THE SQUALESTATINS, NOVEL INHIBITORS OF SQUALENE SYNTHASE PRODUCED BY A SPECIES OF *PHOMA*

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY

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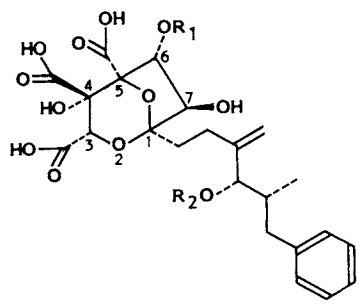
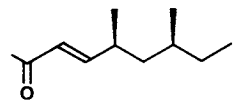
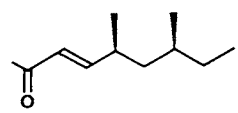
During the screening of fungi for inhibitors of squalene synthase, *Phoma* sp. C2932 was found to produce three structurally related novel inhibitors. These compounds, designated the squalestatins, exhibited potent activity against both mammalian (rat liver) and fungal (*Candida albicans*) squalene synthase. Furthermore, they also had broad spectrum *in vitro* antifungal activity.

High serum cholesterol levels have been established as an important risk factor for atherosclerosis, and cholesterol lowering regimens have been shown to decrease the incidence of coronary heart disease morbidity and mortality[1,2]. In the search for agents which may lower cholesterol levels, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), a major regulatory enzyme in the cholesterol biosynthetic pathway[3], has been selected as a target for inhibition. The discovery of compounds such as mevinolin, from *Aspergillus terreus*[4] with potent inhibitory activity against this enzyme, has led to the development of a new generation of cholesterol lowering agents with proven efficacy in man.

As an alternative strategy for the development of novel cholesterol lowering agents, we have chosen the enzyme squalene synthase [EC 2.5.1.21] as a possible target for therapeutic intervention. Squalene synthase (SQS) catalyses the first pathway specific step in sterol biosynthesis[3,5]. We conducted a screening campaign seeking inhibitors of the enzyme and found potent activity in fermentation broths of a newly isolated species of *Phoma*. Subsequent investigations revealed that inhibition was due to a novel family of compounds, the

Fig. 1. Structures of squalestatins.



Compound	R_1	R_2
1		
2		H
3	H	

squalestatins (Fig. 1). In this paper we describe the taxonomy of the producing strain, fermentation conditions, together with isolation, physico-chemical properties and biological activities of three squalestatins. Full details of the structure elucidation of the squalestatins are described in the accompanying paper[6].

Materials and Methods

Fermentation

Phoma sp. C2932 was maintained on oatmeal agar plates. Liquid cultures were inoculated with 6 mm agar plugs taken directly from plates, or previously stored in distilled water at room temperature. Vegetative seed growth in broth was also used as an inoculum source and could be stored frozen at −20°C.

Seed cultures were inoculated with two agar plugs (or vegetative culture) added to a 250-ml Erlenmeyer flask containing 50 ml of a medium of the following composition: Peptone (Oxoid L34) 10 g, malt extract (Oxoid L39) 21 g, glycerol 40 g, Junlon PW110 (Honeywell and Stein Ltd., Wallington Surrey) 1 g in 1 litre distilled water. The pH of the medium was adjusted to 6.5 by the addition of aq NaOH before autoclaving.

The flasks of inoculated seed medium were incubated at 25°C on an orbital shaker platform, rotating at 250 rpm with a 50-mm throw, for 5 days. The contents of seed flasks were pooled and homogenised in a sterile Waring blender and used at 3% to inoculate 50 ml aliquots of fermentation medium in 250 ml Erlenmeyer flasks. The fermentation medium (FM13) contained glycerol 50 g, soybean oil 30 g and cottonseed flour (Sigma) 10 g in 1 litre distilled water. The pH of the medium was 6.1 to 6.3 before autoclaving. The flasks were incubated with shaking (as above) for 8 days.

Isolation

The culture filtrate from 4 litres fermentation broth was adjusted to pH 2.8 with 20% aq H_2SO_4 and partitioned between an equal volume of EtOAc (3 times). The organic phases were pooled, partitioned with 1% aq $NaHCO_3$ and the aqueous phase, which accounted for the bulk of the SQS-inhibitory activity, was separated. The pH of this aqueous phase was adjusted to 2.8 as above and re-extracted with EtOAc. The organic phase was dried over anhydrous sodium sulfate and concentrated *in vacuo* to a brown oil (500 mg).

Further purification was carried out by countercurrent chromatography (CCC) using an Ito Planet Coil Centrifuge (P.C. Inc). Phases were pumped using a Gilson Model 303 pump fitted with a 50.S pumping head and a Model 804C manometric module. Samples were injected *via* a simple T-piece fitted with a 3-way tap at the "tail" end of the column. A preparative coil (70 m × 2.6 mm i.d.) of capacity 380 ml was used with a solvent system composed of EtOAc - hexane - MeOH - 0.01 N aq H_2SO_4 (6 : 5 : 5 : 6). The centrifuge was operated at 800 rpm with a flow rate of 4 ml/minute. After 4 hours, the centrifuge was stopped and the column was displaced with MeOH at a flow rate of 20 ml/minute. Throughout the run, 20 ml fractions were collected and monitored for SQS inhibitory activity after a dilution of 1 in 5,000.

Active compounds were detected in 3 groups of CCC fractions, representing elution of 300 ~ 500 ml (compound **1**), 1,100 ~ 1,300 ml (compound **2**) and 1,450 ~ 1,600 ml (compound **3**). The samples were concentrated *in vacuo* to pale yellow oils. Compounds **2** and **3** were further purified by CCC under the same conditions described above, but changing the proportion of the solvent system to (3 : 2 : 2 : 3) for **2** and (6 : 1 : 1 : 6) for **3**. The yields of **1**, **2** and **3** were 90 mg, 15 mg and 5 mg, respectively. The oils could be converted into white powders by dissolving in MeOH, chromatographing over ODS-silica eluted with an MeCN - H_2O gradient, concentrating active fractions *in vacuo* and freeze drying.

Determination of Physico-chemical Properties

UV spectra were determined in MeCN using a Hewlett-Packard 8452-A diode array spectrophotometer. IR spectra were determined in Nujol using a Nicolet 5 SXC, or 20 SXB FTIR spectrophotometer. Optical rotations were measured using a Perkin-Elmer 241 polarimeter. High resolution negative ion FAB mass spectrometry was performed on a VG ZAB-2SE mass spectrometer operating at a resolving power of 10,000. A cesium ion gun operating at 30 kV was used to generate spectra. Polyethylene glycol was used as a reference compound and glycerol was used as a matrix. Low resolution EI-MS were obtained on a

Finnigan MAT 8400 mass spectrometer operating at a resolution of 1,000. NMR spectra in CD_3OD were recorded on a Bruker AM500 instrument.

Squalene Synthase Activity

Materials

[1-^{14}C]Isopentenyl pyrophosphate (IPP, 56 Ci/mol) and *trans, trans*-[2-^{14}C]farnesyl pyrophosphate (FPP, 50 Ci/mol) were obtained from Amersham International plc. All other assay reagents were purchased from Sigma.

In Vitro Assay

Squalene synthase activity was determined by a novel assay procedure described in detail elsewhere[7]. Assays were conducted in the wells of microtiter plates in a total volume of 50 μl containing 50 mM MOPS - NaOH; pH 7.4, 10 mM KF, 10 mM $MgCl_2$, 0.5 mM NADPH, 50 mM ascorbate, 20 units/ml ascorbate oxidase, 1 ~ 3 mg/ml rat liver homogenate protein and either 9 μM [1-^{14}C]IPP (25 nCi), or 21 μM [2-^{14}C]FPP (52 nCi). Squalene synthase inhibitors were added as solutions in 25% DMSO. The final concentration of DMSO in all assays was 5%. After incubation at room temperature for 30 minutes, reactions were terminated by the addition of 50 μl propan-2-ol. Portions of each reaction mixture (25 μl) were spotted onto polyester-backed silica gel TLC sheets (Sigma) scored into 2 cm × 2 cm squares. The sheets were washed in 0.1 M ethanolamine containing 1% (w/v) SDS (3 × 30 minutes) to remove reaction products other than squalene. After drying, each square was excised and counted for radioactivity in 10 ml of Optiphase Safe scintillation cocktail (LKB) using an LKB 1219 Rackbeta scintillation counter.

Preparation of Rat Liver Homogenate

Freshly obtained rat livers were rinsed in ice-cold homogenisation buffer (50 mM MOPS - NaOH; pH 7.4, 40 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol) and chopped with a scalpel. The tissue was homogenised (2 ml of buffer per g tissue) in an all-glass Wheaton homogeniser (50 strokes with a loose-fitting pestle) and the homogenate was centrifuged at 5,000 × g for 10 minutes. The supernatant was filtered through 4 layers of muslin and recentrifuged at 15,000 × g for 15 minutes. The supernatant was again filtered (through 2 layers of muslin) and then stored in 1 ml portions at −20°C. Under these conditions, squalene synthase activity in the preparation was stable for several months.

Thin-layer Chromatography

Portions of terminated reaction mixtures (50 μl) produced as above were applied as a streak onto polyester-backed silica gel TLC sheets (Sigma) and chromatographed in propan-1-ol - ammonia - water (6 : 3 : 1). The plates were dried, autoradiographed and radioactive bands were excised and counted as above. The position of IPP, FPP and squalene were determined using radiolabelled standards.

Antifungal Activity

Minimum inhibitory concentrations (MICs) *in vitro* were measured using the agar dilution method with a multipoint inoculater. Iso-sensitest agar (Oxoid) was used for all organisms except the dermatophytes which were tested on SABOURAUD-dextrose agar (Oxoid). The *Candida* spp. were inoculated at 10^3 cfu and incubated at 34°C for 12 hours. The *Aspergillus* spp. were inoculated with 10^5 spores and the *C. neoformans* at 10^5 cfu and incubated at 28°C for 48 hours. The inocula for the dermatophyte species were a mixture of microconidia and hyphal elements and the incubation time was 4 days.

Results

Taxonomy of the Producing Organism

The fungus strain C2932 was isolated from a soil sample collected at Armacao de Pera, Portugal. After 2 ~ 3 weeks growth at 25°C on oatmeal agar the colonies were smoke grey to mouse grey in colour[8] on both the surface and reverse of the colony.

Morphological observations were made of the strain grown at 25°C on oatmeal agar. The fungus had

no sexual reproductive stage but formed pycnidia, thereby placing it in the class Coelomycetes. The fungus produced rostrate pycnidia with loose hyphae and both aseptate and one-septate ellipsoid conidia. The conidia were approximately $5\sim9\times1.5\sim3\,\mu m$ in dimensions (usually $7\sim9\times1.5\sim2.5\,\mu m$). Numerous multiseptate-multicellular, globose structures resembling chlamydospores or pycnidial initials were also observed. Distinct dictyochlamydospores were absent.

From the aforementioned characteristics, the strain was identified as a species of the genus *Phoma*[9] and the identity confirmed by the International Mycological Institute (IMI), England. As it has not been possible to assign the strain to an existing species of *Phoma*, a complete taxonomic description is in preparation for publication elsewhere. The strain has been deposited in the IMI culture collection under the accession number IMI 332962.

Fermentation

A number of complex and synthetic media were evaluated for production of the squalestatins. Amongst the complex media, those containing cottonseed flour supported much the highest activity. Medium FM13 (see Materials and Methods) was chosen for further studies.

In this medium a dense mycelium grew rapidly and darkened to a black or green/black colour after $2\sim3$ days. Chlamydospore production was observed in most liquid cultures (Plate 1). The time course of growth and squalestatin production in a 250-ml flask containing 25 ml culture are shown in Fig. 2. The pH of the culture fell steadily from a starting value of $6.3\sim6.6$ to $5.5\sim6.0$ through the course of the fermentation. SQS inhibitory activity was first detected after $3\sim4$ days and activity continued to increase until at least 8 days after inoculation. At

Plate 1. Light micrograph of mycelium and chlamydospores of *Phoma* sp. C2932 produced in liquid culture (scale: 10 μm).

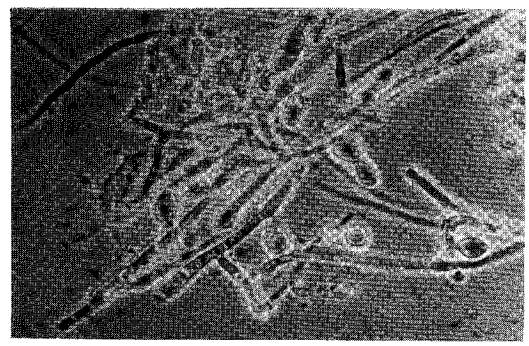
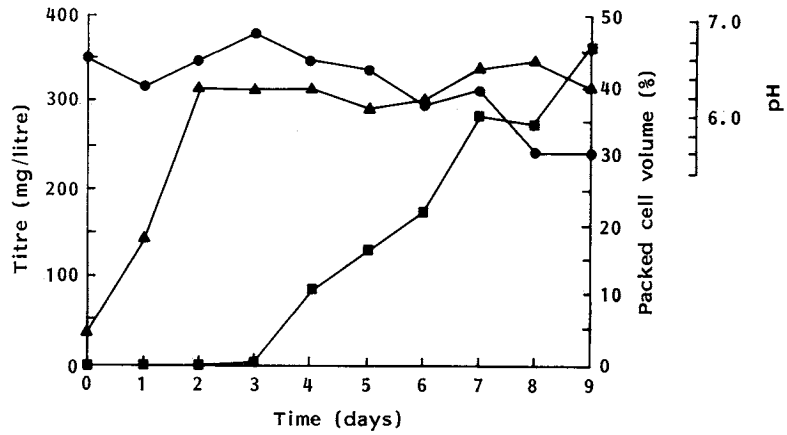


Fig. 2. Profile of squalestatin fermentation.

■ Squalestatin titre, ▲ packed cell volume, ● pH.



this stage the broth filtrate could be diluted 1,000~2,000-fold to produce 50% inhibition in the SQS assay.

Large batches of 250 ml flasks were used to produce 4 litres of broth for isolation of the active components. Three active components were initially purified.

Isolation

Compounds **1**~**3** were purified from the culture filtrate by solvent extraction followed by countercurrent chromatography (CCC), Fig. 3, as described in Material and Methods. The EtOAc extract prior to CCC accounted for *ca.* 70% of the total SQS inhibitory activity of the culture filtrate. CCC was chosen as the principal chromatographic technique in this case because simple phase partition experiments indicated that separation of the individual active components could be optimised by selection of appropriate biphasic solvent systems.

Physico-chemical Properties

The physico-chemical properties of **1**~**3** are summarised in Table 1. The UV absorption maxima for compounds **1** and **2** (210 and 211 nm, respectively) are consistent with the presence of an α,β-unsaturated ester (absent in compound **3**). The IR absorption bands for **1**~**3** (3600~3300, 1735 and 1729 cm^{-1}) are

Fig. 3. Countercurrent chromatography of an ethyl acetate extract derived from the *Phoma* culture filtrate.

Peaks A, B and C correspond to compounds **1**, **2** and **3**, respectively.

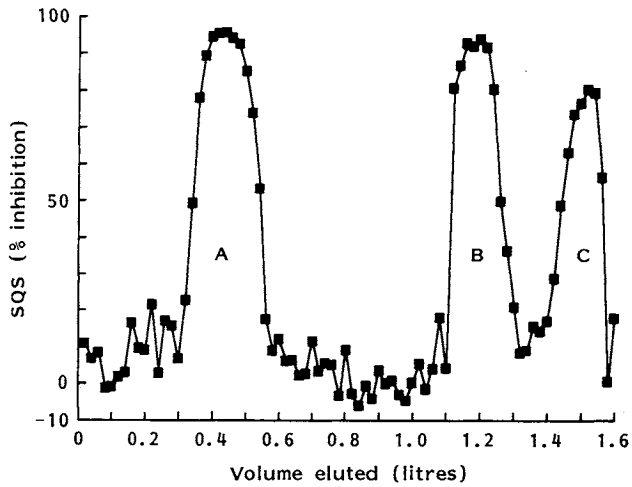


Table 1. Physico-chemical properties of squalestatins **1**~**3**.

	1	**2**	**3**
Appearance	White powder	White powder	White powder
$[\alpha]_D^{20}$ (*c* 1.0, $CHCl_3$)	+10.7°	+10.4°	+7.7°
Molecular formula	$C_{35}H_{46}O_{14}$	$C_{33}H_{44}O_{13}$	$C_{25}H_{30}O_{13}$
MW	690	648	538
EI-MS (*m/z*)	630, 612, 539, 521, 170, 91, 43	497, 170, 91	
HRFAB-MS (*m/z*) ion:			
Observed $(M-H)^-$	689.2789	647.2708	537.1673
Calculated $(M-H)^-$	689.2809	647.2704	537.1608
UV λ_{max}^{MeCN} nm ($E_{1cm}^{1\%}$)	210 (314)	211 (345)	End absorption
IR (Nujol) cm^{-1}	3600~3300 (br), 1729	3600~3300 (br), 1729	3600~3300 (br), 1735

consistent with the presence of hydroxyl and carbonyl groups. The UV, IR and ^{1}H NMR spectra of **1** are shown in Figs. 4, 5 and 6, respectively.

Compounds **1**~**3** are novel, each possessing the highly functionalised bicyclic core; 4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic acid. Their structures have been determined by a combination of spectroscopic, X-ray crystallographic and chemical methods. Full details are described in the accompanying paper[6].

Biological Activities

Squalene Synthase

The assay method allows measurement of squalene production from either [1-^{14}C]IPP as a radiolabelled precursor of FPP or from [2-^{14}C]FPP as a direct substrate for squalene synthase. The use of ascorbate and ascorbate oxidase in the incubation mixture as an oxygen consumption system prevents further conversion of IPP or FPP beyond squalene[7].

Compounds **1**~**3** were potent inhibitors of squalene production from either [1-^{14}C]IPP or [2-^{14}C]FPP (Table 2). IC_{50}'s for compound **1** using FPP as substrate were in the range 4~22 nM. Thin-layer chromatographic analyses of reaction mixtures containing [1-^{14}C]IPP as substrate indicated that **1** caused a marked accumulation of radiolabelled FPP with a concomitant reduction in radioactivity associated with squalene (Table 3).

Fig. 4. UV spectrum of squalestatin **1** (MeCN).

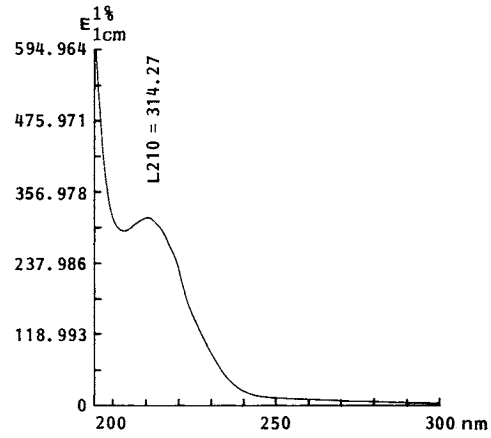


Fig. 5. IR spectrum of squalestatin **1** (Nujol).

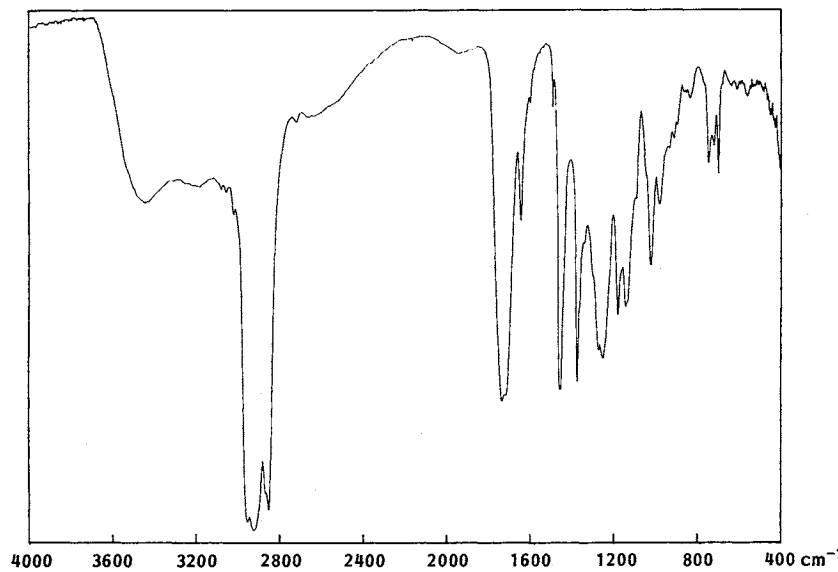


Fig. 6. ^{1}H NMR spectrum of squalestatin **1** (500 MHz, CD_3OD).

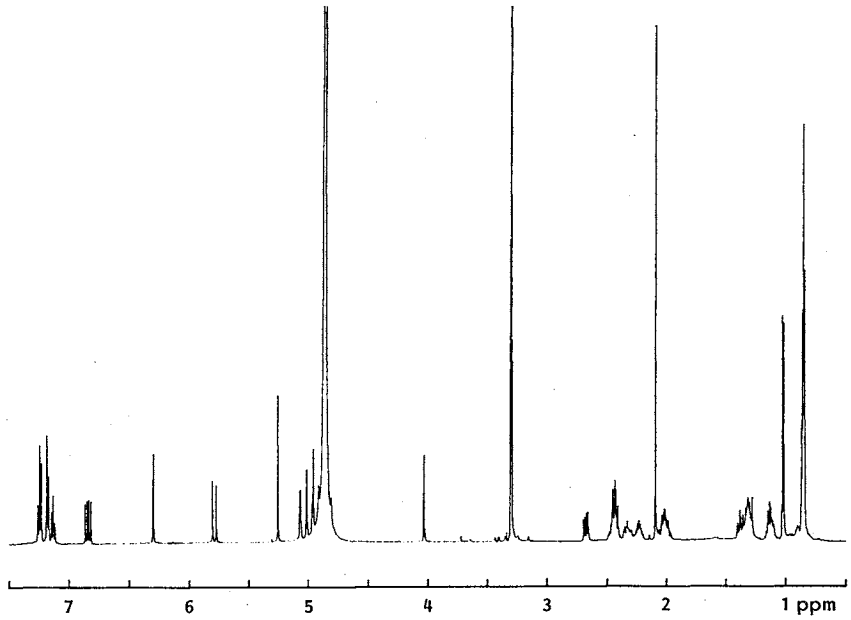


Table 2. Inhibition of squalene production by squalestatins using either [1-^{14}C]IPP or [2-^{14}C]FPP as substrate.

Compound	IC_{50} values (nM)	
	[1-^{14}C]IPP	[2-^{14}C]FPP
1	21.5	15.2
2	20.8	15.1
3	3.9	5.9

Assays were in triplicate in the presence of compounds **1**~**3** over a range of final concentrations. The concentrations of each compound which produced 50% inhibition of squalene production were determined from the dose response curves.

Table 3. TLC analysis of radiolabelled metabolites produced from [1-^{14}C]IPP in the presence or absence of squalestatin **1**.

Rf value	% of total radioactivity	
	Control	+Compound **1**
0.00	9.9	10.5
0.12 (IPP)	29.0	31.8
0.21	0.8	6.1
0.26	1.1	10.2
0.32 (FPP)	2.3	31.8
0.50	2.5	2.7
0.64	1.5	0.2
0.70	0.3	5.1
0.96 (Squalene)	52.4	1.5

The concentration of **1** (300 nM) was sufficient to produce maximal inhibition of squalene biosynthesis. Results are expressed as the percentage of total radioactivity on the TLC sheet associated with each radiolabelled band.

Three other minor intermediates accumulated in the presence of compound **1**. Those at Rf 0.21 and 0.26 were not present in analogous experiments using [2-^{14}C]FPP as substrate (results not shown) and may therefore, represent dimethylallyl pyrophosphate and geranyl pyrophosphate, respectively. The identity of the reaction product at Rf 0.70 remains unknown.

Taken together, the above results demonstrate that **1** is a potent inhibitor of rat liver squalene synthase. Exactly analogous results were obtained with compounds **2** and **3** (data not shown).

Compound **1** was also very active (IC_{50} = 5 nM) against microsomal squalene synthase from *Candida albicans*.

Antifungal Activity

The MICs of **1** for yeasts and fungi are listed in Table 4. The compound exhibited a broad spectrum of activity against a number of important pathogens. *C. neoformans* and the dermatophytic fungi were particularly sensitive. (MICs ~0.5 μg/ml).

Table 4. Activity of squalestatin **1** against fungi.

Test organism	MIC (μg/ml)
Candida albicans C316	8
C. albicans 2005E	4
C. glabrata 2375E	4
C. krusei 2374E	31
C. parapsilosis 2372E	8
C. pseudotropicalis 2371E	4
C. tropicalis 2808E	16
Cryptococcus neoformans 2867E	0.5
Aspergillus flavus C1150	8
A. fumigatus 48238	16
Trichophyton rubrum 1446E	0.5
T. mentagrophytes 857E	0.5
Microsporum canis 1742E	1
Epidermophyton floccosum C1169	0.5

Discussion

Previous attempts to identify novel, potent and selective inhibitors of mammalian squalene synthase have concentrated on the chemical synthesis of substrate and putative transition state analogues of the reaction. Farnesyl pyrophosphate analogues were initially studied by DE MONTELLANO *et al.*[10] and, more recently, by BILLER *et al.*[11] who defined a series of isoprenoid (phosphinyl) phosphonates which demonstrated inhibition of squalene synthase at micromolar concentrations. Presqualene phosphonophosphates[12], ammonium analogues of presqualene pyrophosphate[13], prenyl-substituted cyclobutanones[14] and a series of amphiphilic polyisoprenoid compounds[15] have also been investigated as squalene synthase inhibitors, but only modest activity has been observed.

Squalestatins represent the first novel class of inhibitors of squalene synthase from a natural source. The 2,8-dioxabicyclo[3.2.1]octane ring system occurs in nature[16], but in the squalestatins this bicyclic core is more highly functionalised. They are approximately 100-fold more potent than the most active compounds described in the above literature studies. They may, therefore, have therapeutic potential for the treatment of hypercholesterolaemia in an analogous fashion to the series of HMG CoA reductase inhibitors which have demonstrated cholesterol-lowering activity in man[17]. We have also confirmed that the squalestatins inhibit squalene synthase activity in microsomes of *Candida albicans* with a similar degree of potency. Furthermore, they exhibit potent broad spectrum antifungal activity *in vitro*. This raises the possibility of developing new antifungal agents targeted to the inhibition of fungal squalene synthase.

In addition to their therapeutic potential, the squalestatins should also find use as important research tools in studies of the regulatory mechanisms governing the isoprenoid biosynthetic pathway and of the relationships between this pathway and other cellular processes such as growth and proliferation[3,5,18].

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