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

IV. PREPARATION OF FLUORINATED SQUALESTATINS BY DIRECTED BIOSYNTHESIS

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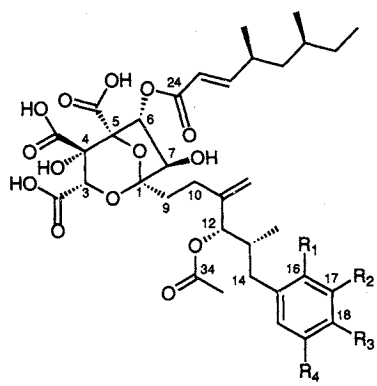
Feeding of fluorinated benzoic acids to fermentations of a *Phoma* sp. resulted in the biosynthesis of a series of novel fluorinated squalestatins. The feeding studies, isolation, structural elucidation and biological activities of these compounds are reported.

During a search for inhibitors of squalene synthase, a group of novel, highly active compounds, the squalestatins, was isolated from *Phoma* sp. C2932[1,2]. Biosynthesis studies using ^{13}C and ^{18}O-labelled precursors showed that the backbone of the major squalestatin (**1**) was derived from two acetate-generated polyketide chains, a 4-carbon unit related to succinate, methionine and atmospheric oxygen[3]. Interestingly, the starter unit for one of the polyketide chains appeared to be benzoic acid, itself derived from phenylalanine. In an attempt to generate novel squalestatins, analogues of this aromatic starter unit were fed to *Phoma* fermentations. This paper reports these feeding experiments and describes the analogues thus generated.

Fig. 1. Structures of Squalestatins (**1**~**7**).



	R_1	R_2	R_3	R_4
1	H	H	H	H
2	H	H	F	H
3	H	F	H	H
4	F	H	H	H
5	H	F	F	H
6	H	F	H	F
7	F	F	H	H

Materials and Methods

Organism

The squalestatin producer *Phoma* sp. C2932 was used for all of the work discussed.

Culture Conditions

Phoma sp. C2932 was maintained as frozen (−70°C) spore suspensions in 5% (w/v) glycerol. To prepare working stocks, a spore suspension was thawed at room temperature and 0.5 ml was used to inoculate a seed culture dispensed as a 50 ml aliquot in a 250-ml Erlenmeyer flask. The seed medium had the following composition: peptone (Oxoid L34) 10 g, malt extract (Oxoid L39) 21 g, glycerol 40 g, Junlon PW110 (Honeywill and Stein Ltd. Wellington, Surrey) 1 g in 1 litre of distilled water. The

pH of the medium was adjusted to 6.5 by the addition of aqueous NaOH prior to autoclaving. The flask of inoculated seed medium was then incubated at 25°C on an orbital shaker platform, rotating at 250 rpm with a 50 mm throw, for 4 days. The culture was then dispensed through a sterile 20-ml plastic syringe to reduce clumping and stored in 1.8 ml aliquots at −20°C.

For shake flask fermentations, 0.5 ml of the thawed culture (room temperature) was used to inoculate 50 ml of the seed medium (dispensed in a 250-ml Erlenmeyer flask and prepared as above). Incubation conditions were as above except that cultures were incubated for 3 days as this was found to be optimal. 1 ml of the seed stage was transferred to the fermentation medium (2% (v/v)) dispensed as 50 ml aliquots in 250-ml Erlenmeyer flasks. The fermentation medium had the following composition: cotton seed flour (Sigma) 20 g, fructose 50 g and soya bean oil 30 g in 1 litre of distilled water. No pH adjustment of this medium was found to be necessary. Flasks were incubated at 25°C and agitated as above.

Feeding Experiments

Potential precursors of squalestatin analogues were routinely added to the fermentation stage after incubation for 3 days. Where the required feed was a solid at room temperature, an aqueous solution was prepared (6.25 mg/ml), adjusted to neutral pH with NaOH and filter sterilised. 2 ml of each precursor was then added to 50 ml of the fermentation medium to give a final concentration of 250 μg/ml. Flasks were harvested following a 7 day fermentation (4 days post feeding).

Radioactive Labelling and Analysis

7-[^{14}C]-Benzoic acid (16 mCi/mmol, 20 μCi/50 ml culture, Amersham International) was added to a 3 day culture of C2932 in fermentation medium (50 ml in a 250-ml flask). 1 ml samples were removed daily and mixed with an equal volume of MeCN containing H_2SO_4 (5 ml/litre). The sample was then shaken and left to stand for 30 minutes. The sample was centrifuged at 10,000 × g for 10 minutes and the supernatant was decanted into a fresh tube. The sample was then centrifuged again and 200 μl of supernatant was chromatographed using the gradient HPLC system. 45 fractions (0.67 ml) were collected over 30 minutes and 10 ml of Optiphase Safe scintillation fluid (LKB) was added to each fraction. Radioactivity was determined by scintillation counting with a ^{14}C programme.

HPLC Analysis

Samples were prepared for HPLC analysis by mixing whole broth with an equal volume of MeCN containing H_2SO_4 (5 ml/litre). Supernatant was removed after centrifugation at 1,200 × g for 30 minutes. Samples were injected onto a Spherisorb C6 5 μm column (15 cm × 4.6 mm i.d.) which was developed with a gradient of 0 ~ 50% MeCN in water containing H_2SO_4 (50 μl/litre) (flow rate: 1 ml/minute) with monitoring at 210 nm.

HPLC-MS Analysis

Samples were prepared for HPLC-MS analysis by mixing whole broth with an equal volume of MeCN containing TFA (5 ml/litre). The samples were centrifuged at 1,200 × g for 30 minutes and the supernatant collected. Aliquots of the supernatant were injected onto a Spherisorb S5 ODS-2 HPLC column (25 cm × 2 mm) and the components of interest were eluted with MeCN-H_2O (55:45) (flow rate: 0.5 ml/minute) containing 0.1% TFA (v/v). The HPLC system was connected *via* a thermospray interface (TSP2) to a Finnigan Mat TSQ 70B mass spectrometer. The thermospray conditions were optimised to: source temperature 250°C, vapouriser temperature 70°C, repeller voltage-10V, mode negative ion TSP filament on, filament emission current 600 μA, conversion dynode voltage-20,000V.

Under these LC conditions **1** and its fluorinated analogues co-eluted, but from the mass spectra it was possible to differentiate between the two compounds.

Product Isolation

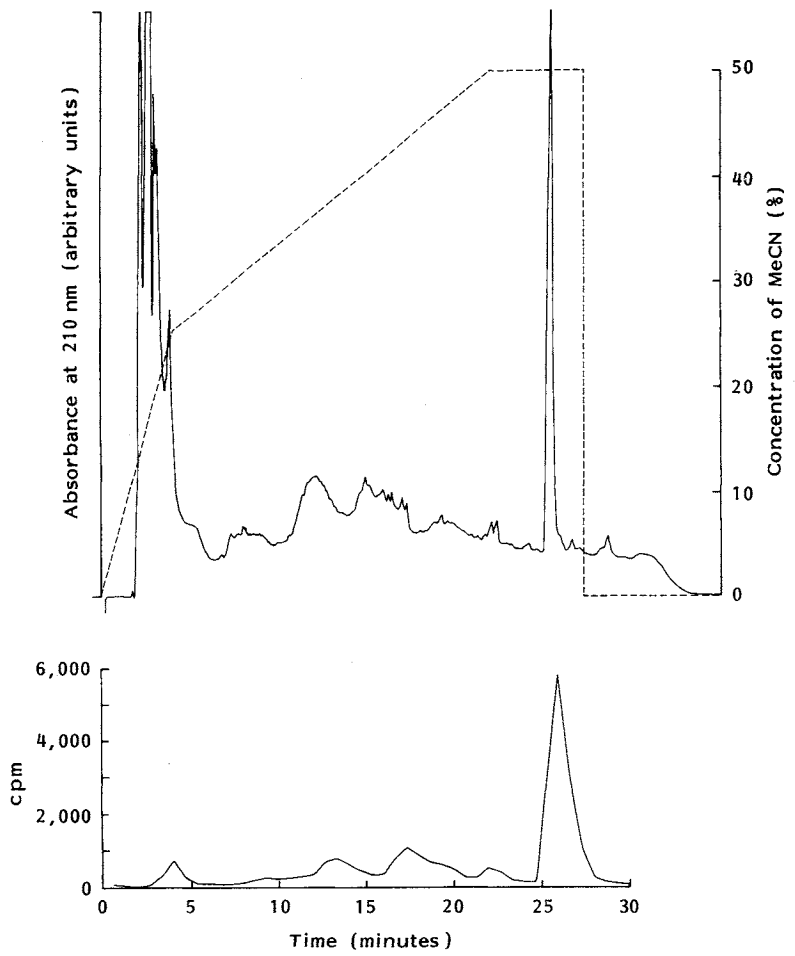
The crude broths were treated in one of two ways. In the first method the cells were extracted twice with water adjusted to pH 10.4 with ammonia solution, and after removal of the cells by centrifugation the pooled supernatants were adjusted to pH 8.2. Alternatively, one volume of MeCN containing H_2SO_4 (150 μl/litre) was added to the broth and the cells were harvested by centrifugation and re-extracted with

MeCN - H_2O (1 : 1). The extracts were pooled, and the MeCN was removed under vacuum. Extracts that were oily were filtered through a cellulose bed. Compounds of interest were then adsorbed onto an Amberlite XAD-16 column. The column was washed successively with water, 1% tetrasodium EDTA (w/v) and water, and then elution was carried out with Me_2CO - H_2O (6 : 4). Fractions containing **1** and its analogues were pooled, diluted and acidified with H_2SO_4 and then loaded onto a Spherisorb S5 ODS-2 HPLC column (25 × 2.1 cm). The column was washed with MeCN - H_2O (25 : 75) + H_2SO_4 (2 ml/litre) and compounds were eluted with MeCN - H_2O (1 : 1) + H_2SO_4 (2 ml/litre). The resulting fractions contained only **1** and analogues. These fractions were concentrated under vacuum.

Compounds **2** and **3** were separated from **1** by preparative HPLC using a Spherisorb S5 ODS-2 column (25 × 2.1 cm) and a mobile phase of MeCN - H_2O (45 : 55) + H_2SO_4 (2 ml/litre). Compounds **4** and **1** were resolved on a Spherisorb C6 column (25 × 2.1 cm) using a mobile phase of MeOH - H_2O (57.5 : 42.5) adjusted to pH 3 using H_3PO_4.

Fig. 2. Incorporation of 7-[^{14}C]-benzoic acid into **1**.

—— Absorbance at 210 nm, ------ concentration of MeCN.



20 μCi of 7-[^{14}C]-benzoic acid was added on day 3 to a growing culture of C2932. A sample was removed at day 4 and was analysed by HPLC. The HPLC trace (detection at 210 nm, 10 μl injection) is shown in the upper panel and the cpm (200 μl injection) are shown in the lower panel. The main peak on the radioactivity trace corresponded to **1**, and the minor peaks corresponded to the other related compounds which had been observed previously[1].

Table 1. Screen for incorporation of substituted benzoic acid compounds.

Analogue fed	**1** titre (mg/litre)	Analogue formed	Analogue titre (mg/litre)	Utilisation of feed (%)
Control (no feed)	400～700	—	—	—
Benzoic acid	157	—	—	100
2-Hydroxybenzoic acid	241	—	—	100
3-Hydroxybenzoic acid	600	—	—	100
4-Hydroxybenzoic acid	296	—	—	100
2,5-Dihydroxybenzoic acid	606	—	—	100
3,4-Dihydroxybenzoic acid	468	—	—	58
2-Aminobenzoic acid	205	—	—	100
3-Aminobenzoic acid	274	—	—	n.d.
4-Aminobenzoic acid	411	—	—	89
3-Amino,4-chlorobenzoic acid	548	—	—	100
3-Nitrobenzoic acid	399	—	—	24
4-Nitrobenzoic acid	475	—	—	100
2-Fluorobenzoic acid	304	**4**	30	9
3-Fluorobenzoic acid	82	**3**	41	24
4-Fluorobenzoic acid	50～75	**2**	10～21	12～51
2,3-Difluorobenzoic acid	284	—	—	0
2,4-Difluorobenzoic acid	289	—	—	0
2,5-Difluorobenzoic acid	250	—	—	0
2,6-Difluorobenzoic acid	278	—	—	0
3,4-Difluorobenzoic acid	128	**5**	Trace	0
3,5-Difluorobenzoic acid	190	**6**	2	0
2,3,4-Trifluorobenzoic acid	281	—	—	0
2,3,6-Trifluorobenzoic acid	347	—	—	0
2,4,5-Trifluorobenzoic acid	339	—	—	0
3,4,5-Trifluorobenzoic acid	195	—	—	0
2,3,4,5-Tetrafluorobenzoic acid	270	—	—	0
2,3,5,6-Tetrafluorobenzoic acid	294	—	—	0
Pentafluorobenzoic acid	354	—	—	2
2-Chlorobenzoic acid	369	—	—	0
3-Chlorobenzoic acid	111	—	—	14
4-Chlorobenzoic acid	56	—	—	7
2-Iodobenzoic acid	250	—	—	36
3-Iodobenzoic acid	130	—	—	0
4-Iodobenzoic acid	119	—	—	0
4-Methylbenzoic acid	87	—	—	50
2-Methoxybenzoic acid	326	—	—	0
3-Methoxybenzoic acid	275	—	—	27
4-Methoxybenzoic acid	209	—	—	100

n.d.: Not determined.

Substituted benzoic acids (250 mg/litre) were added to a 3 day flask culture of C2932. Each culture was examined by HPLC and HPLC-MS to determine whether the substituted benzoic acid had been incorporated into an analogue of **1**. The column labelled 'utilisation' refers to an HPLC determination of substituted benzoic acid utilisation between time of addition (day 3) and harvest (day 7).

Table 2. Inhibition by compounds **1**～**4** of mammalian and fungal squalene synthases.

Compound	SQS activity I_{50} (nM)	CSS activity I_{50} (nM)	Compound	SQS activity I_{50} (nM)	CSS activity I_{50} (nM)
2	32	13	**4**	43	7.7
3	38	4	**1** (range)	4～22	2.4～6.6

The analogues which were isolated were tested in the mammalian squalene synthase (SQS) and the *Candida* squalene synthase (CSS) assays. The ranges of values that have been obtained for **1** are shown for comparison. Compounds **5**, **6** and **7** were not isolated for testing because they were only produced in scant amounts.

Fig. 3. Mass spectra of compounds **1**, **2** and **6**.

The mass spectra from the HPLC-MS analyses of broth extracts with no feeding (A), and after being fed 4-fluorobenzoic acid (B) and 3,5-difluorobenzoic acid (C) are shown accompanied by fragmentation diagrams.

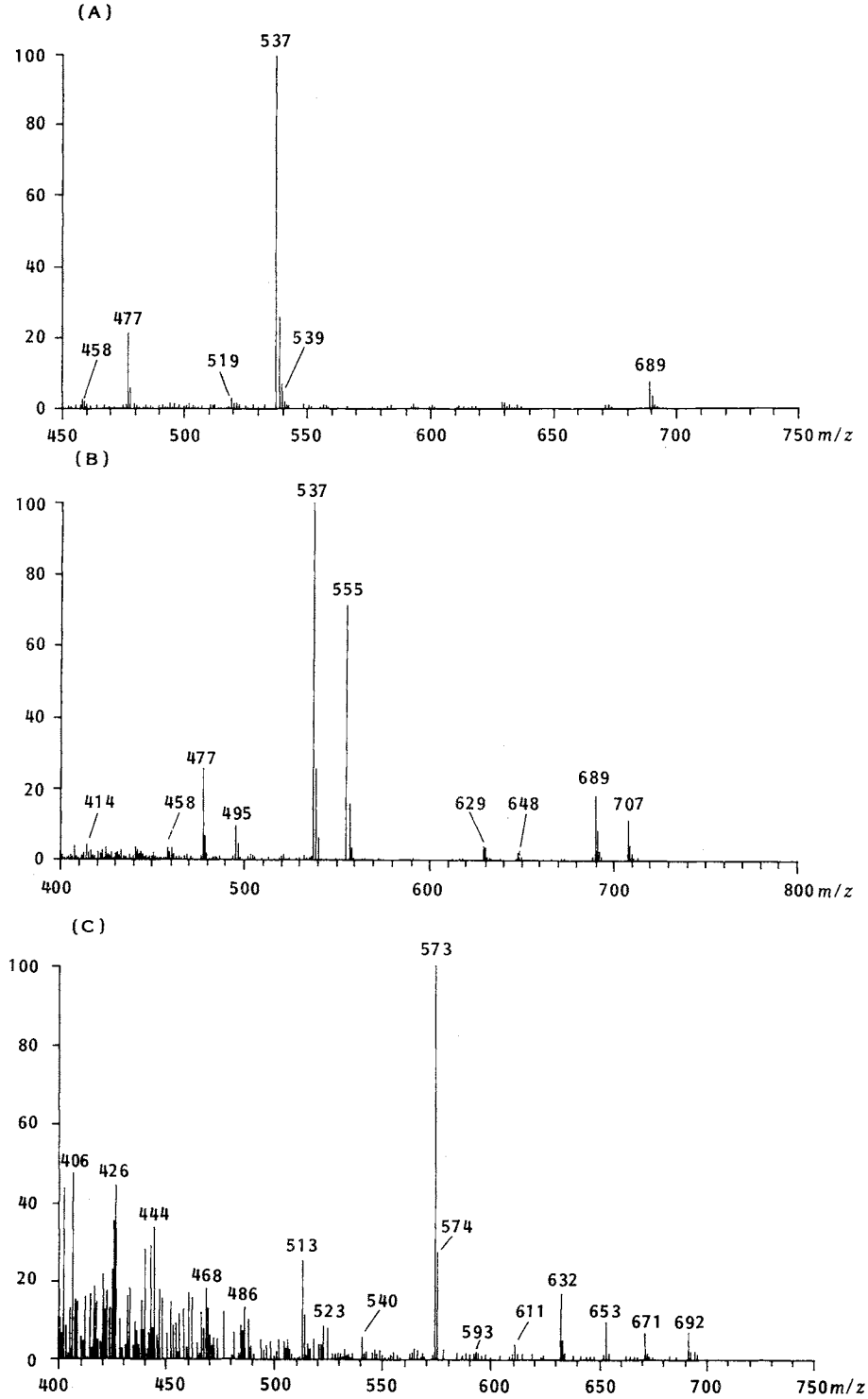
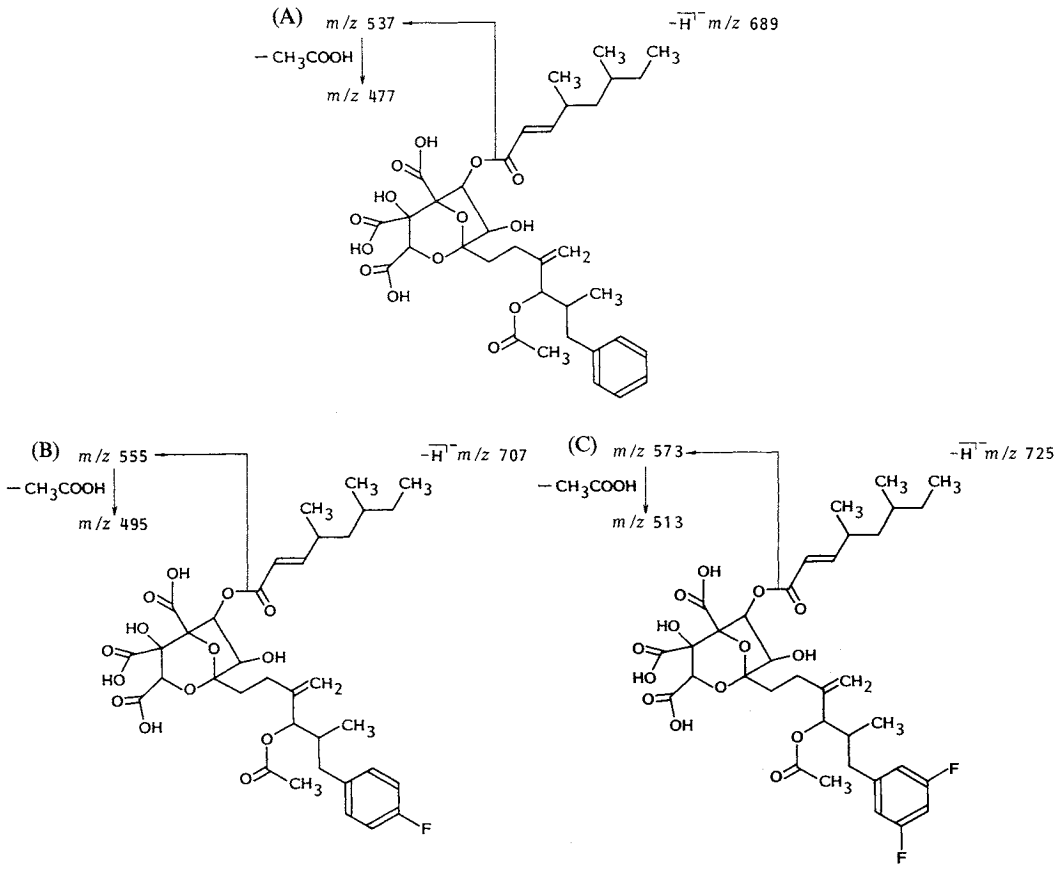


Fig. 3. (Continued)



In (A) the three principal ions from compound **1** are shown. These are also present in (B) because in this HPLC system, **1** and its monofluorinated analogues co-eluted. In (B) and (C) the associated ions shifted up by 18 and 36 mass units respectively, are shown, indicating that the monofluoro- and difluoro-compounds had been successfully incorporated into analogues of **1**. The (M−H) ion of **6** (C) was not observed.

Biological Testing

Inhibition of squalene synthase (rat and *Candida albicans*) was determined as described previously[1].

Results and Discussion

Incorporation of 7-[^{14}C]-Benzoic Acid Into 1

Confirmation that benzoic acid could be incorporated into **1** was obtained by feeding 7-[^{14}C]-labelled material to a growing culture of C2932. If the benzoic acid was added at the time of inoculation, no incorporation was observed, but if the addition was made at day 3,4 or 5 (corresponding to the maximum rate of synthesis of **1**) then incorporation at a level of 7% was observed (Fig. 2). All additions for subsequent experiments were made at day 3.

Generation of Squalestatin Analogues

Feeding experiments were carried out using a broad range of substituted benzoic acid compounds (Table 1). Of those tested, the monofluorobenzoic acids showed good incorporation and two of the

Table 3. ^{1}H NMR Characterisation of fluorinated squalestatins.

Ring position	Compound			
	2	**3**	**4**	**1**
16	7.21 (dd, 8, 5 Hz, 1H)	6.94 (dt, 10, 2 Hz, 1H)	—	7.19 (d, 7 Hz, 1H)
17	6.99 (t, 8 Hz, 1H)	—	7.00 (m, 1H)	7.26 (t, 7 Hz, 1H)
18	—	6.88 (dt, 2, 8 Hz, 1H)	7.18 (m, 1H)	7.14 (t, 7 Hz, 1H)
16[1]	7.21 (dd, 8, 5 Hz, 1H)	7.03 (d, 8 Hz, 1H)	7.28 (dt, 2, 7.5 Hz, 1H)	7.19 (d, 7 Hz, 1H)
17[1]	6.99 (t, 8 Hz, 1H)	7.28 (m, 1H)	7.09 (dt, 1, 7.5 Hz, 1H)	7.26 (t, 7 Hz, 1H)

All other ^{1}H NMR resonances of **2**~**4** are similar to those of **1**[2].

^{1}H NMR spectra were recorded at 500 MHz on a Bruker AM500 spectrometer in MeOD-d_4 solution at 289 K. The assignments shown are of the aromatic protons and are characteristic of the site of fluorination.

Table 4. Screen for incorporation of analogues of possible biosynthetic precursors of benzoate.

Analogue fed	**1** titre (mg/litre)	Analogue formed	Analogue titre (mg/litre)	Utilisation (%)
Control (no feed)	400~700	—	—	—
2-Fluorophenylalanine	85	**4**	2	100
3-Fluorophenylalanine	152	**3**	30	100
4-Fluorophenylalanine	132	**2**	13	100
4-Fluorocinnamic acid	88	**2**	9	67
2-Hydroxycinnamic acid	122	—	—	8
3-Hydroxycinnamic acid	180	—	—	100
4-Hydroxycinnamic acid	108	—	—	100
4-Aminocinnamic acid	146	—		50
2-Nitrocinnamic acid	133	—	—	100
3-Nitrocinnamic acid	164	—	—	100
4-Nitrocinnamic acid	188	—	—	100
2-Fluorobenzaldehyde	143	**4**	72	39
3-Fluorobenzaldehyde	150	**3**	15	100
4-Fluorobenzaldehyde	86	**2**	5	38
2,3-Difluorobenzaldehyde	292	**7**	2.8	0
2,4-Difluorobenzaldehyde	131	—	—	0
3,5-Difluorobenzaldehyde	100	—	—	0
2-Hydroxybenzaldehyde	49	—	—	0
3-Hydroxybenzaldehyde	211	—	—	0
4-Hydroxybenzaldehyde	241	—	—	100
2-Aminobenzaldehyde	156	—	—	100
2-Nitrobenzaldehyde	142	—	—	100
3-Nitrobenzaldehyde	189	—	—	100
4-Nitrobenzaldehyde	138	—	—	46
2-Bromobenzaldehyde	143	—	—	35
3-Bromobenzaldehyde	60	—	—	0
4-Bromobenzaldehyde	61	—	—	n.d.

n.d.: Not determined.

Analogues of presumed biosynthetic precursors of benzoic acid (250 mg/litre) were added to a 3 day flask culture of C2932. Each culture was examined by HPLC and HPLC-MS to determine whether the compound had been incorporated into an analogue of **1**. The column labelled 'utilisation' refers to an HPLC determination of possible biosynthetic precursor utilisation between time of addition (day 3) and harvest (day 7).

difluoro-compounds showed low levels of incorporation.

The squalestatin analogues generated are shown in Fig. 1. Of the six analogues generated, only **2**, **3** and **4** were isolated in sufficient quantities for NMR spectroscopy and biological testing. These three analogues appeared to be marginally less active than **1** against mammalian squalene synthase and **2** and **4** were slightly less active than **1** against the fungal enzyme (Table 2). Clearly, the incorporation of a fluorine atom in the aromatic ring did not have a dramatic effect on the biological activity.

The structures of the monofluorinated squalestatins were determined by HPLC-MS (Fig. 3) and ^{1}H NMR (Table 3) and by comparison with spectroscopic data obtained from **1**[2]. The structure of the difluorinated squalestatins was indicated by HPLC-MS. In addition to the mass spectrometry described, accurate mass negative ion Fast Atom Bombardment with polyethylene glycol calibration ions, was performed on the molecular ion ($(M-H)^-$) of purified **4**. This showed an error of only 1.7 ppm from the calculated value. The ^{1}H NMR assignments of aromatic protons of compounds **1**～**4** are shown in Table 3 and all other ^{1}H NMR resonances of compounds **2**～**4** are similar to those of **1**. In addition to ^{1}H NMR, a ^{19}F NMR spectrum of **2** was recorded at 376 MHz on a Varian VXR400 spectrometer, in MeOD-d_4 solution, at 303 K. The couplings observed (−118.75 ppm, (tt, 8,5 Hz)) are consistent with those in the proton spectrum.

There was no evidence of incorporation of any of the other substituted benzoic acids although a number appeared to be completely metabolised by the microorganism (C2923). In most cases, addition of the benzoic acid analogue reduced the production of **1**. The reason that the mono- and difluorinated benzoates were incorporated may partly have been that the hydrogen and fluorine atoms are nearly isosteric[4,5]. It is not clear whether the failure to detect even low levels of incorporation of the other compounds was due to enzyme specificity or to the failure of uptake of these compounds.

There are several possible biosynthetic routes leading to the formation of benzoic acid prior to incorporation into **1**[6]. Consequently, substituted phenylalanine, cinnamic acid and benzaldehyde compounds were also tested for incorporation into analogues of **1**. As indicated in Table 4, all the monofluorinated derivatives of these compounds were found to be incorporated, as well as one of the difluorinated benzaldehydes. This suggested that the supply pathway for benzoic acid was from phenylalanine *via* cinnamic acid (*i.e. via* the phenylalanine ammonia lyase pathway)[7]. The incorporation of fluorinated benzaldehyde could also suggest that this was part of the 'feeder' pathway, but a simple chemical oxidation to the benzoic acid could not be excluded.

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