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

III. BIOSYNTHESIS

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The biosynthetic origin of the carbon and oxygen atoms of the novel fungal secondary metabolite **1** was studied. Incorporation studies with single and multiple labelled ^{13}C precursors indicated that the major portion of the molecule was derived from two polyketide chains made up of acetate units. One of the chains had benzoic acid (which can be derived from phenylalanine) as a starter unit. The remaining carbons were derived from a four-carbon unit related to succinate and from methionine. Studies with [1-^{13}C,$^{18}O_2$]acetate and $^{18}O_2$ indicated that five of the oxygens, including both of the heterocyclic oxygens, were derived from atmospheric oxygen. The oxygens at the two ester carbonyls were derived from acetate.

A group of novel highly potent inhibitors of squalene synthase, the squalestatins, was isolated from *Phoma* sp. C2932[1] during a screening campaign for inhibitors of this key enzyme in cholesterol biosynthesis. The major component was identified as **1**[2] (Fig. 1). Examination of this unusual structure raised interesting questions about the biosynthetic origin of the carbon backbone and particularly the origin of the high number of oxygen atoms. This paper reports incorporation experiments with ^{13}C and ^{18}O labelled precursors to determine the origins of the carbon and oxygen atoms of **1**.

Materials and Methods

Organism

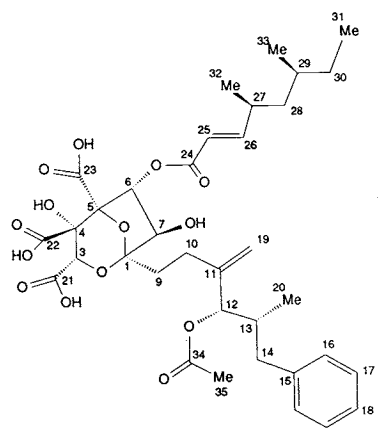
Strain PA13 derived from the squalestatin producer *Phoma* sp. C2932 was employed.

Culture

A seed culture medium (50 ml) in a 250-ml Erlenmeyer flask was inoculated with 0.5 ml of a spore suspension in 5% (w/v) glycerol thawed from storage at −70°C. This was incubated for 3 days at 25°C, 250 rpm on a rotary shaker.

0.5 ml of the inoculum culture was transferred to the production medium (50 ml/250 ml flask) which was incubated at 25°C, 250 rpm. After 3 to 4 days fermentation, at a suitable stage in squalestatin production, cultures received the first addition of ^{13}C-labelled precursor. Incubation was continued up to 7 days, with additional single daily dosing ending at day 6. In the case of [1-^{13}C,$^{18}O_2$]acetate, cultures received a total of five doses over days 4 to 6. Precursors were added in doses of 0.05% (w/v),

Fig. 1. Structure of the major squalestatin (**1**) from *Phoma* sp. C2932.



except for [2,3-$^{13}C_2$]succinate added at 0.025% (w/v). With [1-^{13}C,$^{18}O_2$]acetate, cultures received 0.034% (w/v) labelled material together with 0.108% (w/v) unlabelled acetate.

To achieve incorporation of $^{18}O_2$ into squalestatin, cultures were transferred at day 4 and incubated until day 7 (25°C, 275 rpm) in linked 250-ml conical flasks with ground glass stoppers in an air-tight closed system. Air was replaced by a circulated gas phase which maintained a supply of 40% (v/v) $^{18}O_2$ in nitrogen at 1 atm pressure, in an apparatus designed for the purpose[3].

The seed medium contained lactose 10%, cotton seed flour 2%, Junlon PW110 (Honeywell+Stein, Surrey, UK) 0.1% and was also used as the production medium for [1-^{13}C, $^{18}O_2$]acetate and $^{18}O_2$ studies. In all other experiments the production medium contained lactose 5%, and cotton seed flour 2%.

Chemicals

^{13}C precursors contained >99 atom% ^{13}C at the labelled positions. $^{18}O_2$ (gas) contained 99.7 atom% ^{18}O.

Sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate and L-[*methyl*-^{13}C]methionine were purchased from Sigma Chemical Co., Ltd., UK. Sodium [1,2-$^{13}C_2$]acetate and [*carboxy*-^{13}C]benzoic acid were from Aldrich Chemical Co., Ltd., UK. Merck, Sharp and Dohme Co., Ltd., supplied D,L-[3-^{13}C]phenylalanine, ring labelled [$^{13}C_6$]benzoic acid and $^{18}O_2$ (gas). The precursors [2,3-$^{13}C_2$]succinic acid and [1-^{13}C,$^{18}O_2$]acetate were synthesised at Bristol University, England and supplied by Professor T. J. SIMPSON.

Isolation of Isotopically Labelled 1

Each fermentation broth was subjected to one of two initial extractions: either, the broth was adjusted to pH 10.4 with ammonia solution, centrifuged (5,000×*g*, 4°C, 30 minutes), the cells re-extracted with water adjusted to pH 10.4 with ammonia solution, the supernatants pooled and adjusted to pH 8.6 with conc H_2SO_4; or, the broth was adjusted to pH 2 with conc H_2SO_4, diluted with an equal volume of acetonitrile, centrifuged (5,000×*g*, 4°C, 30 minutes), the cells re-suspended in the same volume of acetonitrile - 76 mM H_2SO_4 (1 : 1), centrifuged (5,000×*g*, 4°C, 30 minutes), the supernatants pooled, adjusted to pH 7 with ammonia solution and the acetonitrile removed by evaporation under reduced pressure.

The initial extract was loaded onto a column of Amberlite XAD-16 which adsorbed **1**. The column was washed successively with water (2 bed volumes), tetrasodium EDTA solution (1%, w/v) (3 bed volumes), and water (2 bed volumes). **1** was eluted with acetone - water (6 : 4) and the fractions containing **1** were pooled, and diluted and acidified with an equal volume of 76 mM H_2SO_4.

This solution was pumped onto a Spherisorb S5 ODS2 HPLC column, the column was washed with acetonitrile - 57 mM H_2SO_4 (1 : 3) (approximately 10 bed volumes) and **1** was eluted with acetonitrile - 76 mM H_2SO_4 (1 : 1). Fractions containing **1** were diluted with water to give a solution containing 20% acetonitrile. **1** was adsorbed on a Spherisorb S5 ODS2 HPLC column and the column was washed with 20~30 bed volumes of water to remove all traces of acid. **1** was eluted from the column with acetonitrile - water (9 : 1), concentrated by evaporation under reduced pressure and freeze-dried to give a white solid.

NMR Experiments

NMR spectra were measured in acetone-d_6 or methanol-d_4 on a Bruker AM500 instrument operating at 125 MHz for ^{13}C. Enrichment percentages were determined either from peak heights (by comparison with spectra of unenriched material recorded under the same conditions) or from integrations (by comparison with unenriched sites). Integrated spectra were recorded using gated decoupling and a long relaxation delay (50 seconds) to ensure accurate quantitation.

Results and Discussion

In order to establish the biosynthetic origin of the carbon and oxygen atoms in **1** produced by strain PA13 a series of incorporation studies was carried out with the substrates below.

[1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]Acetate

The data for incorporation of label into **1** from these three substrates are shown in Tables 1 and 2. The results indicated the incorporation of acetate into a tetraketide chain (carbons 24 to 31), a hexaketide chain (carbons 23, 5, 6, 7, 1, 9 to 13) and a single acetate unit (carbons 34, 35).

With [1,2-$^{13}C_2$]acetate, the NMR coupling patterns indicated a high level of incorporation of intact acetate units into the molecule (estimated 50%). However, with carbon pairs 3, 21 and 4, 22, the coupling patterns indicated a lower (20%) level of adjacent intact acetate derived units. This was taken to indicate metabolism of [1,2-$^{13}C_2$]acetate *via* the tricarboxylic acid cycle to a four carbon unit with consequent label dilution before incorporation at this site. This hypothesis was supported by the comparatively low levels of enrichment observed of carbons 21 and 22 with [1-^{13}C]acetate and carbons 3, 4, 21 and 22 with [2-^{13}C]acetate (Table 1).

Table 1. ^{13}C Chemical shifts[a] and isotopic incorporations into **1**.

Atom No.	δ ^{13}C (ppm)	% Incorporation of ^{13}C in **1** produced from				
		[1-^{13}C]-Acetate	[2-^{13}C]-Acetate	[1-^{13}C]-Benzoate	L-[*Methyl*-^{13}C]-methionine	D,L-[3-^{13}C]-Phenylalanine
1	106.5	7.0±0.8				
3	76.2		3.5±0.3			1.0±0.5
4	75.0		3.5±0.3			1.0±0.5
5	90.4		6.0±0.5			3.0±1.0
6	80.8	7.0±0.8				
7	82.7		6.0±0.5			3.0±1.0
9	34.8		6.0±0.5			3.0±1.0
10	26.4	7.0±0.8				
11	147.7		6.0±0.5			3.0±1.0
12	78.9	7.0±0.8				
13	37.4		6.0±0.5			3.0±1.0
14	40.5			62±3		47±5
15	141.3					
16	130.0					
17	129.1					
18	126.7					
19	111.1				42±2	
20	14.1				42±2	
21	168.0	2.7±0.2	1.8±0.2			
22	170.8	2.7±0.2	1.8±0.2			
23	166.5	7.0±0.8				
24	165.5	7.0±0.8				
25	119.7		6.0±0.5			3.0±1.0
26	156.8	7.0±0.8				
27	35.0		6.0±0.5			3.0±1.0
28	43.9	7.0±0.8				
29	32.7		6.0±0.5			3.0±1.0
30	30.4	7.0±0.8				
31	11.4		6.0±0.5			3.0±1.0
32	20.5				42±2	
33	19.2				42±2	
34	170.2	7.0±0.8				
35	20.9		6.0±0.5			

[a] Spectra were measured in CD_3COCD_3 and referenced to the solvent peak at 29.8 ppm as internal standard. Assignments are based on those reported in CD_3OD[2] and have been fully checked by a range of 2D NMR techniques.

L-[*Methyl*-^{13}C]methionine

The high level of enrichment observed (Table 1) for carbons 20, 32, 33 and 19 indicated the origin of these atoms from *S*-adenosylmethionine.

[2,3-$^{13}C_2$]Succinate

A 1.3% incorporation of label from this substrate at carbons 3 and 4 ($J=36$ Hz, Fig. 2) clearly demonstrated the intact incorporation of carbons 2 and 3 of succinate into **1**. There was also evidence of labelling of the molecule by metabolism of [2,3-$^{13}C_2$]succinate, presumably through oxaloacetate, phospho*enol*pyruvate and pyruvate to [1,2-$^{13}C_2$]acetate ($\sim$0.5% incorporation). In addition, there was a low ($\sim$0.1%) incorporation at carbon 15 and at the adjacent carbon of the aromatic ring, with coupling observed between these atoms ($J=57$ Hz). The introduction of label in these positions could be *via* phospho*enol*pyruvate which is a precursor of dehydroquinate in the aromatic amino acid pathway, and be an indication of the shikimate origin of the aromatic ring.

[*Carboxy*-^{13}C]benzoic Acid, D,L-[3-^{13}C]Phenylalanine and [*Aromatic*-$^{13}C_6$]benzoic Acid

Addition of [*carboxy*-^{13}C]benzoic acid resulted in enrichment at carbon 14 only (Table 1). Strong enhancement of the signal for carbon 14 was observed with D,L-[3-^{13}C]phenylalanine, but in addition the pattern of enrichment observed previously with [2-^{13}C]acetate was seen (Table 1). [2-^{13}C]Acetate could have been formed from [3-^{13}C]phenylalanine *via* the homogentisate catabolic pathway[4] which has been demonstrated in fungi[5].

Feeding of [*aromatic*-$^{13}C_6$]benzoic acid resulted in a spectrum of **1** in which ^{13}C signals for carbons of the aromatic ring were strongly enhanced (30 ± 5% incorporation). In addition, other carbons showed a lower enhancement (2.0 ± 0.5% incorporation). Complex multiplets were observed for the signals for carbons 3 and 4 indicating coupling between these carbons and between these carbons and carbons 21 and 22. This implied incorporation of a $^{13}C_4$ unit which could arise from [*aromatic*-^{13}C]benzoate by catabolism *via* protocatechuate to yield uniformly labelled succinic acid and [1,2-$^{13}C_2$]acetyl-CoA[6]. This hypothesis was supported by the observation in the ^{13}C spectrum of this sample of **1** of the enhanced signals and C-C coupling previously seen from [1,2-$^{13}C_2$]acetate incorporation (Table 2). With metabolism of [*carboxy*-^{13}C]benzoic acid *via* the

Fig. 2. Signals for C3 and C4 in the 125 MHz ^{13}C NMR spectrum of (**1**) labelled by [2,3-$^{13}C_2$]succinate.

+ $J_{3,4}=J_{4,3}=36$ Hz, ■ $J_{3,21}=65$ Hz, ● $J_{4,22}=62$ Hz.

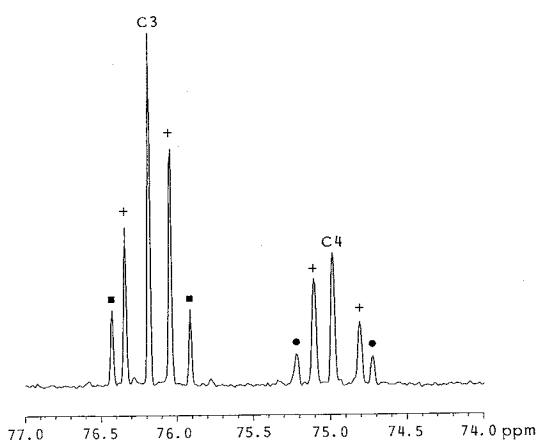


Table 2. One bond ^{13}C-^{13}C coupling constants observed in **1** derived from [1,2-$^{13}C_2$]acetate.

Atom numbers	$^1J_{CC}$ ($\pm$0.5 Hz)
1$\sim$ 9	48.5
3$\sim$21	65.0
4$\sim$22	62.0
5$\sim$23	68.0
6$\sim$ 7	41.5
10$\sim$11	42.5
12$\sim$13	38.5
24$\sim$25	75.5
26$\sim$27	41.5
28$\sim$29	35.0
30$\sim$31	35.0
34$\sim$35	60.0

protocatechuate pathway, label would have been lost as $^{13}CO_2$ which would explain the specific labelling at carbon 14 with this substrate.

The demonstration that carbon 14 of **1** can be labelled with very high efficiency from [*carboxy*-^{13}C]benzoic acid or [3-^{13}C]phenylalanine suggested the derivation of benzoic acid from phenylalanine. The operation of a phenylalanine metabolic pathway that generates benzoic acid *via trans*-cinnamic acid (through the action of phenylalanine ammonia lyase) is well known and important in plant secondary metabolism[7], and has been demonstrated in fungi[5,8]. The implied observation of two functioning pathways for metabolism of phenylalanine in C2392 (*via trans*-cinnamic acid, benzoic acid and protocatechuate or *via* homogentisate) is not uncommon in fungi[5].

[1-^{13}C,$^{18}O_2$]Acetate

On incorporation of [1-^{13}C,$^{18}O_2$]acetate into **1** the signals due to the C-24 and C-34 ester carbonyls showed the presence of isotopically shifted peaks (Fig. 3, Table 3) to indicate the origin of these oxygen atoms from acetate. As the procedure used for the isolation of **1** would probably have resulted in oxygen exchange at the carboxyl groups, no conclusions may be drawn about the origin of the oxygen atoms at these positions.

$^{18}O_2$

The results from this experiment are shown in Fig. 4 and Table 3 and these demonstrate clear signal shifts at carbons 6, 24, 5, 1, 3, 7, 12 and 34. These indicated labelling of the five oxygens attached to carbons

Fig. 3. Sections of the 125 MHz ^{13}C NMR spectrum of **1** labelled by [1-^{13}C, $^{18}O_2$]acetate.

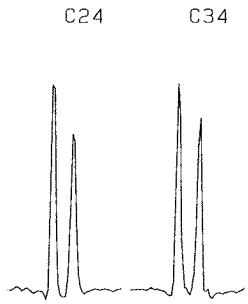


Table 3. ^{18}O Isotope shifts observed in ^{13}C NMR spectra of **1** produced from ^{18}O labelled precursors.

Carbon number	Isotope shift[a]	
	$^{18}O_2$	[1-^{13}C,$^{18}O_2$]Acetate
1	26[b]	—
3	20	—
4	—	—
5	24	—
6	28	—
7	15	—
12	37	—
24	9	36
34	15	37

[a] In ppm $\times 10^{-3}$; all values are negative.
[b] Per ^{18}O.

Fig. 4. Sections of the 125 MHz ^{13}C NMR spectrum of **1** labelled by $^{18}O_2$ gas.

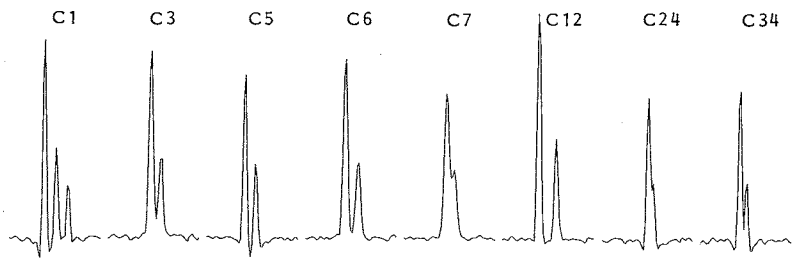


Fig. 5. Part of negative FAB mass spectrum of 1 labelled by $^{18}O_2$.

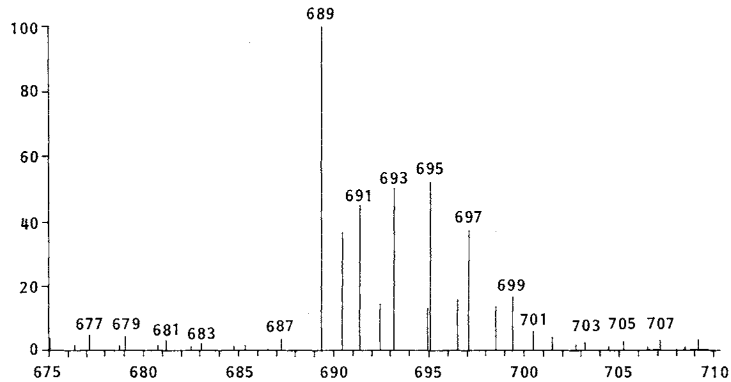
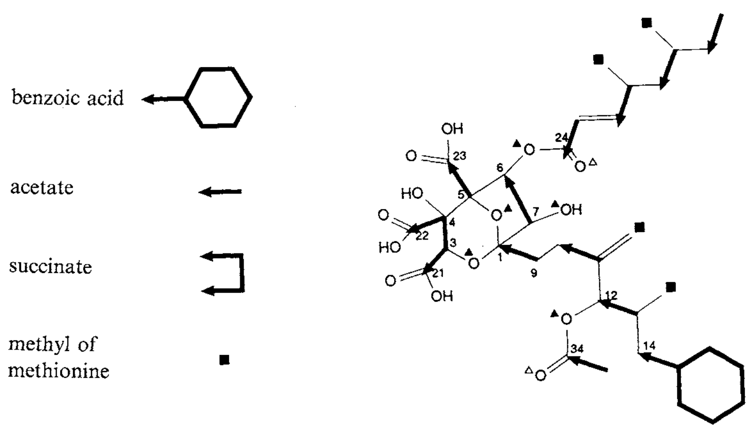


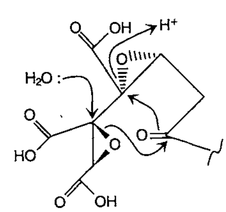
Fig. 6. Biosynthesis of 1.

▲ Oxygen atom derived from atmosphere, △ oxygen atom derived from acetate.



3, 5, 6, 7 and 12. This result was supported by the mass spectrum of this sample of 1 (Fig. 5) which showed a group of molecular ions +2, +4, +6, +8 and +10 mass units greater than unlabelled parent ion, and indicated the presence of up to five ^{18}O atoms per molecule of 1. It appears likely that the oxygen atom on C4 is derived from water.

Fig. 7. Postulated mechanism of formation of bicyclic system of 1.



Concluding Remarks

The results of the incorporation studies are summarised in Fig. 6. The main carbon skeleton of the molecule is presumably built up by polyketide chain extension of the aromatic starter unit benzoic acid, methylation, and the condensation of the α-methylene group of the chain with a four carbon α-keto dicarboxylic acid.

Aromatic starter units are known in polyketide biosynthesis, but are rare[10,11]; examples of the condensation of polyketide intermediates with tricarboxylic acid cycle intermediates are more common[12].

Formation of the bicyclic system with the observed oxygen origin may be postulated from the ketotricarboxylic acid shown in Fig. 7 arising from a trisepoxide. Attack by water as shown could then lead to the bicyclic system lacking oxygen at C7 (ring numbering system). Then subsequent oxidation at C7, tetraketide acylation and acetylation, in unknown order, are required to complete the biosynthesis of **1**. Some support for this hypothesis may be inferred from the isolation of 7-deoxy analogues which will be described in a separate paper on minor metabolites of C2932.

Acknowledgements

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