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NOTE

Ascosteroside D, a new mitochondrial respiration inhibitor discovered by pesticidal screening using insect ADP/ATP carrier protein-expressing *Saccharomyces cerevisiae*

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A new lanostane-type triterpenoid, ascosteroside D, was isolated from a fungus, *Aspergillus* sp. FKI-6682. It inhibited insect ADP/ATP carrier protein (AAC)-expressing *Saccharomyces cerevisiae* in glycerol-containing medium, but did not inhibit *Δaac S. cerevisiae* in glucose-containing medium. It is hypothesized that ascosteroside D inhibits ATP production in mitochondria.

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A number of pesticides that inhibit the function of mitochondria have been found recently. Several commercial insecticidal and acaricidal compounds show inhibitory effects against the electron transport system complexes I and III, for example, fenpyroximate and strobilurin derivatives.[1,2] However, commercial pesticides targeting mitochondria are currently limited to those compounds inhibiting the electron transmission system.

ATP is produced in the mitochondrial inner membrane and transported by an ADP/ATP carrier protein (AAC). If compounds inhibit this crucial AAC, the mitochondria will not be able to produce ATP. Thus, the AAC would appear to be a good pesticidal target. Consequently, a screening system using insect AAC-expressing *S. cerevisiae* was developed by our group to find the compounds, especially those with a novel mechanism of action.[3] Two new mitochondrial inhibitors, ascosteroside C (**1**) and trichopolyn VI, have been found by this system to date.[4,5]

Further investigation of a cultured broth of *Aspergillus* sp. FKI-6682, producer of **1**, led us to discover a new mitochondrial inhibitor, named ascosteroside D (**2**) (Figure 1). *Aspergillus* sp. FKI-6682, isolated from a soil sample collected in Haha-jima, Japan, was cultured on an agar slant consisting of 0.1% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% yeast extract and 1.5% agar (adjusted to pH 6.0 before sterilization).[4] A loopful of spores was inoculated into seed medium (100 ml), consisting of 2.0% glucose, 0.5% Polypepton (Nihon Pharmaceutical Co., Tokyo, Japan), 0.2% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$ and 0.1% agar (adjusted to pH 6.0 before sterilization), in each of five 500 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker (210 r.p.m.) at 27 °C for 2 days. The seed culture (25 ml) was inoculated into each of 20 culture bags (Ulpack 47, HOKKEN Co. Ltd, Tochigi, Japan) containing a production medium (500 g of wet rice). Static fermentation was continued at 27 °C for 14 days.

The stationary culture (10 kg) was extracted with acetone (16 l). After filtration, the filtrate was concentrated *in vacuo* to remove acetone. The obtained aqueous solution was applied to an HP20 chromatography column (600 ml resin, Mitsubishi Chemical Co., Tokyo, Japan). After washing with H_2O (2.0 l) and 50% MeOH aq. (2.0 l), the active fraction eluted with MeOH was concentrated *in vacuo* to afford crude material. The active fraction was applied to an ODS chromatography column (300 ml resin, YMC Co., Kyoto, Japan). After being washed with H_2O (1.0 l), the column was eluted stepwise with 20, 40, 60, 80, 90 and 100% MeOH (each 1.0 l). The active material, eluted with 80% and 90% MeOH, was concentrated *in vacuo*. The remaining aqueous solution (200 ml) was extracted three times with ethyl acetate (200 ml) and the organic

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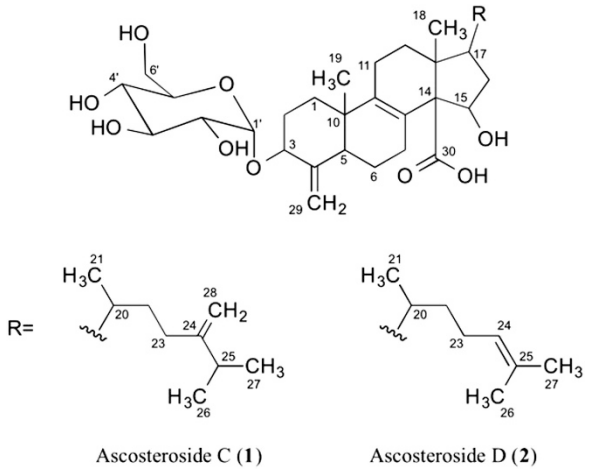

Figure 1 Structures of ascosterosides C (**1**) and D (**2**).

layer was concentrated *in vacuo* to dryness. The extract (1.42 g) was applied to a silica gel chromatography column (125 ml resin, Merck KGaA, Darmstadt, Germany) and eluted stepwise with a solvent mixture of $CHCl_3$:MeOH (100:0, 100:1, 100:2 100:5, 100:10, 35:8, 1:1, 0:100 and 0:100 with 0.1% trifluoroacetic acid). The active compound was eluted with $CHCl_3$:MeOH (35:8) and dried *in vacuo*. Finally, the active material (171 mg) was purified by high-performance liquid chromatography (Pegasil ODS SP 100, 10 i.d.×250 mm, Senshu Scientific Co., Tokyo, Japan) with an isocratic solvent system of 50% aqueous acetonitrile at a flow rate of 4.0 ml min^{-1}. The peaks with the retention time of 16–19 min and 23–27 min were collected and freeze-dried to afford **2** (24.0 mg) and **1** (76.0 mg), respectively.

Compound **2** was obtained as a white powder; soluble in MeOH, $CHCl_3$, acetonitrile and dimethyl sulfoxide; $[\alpha]_D^{23}$ +31.4° ($c=0.1$, MeOH); IR (KBr) λ_{max} 3434, 2956, 2871, 1691, 1452, 1378 and 1027 cm^{-1}; and UV (MeOH) λ_{max} nm (ε) 203 (13680) and 231 (sh, 2530). The similarity of these physico-chemical characteristics with those of **1** strongly suggested that these congeners are analogs. The molecular formula of **2** was elucidated as $C_{35}H_{54}O_9$ from the $[M+Na]^+$ ion at *m/z* 641.3662 (calcd. for $[M+Na]^+$, *m/z* 641.3660) in high-resolution electrospray ionization mass spectrometry analysis, indicating that the structure of **2** differs from that of **1** by a CH_2.

The 1D NMR spectral data of **2** in CD_3OD are shown in Table 1. The ^1H and ^{13}C NMR and HSQC spectra of **2** indicated the presence of five methyls, 10 methylenes including one oxymethylene and one sp^2 exomethylene, 10 sp^3 methines including five oxymethines, one anomeric methine and one olefinic methine, three sp^3 quaternary carbons, four fully substituted olefinic carbons and one carbonyl carbon.

The ^1H and ^{13}C NMR spectra of **2** resembled those of **1**. However, the sp^2 carbon at C-24, sp^3methine at C-25 and exomethylene at C-28, as well as two doublet methyls at C-26 and C-27 of **1**, were not observed. Instead of the above signals in **1**, sp^2 olefinic methine at C-24, sp^2 carbon at C-25 and two singlet olefinic methyls at C-26 and C-27 appeared in **2**. These chemical shifts strongly suggested that the structure of **2** is almost the same as that of **1** except for a side chain. The ^1H-^{13}C HMBC correlations from H_2-23 (δ_H 1.91, 2.01) to C-24 (δ_C 126.0); from H-24 (δ_H, 5.10) to C-26 (δ_C 17.7) and C-27 (δ_C 25.9); from H_3-26 (δ_H 1.60) to C-24, C-25 (δ_C 131.9) and C-27; and from H_3-27 (δ_H 1.68) to C-24, C-25 and C-26 showed that **2** has 6-methyl-hep-5-en-2-yl group instead of the 6-methyl-5-methylidene-heptan-2-yl group in **1.** Therefore, the structure of **2** was elucidated as shown in Figure 2 and **2** was named ascosteroside D.

Table 1 NMR spectroscopic data for ascosterosides D (1) and C (2) in CD_3OD at 400 MHz for ^1H and 100 MHz ^{13}C

	1		*2*[a]	
Position	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	36.3	1.47 (m)	36.3	1.47 (m)
		1.88 (m)		1.88 (m)
2	29.5	1.48 (m)	29.5	1.49 (m)
		2.11 (m)		2.11 (m)
3	76.7	4.11 (m)	76.7	4.02 (m)
4	151.5		151.6	
5	47.9	2.05 (m)	47.9	2.05 (m)
6	22.1	1.62 (m)	22.1	1.60 (m)
		1.78 (m)		1.77 (m)
7	27.1	1.97 (m)	27.2	1.95 (m)
		2.76 (m)		2.70 (m)
8	128.7		128.9	
9	140.8		140.8	
10	40.6		40.6	
11	24.6	2.15 (m)	24.6	2.17 (m)
		2.29 (m)		2.29 (m)
12	34.0	1.73 (m)	34.0	1.70 (m)
		2.18 (m)		2.21 (m)
13	47.5		47.5	
14	67.8		68.1	
15	73.2	4.58 (d, 6.4)	73.4	4.62 (d, 6.8)
16	44.6	1.50 (m)	44.6	1.47 (m)
		2.65 (m)		2.68 (m)
17	51.9	1.45 (m)	51.8	1.45 (m)
18	18.9	1.12 (s)	19.0	1.11 (s)
19	19.3	0.96 (s)	19.4	0.95 (s)
20	36.6	1.58 (m)	36.7	1.60 (m)
21	19.2	0.95 (d, 6.6)	19.2	0.97 (d, 6.4)
22	37.3	1.07 (m)	36.1	1.18 (m)
		1.43 (m)		1.58 (m)
23	25.6	1.91 (m)	32.0	1.92 (m)
		2.01 (m)		2.11 (m)
24	126.0	5.10 (t)	157.7	
25	131.9		34.9	2.25 (qq, 6.6)
26	17.7	1.60 (s)	22.3	1.03 (s)
27	25.9	1.68 (s)	22,5	1.05 (s)
28	–	–	106.9	4.67 (s)
				4.73 (s)
29	104.6	4.68 (s)	104.5	4.68 (s)
		5.27 (s)		5.26 (s)
30	178.9		179.4	
1′	96.9	5.01 (d, 4.0)	96.9	5.01 (d, 4.0)
2′	73.6	3.42 (dd, 4.0, 9.7)	73.6	3.42 (dd, 3.8, 9.5)
3′	75.2	3.77 (dd, 9.7, 9.3)	75.2	3.77 (dd, 9.5, 9.5)
4′	71.9	3.31 (dd, 9.3, 9.2)	71.9	3.32 (dd, 9.5, 9.5)
5′	74.0	3.67 (m)	74.0	3.66 (m)
6′	62.7	3.66 (m)	62.7	3.66 (m)
		3.74 (m)		3.73 (m)

[a]Reported data in ref. 4.

The inhibitory effect of **2** against mitochondrial function was evaluated using the paper disc method on agar plates with endogenous AAC-disrupted and *Acyrthosiphon pisum* AAC-expressing *S. cerevisiae* and AAC-disrupted empty vector-carrying *S. cerevisiae*

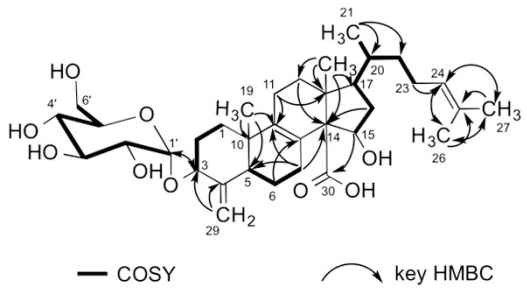

Figure 2 Key correlation of ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC in ascosteroside D (**2**).

Table 2 Selective growth inhibition activity of ascosterosides D (1) and C (2) against recombinant *S. cerevisiae*

Compound	*μg per disc*	*Δaac* S. cervisiae	
		A. pisum-*AAC expressing strain*	Empty vector strain
Ascosteroside D (**1**)	10	15.3	–
	3	13.5	–
	1	12.6	–
	0.3	10.5	–
	0.1	–	–
Ascosteroside C (**2**)	10	19.0	–
	3	15.8	–
	1	14.2	–
	0.3	11.5	–
	0.1	10.1	–

Results of paper disc assays for the empty vector strain (endogenous AAC-disrupted and single-copy type yeast shuttle vector pRS314YA2P-expressing strain) and *A. pisum* strain (endogenous AAC-disrupted and *A. pisum* AAC-expressing). Test compounds were dissolved in MeOH at appropriate concentrations. Inhibition zone diameter (millimeter). –; No inhibition. correlation of ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC in ascosteroside D (**2**).

(*Δaac S. cerevisiae*).[3,6] The *A. pisum aac*-transformed *S. cerevisiae* grown in glycerol-containing medium can only produce ATP in its mitochondria. The *Δaac S. cerevisiae* grown in glucose-containing medium cannot produce ATP in its mitochondria but it can produce ATP in the cytosol. If any compound inhibits the growth of insect AAC-expressing *S. cerevisiae* more than *Δaac S. cerevisiae*, this suggests that it inhibits mitochondrial function.[4,5]

Sterile filter discs impregnated with each compound solution (10 μl) were placed on the agar plate and the plates incubated at 30 °C for 48 h. After incubation, the inhibition zones were measured. Compound **2** inhibited the growth of insect AAC-expressing *S. cerevisiae* in glycerol-containing medium (Table 2). However, it did not inhibit *Δaac S. cerevisiae* in glucose-containing medium. Compound **1** showed similar results.[4] This suggests that **2** inhibited the mitochondrial function as did **1**. Cell growth inhibition assays were performed against several tumor cell lines using the method employed for evaluating **1**.[4] The IC_{50} values of **2** were 47, 46, 119, 142 and 67 μM against HeLa S3, HT29, A549, H1299 and Panc1 cells, respectively. Conversely, **1** did not inhibit cell growth, even at a concentration of 100 μM. These results suggested that the difference in structure between **1** and **2** might be important for influencing biological activity.

Antimicrobial activities of **2** against 11 microorganisms, *S. cerevisiae* ATCC9763, *Candida albicans* ATCC64548, *Mucor racemosus* IFO4581, *Aspergillus niger* ATCC6275, *Staphylococcus* aureus ATCC6538p, *Bacillus subtilis* ATCC6633, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* IFO3080, *Xanthomonas campestris* pv. oryzae KB88, *Acholeplasma laidlawii* PG8 and *Kocuria rhizophila* ATCC9341, were evaluated using the paper disc method.[7] Compound **2** inhibited the growth of *S. cerevisiae* and *C. albicans* at 0.1 μg per disc, whereas it did not show antimicrobial activity against the other microorganisms.

In conclusion, **2** was isolated from the fungus *Aspergillus* sp. FKI-6682 as a new mitochondrial inhibitor, which could inhibit insect AAC-expressing *S. cerevisiae* in glycerol-containing medium. However, **2** did not inhibit *Δaac S. cerevisiae* in glucose-containing medium. These results suggest that **2** inhibited ATP production in mitochondria in the same manner as ascosteroside C (**1**).[4]

DEDICATION

We dedicate this article to the pioneering work of Professor Dr. Hamao Umezawa.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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