used in this study are shown in Table I, and were preincubated for one or two weeks on an agar culture broth [yeast extract (3 g), malt extract (3 g), peptone (5 g) and glucose (10 g) with agar (10 g) in water (1 l)]. Mycelia were collected, suspended in an NaCl solution (0.9% with 0.05% Tween), and inoculated into new agar plates (5×10^4 cells/ml).

Pieces of paper disc (Whatman AA discs, 6 mm diameter) were soaked in an ethanol solution (10 mg/ml) of a sample. These pieces corresponding to 25 μl of a sample were placed on the surface of the agar culture broth. A piece of control disc containing only ethanol was also placed on the same agar plate. The whole system was kept at 30°C and, after a week, the antifungal activity was measured.

Measurement of the minimum inhibitory concentration (MIC) of the crude saponin fraction and isolated saponin. Fungi were prepared as already mentioned for the paper disc method. Fungal spores were suspended (5×10^4 cells/

ml) in tubes containing broth (5 ml) without agar, the saponins being added at levels of 1–200 μg/ml. The tubes were incubated for one weeks at 30°C and the MIC was determined.

Chromatography. Reversed phase silica gel column chromatography was carried out on octadecyl silica gel (YMC-GEL ODS-A; 230 × 18 mm i.d.; Yamamura Chemical Laboratories Co., Ltd.). High-performance liquid chromatography (HPLC) was performed by using a Waters model 510 pump with a Rheodyne model 7125 injection valve. The column used was a YMC Pack AM-323-7 (7 μm, 250 × 10.0 mm i.d.; Yamamura Chemical Laboratories Co.,Ltd.). Detection was carried out by an RI detector (ERC-7520; Erma Optical Works Ltd.).

Identification of individual component sugars of the isolated saponin. The saponin was hydrolyzed with 2 N trifluoroacetic acid (100°C, 6 hr). The hydrolysis products were dissolved in distilled water and then analyzed by HPLC, using a Shimadzu LC-6A pump. The column was a TSK-gel Sugar AXG (150 × 4.6 mm i.d.; Tosoh), temperature was 70°C and flow rate was 0.4 ml/min. Detection was carried out by a fluorescence detector (Shimadzu RF-533; reaction reagent, 1% arginine–3% boric acid; flow rate, 0.5 ml/min; temperature, 150°C; excitation, 320 nm and emission, 430 nm).

The saponin (1 mg) was methylated by a slight modification of Hakomori's method[8] and then hydrolyzed. The hydrolyzate was reduced with $NaBH_4$, and the liberated alditol acetates analyzed by GC-MS.

Spectroscopy. NMR spectra were recorded on a JEOL GSX-400 spectrometer (^1H-NMR at 400 MHz, ^{13}C-NMR at 100 MHz). Mass spectra were obtained with a JEOL JMS HX-105. Optical rotation $[\alpha]_D$ was measured with a Jasco J-20 spectrometer at 589 nm wavelength, and IR spectra were recorded with a Jasco A-202 instrument.

Results and Discussion

Antifungal activity of the crude saponin fraction obtained from asparagus

The crude saponin fraction was obtained from the bottom cut of white asparagus, which had been discarded by the food factory due to its bitter taste. The antifungal activities of this fraction against several fungi were determined by the paper disc method, and are shown in Table I. This fraction had clear antifungal activity against certain fungi, namely *Candida albicans*, *Cryptococcus albidus*, *Epidermophyton floccosum*, *Microsporum gypseum* and *Trichophyton* spp. On the other hand, this fraction was ineffective against other fungi, *e.g.*, *Rhizopus* and *Chaetomium*. Within the *Aspergillus* genus, some species were sensitive, *e.g.*, *luchuensis* and *repens*, but the others were insensitive. Thus, this antifungal activity was very specific. To examine this further, an attempt was made to separate and isolate the active principles.

Table I. ANTIFUNGAL ACTIVITIES OF THE CRUDE SAPONIN FRACTION FROM *Asparagus officinalis* L.

Fungus		Activity
Aspergillus candidus	AHU 7017	–
Aspergillus chevalieri	AHU 7443	–
Aspergillus fumigatus	IFO 9733	–
Aspergillus luchuensis	AHU 7092	±
Aspergillus niger	IFO 4091	–
Aspergillus oryzae	S-01	–
Aspergillus repens	AHU 7458	±
Aspergillus sulphureus	AHU 7182	–
Aspergillus tamarii	AHU 7367	–
Aspergillus terreus	FERM S-3	–
Aspergillus versioclor	AHU 7200	–
Aspergillus wentii	AHU 7207	–
Candida albicans	IFO 1061	+
Chaetomium globosum	FERM S-11	–
Cryptococcus albidus	IFO 1420	+
Epidermophyton floccosum	IFO 9045	+
Fusarium oxysporum	IFO 31213	–
Microsporum gypseum	IFO 8307	+
Mucor racemosus	IFO 4581	±
Penicillium funiculosum	FERM S-6	–
Penicillium italicum	IFO 9419	+
Rhizopus stolonifer	IFO 30816	–
Sporothrix schenckii	IFO 8158	–
Trichophyton mentagrophytes	IFO 5809	+
Trichophyton rubrum	IFO 5808	+
Trichophyton tonsurans	IFO 5945	+
Trichophyton violaceum	IFO 31064	+

Isolation of antifungal principle from the crude saponin fraction

The crude saponin fraction of asparagus was subjected to ODS column chromatography. The main saponin-containing fraction, which was almost inactive, was eluted first, and thereafter, the active fraction was eluted with methanol–water (8 : 2). This fraction was subjected to reversed phase silica gel HPLC. Two main constituents (AS-1 and AS-2) were separated and eluted (Fig. 1). The antifungal

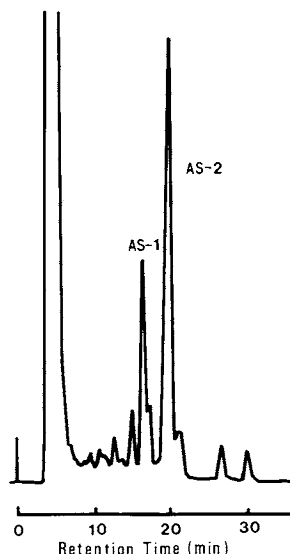

Fig. 1. Liquid Chromatograms of the Antifungal Fraction from Asparagus.

The mobile phase was methanol–water (88 : 12, v/v).

activity was detected in fraction AS-1. The fraction containing AS-1 was collected and, following removal of the solvent, afforded pure saponin (17 mg).

Structural elucidation of AS-1

The physical properties of AS-1 were as follows: Mp 182–184°C, $[\alpha]_D^{24}$ $-26.8°$ ($c=0.1$, MeOH). IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 980, 915, 890 and 850 (intensity 915 > 890 showed the presence of 25S-spiroketal[9]). FAB-MS showed ion peaks at m/z 873 ($[M+H]^+$) and 895 ($[M+Na]^+$), indicating a molecular weight of 872. *Anal.* Found: C, 58.16; H, 8.33%. Calcd. for $C_{44}H_{72}O_{17} \cdot 2H_2O$: C, 58.15; H, 8.37%. Acid hydrolysis of AS-1 gave xylose (t_R, 61.58 min) and glucose (81.05 min). One 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, one 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl glucitol and 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylitol were identified following the methylation, methanolysis and reduction of AS-1. The high-resolution EI-MS of the hydrolysis product of AS-1 showed a molecular ion at m/z 416.328 indicating $C_{27}H_{44}O_3$. ^{13}C-NMR data (Table II) of this showed the aglycone to be of an identical pattern to that of

Table II. ^{13}C-NMR SPECTRAL ASSIGNMENTS FOR AS-1 (δ_C ppm)

	Aglycone moiety			Sugar moiety	
	AS-1[a]	Aglycone[b]	Reference[c]		
C-1	30.8	29.9	29.9		
C-2	26.8	27.8	27.8	Glc-1′	101.7
C-3	80.6	67.1	67.0	Glc-2′	81.7
C-4	30.7	33.5	33.6	Glc-3′	75.3
C-5	36.6	36.5	36.5	Glc-4′	76.2
C-6	27.5	26.5	26.6	Glc-5′	76.3
C-7	26.8	26.5	26.6	Glc-6′	61.7
C-8	35.5	35.3	35.5		
C-9	40.3	40.3	40.3		
C-10	35.2	35.3	35.3	Glc-1″	105.4
C-11	21.1	20.9	20.9	Glc-2″	77.1
C-12	40.2	39.8	39.9	Glc-3″	77.9
C-13	40.9	40.7	40.6	Glc-4″	71.9
C-14	56.4	56.5	56.4	Glc-5″	78.6
C-15	32.1	31.7	31.7	Glc-6″	63.0
C-16	81.3	81.0	80.9		
C-17	61.7	62.3	62.1		
C-18	16.6	16.5	16.5		
C-19	24.0	23.9	23.9	Xyl-1‴	105.5
C-20	42.5	42.1	42.1	Xyl-2‴	75.0
C-21	14.9	14.3	14.3	Xyl-3‴	78.4
C-22	109.7	109.7	109.5	Xyl-4‴	70.8
C-23	27.0	27.1	27.1	Xyl-5‴	67.4
C-24	26.4	25.9	25.8*		
C-25	26.2	25.8	26.0*		
C-26	65.1	65.1	65.0		
C-27	16.3	16.0	16.1		

[a] Solvent: d_5-pyridine, 25°C.
[b] Solvent: $CDCl_3$, 25°C.
[c] Sarsasapogenin[10]: solvent, $CDCl_3$.
* These assignments may be interchangeable.

sarsasapogenin,[10] thus confirming the assignment of sarsasapogenin. Proton assignments of the carbohydrate moieties were made by ^1H-NMR and by ^1H–^1H COSY and ^{13}C–^1H COSY techniques (Table III). Three anomeric protons at δ_H 4.47 ppm (d, $J=8$ Hz), δ_H 4.70 ppm (d, $J=8$ Hz) and δ_H 4.34 ppm (d, $J=8$ Hz) were identified, consistent with two β-linked glucose moieties and one β-linked xylose moiety, respectively.

The linkages of the carbohydrate moieties were estimated by Nuclear Overhauser Effect (NOE) experiments (Fig. 2), which were carried at $-25°C$ in d_4-methanol. Irradiation at δ_H 4.72 ppm (the anomeric proton of the terminal

Table III. ^{1}H-NMR SPECTRAL DATA FOR AS-1 (δ_H ppm)

Glc-1′	4.47 (d, $J=8$)
Glc-2′	3.62 (dd, $J=8$, 9)
Glc-3′	3.71 (dd, $J=9$, 9)
Glc-4′	3.52 (dd, $J=9$, 9)
Glc-5′	3.38 (m)
Glc-6′	3.81 (dd, $J=4$, 12)
	3.87 (dd, $J=2$, 12)
Glc-1″	4.70 (d, $J=8$
Glc-2″	3.17 (dd, $J=8$, 9)
Glc-3″	3.36 (dd, $J=9$, 9)
Glc-4″	3.21 (dd, $J=8$, 9)
Glc-5″	3.25 (m)
Glc-6″	3.66 (dd, $J=5$, 12)
	3.84 (dd, $J=2$, 12)
Xyl-1‴	4.34 (d, $J=8$)
Xyl-2‴	3.19 (dd, $J=8$, 9)
Xyl-3‴	3.31 (dd, $J=9$, 9)
Xyl-4‴	3.49 (m)
Xyl-5‴	3.25 (dd, $J=10$, 12)
	3.90 (dd, $J=5$, 10)

Solvent: d_4-MeOH with TMS, 25°C.

glucose residue) showed negative NOE at 3.64 ppm, which was assigned to the H-2′ position of the inner glucose residue, whilst irradiation at 4.30 ppm (xylose) showed negative NOE at 3.50 ppm, which was assigned to the H-4′ position. Further irradiation at 4.50 ppm (inner glucose) showed NOE at 4.10 ppm, the signal corresponding to the H-3 position of aglycone.

From all the foregoing studies, the structure of new saponin AS-1 was estimated to be 3-*O*-[{β-D-glucopyranosyl(1→2)}{β-D-xylopyranosyl(1→4)}-β-D-glucopyranosyl]-(25*S*), 5β-spirostan-3β-ol (Fig. 3) and can be considered as a newly isolated saponin.

Measurement of MIC

The minimum inhibitory concentrations of the crude saponin fraction and AS-1 to several fungi are shown in Table IV. The MICs of the crude saponin fraction ranged from 5 to 20 μg/ml for most fungi, with *Mucor racemosus*, *Aspergillus luchuensis* and *A. repens* showing higher figures. Some species of *Aspergillus* were insensitive, the crude fraction having only weak antifungal activity against this genus. Isolated AS-1 showed lower MICs for almost all fungi. The antifungal activity of saponins isolated from *Dolichos kilimandscharicus* has been

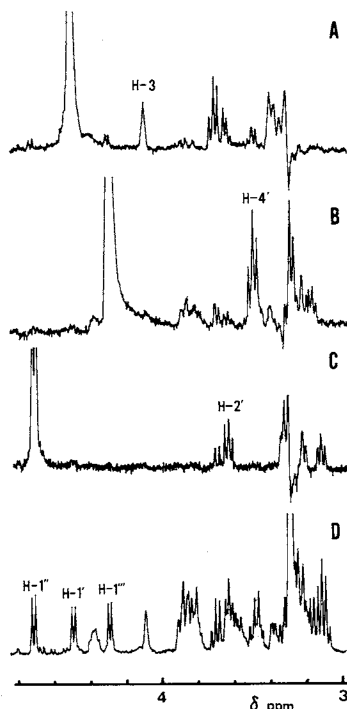

Fig. 2. ^{1}H-NMR Spectra of AS-1.

Part of the sugar moieties is shown. A, NOE difference spectrum of AS-1 irradiated at H-1′ (inner glucose residue of AS-1, δ 4.50 ppm); B, at H-1‴ (xylose residue, δ 4.30 ppm); C, at H-1″ (terminal glucose residue, δ 4.72 ppm); D, normal ^{1}H-NMR spectrum of AS-1. All spectra were recorded at −25°C in d_4-methanol with TMS as the internal standard.

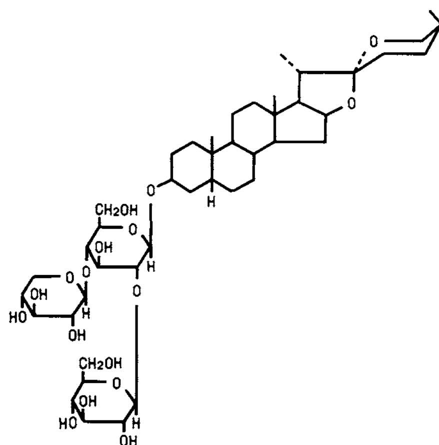

Fig. 3. Structure of AS-1.

Table IV. Minimum Inhibitory Concentration (MIC) of the Crude Saponin Fraction and Isolated Saponin, AS-1, from *Asparagus officinalis* L. (μg/ml)

Fungus	Crude[a]	AS-1
Aspergillus luchuensis	200 <	—
Aspergillus repens	100–200	—
Candida albicans	5–10	20–30
Cryptococcus albidus	5–10	4–5
Epidermophyton floccosum	5–10	0.5–1
Microsporum gypseum	5–10	1–2
Mucor racemosus	50–100	8–10
Penicillium italicum	10–20	10–20
Trichophyton mentagrophytes	5–10	8–9
Trichophyton rubrum	5–10	2–3
Trichophyton tonsurans	10–20	1–2
Trichophyton violaceum	10–20	4–5

[a] Crude saponin fraction.

found to range from 2.5 to 5 μg.[4)] Whilst these activity values were estimated by a different method, the values obtained appear to be of a similar order to that of AS-1. The MICs of AS-1 against *Epidermophyton floccosum* and *Mucor racemosus* were ten times lower than that associated with the crude fraction, but the MICs of the other fungi were generally not much different for either the crude saponin fraction or AS-1. The MIC to *Candida albicans* was higher for AS-1 than for the crude saponin fraction. It is concluded, therefore, that there must be additional antifungal principles in asparagus. These antifungal principles in the waste parts of asparagus are currently under investigation.

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