Ascosteroside, a New Antifungal Agent from *Ascotricha amphitricha*

II. Isolation and Structure Elucidation

JOHN E. LEET*, STELLA HUANG, STEVEN E. KLOHR
and KIMBERLY D. MCBRIEN

Bristol-Myers Squibb Company, Pharmaceutical Research Institute,
5 Research Parkway, P.O. Box 5100, Wallingford, Connecticut 06492, U.S.A.

(Received for publication September 18, 1995)

The novel antifungal agent ascosteroside (**1**) was isolated from cultured broth of *Ascotricha amphitricha* (ATCC 74237). The structure based on spectroscopic data was determined to be an α-linked glycoside of a lanostane-type triterpenoid.

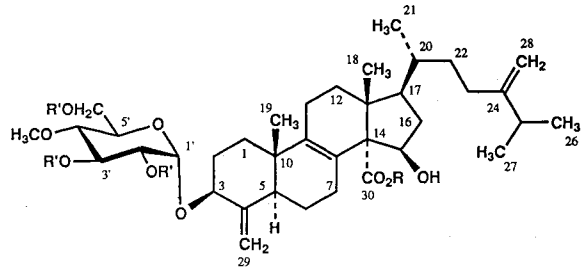
In the course of screening for novel antifungal agents from microorganisms, a strain of *Ascotricha amphitricha* (Ascomycetes) was selected for further study. Whole broth extracts of the culture were active against *Saccharomyces cerevisiae* as described in the preceding paper[1]. This investigation led to the discovery of the new antifungal agent ascosteroside (**1**). In the preceding paper, we reported the taxonomy of the producing organism, fermentation and biological activity of this compound. Described herein is the isolation and structural elucidation of ascosteroside.

Isolation and Purification

The isolation of ascosteroside was guided by the use of *S. cerevisiae* as the test organism in an antifungal screen. Fermentation broth (pH 8.6, 20 liters), prepared as described in the preceding paper, was extracted with EtOAc (10 liters). Upon filtration and phase separation, the organic layer upon evaporation yielded 2 g of crude solids. This extract was dissolved in 10 ml of 10% aq MeOH and the solution was washed three times with equal volumes of *n*-hexane. The *n*-hexane extract was found by ^1H and ^{13}C NMR to contain large amounts of ergosterol, a common fungal sterol[2]. The remaining aqueous methanol phase was diluted to 35% aq MeOH and extracted three times with equal volumes presaturated chloroform. The chloroform fraction (0.56 g) was dissolved in 2 ml chloroform-methanol 1:1 and applied to a column of Sephadex LH-20 (100 g) packed in chloroform-methanol 1:1. Fractions measuring 8 ml each were collected at an elution flowrate of 2~3 ml/minute, using the above solvent composition, and were consolidated on the basis of silica gel TLC ($CHCl_3$-MeOH 9:1, ceric sulfate spray plus heating for detection). Final purification of the active fraction was accomplished by reverse phase preparative HPLC (Dynamax C18 column, 21.4 mm inner diameter × 25 cm length, 8 μm particle size, 60 Å pore size). The mobile phase was acetonitrile-water 1:1 with a linear gradient to acetonitrile over 30 minutes at a flowrate of 10 ml/minute. UV detection (230 nm) was used. The major peak (22 minutes) was collected and the solvent evaporated to yield 19 mg of pure ascosteroside (**1**).

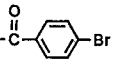
Characterization and Structure Elucidation

Ascosteroside was isolated as a colorless amorphous solid. It is soluble in DMSO, methanol, ethanol, chloroform, acetone, slightly soluble in ethyl acetate, acetonitrile, and practically insoluble in *n*-hexane, benzene, and water. The molecular formula for **1** was established as $C_{37}H_{58}O_9$ by HRFAB-MS ($[M+Na]^+$ *m/z* 669.3964, calcd 669.3978), indicating nine degrees of unsaturation in the molecule. A key fragment ion



(**1**) R=H, R′=H

(**2**) R=CH_3, R′=H

(**3**) R=H, R′= 

observed at m/z 453.3355 ($C_{30}H_{45}O_3$) was due to facile cleavage of a sugar species (neutral loss of 176 mass units, $C_7H_{12}O_5$) and loss of water. The IR spectrum (Fig. 1) displayed carbonyl absorption at 1700 cm^{-1}, suggesting carboxylic acid functionality. Supporting evidence for a carboxylic acid group resulted from conversion to the methyl ester (**2**) using etherial diazomethane (IR (KBr) $\nu_{C=O}$ 1710 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 3.51. The UV spectrum (Fig. 2) showed mainly end absorbance at 204 nm, with weak maxima at 235 and 294 nm. The physico-chemical properties of ascosteroside (**1**) are summarized in Table 1.

Apparent in the ^1H NMR spectrum (Fig. 3) were two methyl singlets (δ 0.82, 1.00), four methyl doublets (δ 0.89, 0.97, 0.98), one *O*-methyl (δ 3.41), four olefinic singlets (δ 4.59, 4.63, 4.70, 5.11), six oxygenated methine resonances (δ 3.91, 4.41 (aglycone); 3.21, 3.64, 2.92, 3.38 (sugar), and one anomeric proton (δ 4.83), consistent with the presence of one sugar unit. Also present were numerous overlapping resonances (δ 1.0~2.7) corresponding to 22 protons, 18 of which were methylenes. The ^{13}C NMR data (Fig. 4) revealed 36 signals, composed of six methyls (two methyl resonances were degenerate), twelve methylenes, eleven methines, and eight quaternary carbons, as indicated through the DEPT experiment. These included one carbonyl (δ 176.2), three olefinic groups, of which two highly polarized units (δ 103.5, 150.3; 106.6, 155.8) were exocyclic methylenes, one anomeric carbon (δ 95.1), and nine aliphatic methylene carbons (δ 20~44). Given the number of aliphatic methylenes, two angular methyl groups, and four rings as defined by unsaturation requirements, a C_{30} aglycone with a sterol-type skeleton was considered.

Fig. 1. IR (KBr) spectrum of ascosteroside.

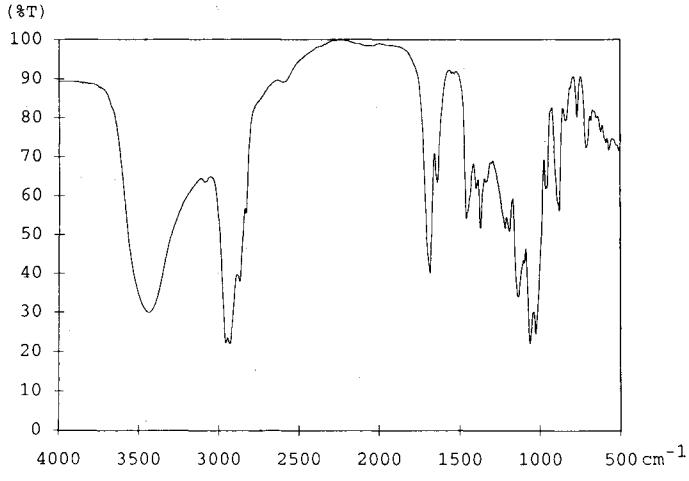


Table 1. Physico-chemical properties of ascosteroside (**1**).

Appearance	Colorless amorphous solid
MP	130~132°C
$[\alpha]_D^{20}$	+43° (*c* 0.2, MeOH)
Molecular formula	$C_{37}H_{58}O_9$
Molecular weight	646.4066
HRFAB-MS (m/z)	
Found:	669.3964 $[M+Na]^+$
Calcd:	669.3978
MS (IONSPRAY) (m/z)	647 (M+H), 471 (M−$C_7H_{12}O_5$+H, aglycone+H), 453 (aglycone−H_2O+H), 425 (aglycone−COOH), 408 (aglycone−COOH−H_2O+H)
UV λ_{max}^{MeOH} nm (ε)	204 (8000), 235 (sh, 2100), 294 (300)
IR ν_{max} (KBr) cm^{-1}	3448, 2960, 2934, 1700, 1652, 1466, 1382, 1196, 1146, 1066, 1030, 972, 886
CD λ ($\Delta\varepsilon$) (MeOH)	231 (−13.3)
TLC[a] (Rf)	0.16
HPLC[b] (Rt)	24.6 minutes

[a] Silica gel plates; $CHCl_3$ - MeOH (9 : 1), ceric sulfate spray plus heating; spot turns dark blue-gray.

[b] Column: Rainin "Short-One" C18 (3μ) 10 cm L.; eluant: acetonitrile - 0.01 M potassium phosphate (monobasic) buffer pH 3.5 gradient (according to D. J. Hook *et. al.* J. Chromatogr. 385: 99~108, 1987); flow rate 1.2 ml/minute; UV detection at 230 nm.

Fig. 2. UV spectrum of ascosteroside (MeOH).

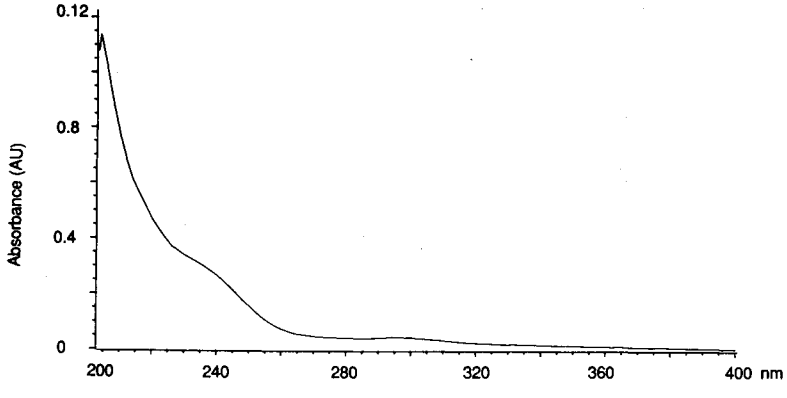


Fig. 3. ^{1}H NMR spectrum of ascosteroside (500.13 MHz, DMSO-d_6).

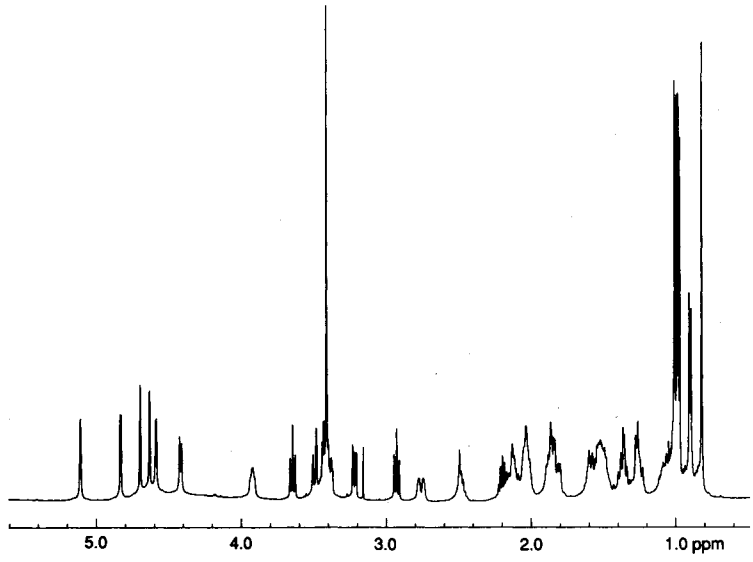
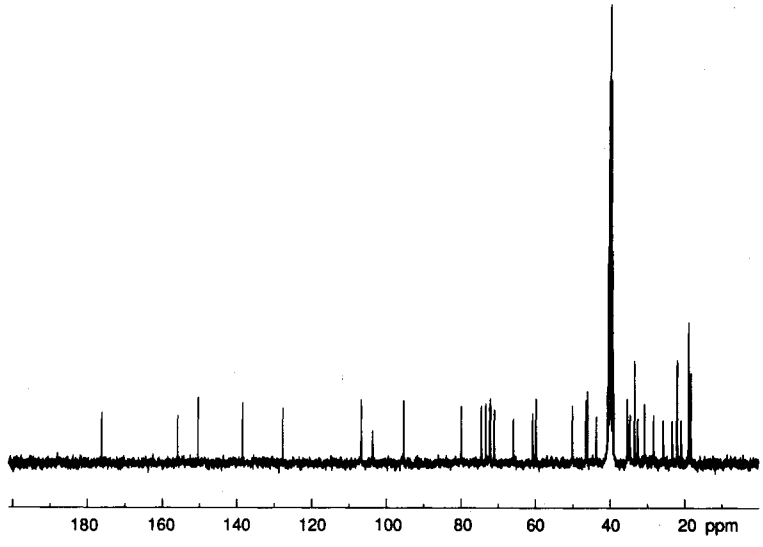


Fig. 4. ^{13}C NMR spectrum of ascosteroside (125.76 MHz, DMSO-d_6).



Furthermore, the ^{13}C NMR data suggested a lanostane-type triterpenoid[2,3].

The gross structure of ascosteroside (**1**) was deduced primarily through 2D NMR, including COLOC (correlation spectroscopy for long-range couplings), HMBC (heteronuclear multiple bond correlation) and NOE experiments. The ^{1}H and ^{13}C assignments appear in Table 2. Placement of one of the exocyclic methylene groups at C-4 was established by long range ^{1}H-^{13}C coupling (*e.g.* 3-bond) of the olefinic protons (δ 4.59, 5.11) to carbons C-3 and C-5; the C-5 proton (δ 1.84) in turn showed a correlation with the exocyclic methylene carbon C-29 (δ 103.5). Placement of the second exocyclic methylene on the side chain was indicated by long range couplings of the methylene protons (δ 4.63, 4.70) to carbons C-23 and C-25 (δ 30.5, 33.2, respectively). The tetrasubstituted double bond was placed at the B/C ring juncture, indicated by 3-bond correlations between C-9 (δ 138.4) and the C-19 angular methyl protons (δ_H 0.82), H-7α (δ 1.86) and H-12β (δ 1.58), and by a correlation between C-8 (δ 127.6) and H-6 (δ 1.52). The carboxylic acid group was placed at C-14 (δ 65.7), as evidenced by long range coupling of the carbonyl carbon (δ 176.2) to the C-15 methine (δ_H 4.41). In addition C-14 shows correlations with the C-18 angular methyl protons (δ 1.00) and H-12β (δ 1.58). A secondary hydroxyl group

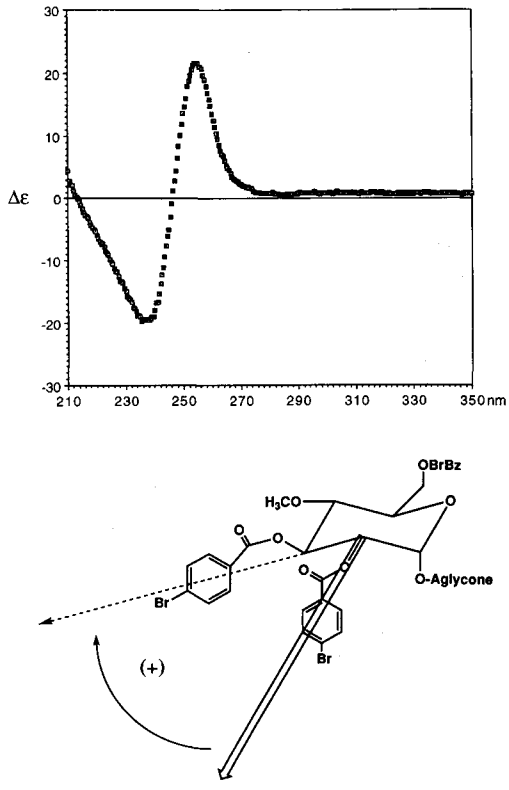
Table 2. Ascosteroside (**1**): ^{1}H and ^{13}C NMR data (DMSO-d_6).

Carbon	^{13}C ppm (mult)	^{1}H ppm (mult, J (Hz))	long-range ^{1}H-^{13}C correlations
Aglycone			
1	34.8 (t)	α 1.28 (m)	C2, C3, C19
		β 1.80 (m)	C3, C5, C10
2	28.1 (t)	α 2.05 (m)	C3, C4, C10
		β 1.26 (m)	C1, C10
3	74.4 (d)	3.91 (m)	C2, C4, C1′
4	150.3 (s)		
5	46.3 (d)	1.84 (m)	C1, C4, C6, C10, C19, C29
6	20.7 (t)	1.52 (m)	C5, C8, C10
7	25.5 (t)	α 1.86 (m)	C8, C9
		β 2.75 (d br, 13.2)	C5, C6, C8
8	127.6 (s)		
9	138.4 (s)		
10	39.6 (s)		
11	23.1 (t)	α 2.12 (m)	C9, C13
		β 2.01 (m)	C8, C9
12	32.3 (t)	α 2.10 (m)	C18
		β 1.58 (m)	C9, C11, C13, C14
13	45.9 (s)		
14	65.7 (s)		
15	70.9 (d)	4.41 (d, 7.1)	C13, C16, C17, C30
16	43.5 (t)	α 2.49 (m)	C13, C17
		β 1.36 (m)	C13, C15, C17, C20
17	50.0 (d)	1.32 (m)	C16, C18
18	18.1 (q)	1.00 (s)	C12, C13, C14, C17
19	18.7 (q)	0.82 (s)	C1, C5, C9, C10
20	35.2 (d)	1.52 (m)	
21	18.7 (q)	0.89 (d, 6.4)	C17, C22
22	34.4 (t)	1.08 (m)	
		1.48 (m)	C17, C20, C21, C23
23	30.5 (t)	1.81 (m)	C20, C24, C28
		2.03 (m)	C20, C22, C24, C28
24	155.8 (s)		
25	33.2 (d)	2.19 (septet, 6.8)	C23, C24, C26, C27, C28
26	21.8 (q)	0.97 (d, 6.8)	C24, C25
27	21.8 (q)	0.98 (d, 6.8)	C24, C25
28	106.6 (t)	4.63 (s)	C23, C25
		4.70 (s)	C23, C25
29	103.5 (t)	4.59 (s)	C3, C5
		5.11 (s)	C3, C4, C5
30	176.2 (s)		
4′-OMe-glucose			
1	95.1 (d)	4.83 (d, 3.5)	C3, C3′
2′	72.1 (d)	3.21 (dd, 3.5, 9.6)	C3′
3	73.2 (d)	3.64 (t, 9.2)	C2′, C4′, C5′
4′	79.7 (d)	2.92 (t, 9.2)	C2′, C6′
5′	71.9 (d)	3.38 (m)	
6′	60.6 (t)	3.43 (m)	C4′
		3.49 (d, 11.4)	
7′	59.7 (q)	3.41 (s)	C4′

was placed at C-15 (δ_C 70.9, δ_H 4.41) since H-15 showed 3-bond correlations with C-13 (δ 45.9), C-17 (δ 50.0) and the carboxylic acid carbonyl, C-30 (δ 176.2).

The glycosidic unit was shown through ^{1}H-^{1}H coupling constants and COSY data to have the glucopyranose configuration. A small ^{1}H-^{1}H coupling (J=3.5 Hz) between the anomeric proton (δ 4.83) and the adjacent axial methine proton (H-2′, δ 3.21) indicated α-linkage. A methoxyl group (C-7′ δ 3.41) showed long range coupling with C-4′ (δ 79.7), supporting placement at that position. Linkage of the sugar to C-3 of the aglycone was established by ^{1}H-^{13}C long range coupling between the anomeric carbon (δ 95.1) and the C-3 methine (δ 3.91) and the anomeric proton (δ 4.83) with C-3 (δ 74.4). The above data indicate that ascosteroside (**1**) consists of an α-linked 4-*O*-methyl glucoside of a lanostane-type triterpenoid.

Fig. 5. CD curve of ascosteroside 2′, 3′, 6′ tri-*p*-bromobenzoate.

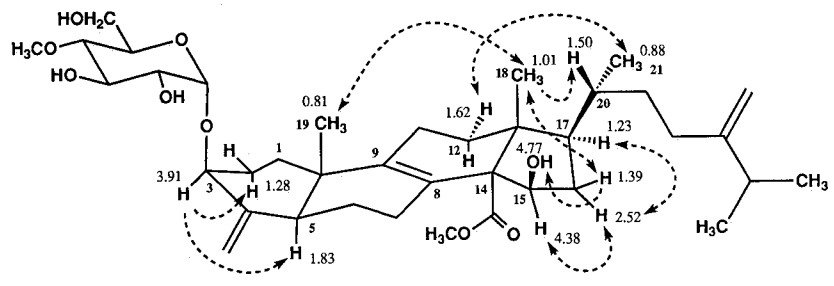


The absolute configuration of the 4-*O*-methyl glucoside portion of ascosteroside was determined by application of the exciton chirality method[4]. The 2′, 3′, 6′ tri-*p*-bromobenzoate derivative (**3**) of ascosteroside was prepared for circular dichroism (CD) measurements. A split CD curve was centered at 246 nm, having a positive 1st Cotton effect at 257 nm, and a negative 2nd Cotton effect at 235 nm. The large amplitude observed (+44) is due mainly to the contribution of the 2′, 3′ dibenzoate chromophore interaction (Fig. 5). A positive chirality between the 2′, 3′ vicinal dibenzoates is thus indicative of the D-configuration for the 4-*O*-methyl

Table 3. Ascosteroside methyl ester (**2**): ^{1}H, ^{13}C, NOE NMR data (DMSO-d_6).

Carbon	^{13}C ppm	^{1}H ppm (mult, J (Hz))	^{1}H-^{1}H NOE
Aglycone			
1	34.8	α 1.28 (m)	
		β 1.77 (m)	
2	28.1	α 2.03 (m)	
		β 1.27 (m)	
3	74.4	3.91 (m)	H1α, H5, H29a,b, H1′, H2′, H5′
4	150.2		
5	46.2	1.83 (m)	
6	20.6	1.57 (m)	
7	25.5	α 1.80 (m)	H6, H7β, H15
		β 2.74 (d br, 13.4)	H6, H7α, H15, C15-OH
8	127.1		
9	138.9		
10	39.6		
11	23.0	α 2.14 (m)	
		β 2.04 (m)	
12	32.5	α 1.89 (m)	H11α,β, H12β, H17, H21
		β 1.62 (dd, 8.4, 12.4)	H11α,β, H12α, H17, H18, H21
13	46.3		
14	66.3		
15	70.7	4.38 (t, 5.5)	H7α,β, H16α,β, C15-OH
		OH, 4.47 (d, 4.8)	
16	43.6	α 2.52 (m)	H15, H16β, H17
		β 1.39 (dd, 9.2, 13.4)	H15, H18, H16α, C15-OH
17	50.2	1.23 (m)	H12α,β, H16α, H22α,β, H23
18	18.0	1.01 (s)	H11β, H12α,β, H16β, H17, H19, H20, H21, H23
19	18.6	0.81 (s)	H2β, H6, H11α,β, H18, H29a,b
20	35.2	1.50 (m)	
21	18.6	0.88 (d, 6.4)	H12α,β, H17, H18, H20, H23
22	34.4	1.06 (m)	
		1.48 (m)	
23	30.6	1.85 (m)	
		2.00 (m)	
24	155.8		
25	33.2	2.19 (septet, 6.8)	
26	21.7	0.97 (d, 6.8)	
27	21.8	0.98 (d, 6.8)	
28	106.6	a. 4.69 (s)	
		b. 4.63 (s)	
29	103.6	a. 4.58 (s)	
		b. 5.10 (s)	H19, H29a, H3′
30	174.9		
31	51.8	3.51 (s)	
4′-OMe-glucose			
1′	95.2 (d)	4.83 (d, 3.5)	
2′	72.1 (d)	3.22 (dd, 3.0, 9.6)	
3′	73.1 (d)	3.64 (t, 9.2)	
4′	79.7 (d)	2.93 (t, 9.2)	
5′	71.9 (d)	3.38 (m)	
6′	60.6 (t)	3.43 (m)	
		3.47 (m)	
		OH, 4.52 (t, 5.5)	
7′	59.7 (q)	3.41 (s)	

Fig. 6. Ascosteroside methyl ester: Key ^{1}H-^{1}H NOE's.



glucose unit.

The relative configuration of the aglycone portion of ascosteroside was determined by NOE difference spectroscopic measurements on the methyl ester derivative (**2**), and the results are summarized in Table 3. The trans A/B ring juncture was supported by NOE's between triaxial protons H-3 (δ 3.91) and H-1α (δ 1.28), H-5 (δ 1.83). This is also consistent with linkage of the sugar at the 3β position. A strong NOE between the two angular methyl groups H-18 (δ 1.01) and H-19 (δ 0.81), together with NOE's between the H-18 methyl group and H-20 (δ 1.50), H-21 (Me, δ 0.88) indicated that the two angular methyls and the C-17 side chain reside on the same face of the tetracyclic triterpene system. In addition, the strong

NOE (*e.g.* close proximity) between the angular methyls is consistent with a trans C/D ring juncture. No NOE was observed between the carbomethoxyl (δ 3.51) and the angular methyl H-18 (δ 1.01). NOE's were observed between H-16β (δ 1.39) and angular methyl H-18, and the C-15 OH proton (δ 4.77). Proton H-16α (δ 2.52) in turn, displayed NOE's with H-15 (δ 4.38) and H-17 (δ 1.23). The C-15 hydroxyl group is thus assigned to the β position. Finally, the observed NOE's between H-21 (Me δ 0.88) and H-12β (δ 1.62), and between H-20 (δ 1.50) and H-18 (δ 1.01) establishes the relative configuration at C-20. In summary, the NOE data for ascosteroside methyl ester are in agreement with the lanostane configuration as drawn (Fig. 6).

Discussion

Ascosteroside (**1**) is a new antifungal agent isolated from fermentation broths of *Ascotricha amphitricha*. Structural studies have shown that the compound is a glycosylated C_{30} tetracyclic triterpene having a lanostane nucleus. The aglycone is unusual in that it contains an exocyclic methylene group at C-4, a carboxylic acid group at C-14, and a hydroxyl group at C-15. These features distinguish it from other fungal lanostane triterpenoids such as sulphurenic acid, eburicoic acid and the polyporenic acids (*Polyporus* sp., Polyporaceae, Basidiomycetes)[5]. The 4-methylene sterols are very unusual. Only two examples, theonellasterol and conicasterol, from marine sponges, have been published[6]. Furthermore, relatively few lanosterol-type sterols having a 14-carboxy group have been isolated from natural sources: lyofoligenic acid[7], from the plant *Lyonia ovalifolia*, penasterol[8], from the marine sponge *Penares* sp., and penasterone and acetylpenasterol[9] from *Penares incrustans*. Ascosteroside showed *in vitro* activity against *S. cerevisiae* and *Candida albacans*. Interestingly, the methyl ester derivative (**2**) was less active against *S. cerevisiae*, suggesting that the carboxylic acid group is important for antifungal activity.

Experimental

General

All ^1H and ^{13}C NMR spectra were recorded using a Bruker AM-500 operating at 500.13 and 125.76 MHz, respectively, using a 5-mm broadbanded probe. Chemical shifts are reported in ppm relative to solvent (DMSO-d_6 δ_H 2.49, δ_C 39.6). Low resolution mass spectrometric analyses were performed with a SCIEX API-III tandem quadrupole mass spectrometer, using an IONSPRAY interface. Accurate mass measurements were obtained by peak matching using a Kratos MS50 mass spectrometer with a cesium iodide saturated glycerol solution as the reference. MS/MS measurements were performed with a Finnigan TSQ-70 triple quadrupole mass spectrometer in the positive ion FAB mode, using a *m*-nitrobenzyl alcohol matrix, and Argon as the primary particle source. UV absorption spectra were determined using a Hewlett Packard 8452A diode array spectrophotometer. IR spectra were obtained on KBr discs using a Perkin-Elmer 1800 Fourier transform spectrometer. Specific rotations were recorded with a Perkin-Elmer 241 polarimeter. Circular dichroism spectra were obtained using a Jasco J-720 spectropolarimeter.

Preparation of Ascosteroside Methyl Ester (2)

Ascosteroside (**1**) (21 mg, 0.03 mmol) was dissolved in a few drops methanol and mixed with excess etherial diazomethane (3 ml, prepared from Diazald reagent according to Aldrich Co. technical bulletin AL-121, 1982). The contents were allowed to stand at 10°C for 18 hours. The mixture was concentrated under nitrogen gas and dried. Final purification was accomplished by preparative HPLC (C-18) as described previously, using a mobile phase of acetonitrile - water 7 : 3, followed by a linear gradient to 100% acetonitrile over 30 minutes. The major peak (24 minutes) was collected and solvent removed *in vacuo* to yield 12 mg of pure ascosteroside methyl ester (**2**): $C_{38}H_{60}O_9$: TLC Rf 0.35 ($CHCl_3$ - MeOH 9 : 1); $[\alpha]_D$ +53° (*c* 0.17, MeOH); HRFAB-MS *m/z* 683.4113 ($[M+Na]^+$; calcd 683.4135); IR ν_{max} (KBr) 3440, 2936, 2874, 1710, 1646, 1464, 1384, 1204, 1140, 1068, 1030, 972, 896, 778 cm^{-1}; ^1H NMR, ^{13}C NMR (Table 3).

Preparation of Ascosteroside 2′, 3′, 6′ tri-*p*-Bromobenzoate (3)

Ascosteroside (**1**) (5 mg) was dissolved in 1 ml pyridine and mixed with excess *p*-bromobenzoyl chloride (10 mg) and dimethylaminopyridine (2 mg). The contents were heated to 60°C, with stirring, for 48 hours. Purification of the crude product by silica gel preparative TLC (0.5 mm Merck plate) using hexane - acetone 7 : 3 as the developing solvent afforded the 2′, 3′, 6′ tri-*p*-bromobenzoate (**3**) (1.2 mg): $C_{58}H_{67}O_{12}Br_3$: TLC Rf 0.36 (Hexane - Acetone 7 : 3); HRFAB-MS *m/z* 1199.2307 ($[M+Li]^+$; calcd 1199.2342); MS/MS *m/z* 1201 $[M+Li]^+$, 1157 $[M-COOH+Li]^+$, 1139 $[M-COOH-H_2O+Li]^+$, 725 $[C_{28}H_{22}O_8Br_3]^+$; CD λ ($\Delta\varepsilon$) (MeOH) 257 (+20.3), 246 (0), 235 (−23.9); UV λ_{max}^{MeOH} nm ($\log\varepsilon$) 206 (4.54), 246 (4.66); ^1H NMR (500 MHz, $CDCl_3$) δ 0.86 (3H, s, H-19), 0.91 (3H, d, J=6.3 Hz, H-21), 1.00 (6H, d, J=6.8 Hz, H-26, 27), 1.09 (3H, d, J=6.8 Hz, H-18), 1.10～2.30 (broad envelope, 20H), 2.73 (1H, m, H-7), 2.84 (1H, m, H-16), 3.42 (3H, s, 4′-OMe), 3.55 (1H, t, J=9.3 Hz, H-4′), 3.97 (1H, m, H-3), 4.26 (1H, m, H-5′), 4.69 (1H, dd, J=5.5, 11.9 Hz, H-6′), 4.54 (1H, d, J=7.1 Hz, H-15), 4.63 (1H, m, H-6′), 4.63 (1H, s, H-29), 4.69 (1H, s, H-28), 4.71 (1H, s, H-28), 5.12 (1H, dd, J=3.7, 10.2 Hz, H-2′), 5.33 (1H, s, H-29), 5.37 (1H, d, J=3.7 Hz, H-1′), 6.00 (1H, t, J=9.5 Hz, H-3′), 7.51, 7.54,

7.57, 7.80, 7.86, 7.89 (2H ea, d, $J=8.5$ Hz, Ar-H's, 3 × bromobenzoate groups).

Acknowledgments

The authors thank D. R. GUSTAVSON, K. S. LAM, S. LOWE, and S. FORENZA for fermentation support, S. W. MAMBER for bioassay guidance; B. S. KRISHNAN, J. L. WHITNEY, K. J. VOLK, K. J. EDINGER, R. A. DALTERIO, and S. J. SCHADTLE for analytical measurements, and J. GOLIK, D. R. SCHROEDER, and T. W. DOYLE for helpful discussions.

References

1) GORMAN, J. A.; L.-P. CHANG, J. M. CLARK, D. R. GUSTAVSON, K. S. LAM, S. W. MAMBER, D. PIRNIK, C. RICCA, P. B. FERNANDES & J. O'SULLIVAN: Ascosteroside, a new antifungal agent from *Ascotricha amphitricha* I. Taxonomy, fermentation and biological activities. J. Antibiotics 49: 547~552, 1996
2) SMITH, W. B.: The Carbon-13 spectra of steroids on the way to ecdysone. Org. Magn. Reson. 9: 644~648, 1977
3) KNIGHT, S. A.: Carbon-13 NMR spectra of some tetra- and pentacyclic triterpenoids. Org. Magn. Reson. 6: 603~611, 1974
4) HARADA, N. & K. NAKANISHI: Circular Dichroic Spectroscopy—Exciton Coupling in Organic Stereochemistry. pp. 164~181, University Science Press, Mill Valley, CA, 1983
5) TURNER, W. B. & D. C. ALDRIDGE: Fungal Metabolites II. pp. 304~366, Academic Press, New York, 1983
6) KHO, E.; D. K. IMAGAWA, M. ROHMER, Y. KASHMAN & C. DJERASSI: Sterols in Marine Invertebrates. 22. Isolation and structure elucidation of conicasterol and theonellasterol, two new 4-methylene sterols from the red sea sponges *Theonella conica* and *Theonella swinhoei*. J. Org. Chem. 46: 1836~1839, 1981
7) SAKAKIBARA, J.; Y. HOTTA, M. YASUE, Y. IITAKA & K. YAMAZAKI: X-Ray structure of lyofoligenic acid, a novel B-homotriterpene. J. Chem. Soc. Chem. Commun. 839, 1974
8) CHENG, J.; J. KOBAYASHI, H. NAKAMURA, Y. OHIZUMI, Y. HIRATA & T. SASAKI: Penasterol, a novel antileukemic sterol from the Okinawan marine sponge *Penares* sp. J. Chem. Soc. Perkin Trans. I: 2403~2406, 1988
9) SHOJI, N.; A. UMEYAMA, S. MOTOKI, S. ARIHARA, T. ISHIDA, K. NOMOTO, J. KOBAYASHI & M. TAKEI: Potent inhibitors of histamine release, two novel triterpenoids from the Okinawan marine sponge *Penares incrustans*. J. Nat. Prod. 55: 1682~1685, 1992