Antifungal Antibiotics from *Calcarisporium thermophilum*: A New Source of 15-Azahomosterol Derivatives

Paul Chrisp and Paul M. Dewick

Department of Pharmaceutical Sciences, University of Nottingham, Nottingham, NG 7 2RD, U.K.

and

F. Tom Boyle

ICI Pharmaceuticals plc, Alderley Park, Macclesfield, Cheshire, SK 10 4TG, U.K.

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Two antifungal metabolites isolated from *Calcarisporium thermophilum* were identified as 15-azahomosterols related to the compounds previously isolated from *Geotrichum flavo-brunneum*. By full spectral comparison with authentic 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (A 25822 B) from *G. flavo-brunneum,* the *Calcarisporium* metabolites were characterized as the 4α-methyl- and 4,4-dimethyl-analogues of A 25822 B. Several minor members of the series were also detected, and tentatively identified by MS analysis. The 15-azahomosterols exhibited good antifungal activity towards *Candida parapsiliosis,* though the activities were somewhat lower than that of the 4-demethyl derivative A 25822 B. *Calcarisporium thermophilum* is the second microorganism known to synthesize these unusual 15-azahomosterol derivatives.

Introduction

Routine screening of microorganisms for antifungal antibiotics showed that crude mycelial extracts from *Calcarisporium thermophilum* ATCC 11485, a member of the Fungi Imperfecti, demonstrated good activity against *Candida* and dermatophytes. Preliminary spectral analysis suggested the activity was due to compounds of steroidal or triterpenoid nature, and a relationship to the novel 15-azahomosterols isolated earlier [1–4] from another member of the Fungi Imperfecti, *Geotrichum flavo-brunneum,* was recognized. The major *Geotrichum* azasterol, A 25822 B, has been unequivocally identified by X-ray analysis [1] as 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**1**), and six further compounds were tentatively identified by comparison with this compound [3]. A 25822 B appears to interfere with steroid biosynthesis at three different steps, *via* inhibition of Δ^{14}-sterol reductase, Δ^{24}-sterol methyltransferase, and $\Delta^{24(28)}$-sterol reductase [5–10]. Of these enzymes, the Δ^{14}-reductase appears to be the most sensitive to the inhibitor [7, 8], so inhibition of steroid me-

Reprint requests to Dr. P. M. Dewick.

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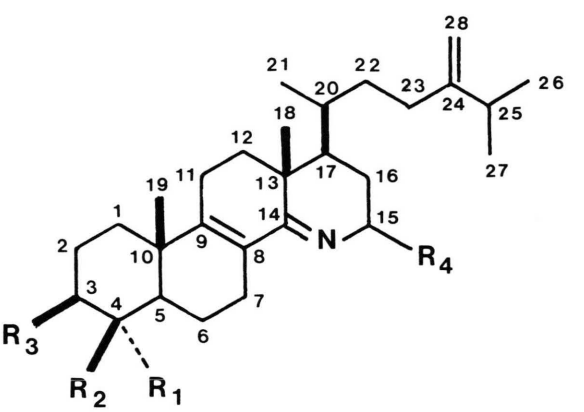
Fig. 1. Structures of 15-azahomosterol derivatives.



	R_1	R_2	R_3	R_4
1 A 25822 B	H	H	OH	H
2 AzX	Me	H	OH	H
3 A 25822 A	Me	Me	OH	H
4 A 25822 M	H	H	OAc	H
5	Me	Me	OH	=O
6 A 25822 L	H	H	OH	=O
7	Me	H	OH	OH
8 A 25822 D	H	H	OH	OH

Note: The above numbering system does not conform to rules of systematic nomenclature, but is used in the present paper to correlate with normal steroids.

tabolism through this means offers a novel mode of action for potential antifungal agents. Thus new examples of this group of compounds, and further study of their antifungal activity are of considerable interest. To facilitate the isolation, purification and identification of the active compounds in *C. thermophilum,* A 25822 B was obtained from cultures of *G. flavo-brunneum* ATCC 28804, and full spectral analysis was carried out. This has allowed characterization of the two main 15-azahomosterols from *C. thermophilum* as the 4α-methyl- and 4,4-dimethyl-homologues, (**2**) and (**3**) respectively, of A 25822 B.

Materials and Methods

UV spectra were recorded in EtOH solution. NMR spectra were recorded at 250 MHz (^{1}H) and 62.89 MHz (^{13}C) using a Bruker WM 250 spectrometer. Solutions were made up in $CDCl_3$ or $CDCl_3$–DMSO-d_6, and TMS was used as internal standard. Electron impact mass spectra were obtained using an AEI MS 902 spectrometer at 70 eV, with an ion chamber temperature in the range 150–200 °C.

GC-MS analysis was performed using a Hewlett-Packard 5790 gas chromatograph connected to a VG 7070 E mass spectrometer operating in electron impact mode. A Chrompak 5% CP Sil 8 column was employed, with a column temperature of 250 °C and a carrier gas flow rate of 10 ml/min.

Analytical TLC was carried out using Merck aluminium sheets coated with silica gel 60 F 254. Glass plates coated with 0.5 mm layers of Merck silica gel GF 254 were used for preparative work. Bands were eluted from the plates with chloroform–methanol 5:1. The 15-azahomosterols were located by viewing under UV light (254 nm), or by spraying the edges of the plate with Dragendorff's reagent or potassium chloroplatinate (equal vols. 6% aq. potassium iodide and 0.3% hexachloroplatinic acid).

Preparative medium pressure liquid chromatography (MPLC) was achieved using a Merck Lobar size C (440-37) LiChroprep Si 60 (40–63 μm) silica column. Solvents were pumped through at a rate of 20 ml/min, monitoring the eluate with a UV detector at 254 nm and collecting 8–10 ml fractions. Preparative flash column chromatography was carried out using a silica gel column (Merck silica gel 60, 230–400 mesh; 15 × 2 cm), eluted at a pressure of 5 psi with nitrogen gas. Fractions (10 ml) were collected and monitored by TLC analysis. HPLC analysis was performed using Hichrom S 5 O DS 2 reversed phase or Hichrom Spherisorb-S 5 W silica columns, 4.6 × 250 mm, with a flow rate of 0.5–1 ml/min, monitoring the eluate with a UV detector at 239 nm.

Isolation of A 25822 B from Geotrichum flavo-brunneum

Geotrichum flavo-brunneum ATCC 28804 was maintained on nutrient agar slants at 25 °C in the dark, using the medium described by Chamberlin [11] and Jones [12]. They were subcultured every 4–6 weeks. Sections of mycelia (7–10 days old) were transferred to 250 ml Erlenmeyer flasks containing 50 ml vegetative medium (sucrose, 25 g; edible molasses, 36 g; corn steep liquor, 6 g; KH_2PO_4, 2 g; NZ amine B, 2 g; H_2O to 1 l) and were incubated at 25 °C in the dark for 48 h on an orbital shaker at 250 rpm. Viable growth was then filtered and used to inoculate 1 l Erlenmeyer flasks each containing 250 ml production medium (glucose, 25 g; soluble starch, 10 g; peptone, 10 g; NZ amine B, 4 g; edible molasses, 5 g; $MgSO_4 \cdot 7H_2O$, 5 g; $CaCO_3$, 2 g; L-glutamine, 4 g; H_2O to 1 l). These cultures were incubated as above for 96 h.

The fungal cell mass from 2–3 l production medium was collected by filtration on cellulose filter pads using a filter aid (Celite Hyflo-Super-Cel; Koch-Light), and then suspended in $CHCl_3$–MeOH, 4:1 (1–1.5 l). Negligible amounts of azasterol were found in the medium, and the filtered broth was discarded. The cell suspension was stirred at room temperature for 15 min, and filtered. The filtrate was dried over $MgSO_4$, filtered, and evaporated to a viscous brown oil. This was suspended in ether (50 ml), filtered, and again evaporated to a brown oil. This crude material was fractionated by MPLC, eluting initially with $CHCl_3$–MeOH, 9:1 (260–280 ml) to remove non-polar sterols, then $CHCl_3$–MeOH, 5:1 (120–160 ml) and finally $CHCl_3$–MeOH–H_2O, 14:6:1 (*ca.* 400 ml). The azasterol fraction eluted with $CHCl_3$–MeOH, 5:1. Flash column chromatography may be used as an alternative to MPLC in the preliminary fractionation. $CHCl_3$–MeOH mixtures (9:1, 50–70 ml; 5:1, 60–80 ml; 3:1,

70–100 ml) and then $CHCl_3$–MeOH–H_2O, 14:6:1 (*ca.* 150 ml) were employed consecutively, the azasterol fraction again eluting with $CHCl_3$–MeOH, 5:1. In both procedures, 8–10 ml fractions were collected and monitored by TLC ($CHCl_3$–MeOH, 5:1), combining appropriate fractions. Further purification of 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (A 25822 B, **1**) was achieved by a second MPLC process as above. The purity of the product was monitored by TLC ($CHCl_3$–MeOH, 5:1; $CHCl_3$–MeOH–H_2O, 14:6:1) and by HPLC (Hichrom S5O DS2; MeOH–H_2O, 1:1). Yields of **1** were typically 12–20 mg/l.

Isolation of 15-azahomosterols from Calcarisporium thermophilum

Calcarisporium thermophilum ATCC 11485 was maintained on malt agar slants (malt extract, 20 g; yeast extract, 1 g; agar, 20 g; H_2O to 1 l) at 30 °C in the dark, subculturing every 4–6 weeks. Mycelium (10–14 days old) was transferred to 2 l surface culture vessels (Glaxo vessels) each containing 250 ml production medium ($NaNO_3$, 2 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; yeast extract, 1 g; L-glutamine, 4 g; glucose, 50 g; minor element solution, 1 ml; H_2O to 1 l. Minor element solution: $FeSO_4 \cdot 7H_2O$, 1 g; $CuSO_4 \cdot 5H_2O$, 0.15 g; $ZnSO_4 \cdot 7H_2O$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; K_2MoO_4, 0.1 g; H_2O to 1 l) and the fungus was grown as a surface static culture at 30 °C in the dark for 21 days.

The rafts of mycelia were harvested by decanting off the liquid medium (4–5 l) and filtering through cellulose filter pads. The filtrate contained negligible amounts of azasterols and was discarded. The mycelial mass was immersed in liquid nitrogen, and while frozen was pulverized in a mortar. The mycelial fragments were suspended in $CHCl_3$–MeOH, 4:1 (2–2.5 l), and stirred at room temperature for 30 min. The suspension was filtered, and the filtrate dried over $MgSO_4$. After filtering, the organic phase was evaporated to yield a brown oil, which was then suspended in ether (50 ml). This was again filtered and evaporated to yield a yellow-brown residue. The crude extract was fractionated by MPLC, eluting with $CHCl_3$–MeOH, 9:1 (270–280 ml) to remove non-polar sterols, which typically eluted in the first two or three 8 ml fractions (8–24 ml solvent). The azasterol complex also eluted with this solvent system, typically appearing in fraction 5. $CHCl_3$–MeOH, 5:1 (160 ml) and $CHCl_3$–MeOH–H_2O, 14:6:1 (*ca.* 400 ml) were used for further development of the column. Flash column chromatography eluting successively with $CHCl_3$–MeOH mixtures (9:1, 60–90 ml; 7:1, 50 ml; 5:1, 70–80 ml; 3:1, 70–100 ml) and finally $CHCl_3$–MeOH–H_2O, 14:6:1 (*ca.* 150 ml) may also be used to isolate the azasterols from the mycelial extract. The complex again eluted with $CHCl_3$–MeOH, 9:1, typically in fractions 6–9 (60–90 ml solvent). Fractions were collected and monitored by TLC (Et_2O–EtOH, 10:1), combining appropriate fractions. The purity of fractions was also analyzed by HPLC (Spherisorb silica S5W; CH_2Cl_2–MeOH, 20:1). Neither MPLC nor flash chromatography resolved the two main components of the azasterol complex, and these were separated by preparative TLC (Et_2O–EtOH, 10:1). Bands at R_f 0.70 and 0.75 were separately eluted with $CHCl_3$–MeOH, 5:1 (3 × 25 ml), and rechromatographed as necessary to obtain pure samples of 4α-methyl-15-aza-24-methylene-D-homocholestan-8,14-dien-3β-ol (**2**) from the lower band, and 4,4-dimethyl-15-aza-24-methylene-D-homocholestan-8,14-dien-3β-ol (**3**) from the upper band. Yields of **2** and **3** were typically 2 mg and 0.9 mg respectively from 1 l production medium.

Results and Discussion

Chemical characterization of 15-azahomosterols

(i) UV spectra

The 15-azahomosterols typically exhibit UV absorption in the range 235–239 nm, due to the presence of the conjugated imine group [1, 3]. However, under acidic conditions, protonation results in a bathochromic shift in the spectrum, with the major absorption now at about 278 nm. This characteristic served as a valuable method for identifying the presence of these compounds in chromatographic fractions. Pure samples of A 25822 B (**1**), **2** and **3** all had UV absorptions at 238 nm in neutral conditions, and at 279 nm on addition of a few drops of dilute (10%) hydrochloric acid.

(ii) Mass spectra

The electron impact mass spectrum of A 25822 B (**1**) showed a molecular ion peak at m/z 411

(100%) with the major fragment ion at *m/z* 396 (49%), corresponding to loss of a methyl group, most likely the C-19 methyl due to stabilization of the resultant ion by the conjugated imine system [13]. Loss of a mono-unsaturated sidechain (C_9H_{17}) is denoted by a peak at *m/z* 286 (12%), and loss of hydroxyl resulted in a fragment at *m/z* 394 (5%). The position of the double bond is indicated by a number of fragments, notably *m/z* 327 (2%) (loss of C_6H_{12}) and 312 (4%) (loss of C_6H_{12} and CH_3). These fragments arise from a McLafferty rearrangement with scission of the bond between C-22 and C-23, characteristic of sidechains with a 24-methylene group [14–17], but are of much weaker intensity than in sterols with the same sidechain. A fragment at *m/z* 326 (loss of C_6H_{12} + H) was also observed. An accurate mass measurement (M^+ at *m/z* 411.3454) confirmed its molecular formula ($C_{28}H_{45}NO$ requires *m/z* 411.3499).

The mass spectrum of compound **2** suggested it to be a monomethyl analogue. It gave a molecular ion peak at *m/z* 425 (100%) with a major fragmentation ion at *m/z* 410 (34%) due to loss of a methyl group. Loss of hydroxyl also occurred giving a peak at *m/z* 408 (6%). A sidechain including the 24-methylene group was implicated by the existence of fragments at *m/z* 341 (2%) (loss of C_6H_{12}), 340 (5%) (loss of C_6H_{12} + H), 326 (1%) (loss of C_6H_{12} + Me), and 300 (5%) (loss of C_9H_{17}). Accurate mass measurement (M^+ at *m/z* 425.3658) confirmed the molecular formula as $C_{29}H_{47}NO$ (calculated 425.3656).

Compound **3** appeared to be a dimethyl analogue of **1.** It gave a molecular ion peak at *m/z* 439 (100%), with fragments at *m/z* 424 (43%) (loss of methyl), 422 (5%) (loss of OH), 354 (6%) (loss of C_6H_{12} + H), 340 (6%) (loss of C_6H_{12} + Me) and 314 (8%) (loss of C_9H_{17}), again suggesting the same sidechain in all three compounds.

(iii) ^{1}H NMR spectra

High resolution ^{1}H NMR spectral data for the three compounds **1, 2** and **3** are given in Table I.

All three compounds gave two intense low field peaks, an unresolved singlet and a doublet with small coupling constant (*J ca.* 1 Hz), characteristic of the exo methylene group of the sidechain (H-28). Protons at position 15 were also readily assignable, and essentially unchanged in the spectra of each compound, except for possible solvent effects. The coupling patterns were consistent with $J_{15\beta,16\alpha}$ being negligible, and indeed, models suggest these protons are roughly at right angles.

Signals arising from the 3α (axial) protons varied markedly. In A 25822 B, this is a complex multiplet, approximately a triplet of triplets (*J* = 11, 4 Hz), due to coupling with two adjacent axial and two adjacent equatorial protons, respectively. The major *C. thermophilum* compound **2** had H-3 as a

Table I. ^{1}H NMR chemical shifts and coupling constants for 15-azahomosterols.

	15-Aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**1**)	4α-Methyl-15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**2**)	4,4-Dimethyl-15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**3**)
H-3α (ax)	3.70 (tt, *J* = *ca.* 11, 4)	3.11 (td, *J* = 10.5, 4.6)	3.25 (dd, *J* = 11.6, 4.4)
H-4α (eq)	2.98 (dt, *J* = *ca.* 13, 4)		
H-4β (ax)	2.59 (dt, *J* = *ca.* 13, 11)	unassigned	
H-15α (ax)	3.62 (ddd, *J* = *ca.* 17.5, 8, 4.5)	3.54 (ddd, *J* = *ca.* 17.5, 8, 4.5)	3.54 (ddd, *J* = *ca.* 17.5, 8, 4.5)
H-15β (eq)	4.15 (dd, *J* = *ca.* 17.5, 4.5)	4.00 (dd, *J* = *ca.* 17.5, 4.5)	4.00 (dd, *J* = *ca.* 17.5, 4.5)
H-18	1.06 (s)	0.99 (s)	0.96 (s)
H-19	1.13 (s)	1.06 (s)	1.08 (s)
H-21	0.99 (d, *J* = *ca.* 7)	0.97 (d, *J* = 6.9)	0.96 (d, *J* = 6.7)
H-26	1.01 (d, *J* = *ca.* 7)	1.03 (d, *J* = 6.8)	1.03 (d, *J* = 6.9)
H-27	1.01 (d, *J* = *ca.* 7)	1.02 (d, *J* = 6.8)	1.02 (d, *J* = 6.6)
H-28	4.75 (s)	4.74 (s)	4.74 (s)
	4.63 (d, *J* < 1)	4.66 (d, *J* = 1.3)	4.66 (d, *J* < 1)
H-29 (eq)		1.01 (d, *J* = 6.5)	1.01 (s)
H-30 (ax)			0.83 (s)

Solvents: (**1**), $CDCl_3$–DMSO-d_6; (**2**) and (**3**), $CDCl_3$. Chemical shifts in ppm from TMS; coupling constants (*J*) in Hz.

doublet of triplets (J = 4.6, 10.5 Hz) indicating coupling to two axial protons, but only one equatorial proton. Compound **3** had H-3 as a doublet of doublets (J = 11.6, 4.4 Hz), and was thus coupled to one axial proton and one equatorial proton. Thus, **2** and **3** are probably the 4α-methyl- and 4,4-dimethyl-analogues of **1,** alternative 2-methylated derivatives being biosynthetically unacceptable. These proposals are borne out by the methyl signals. A 25822 B (**1**) has five methyl groups, appearing as three singlets and two coincident doublets. Allowing for slight solvent effects, these signals are also found in the spectrum of **2,** together with an additional doublet at δ 1.01 for the 4α (equatorial) methyl group. Compound **3** differed from **1** in giving two new methyl singlet peaks at δ 1.01 and 0.83, assignable to 4α (equatorial) and 4β (axial) methyls respectively.

The remainder of the proton spectra exhibited a broad envelope of absorption due to methine and methylene protons. Further assignments were not possible, even for H-4β in **2,** and a COSY spectrum failed to define this signal.

(iv) ^{13}C NMR spectra

The ^{13}C NMR spectral assignments are given in Table II. Of particular value in making these assignments was comparison with published spectra for 24-methylenecholesterol [18] and cycloeucalenol [19], which both possess the 24-methylene sidechain, and for the acetates of cycloartanol, cycloeucalenol, and pollinastanol [19], which represent an homologous series of 4-demethyl, 4α-monomethyl and 4,4-dimethyl steroids respectively. This allowed the sidechain carbons C-23–C-28 to be

Table II. ^{13}C NMR chemical shifts of 15-azahomosterols.

	15-Aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**1**)	4α-Methyl-15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**2**)	4,4-Dimethyl-15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**3**)
C-1	33.60	34.60	35.46
C-2	30.70	30.88	27.66
C-3	68.49	75.97	78.54
C-4	37.04	38.94	39.00
C-5	38.87	46.42	49.82
C-6	16.44	18.65	18.45
C-7	30.54	26.69	29.71
C-8	121.80	126.33	126.69
C-9	183.3	183.2	183.2
C-10	38.21	37.79	37.67
C-11	21.42	20.67	18.92
C-12	25.65	27.22	28.28
C-13	38.48	38.11	38.50
C-14	166.94	166.20	166.20
C-15	45.85	49.91	50.57
C-16	23.63	20.79	20.01
C-17	45.36	47.69	47.93
C-18	17.24*	17.03*	16.95*
C-19	18.51	19.69	20.62
C-20	29.89	30.65	30.68
C-21	20.78*	21.40*	21.43*
C-22	32.26	33.20	33.26
C-23	31.13	31.45	31.50
C-24	154.88	156.22	156.34
C-25	33.05	33.72	33.74
C-26	21.67	21.95	21.96
C-27	21.23	21.75	21.78
C-28	106.54	106.49	106.44
C-29		15.01	28.00
C-30			15.54

Solvents: (**1**), $CDCl_3$–DMSO-d_6; (**2**) and (**3**), $CDCl_3$. Chemical shifts given in ppm from TMS.
* Tentative assignments and may be interchanged.

identified, signals appearing essentially unchanged from those of the model compounds. Carbons 20–22 were, however, shifted somewhat due presumably to the presence of the 6-membered D-ring in the azasterols. Indeed, without further information, the assignment for C-21 is only tentative. Slight differences in chemical shifts between the *Geotrichum* and *Calcarisporium* compounds were ascribed to solvent effects.

The conjugated imine system gave three quaternary signals at approximately δ 122, 167 and 182 ppm in the spectrum of **1.** In the spectra of the *Calcarisporium* compounds, only a δ 126 signal was observed in initial studies. However, by the addition of sodium dithionite (Hydros) to the sample, signals for C-9 and C-14 were resolved. Paramagnetic impurities were assumed to be weakening these signals to the point of non-detection. Carbon 4 appeared as a methylene carbon at δ 37.04 in **1,** a methine at δ 38.94 in **2,** and a quaternary carbon at δ 39.00 in **3,** in good agreement with relative values for the series of 4-demethyl, 4α-monomethyl and 4,4-dimethyl compounds mentioned above [19]. The methyl signals due to the 4-substitution were easily and unequivocally assigned. An extra methyl signal at δ 15.01 was present in the spectrum of **2,** and was assigned to the 4α (equatorial) methyl group, C-28. Two additional methyl peaks at δ 15.54 and 28.00 were apparent in the spectrum of **3.** These chemical shifts agree well with those in model compounds [19] in which a mono 4α-methyl has a shift of δ 14–15, and the 4,4-dimethyl derivative has C-4β (axial) at *ca.* δ 15 and C-4α (equatorial) at δ 25–28.

Thus from a combination of spectroscopic data, and an unequivocal structure for A25822B (**1**) based on X-ray crystallography, the *Calcarisporium* compounds are confirmed as the corresponding 4α-monomethyl- and 4,4-dimethyl-analogues (**2**) and (**3**) respectively. The dimethyl compound (**3**) has been reported as a constituent of *Geotrichum flavo-brunneum,* and was designated A25822A [1, 3]. The presence of trace amounts of the 4α-monomethyl compound (**2**) in *G. flavo-brunneum* has also been suggested, and this compound was designated AzX, though few details are available [20]. In the present studies, mass spectra from partially-purified samples of A25822B showed traces of a higher molecular weight constituent at *m/z* 425, consistent with the 4α-monomethyl analogue. A synthesis of A25822A (**3**) has recently been described [21], thus confirming the proposed structure.

Minor 15-azahomosterols from Calcarisporium thermophilum

Based on preliminary MS evidence, *Calcarisporium thermophilum* produces a range of 15-azahomosterol derivatives, of which the 4α-methyl- and 4,4-dimethyl-analogues of A25822B are the predominant metabolites. A minor constituent isolated had a molecular ion peak at *m/z* 411, and was chromatographically identical to A25822B (**1**) from *Geotrichum flavo-brunneum.* A second material was observed as a contaminant at *m/z* 453 in impure samples of **2** and **3,** a molecular weight corresponding to that of the 3-O-acetyl derivative (**4**) of A25822B isolated from *G. flavo-brunneum* and designated A25822M [3].

Further evidence for the presence of other azasterols was obtained by GC-MS analysis of the crude azasterol fraction, derivatized as trimethylsilyl ethers (Table III). Five significant bands were observed, the most abundant compound having a molecular ion peak at *m/z* 497 (100%) with [M-methyl] at 482 (30%), and thus corresponding to the mono-TMS ether of **2.** The TMS ether of compound **3** was the second major compound, and similarly gave a molecular ion peak at *m/z* 511

Table III. GC-MS data for 15-azahomosterol fraction isolated from *Calcarisporium thermophilum.*

R_t (min:sec)	Rel. peak ht	M^+ TMS deriv.	M^+ underivatized	Suggested structure [relationship to **1**]	
7:39	12	585	441	4α-methyl-15-hydroxy-	(**7**)
8:46	100	497	425	4α-methyl-	(**2**)
10:02	30	511	439	4,4-dimethyl stereoisomer	
10:15	46	511	439	4,4-dimethyl-	(**3**)
11:42	10	525	453	4,4-dimethyl-15-keto-	(**5**)

(100%) with [M-methyl] at 496 (30%). A third peak, clearly different from the TMS ether of **3,** gave however an almost identical mass spectrum, and is tentatively suggested to be derived from a stereoisomer of **3,** though this has not been confirmed.

The slowest moving component had a molecular ion at *m/z* 525 (100%) with [M-methyl] at 510 (35%), and corresponds to a mono-TMS ether of the compound with molecular weight 453 detected above. If these materials are in fact analogous, then the 3-O-acetyl derivative (**4**) may be excluded since a TMS ether is produced. An alternative structure corresponding to this mass is the 15-keto derivative (**5**) of **3,** which would be the 4,4-dimethyl analogue of A 25822 L (**6**), reported in *G. flavo-brunneum* [3]. The fastest moving component, again present in relatively small amounts, gave a molecular ion peak at *m/z* 585 (89%) with [M-methyl] at 570 (25%) and [M-OTMS] at 496 (100%), and is likely to be a di-TMS ether. The parent diol with molecular weight 441 may be the 15-hydroxy derivative (**7**) of **2,** which would be the 4α-methyl analogue of A 25822 D (**8**) from *G. flavo-brunneum* [3]. Obviously, further data are required to characterize these azasterol constituents.

Biological activity

In a broth dilution test, the antifungal activities of the 15-azahomosterols **1, 2** and **3** against *Candida parapsiliosis* were compared (Table IV). Amphotericin B was used as reference compound. Both the 4-demethyl and 4α-monomethyl compounds were more active than amphotericin B, and the 4,4-dimethyl compound, although inferior, was significantly active. Clearly, addition of methyl groups on to position 4 reduces activity accordingly. Gordee *et al.* [4] also found the dimethyl compound A 25822 A to be less active than A 25822 B. Preliminary studies also show these compounds to be active against *Cladosporium herbarum* and *Aspergillus niger* [22].

Table IV. Minimum inhibitory concentrations of 15-azahomosterols against *Candida parapsiliosis.*

Compound	MIC [μg/ml]
15-Aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**1**)	0.03–0.05
4α-Methyl-15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**2**)	1.2
4,4-Dimethyl-15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (**3**)	5.6
Amphotericin B	3.1

Calcarisporium thermophilum is only the second known source of 15-azahomosterol derivatives. The major metabolites are 4α-methyl- and 4,4-dimethyl-analogues of A 25822 B, the principal azasterol from *Geotrichum flavo-brunneum* cultures. Although these methyl derivatives have been reported previously as constituents of *G. flavo-brunneum,* they were present only in relatively small amounts, and the biosynthetic sequence in *G. flavo-brunneum* seems to favour demethylated azasterols. In *C. thermophilum,* however, biosynthesis favours the production of azasterols retaining methyl groups at position 4. Of the minor azasterol constituents detected in *C. thermophilum,* mass spectral measurements suggest these also contain methyl groups at position 4, apart from A 25822 B which was present in trace amounts. Although yields of azasterols from *C. thermophilum* are substantially lower than those obtained from *G. flavo-brunneum,* this organism offers another source of natural azasterols differing in structure from the *Geotrichum* compounds, and an alternative opportunity to study their biosynthesis and metabolism. The *Calcarisporium* 15-azahomosterols show a high level of antifungal activity, particularly against *Candida,* and is within the range required for clinically useful antifungal drugs. However, the presence of methyl groups at position 4 appears to reduce the antifungal activity of the azasterol relative to the 4-demethyl compound. A consequence of introducing methyl groups at position 4 is modification of the shape of the azasterol in the A-ring region, destroying its relatively flat nature. This may affect binding to the enzyme systems involved and modulate the degree of inhibition.

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