Novel Squalestatins Produced by Biotransformation

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Microorganisms were screened for the ability to modify the squalene synthase inhibitor squalestatin 1.

Biotransformation of 1 by two actinomycetes, S15106 and S15138, yielded three products hydroxylated on the 4,6-dimethyl-oct-2-enoyl side chain either at the 6 position (5) or 7 position (4 two diastereoisomers), and lacking the acetyl ester from the C-1 side chain. Many strains were found to hydrolyse the 4,6-dimethyl-oct-2-enoyl or acetyl esters to yield squalestatins 2 or 3.

The 3-methyl ester (6) of 1 was obtained using *Fusarium* sp. F13945. This fungus also produced a farnesoic acid derivative, possibly in response to inhibition of its squalene synthase by 1. The biotransformation products of 1 all retained potent squalene synthase inhibitory activity.

The squalestatins are a novel family of compounds isolated from cultures of *Phoma* sp. which act *in vivo* as cholesterol-lowering agents[1~3]. Squalestatins act upon squalene synthase and it has been shown that 1 is a potent competitive inhibitor of the enzyme[4]. Compounds having the same 2,8-dioxabicyclo[3.2.1]-octane-4,6,7-trihydroxy-3,4,5-tricarboxylic acid core structure have been isolated from other fungi and reported under the name zaragozic acids[5].

As part of a programme to explore the effect of structural modifications on the biological activities of these compounds, microorganisms were screened for the ability to convert squalestatins to novel derivatives. This paper reports the microbial modification of 1 *via* hydroxylation of the 4,6-dimethyl-oct-2-enoyl side chain, and hydrolysis of the two ester functionalities. 2,3-Dihydrofarnesoic acid was also produced by a *Fusarium* sp. F13945 only in the presence of squalestatin 1.

Materials and Methods

Materials

Compound 1 was isolated from large scale cultures of *Phoma* sp. and the tri-potassium salt was prepared as described elsewhere[6].

Microorganisms

Microorganisms were obtained from various culture collections or were isolated from environmental samples. The actinomycete S15138 which produced 4 and 5 was deposited as NCIMB40434.

Culture Conditions

For biotransformation screening, a two stage fermentation was employed. Actinomycete seed cultures were

Fig. 1. Structures of squalestatins and products of biotransformation reactions.

Compound	R₁	R₂	R₃
1			H
2		H	H
3	H		H
4a,b (2 isomers)		H	H
5		H	H
6			CH₃
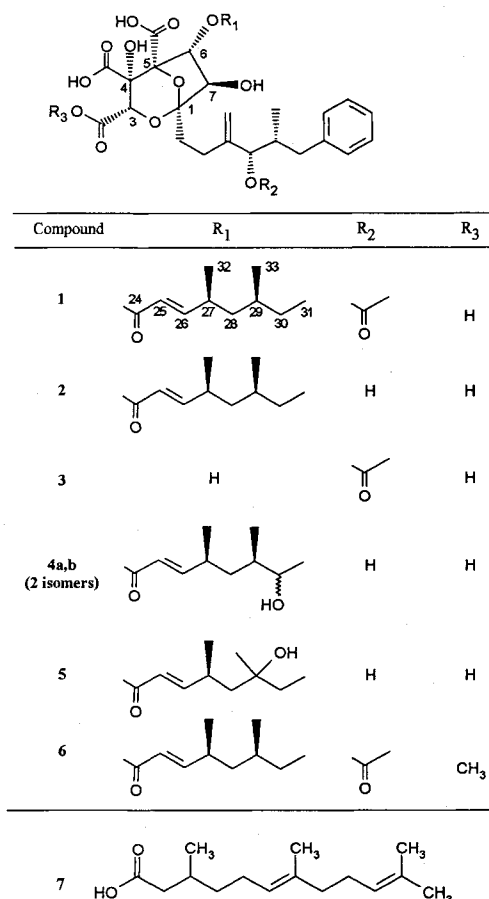
developed in MY or SV2 medium and fungal seed cultures were developed in FS. Seed cultures were inoculated with a spore suspension or surface growth from a slope or, in the case of some fungal cultures, agar plugs[1] and incubated for up to 5 days. Shaken flask cultures were grown in 25 or 50 ml of medium in 250 ml Erlenmeyer flasks (50 mm throw, 250 rpm). Final stage flasks or fermenters were inoculated at 2~3% (v/v). Media MY, SM17 and BHA5 were used for the final stage. Cultures were allowed to grow for 1 (actinomycetes) or 3 days (fungi) before addition of the tripotassium salt of 1 to give a final concentration of 0.1 or 0.5 mg/liter. Cultures were incubated for up to 7 days after substrate addition. Fungi were incubated at 25°C and actinomycetes at 28°C throughout.

Fermenter cultures were grown in a vessel of 4 liters working volume with a stirrer speed of 500 rpm and aeration at 3 liters/minute. PPG2000 antifoam was added, as required, to control foaming.

Media

SV2 consisted of glucose 1.5%, glycerol 1.5%, soy peptone (Lab M) 1.5%, NaCl 0.3%, $CaCO_3$ 0.1%, pH 7.0.

FS consisted of peptone (Oxoid L34) 1.0%, malt extract 2.1%, glycerol 4.0%, Junlon PW110 (Honeywell and Stein Ltd) 0.1%. Junlon was dispersed using a Waring blender and the pH was adjusted to 6.5 before autoclaving.

MY consisted of malt extract 2.4%, yeast extract 0.5% and polypropylene glycol 2000 0.063% dissolved in tap water and adjusted to pH 7.8 with NaOH (1 M).

SM17 consisted of glucose 0.2%, glycerol 4.0%, soluble starch (BDH) 0.2%, Arkasoy soya flour 0.5%, peptone (Oxoid L37) 0.5%, yeast extract (Oxoid) 0.5%, NaCl 0.5%, $CaCO_3$ 0.2%, dissolved in tap water.

BHA5 consisted of glucose 2.5%, malt extract 7.5%, phytone 2.5%, dissolved in tap water.

Analysis of Substrate and Products

Samples of fermentation broth were mixed 1:1 (v/v) with acetonitrile containing H_2SO_4 (0.5% v/v), allowed to stand for 30 minutes, centrifuged (10,000 × g, 5 minutes) and the clear supernatant assayed by HPLC for the substrate and biotransformation products. Two

systems were employed each using a 5 μm Spherisorb C6 column (150 × 4.6 mm) with UV detection at 210 nm. Substrate utilisation, and production of the desacetyl derivative, was measured using a mobile phase of acetonitrile-water (1:1) acidified with H_2SO_4 (0.015% v/v) at a flow rate of 3 ml/minute. More detailed analyses were carried out using a linear gradient from 0 to 50% acetonitrile in water acidified with H_2SO_4 (0.015% v/v) over 15 minutes. The acetonitrile content was held at 50% for a further 10 minutes (flow rate 1 ml/minute).

Mass Spectrometry

MS analysis was carried out using negative ion FAB with a glycerol-thioglycerol (1:1) matrix. The source was fitted to a Finnigan Mat 8400 mass spectrometer operating at a resolving power of 1000. Xenon was used as the FAB gas and the atom gun was operated at 9 kV and 1 mA. Under the above conditions this class of compounds afforded strong $[M-H]^-$ molecular ions coupled with structurally informative fragment ions.

NMR Spectroscopy

NMR spectra were recorded in CD_3OD on a Bruker AM500 instrument. Chemical shifts are reported in ppm referenced to CHD_2OD (3.31 ppm) for ^1H and CD_3OD (48.9) for ^{13}C as internal standards. Coupling constants are given in Hz (±0.5). The number of protons carried by each carbon was determined using DEPT.

Isolation of Products

Biotransformation reactions were carried out as summarised in Table 1.

Streptomyces rimosus: MeCN (16 ml) and H_2SO_4 (0.04 ml) were added to the culture, cells were removed by filtration and 250 mg of Partisil Bio Prep 40 C_{18} (P40, Whatman International Ltd., Maidstone) and water (50 ml) were added to the filtrate. After 1 hour the adsorbent was removed by filtration and washed with water. Adsorbed components were eluted with MeCN (3 ml). The concentrated eluate (1 ml) was diluted with a mobile phase of MeCN-H_2O-H_2SO_4 (52.5:47.5:0.2) (3 ml) and purified by preparative chromatography on Spherisorb S5 ODS2 (25 × 2.1 cm) at 20 ml/minute. 2 (2 mg) eluted from the column between 10.4 and 11.2 minutes.

Table 1. Culture conditions for biotransformation of squalestatin (1).

Product	Organism	Vol/Vessel	Medium	1 added (days)	Harvest (days)	Volume extracted
2	Streptomyces rimosus NRRL2455	25 ml/flask	MY	1*	5	16 ml
3	S. lavendulae CBS414.59	25 ml/flask	MY	1*	8	300 ml
4	Actinomycete S15106	50 ml/flask	MY	1	8	800 ml
4 & 5	Actinomycete S15138	50 ml/flask	MY	1	8	1.5 liters†
4 & 5	Actinomycete S15138	50 ml/flask	SM17	1	8	1.7 liters†
6 & 7	Fusarium sp. F13945	4 liter/fermenter	BHA5	3	8	4.0 liters

* 0.1 g/liter of tripotassium salt, otherwise 0.5 g/liter.
† Cultures pooled for extraction.

Streptomyces lavendulae: 300 ml culture was mixed with MeCN (300 ml) and H_2SO_4 (0.09 ml) and adsorbed to and eluted from P40 as described above to yield a concentrate (40 ml) which was purified by preparative chromatography on Spherisorb S5 ODS2 (25 × 2.1 cm) with a mobile phase of MeCN - H_2O - H_2SO_4 (30 : 70 : 0.2) (25 ml/minute) in eight portions. 3 (4 mg total) eluted from the column between 13.3 ~ 14.6 minutes.

Actinomycete S15106: 800 ml culture was adjusted to pH 2 with H_3PO_4 and centrifuged (5,000 × g, 30 minutes). The supernatant was stirred with P40 (5 g) and this was then removed by filtration and washed with water. Adsorbed material was eluted with MeCN (150 ml) and this combined with extracts of the pellet (250 ml MeCN, 150 ml MeCN). The combined extracts were clarified, concentrated under vacuum to 30 ml, acidified with H_2SO_4 (0.06 ml) and further purified by chromatography on a column of P40 (20 × 7.5 cm) with a mobile phase of MeCN - H_2O - H_2SO_4 (50 : 50 : 0.2). The fraction eluting between 1.3 ~ 1.63 liters was further separated by preparative chromatography on Spherisorb S5 C6 (25 × 2.1 cm) with a mobile phase of MeCN - H_2O - H_2SO_4 (32.5 : 67.5 : 0.2) at 25 ml/minute in five portions. Combined fractions collected from 31.3 ~ 33.0 minutes and a second set collected from 33.2 ~ 34.3 minutes yielded two diastereoisomers **4a** (< 1 mg) and **4b** (< 1 mg) respectively.

Actinomycete S15138: Cultures grown in both media were pooled, an equal volume of MeCN added (3.2 liters) and the mixture acidified with H_2SO_4 (12.8 ml). Cells were removed by centrifugation (5,000 × g, 30 minutes) and discarded and the MeCN removed from the supernatant under vacuum. The aqueous residue was passed through a column of Amberlite XAD16 (22 × 3.6 cm, Rohm and Haas) which was then washed sequentially with water (200 ml), disodium edetate (1% w/v, 200 ml) and water (200 ml). Adsorbed components were eluted with acetone - water (6 : 4). The eluate was diluted with an equal volume of water, adjusted to pH 2 with H_2SO_4 and pumped onto an HPLC column of Spherisorb S5 ODS2 (25 × 2.1 cm). This column was then washed with MeCN - H_2O - H_2SO_4 (20 : 80 : 0.2) and adsorbed material was eluted with MeCN - H_2O - H_2SO_4 (35 : 65 : 0.2). Solutes were recovered from the eluate and further purified by preparative chromatography on Spherisorb S5 ODS2 with a mobile phase of MeCN - H_2O - H_2SO_4 (27.5 : 72.5 : 0.2) at 20 ml/minute in five portions. Combined fractions collected from 43.2 ~ 45.0 minutes yielded **5** (3.8 mg). Combined fractions collected from 50 ~ 54 minutes yielded a 78 : 22 mixture of the two isomers of **4** (25.9 mg).

Fusarium sp. F13945: The culture was centrifuged (5,000 × g, 30 minutes) and the cells extracted with MeCN (1.5 liters). The extract was reduced under vacuum to 30 ml and acidified with H_2SO_4 (0.06 ml) and fractionated (8.4 liters, 1.6 liters, 1.2 liters, 3 liters) on a column of P40 (40 × 7.5 cm) in MeCN - H_2O - H_2SO_4 (50 : 50 : 0.2). Water (1.6 liter) and H_2SO_4 (2 ml) were

added to the 1.6 liter fraction and this was pumped onto an HPLC column of Spherisorb S5 C6 (25 × 2.1 cm) which was then developed with MeCN - H_2O - H_2SO_4 (50 : 50 : 0.2) at 25 ml/minute. This yielded 25 mg of **6**. Similar treatment of the 3 liters fraction from P40 yielded 12 mg of **7**.

Solutes were recovered from column eluates by pumping a 1 : 1 dilution of the sample in water through a cleaned, water-equilibrated column of Spherisorb S5ODS2 (25 × 2.1 cm). The column was washed with water until free of H_2SO_4 and adsorbed material was eluted with MeCN - H_2O (9 : 1) or (7 : 3). MeCN was removed from the eluate by evaporation and the concentrate was either lyophilised to give final product or subjected to further chromatography.

Squalene Synthase Assays

Inhibition of rat liver squalene synthase was measured using procedures described in detail elsewhere[1,8]. A similar procedure using a microsomal preparation derived from *Candida albicans* ATCC 10231 was used to measure inhibition of fungal squalene synthase.

In Vivo Cholesterol Lowering Activity

Cholesterol lowering activity was measured in marmosets using procedures previously described[3].

Results and Discussion

Screening for Modification of Squalestatin 1

Over 3,500 microbial cultures were screened for biotransformation of squalestatin **1**. The reactions observed are discussed below.

Biotransformation of 1 to 2

Biotransformation of **1** (as the K_3 salt) to the desacetyl derivative (**2**) was detected in 36% of the actinomycetes, 20% of the fungi and 18% of the bacteria among the 3533 microbial cultures screened. Product identification was based upon HPLC retention time (Rt) comparison with an authentic standard but in order to confirm identification, the product was isolated from a culture of *Streptomyces rimosus* NRRL 2455. The MS and NMR data were identical with those for authentic material[2].

Biotransformation of 1 to 3

Hydrolysis of the 4,6-dimethyl-oct-2-enoyl ester of **1**, to yield **3**, was observed in cultures of 1 actinomycete, 4 fungi and 9 bacteria. Presumptive identification of the product was based on gradient HPLC comparison with an authentic standard but in one case the material produced by biotransformation with *Streptomyces lavendulae* was isolated and its identity confirmed by

comparison (MS, NMR) with authentic material[2].

Hydroxylated Derivatives of 2 Produced by Actinomycetes S15106 and S15138

Actinomycetes S15106 and S15138 hydrolysed the acetyl ester on the C-1 side-chain of **1** converting $50 \sim 80\%$ to product **2**. A small proportion ($<5\%$) of the substrate was additionally modified by S15106 to two new products. The mass spectra of these (Fig. 3) showed an intense $(M-H)^-$ ion at m/z 663 with characteristic fragment ions at m/z 619, 495, 433 and 185 which suggested that, in addition to the loss of the acetyl group, hydroxylation of the 4,6-dimethyl-oct-2-enoyl ester side chain had taken place. Examination of the ^1H NMR spectra of these compounds (Table 2) showed the replacement of the triplet, assigned to the 31 position, by a doublet. Each also contained a signal consistent with the presence of a new CH–O group. These data

Table 2. ^1H NMR data for novel squalestatins produced by biotransformation.

Position No	Compounds		
	4a	4b	5
25	5.80 (br d, 15.5)	5.81 (br d, 15.5)	5.77 (dd, 1, 15.5)
26	6.80 (dd, 9, 15.5)	6.81 (dd, 9, 15.5)	6.98 (dd, 8.5, 15.5)
27	2.41~2.51 (m)[a]	2.41~2.51 (m)[a]	2.58 (m)
28	1.56 (ddd, 3.5, 10.5, 13.5)	1.51 (ddd, 4, 10, 13.5)	1.59 (dd, 8, 14.5)
	1.12 (ddd, 4.5, 10.5, 13.5)	1.19 (ddd, 5, 10, 13.5)	1.51 (dd, 4.5, 14.5)
29	1.42 (m)	1.42 (m)	—
30	3.53 (m)	3.61 (m)	1.47 (q, 7.5)
31	1.08 (d, 6.5)	1.09 (d, 6.5)	0.87 (t, 7.5)
32	1.06 (d, 6.5)	1.05 (d, 6.5)	1.07 (d, 6.5)
33	0.85 (d, 6.5)	0.86 (d, 6.5)	1.11 (s)

The chemical shifts of the protons not listed here are within ± 0.03 ppm of those of $2^{2)}$.

[a] Overlaps with other signals not given in this table.

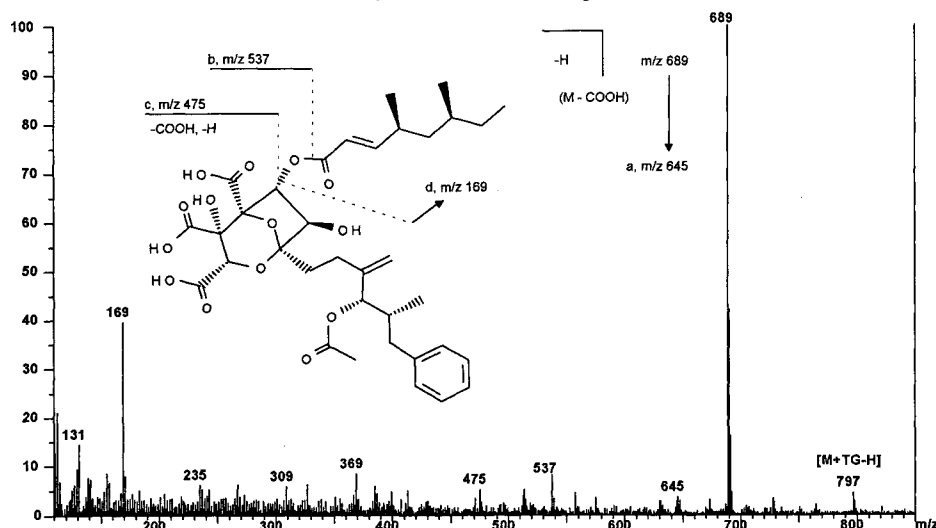Fig. 2.   Negative ion FAB mass spectrum of **1**.
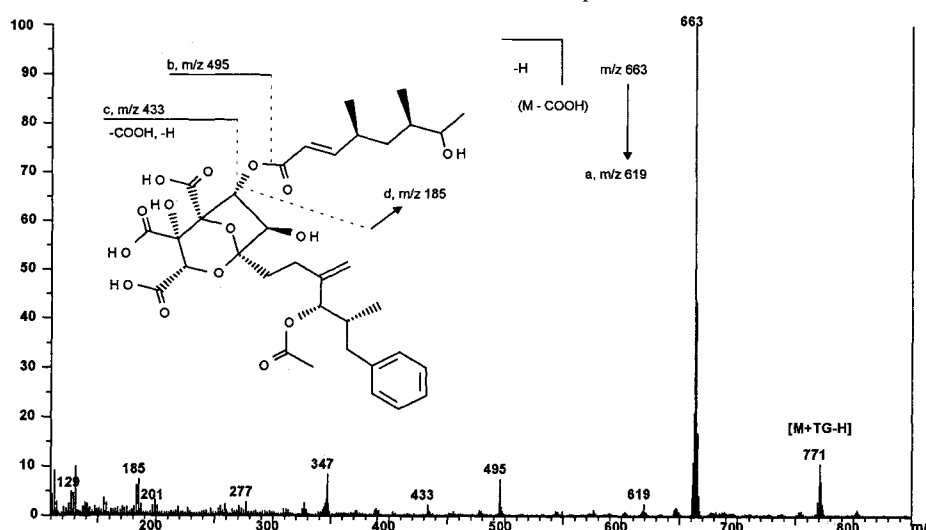

Fig. 3.   Negative ion FAB mass spectrum of **4**.

Table 3. Activity of 1~6 against squalene synthase from two sources.

Product	IC$_{50}$ (nM) for squalene synthase	
	Rat liver homogenates	Candida microsomes
1	12	5
2	5	7
3	26	30
4a	nd	3[a]
4b	12.0	nd
4a-4b (78:22)	6	6
5	9	10
6	7	6

nd Not determined.
[a] Material available for Candida tests contained 25% product 5.

established the point of hydroxylation as C-30, the two compounds being the stereo-isomers at this position (4).

Biotransformations with S15138 produced larger amounts of 4, together with a small amount of a further novel squalestatin. The mass spectrum of this compound was essentially the same as that for 4 (Fig. 3). The ^{1}H NMR spectrum (Table 2) showed the replacement of the doublet, assigned to the 33 position, by a singlet. These data established the point of hydroxylation as C-29. The configuration at the newly hydroxylated carbon has not been determined.

Products Isolated from Cultures of Fusarium sp. F13945

A culture of Fusarium sp. F13945 converted 1 to a product found by HPLC-MS to have a molecular weight 14 mass units greater than the free acid of 1 and Rt typical of a mono-methyl ester of 1. Following isolation, this biotransformation product was shown by NMR and MS, to be identical to a synthetic sample of 6[9].

A 4 liters culture grown to provide sufficient material for isolation and characterisation of this biotransformation product yielded an additional compound. The NMR spectra (^{1}H, ^{13}C and DEPT) of this material are consistent with structure 7 (Fig. 1). The ^{13}C spectrum was assigned by comparison with a range of related compounds[10]. ^{1}H NMR data for 7: δ 0.96 (3H, d, J=6.5, 3-CH$_3$), 1.24 (1H, m, $\frac{1}{2}$ 4-CH$_2$), 1.38 (1H, m, $\frac{1}{2}$ 4-CH$_2$), 1.60 (3H, br s), 1.61 (3H, br s), 1.67 (3H, br d, J=1.0), 1.87~2.13 (8H, m, $\frac{1}{2}$ 2-CH$_2$, 3-CH, 5-CH$_2$, 8-CH$_2$, 9-CH$_2$), 2.29 (1H, dd, J=6.0, 14.5, $\frac{1}{2}$ 2-CH$_2$), 5.09 (1H, m, 10-CH), 5.11 (1H, m, 6-CH). ^{13}C NMR δ 177.0 (C-1), 136.0 (C-7), 132.0 (C-11), 125.4 (C-6 or C-10), 125.3 (C-6 or C-10), 42.5 (C-2), 40.8 (C-8), 37.7 (C-4), 31.0 (C-3),

27.6 (C-9), 26.2 (C-5), 25.8 (C-12), 19.9 (3-CH$_3$), 17.7 (11-CH$_3$), 16.0 (7-CH$_3$).

Compound 7 was found only in cultures fed with 1. It is not squalestatin-related so its production appears to be a response of Fusarium to the presence of 1. The most likely explanation is that inhibiting squalene synthase caused a build up of its substrate, farnesyl pyrophosphate (FPP), which Fusarium converted to 7. Reports of experiments with radiolabelled precursors show that rat hepatocytes[3], Hep G2 cells[5] and mice[5] exposed to squalene synthase inhibitors also accumulate FPP and related metabolites.

Biological Activity of the Compounds Isolated

The concentration of compounds 4~6 causing 50% inhibition of squalene synthase activity (IC$_{50}$) was comparable with squalestatins 1~3 for both rat and Candida enzymes (Table 3). 4, as a mixture of isomers (78:22) was evaluated in single dose lipid lowering studies in marmosets. At 1 mg/kg iv 4 had no significant effect on serum cholesterol. A dose of 8 mg/kg iv produced a significant lowering of lipid levels (44%) 2 days after dosing, which returned to baseline levels 7 days after dosing.

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