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An Antifungal Saponin from White Asparagus (*Asparagus officinalis* L) Bottoms

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Abstract: An antifungal saponin was isolated from the bottom cut of white asparagus (*Asparagus officinalis* L), which is unusable in food processing. The structure of the saponin was identified as 3-*O*-[{α-L-rhamnopyranosyl (1 → 2)} {α-L-rhamnopyranosyl (1 → 4)}-β-D-glucopyranosyl] (25*S*) spirost-5-ene-3β-ol from chemical and spectral data. This saponin was shown to be identical with collettinside III from *Dioscorea collettii*, and to inhibit the growth of some kinds of fungi at μg ml^{-1} levels.

Key words: *Asparagus officinalis*, steroidal saponin, collettinside III, antifungal, *Candida albicans*.

INTRODUCTION

Asparagus is a familiar food as well as a medicinal plant in India and other countries. White asparagus is mostly processed into the canned product. For this purpose, the 'bottom cut' must be discarded because of its bitter taste, which is attributed to saponin constituents. Kawano *et al* (1975, 1977) investigated the bitter components in asparagus and reported their structures to be furostanol saponins. Goryanu *et al* independently isolated and characterised nine steroidal saponins (asparagosides A to I) from *Asparagus officinalis*, whose structures were summarized in a review (Price *et al* 1987). Further, many saponins were isolated from other species in the *Asparagus* genus (Price *et al* 1987; and references therein).

Many kinds of biological activities of saponins have been reported to date (Price *et al* 1987, Kitagawa *et al* 1985). However, there are only a few reports on the biological activities of asparagus saponins (Sati *et al* 1985, Pant *et al* 1988). In our previous paper (Shimoyamada *et al* 1990), a crude saponin fraction from white asparagus bottom cut strongly inhibited the growth of some kinds of fungi. Interestingly, *Candida albicans*, which is responsible for contamination in food processing and candidiasis, and some species of *Trichophyton*, *Microsporum* and *Epidermophyton*, which are responsible for so-called ringworm, were suppressed effectively. A new antifungal saponin, tentatively designated AS-1, was isolated as the active principle. AS-1 suppressed the growth of *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton* spp, etc at levels of 0·5–5 μg ml^{-1}. However, its activity against *Candida albicans* was relatively lower than the crude saponin fraction (minimum inhibitory concentration (MIC) 30 μg ml^{-1} for AS-1).

We attempted, therefore, to isolate and characterize further antifungal constituents from white asparagus bottoms, especially those suppressing the growth of *Candida albicans*.

MATERIALS AND METHODS

Material

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

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Preparation and fractionation of crude saponin fraction from asparagus

The crude saponin fraction was prepared by the method described in the previous paper (Shimoyamada *et al* 1990). The material obtained was dispersed in methanol/water (60 : 40, v/v), subjected to ODS column chromatography (YMC GEL ODS-A; 230 × 18 mm id), equilibrated with methanol/water (60 : 40, v/v) and eluted with methanol/water (50 : 50, v/v), then (70 : 30, v/v) and finally methanol. Each eluted fraction was monitored with TLC and antifungal activity against *Candida albicans*.

Thin-layer chromatography (TLC)

The TLC plate was a Kieselgel 60F-254 (0·25 mm thickness, Merck). It was developed with chloroform/methanol/water (7 : 3 : 1, v/v/v; upper layer). The developed plate was dried and sprayed with a detecting reagent and heated as necessary. Detecting reagents were 100 ml litre^{-1} sulphuric acid and Ehrlich's reagent prepared by dissolving the 4-dimethylaminobenzaldehyde (1 g) in a conc hydrochloric acid/methanol (10 : 75, v/v) mixture (85 ml), and 20 μl ml^{-1} erythrocyte dispersed in phosphate buffered saline (PBS).

Measurement of antifungal activity of isolated saponin

The method previously described (Shimoyamada *et al* 1990), now outlined, was used. The fungi 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 litre)). Fungal spores were suspended (5×10^4 cells ml^{-1}) in tubes containing broth (5 ml) without agar, the saponins being added at appropriate levels. The tubes were incubated for one week at 30°C and the MIC was determined.

High-performance liquid chromatography (HPLC)

HPLC was performed using a Waters 510 pump or a Jasco BIP-I HPLC pump with Rheodyne model 7125 injection valve. The columns were a YMC Pack ODS AM-303 (5 μm, 250 × 4·6 mm id) and a YMC Pack ODS AM-323-7 (7 μm, 250 × 10·0 mm id). Detection was carried out by a Hitachi variable wavelength UV monitor (210 nm) or a Jasco 830-RI detector.

Acid hydrolysis of isolated saponin and separation of aglycone and constituted sugars

A saponin (20 mg for analysing aglycone and 2 mg for sugars) was previously dissolved in 0·5 ml of methanol and mixed with 2·5 ml of 3 M trifluoroacetic acid solution, then heated at 100°C for 6 h. The hydrolysate was evaporated and the dried sample dispersed in 2 ml of water and washed three times with an equal volume of diethyl ether. The ether-soluble fraction was identified by TLC and ^{13}C-NMR experiments.

The water-soluble fraction obtained from acid hydrolysis of a saponin was transferred to aminopyridyl sugars following Kondo *et al* (1990) with a slight modification and applied to HPLC. Sugar analyses were carried out by a Waters LC Module 1 (Millipore Co) and a Waters M470 scanning fluorescence detector (ex, 310 nm, em, 380 nm, Millipore Co). The column was a Palpak Type A (8 μm, 150 × 4·6 mm id; Takara Co), and the mobile phase was 0·4 M potassium borate buffer (pH 9·0)/acetonitrile (90 : 10).

Spectroscopy

NMR spectra were recorded on a JEOL GSX-400 (^{1}H at 400 MHz, ^{13}C at 100 MHz) and a GX-270 (^{1}H at 270 MHz, ^{13}C-NMR at 67·8 MHz) spectrometer. Mass spectra were obtained with a JEOL JMS HX-105. IR spectra were recorded with a System 2000 FT-IR spectroscopy (Perkin Elmer) by KBr methods.

RESULTS AND DISCUSSION

Fractionation and isolation of antifungal constituents in crude saponin fraction from asparagus

The crude saponin fraction prepared from the bottom cut of white asparagus was first applied to an ODS column and three fractions (F1, F2 and F3; Fig 1) were separated. Each fraction was then concentrated under reduced pressure and analysed with TLC. F1 (methanol/water, 60 : 40, v/v) consisted of non-saponin

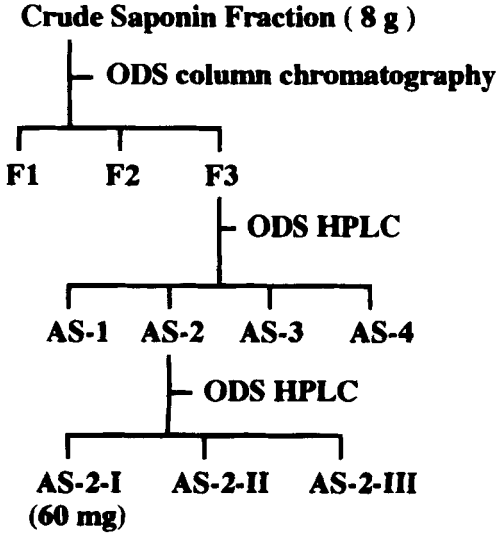

Fig 1. Isolation procedures of asparagus saponins.

components and F2 (methanol/water, 70 : 30, v/v) of saponins which showed low-R_f values on TLC. These constituents were considered furostanol saponins because they were positive to Ehrlich's reagent (Kawano *et al* 1975). F3 (methanol fraction) consisted of saponins which showed high R_f values and were negative to Ehrlich's reagent. As the antifungal activity of each fraction against *Candida albicans*, only F3 showed the high antifungal activity. This fraction was collected to provide the active fraction.

The active fraction was successively applied to the reversed-phase HPLC. The mobile phase used was methanol/water, 88 : 12 (v/v) and eluents were monitored with RI detector or UV detector (210 nm). Four main fractions designated AS-1, AS-2, AS-3 and AS-4 were obtained and their anti-*Candida* activities were measured (Table 1). AS-1 inhibited the growth of *Candida albicans* at levels almost equal to those in the previous paper (Shimoyamada *et al* 1990) and AS-2 inhibited it to about 8 $\mu g\ ml^{-1}$. However, AS-3 and AS-4 did not inhibit it, AS-2 fraction was collected, concentrated under reduced pressure and lyophilised.

AS-2 fraction obtained from HPLC was shown to consist of more than two components by NMR spectroscopy. Then this fraction was rechromatographed on HPLC with reconstituted elution systems (acetonitrile/water, 44 : 56, v/v). AS-2 fraction appeared to consist of three components, tentatively named AS-2-I, II and III. In these constituents, AS-2-I showed strong anti-*Candida* activity, AS-2-I was isolated (*c* 60 mg from 2·3 kg of dried white asparagus bottom).

Structural elucidation of antifungal saponin

In order to estimate the structure of AS-2-I, this constituent was subjected to mass and other spectroscopy procedures. FAB-MS of AS-2-I showed ion peaks at *m/z* 869 ($[M + H]^+$) and 891 ($[M + Na]^+$) indicating a molecular weight of 868. Then, AS-2-I was hydrolysed with 3M TFA. An aglycone moiety obtained was removed by extraction with diethyl ether from hydrolysate; the residue afforded the constituent sugar fraction. Comparing the ^{13}C-NMR spectrum of aglycone moiety (Table 2), the aglycone of AS-2-I was shown to be the mixture of yamogenin (25*S*-spirost-5-ene-3β-ol) and diosgenin (25*R*-spirost-5-ene-3β-ol). On the other hand, the IR spectrum of AS-2-I showed bands at 3412 (O–H), 2937 (C–H), 1630, 1043 (C–O–C), 988, 920, 897 and 838 cm^{-1}. As the band at 920 cm^{-1} was stronger

TABLE 1
Anti-*Candida* activities of asparagus saponins

	MIC[a] ($\mu g\ ml^{-1}$)
Crude saponin	32
AS-1	32
AS-2	8
AS-3	>128
AS-4	>128

[a] MIC, minimum inhibitory concentration.

TABLE 2
^{13}C NMR spectral data for AS-2-I and its aglycone

	AS-2-I[a]	*Aglycone*[b]	*Yamogenin*[c]
Ag-1	37·6	37·3	37·3
2	30·3	31·5[d]	31·4
3	78·2	71·8	71·6
4	39·0	42·3	42·3
5	140·9	140·9	140·9
6	122·0	121·5	120·3
7	32·4	32·1	32·0
8	31·8	31·4[d]	31·4
9	50·4	50·9	50·1
10	37·2	36·7	36·6
11	21·2	20·9	20·9
12	39·9	39·8	39·8
13	40·5	40·3	40·3
14	56·7	56·6	56·5
15	32·3	31·9	31·8
16	81·3	80·9	80·8
17	62·7	62·2	62·1
18	16·4	16·3	16·3
19	19·5	19·5	19·4
20	42·6	42·2	42·2
21	15·0	14·6	14·3
22	110·0	109·4	109·7
23	27·6	26·0	26·0
24	26·3	25·8	25·8
25	26·5	27·1	27·1
26	65·2	65·2	65·2
27	16·5	16·1	16·1
glc-1	100·3		
2	78·1		
3	77·8		
4	78·7		
5	76·9		
6	61·3		
rha-1	102·9		
2	72·4		
3	72·7		
4	74·0		
5	70·5		
6	18·7		
rha-1′	102·2		
2′	72·4		
3′	72·6		
4′	73·8		
5′	69·7		
6′	18·6		

[a] Solvent: d_5-pyridine.
[b] Solvent: $CDCl_3$.
[c] Solvent: $CDCl_3$ (Agrawal *et al* 1985).
[d] May be interchangable.

than at 897 cm^{-1}, the aglycone moiety was considered to be a 25 *S*-spiroketal, namely, a yamogenin (Brain *et al* 1968). During acid hydrolyses of saponins, the 25*S*-type steroidal aglycone (yamogenin) is reported to be isomerized to 25*R*-type aglycone (diosgenin) (Kawano *et al* 1977).

The sugar fraction was analyzed by HPLC equipped with a fluorescence detector following derivatisation to aminopyridylation. AS-2-I hydrolysate consisted of glucose and rhamnose (1 : 2; molar ratio).

The ^{13}C-NMR spectrum of AS-2-I (Table 2) was compared with those of yamogenin, glucose and rhamnose. On the aglycone (yamogenin) moiety, one great downfield-shifted signal was assigned to C-3 (δ 78·2) and two upfield-shifted ones were assigned to C-2 and C-4 (δ 30·3 and 39·0, respectively). These data showed the sugar chain linked to C-3 of yamogenin. For the glucose moiety, two downfield-shifted signals assigned to C-2 and C-4 (78·1 and 78·7, respectively) and other signals upfield-shifted suggested that two rhamnose moieties were linked to C-2 and C-4 of glucopyranose moiety.

On ^{1}H-NMR spectrum (Table 3), three anomeric protons at δ 4·94 (*d*, J = 7 Hz), 5·84 and 6·38 (both singlet like) were consistent with one β-linked glucopyranose moiety and two α-linked rhamnopyranose moieties, respectively.

From the overall above data, the structure of AS-2-I was elucidated to be 3-*O*-[{α-L-rhamnopyranosyl (1 → 2)} {α-L-rhamnopyranosyl (1 → 4)}-β-D-glucopyranosyl] (25*S*) spirost-5-ene-3β-ol (Fig 2). This structure coincides with that of collettinside III from *Dioscorea collettii* (Liu *et al* 1983).

TABLE 3
^{1}H NMR spectral data of sugar moieties of AS-2-I[a]

glc-1	4·94	(*d*, J = 8)
2	4·20	(*dd*, J = 8, 9)
3	4·18	(*dd*, J = 9, 9)
4	4·35	(*dd*, J = 9, 9)
5	3·64	(*m*)
6	4·07	(*dd*, J = 3, 9)
	4·20	(*dd*, J = 4, 9)
rha-1	5·84	(*d*, J = 2)
2	4·68	(*dd*, J = 2, 3)
3	4·55	(*dd*, J = 3, 9)
4	4·35	(*dd*, J = 9, 9)
5	4·92	(*m*)
6	1·61	(*d*, J = 6)
rha-1′	6·38	(*d*, J = 2)
2′	4·83	(*dd*, J = 2, 4)
3′	4·63	(*dd*, J = 4, 9)
4′	4·37	(*dd*, J = 9, 9)
5′	4·95	(*m*)
6′	1·76	(*d*, J = 6)

[a] Solvent, d_5-pyridine.

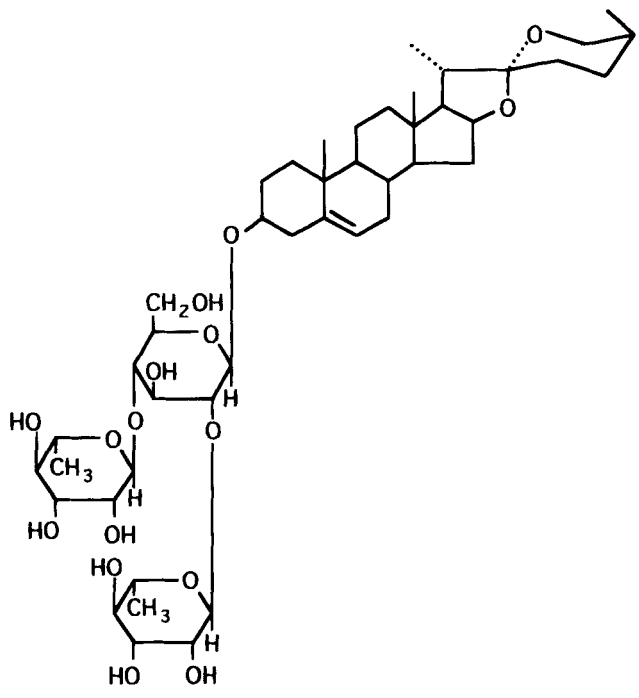

Fig 2. Structure of AS-2-I.

This constituent was first reported as yamoscin from the partial hydrolysate of saponin fraction from *Trigonella foenum-graecum* (Bogacheva *et al* 1976) and as 25*S*-isomer of dioscin from *Liriope platyphylla* (Watanabe *et al* 1983).

Measurement of minimum inhibitory concentration (MIC)

The MICs of AS-2-I were estimated against certain fungi (Table 4). AS-2-I showed very low MIC against *Microsporum gypseum*, *Trichophyton rubrum* and *Epidermophyton floccosum*, and a little higher MIC against *Candida albicans* and *Trichophyton mentagrophytes*.

Takechi *et al* (1991, 1992) and Takechi and Tanaka (1993) estimated the concentration causing 50% growth inhibition (GID_{50}) of natural or synthesised steroidal

TABLE 4
Antifungal activities of AS-2-I

Fungi	*MIC*[a] (μg ml^{-1})
Candida albicans	10
Microsporum gypseum	0·5
Trichophyton rubrum	0·5
Trichophyton mentagrophytes	5
Epidermophyton floccosum	1
Aspergillus oryzae	>100

[a] MIC, minimum inhibitory concentration.

saponins. Dioscin, 25*R*-isomer of AS-2-I, showed the GID_{50} of 0·9 μM against *Trichophyton mentagrophytes* and may be slightly more active than AS-2-I (*c* 6 μM), although there are some differences in the determination and expression of antifungal activities (Takechi *et al* 1991). The configuration having a 25-methyl group may influence the activity of saponins to some extent. The GID_{50} of other diosgenyl glycosides which have one sugar chain consisting of two or three moieties was about 5–6 μM against *Trichophyton mentagrophytes*. These values are almost equal to that of AS-2-I (Takechi *et al* 1992).

On the other hand, AS-2-I did not suppress *Aspergillus oryzae*. Further, we have some data that the crude saponin fraction from asparagus did not inhibit the growth of *Saccharomyces cerevisiae* (unpublished data). These saponins may selectively suppress some fungi.

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