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

II. STRUCTURE ELUCIDATION

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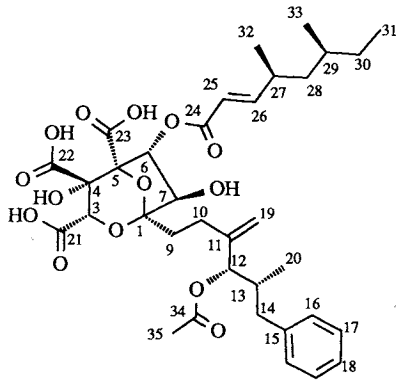
Three novel fungal metabolites **1**~**3** isolated from cultures of a *Phoma* sp. C2932, are potent and selective inhibitors of squalene synthase. Their structures have been determined by a combination of spectroscopic, X-ray crystallographic and chemical methods; these natural products incorporate the highly functionalised bicyclic core, [1*S*-(1α,3α,4β,5α,6α,7β)]-4,6,7-trihydroxy-2,8-dioxabicyclo-[3.2.1]octane-3,4,5-tricarboxylic acid.

During a screening programme for inhibitors of squalene synthase, three structurally related novel inhibitors **1**~**3** were isolated from cultures of *Phoma* sp. C2932. The isolation and physico-chemical properties of this novel class of compounds, designated the squalestatins, together with taxonomy of the producing organism are reported in the preceding paper[1]. In this paper their structure elucidation is described.

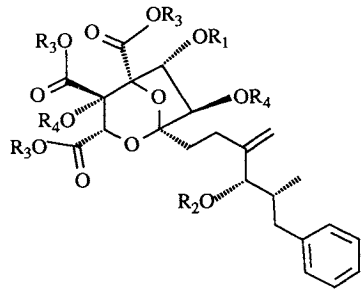
Results and Discussion

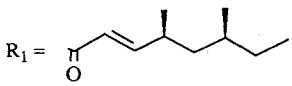
The low resolution negative ion FAB-MS of **1** produced an intense $(M-H)^-$ ion at *m/z* 689 with characteristic fragment ions at *m/z* 645, 537, 475 and 169 (Fig. 1). The molecular formula of **1**, $C_{35}H_{46}O_{14}$, was determined from the high resolution measurement of the $(M-H)^-$ ion (*m/z* 689.2789, $(M-H)^-$, $C_{35}H_{45}O_{14}$, Δ 2.0 mmu of calculated). Initial one dimensional ^1H and ^{13}C (fully decoupled and DEPT) NMR spectra of **1** (Table 1) indicated the fragments given in Fig. 2. The presence of five exchangeable protons and two further rings could therefore be inferred from the molecular formula. Derivatisation enabled the exchangeable protons to be identified as three carboxylic acids and two alcohols. Thus treatment of **1** with diazomethane gave the trimethyl ester (**4**) which could be converted into the corresponding diacetate (**5**) with Ac_2O in the presence of *N*,*N*-dimethylaminopyridine. Comparison of the spectra of **4** and **5** showed that only one proton signal shifted sufficiently (δ 4.05 to δ 5.26) to indicate acylation of a secondary alcohol[2] (Table 1). The conclusion that the other alcohol was therefore tertiary was supported by a down field acylation shift of one of the quaternary carbons (δ 74.3 to δ 79.8)[3].

A number of two dimensional experiments carried out on **1** enabled the two side-chains to be pieced together. The ^1H-^1H phase sensitive double quantum filtered (PS-DQF) COSY spectrum defined most of the alkyl side-chain (C-9 to C-14) with small cross peaks being observed for the allylic couplings to the terminal methylene. Once all the one bond ^1H-^{13}C correlations had been determined (Table 1), the position of the phenyl ring was located from the long-range ^1H-^{13}C correlation data (Fig. 3) obtained by the



1



2	R_1 = 	R_2=H	R_3=H	R_4=H
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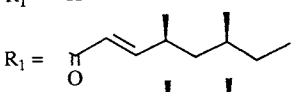
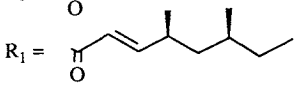
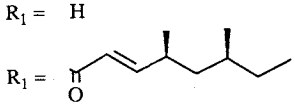
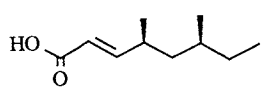
4	R_1 = 	R_2=Ac	R_3=Me	R_4=H
5	R_1 = 	R_2=Ac	R_3=Me	R_4=Ac
6	R_1 = H	R_2=Ac	R_3=Me	R_4=H
7	R_1 = 	R_2=H	R_3=Me	R_4=H



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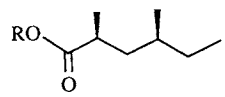
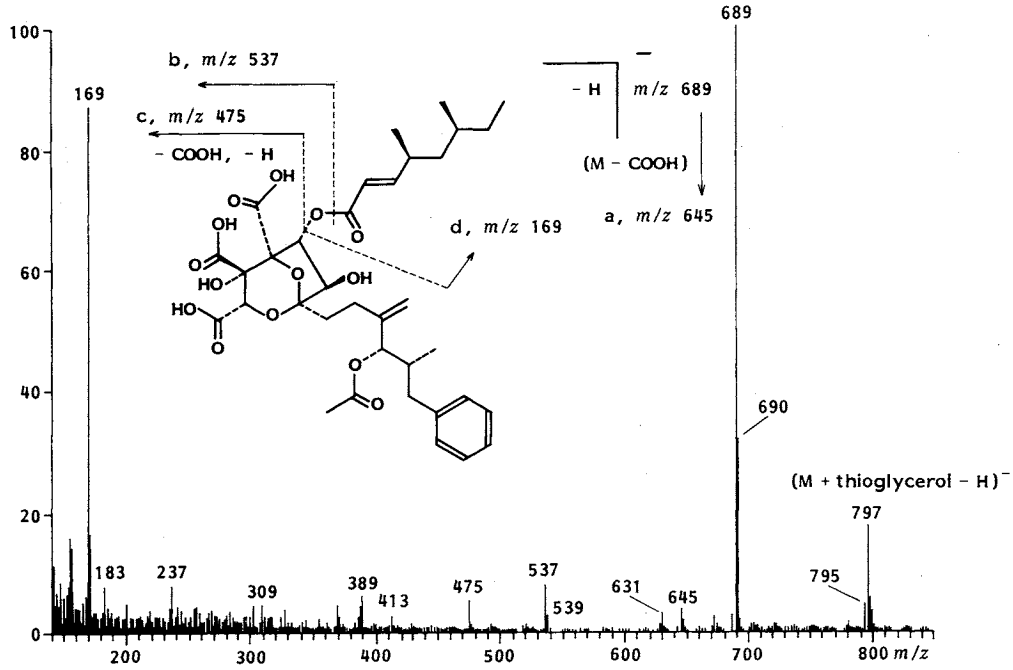


9 R=H
10 R=Me

inverse method (heteronuclear multiple-bond correlation (HMBC))[4].

The ester side-chain had a molecular formula of $C_{10}H_{18}O_2$ as evidenced by the strong fragment ion at *m/z* 169 in the mass spectrum of **1** but its structure could not be deduced from the ^{1}H-^{1}H PS-DQF COSY spectrum due to signal overlap in the δ 1.0~1.5 region. However it was easily determined from

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



the long-range ^{1}H-^{13}C correlation data (Fig. 3). The inverse method (HMBC) used to obtain these data proved invaluable because its improved proton resolution, when compared with normal mode equivalents, enabled the methyl groups to be resolved.

The low resolution negative ion FAB-MS of **2** produced an intense $(M-H)^-$ ion at *m/z* 647 with characteristic fragment ions at *m/z* 603, 495, 433 and 169. The molecular formula of **2**, $C_{33}H_{44}O_{13}$, was determined from high resolution measurement of the $(M-H)^-$ ion (*m/z* 647.2708, $(M-H)^-$, $C_{33}H_{43}O_{13}$, Δ 0.4 mmu of calculated). Comparison of the NMR spectra of **1** and **2** (Table 2) confirmed that they differed only by loss of an acetyl group and that this was attached to the oxygen at C-12.

The low resolution negative ion FAB-MS of **3** produced an intense $(M-H)^-$ ion at *m/z* 537 with a characteristic fragment ion at *m/z* 493. The molecular formula of **3**, $C_{25}H_{30}O_{13}$, was determined by high resolution measurement of the $(M-H)^-$ ion (537.1673, $(M-H)^-$, $C_{25}H_{29}O_{13}$, Δ 6.5 mmu of calculated). Comparison of the spectra of **1** and **3** (Table 2) shows that the latter lacks the ester side-chain, the loss of which leaves another secondary alcohol.

The point of attachment for the alkyl side-chain was determined from a heteronuclear NOE difference experiment carried out on **3**. Irradiation of the protons attached to C-9 (δ 2.06) gave an enhancement only at the ketal carbon C-1 (δ 106.6). This carbon was also the only one enhanced when the proton at C-7 (δ 4.07) was irradiated.

It was now clear that the core of these molecules was formed by a small bicyclic system. The predictive value of chemical shifts and coupling constants would therefore be of limited value due to the fixed geometries in such systems.

In order to gain more information about the bicyclic core Secondary Isotope Multiplet NMR of Partially Labelled Entities (SIMPLE)[5,6] was applied to ester **6** as carboxylic acids are incompatible with

Table 1. ^{1}H and ^{13}C NMR data[a] in $CDCl_3$ for **1**, **4** and **5**.

Atom	1		4		5	
	δ_C	δ_H[b]	δ_C	δ_H	δ_C	δ_H
1	105.8	—	105.7	—	106.2	—
3	75.1	5.03 (s)	75.4	5.26 (s)	74.4	5.10 (s)
4	74.2	—	74.3	—	79.8	—
5	88.5	—	88.6	—	87.4	—
6	80.7	5.92 (s)	81.4	5.81 (d, 2)	77.3	6.49 (d, 2)
7	81.6	4.05 (s)	81.7	4.05 (d, 2)	78.9	5.26 (d, 2)
9	33.6	2.01 (m), 2.14 (m)	33.9	2.06~2.19 (m)	33.0	2.05~2.15 (m)
10	25.4	2.21 (m), 2.31 (m)	25.1	2.32 (m), 2.38~2.49 (m)	24.8	2.28~2.36 (m)
11	145.4	—	145.3	—	144.9	—
12	79.3	5.06 (d, 4)	78.8	5.10 (d, 5)	78.8	5.11 (d, 5)
13	36.7	2.08 (m)	36.6	2.06~2.19 (m)	36.8	2.05~2.15 (m)
14	39.7	2.33 (dd, 13, 9), 2.69 (dd, 13, 5)	39.7	2.34 (dd, 14, 9), 2.70 (dd, 14, 5)	39.7	2.34 (dd, 13, 9), 2.71 (dd, 13, 5)
15	140.1	—	140.2	—	140.2	—
16	128.9	7.11 (d, 7)	128.9	7.15 (d, 7)	128.9	7.15 (d, 7)
17	128.1	7.24 (t, 7)	128.1	7.26 (t, 7)	128.1	7.27 (t, 7)
18	125.8	7.14 (t, 7)	125.8	7.17 (t, 7)	125.8	7.18 (t, 7)
19	111.4	4.94 (s), 4.96 (s)	111.5	4.97 (s), 5.00 (s)	111.6	4.97 (s), 5.00 (s)
20	13.5	0.81 (d, 7)	13.6	0.83 (d, 7)	13.7	0.83 (d, 7)
21	169.1	—	166.7	—	165.9	—
22	171.0	—	169.4	—	165.1[c]	—
23	166.7	—	165.1	—	164.5[c]	—
24	167.0	—	166.7	—	164.0	—
25	117.8	5.78 (d, 16)	117.8	5.75 (dd, 16, 1)	117.8	5.73 (dd, 16, 1)
26	157.9	6.88 (dd, 16, 8)	157.7	6.84 (dd, 16, 9)	157.2	6.86 (dd, 16, 8)
27	34.2	2.40 (m)	34.3	2.38~2.49 (m)	34.3	2.41 (m)
28	42.9	1.08 (m), 1.36 (m)	43.0	1.08~1.18 (m), 1.37 (m)	43.0	1.07~1.16 (m), 1.25~1.40 (m)
29	31.6	1.29 (m)	31.7	1.25~1.34 (m)	31.6	1.25~1.40 (m)
30	29.4	1.09 (m), 1.27 (m)	29.6	1.08~1.18 (m), 1.25~1.34 (m)	29.5	1.07~1.16 (m), 1.25~1.40 (m)
31	10.9	0.83 (t, 7)	10.9	0.85 (t, 7)	10.9	0.84 (t, 7)
32	19.8	1.00 (d, 7)	20.1	1.04 (d, 7)	20.0	1.03 (d, 7)
33	18.8	0.82 (d, 7)	18.7	0.84 (d, 7)	18.7	0.83 (d, 7)
34	171.5	—	170.0	—	169.9	—
35	20.9	2.09 (s)	20.9	2.10 (s)	20.8	2.11 (s)
21-OMe	—	—	52.4	3.76 (s)	52.6	3.80 (s)
22-OMe	—	—	53.6	3.93 (s)	53.3[d]	3.90 (s)[d]
23-OMe	—	—	52.7	3.81 (s)	52.3[d]	3.72 (s)[d]
4-OAc	—	—	—	—	20.9	2.09 (s)
					168.3	—
7-OAc	—	—	—	—	20.6	2.17 (s)
					169.1	—

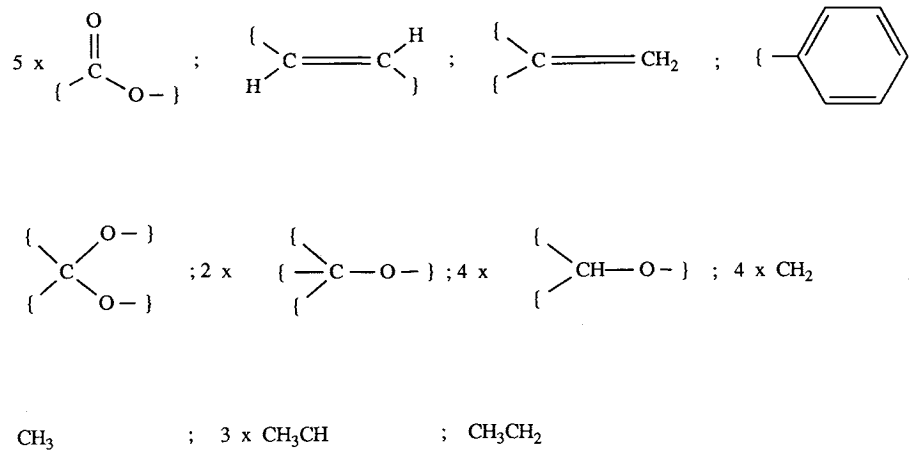
[a] Chemical shifts are given in ppm referenced to $CHCl_3$ at 7.26 ppm for ^{1}H and $CDCl_3$ at 76.81 ppm for ^{13}C as internal standards. Coupling constants are quoted to the nearest Hz.

[b] Line width *ca.* 2 Hz (probably due to aggregation). Chemical shifts for overlapping multiplets are derived from 2D experiments.

[c,d] Assignments may be reversed.

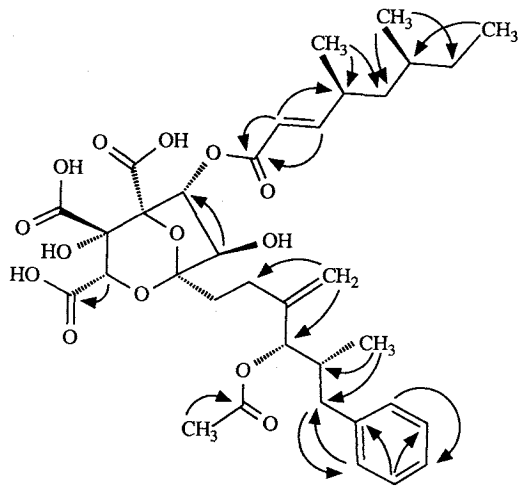
this technique. Trimethyl ester **6** was prepared by esterification of **3** with diazomethane and the results of the SIMPLE experiment are given in Table 3. The β (two bond) deuterium isotope shifts confirmed that C-4, C-6 and C-7 carried alcohol groups. Of these carbons, only C-6 and C-7 also showed a γ (three

Fig. 2. Fragments identified from the initial 1D NMR spectra of **1**.



bond) isotope shift. They must therefore be attached to each other. As one of the quaternary carbons, C-5, showed two γ shifts it must be attached to two of the alcohol bearing carbons. These must be C-4 and C-6, as C-7 could be ruled out because no enhancement was observed at C-5 in the heteronuclear NOE experiments. Although there was no measurable isotope shift at C-1 a direct bond to C-7 was now the only way to fit the heteronuclear NOE data. A similar disposition of atoms in the fructose residue of sucrose also shows no γ isotope shift[7]. Accordingly the γ shifts observed on C-3 and C-22 must be due to their attachment to C-4. The remaining groups could now be unambiguously positioned leaving only the stereochemistry to be determined.

Fig. 3. Long-range ^{1}H-^{13}C correlations for **1** obtained by the inverse method (HMBC).



A strong NOE between the protons at C-3 and C-6 was observed in a ^{1}H-^{1}H NOESY spectrum of the trimethyl ester (**4**). Both these protons must therefore be *endo*. The coupling constant (2 Hz) between the C-6 and C-7 protons established that the latter was *exo*[8].

In order to establish the full relative stereochemistry of this series it was necessary to undertake a single-crystal X-ray diffraction study. In the absence of a suitable crystalline derivative of compound **1**, a study was carried out using the crystalline allylic alcohol (**7**). This material was prepared by selective hydrolysis of the allylic acetate present in **4** using concentrated aq HCl in MeOH and was identical with the trimethyl ester obtained by diazomethane treatment of **2**. Due to the small size of the crystal and the weak intensities of the collected data, the resolution was limited and several attempts were made to solve the structure. A recognisable fragment, based on the expected structure derived from the NMR studies was located and successfully expanded to reveal the complete structure, including a water molecule hydrogen bonded to the C-7 hydroxyl group. The results are illustrated in Fig. 4 and summarised in Table 4.

Table 2. ^{1}H and ^{13}C NMR data[a] in CD_3OD for **1**, **2** and **3**.

Atom	1		2		3	
	δ_C	δ_H[b]	δ_C	δ_H	δ_C	δ_H
1	106.8	—	107.1	—	106.6	—
3	76.6	5.27 (s)	76.6	5.27 (s)	76.5	5.16 (s)
4	75.6	—	75.6	—	75.7	—
5	91.1	—	91.0	—	93.2	—
6	81.0	6.31 (d, 2)	81.0	6.31 (d, 2)	79.3	5.14 (d, 2)
7	82.5	4.04 (d, 2)	82.3	4.08 (d, 2)	84.1	4.07 (d, 2)
9	34.9	2.00 (m), 2.05 (m)	35.1	1.98~2.13 (m)	35.1	2.06 (m)
10	26.5	2.34 (m), 2.40~2.51 (m)	26.0	2.28 (m), 2.39~2.51 (m)	26.3	2.34 (m), 2.47 (m)
11	147.7	—	152.0	—	148.0	—
12	80.1	5.08 (d, 5)	78.6	3.92 (d, 5)	80.5	5.10 (d, 5)
13	37.7	2.24 (m)	39.0	1.98~2.13 (m)	37.6	2.25 (m)
14	40.9	2.44 (dd, 13, 9), 2.68 (dd, 13, 6)	41.2	2.37 (dd, 13, 9), 2.76 (dd, 13, 5)	40.9	2.41 (dd, 13, 9), 2.71 (dd, 13, 6)
15	141.6	—	142.5	—	141.5	—
16	130.2	7.19 (d, 7)	130.2	7.21 (d, 7)	130.1	7.19 (d, 7)
17	129.3	7.26 (t, 7)	129.2	7.24 (t, 7)	129.3	7.26 (t, 7)
18	126.9	7.14 (t, 7)	126.6	7.13 (t, 7)	126.9	7.15 (t, 7)
19	111.5	4.97 (s), 5.02 (s)	110.7	5.00 (s), 5.10 (s)	111.4	4.98 (s), 5.03 (s)
20	14.1	0.87 (d, 7)	14.0	0.83 (d, 7)	14.1	0.85 (d, 7)
21	170.1	—	170.1	—	170.4	—
22	172.5	—	172.4	—	172.9	—
23	168.5	—	168.4	—	169.4	—
24	166.5	—	166.5	—	—	—
25	119.8	5.80 (d, 16)	119.8	5.78 (d, 16)	—	—
26	157.6	6.85 (dd, 16, 8)	157.6	6.84 (dd, 16, 8)	—	—
27	35.6	2.40~2.51 (m)	35.6	2.39~2.51 (m)	—	—
28	44.4	1.09~1.19 (m), 1.39 (m)	44.4	1.09~1.19 (m), 1.39 (m)	—	—
29	33.1	1.27~1.37 (m)	33.1	1.26~1.36 (m)	—	—
30	30.8	1.09~1.19 (m), 1.27~1.37 (m)	30.8	1.09~1.19 (m), 1.26~1.36 (m)	—	—
31	11.4	0.86 (t, 7)	11.4	0.86 (t, 7)	—	—
32	20.5	1.03 (d, 7)	20.5	1.03 (d, 7)	—	—
33	19.2	0.87 (d, 7)	19.2	0.85 (d, 7)	—	—
34	172.1	—	—	—	172.1	—
35	20.9	2.10 (s)	—	—	20.9	2.11 (s)

[a] Chemical shifts are given in ppm referenced to CHD_2OD at 3.31 ppm for ^{1}H and CD_3OD at 48.9 ppm for ^{13}C as internal standard. Coupling constants are quoted to the nearest Hz. DEPT and ^{1}H-^{13}C correlation data are in accord with these assignments.

The X-ray crystal structure determination showed that the two asymmetric centres in the dimethyloctenoate side-chain and the quaternary C-1 centre in the bicyclic core possessed the same relative stereochemistry. The absolute configuration was determined by a degradation study. Alkaline hydrolysis of **1** gave the novel 4,6-dimethyl-2-octenoic acid (**8**). Oxidation of **8** with ruthenium trichloride-sodium periodate gave 2,4-dimethylhexanoic acid (**9**), the methyl ester (**10**) of which had an optical rotation of $+33°$ (liquid, path length (l) = 0.1 dm). The optical rotation of methyl (2*S*,4*S*)-2,4-dimethylhexanoate is reported as $+32.2°$ (liquid, l = 0.2 dm, d = 0.871)[9]. The absolute configuration of (*E*)-4,6-dimethyl-2-octenoic acid (**8**) is therefore (4*S*,6*S*), and hence the absolute stereochemistry of **1** and **2** is [1*S*(4*S*,5*R*), 3*S*,4*S*,5*R*,6*R*(2*E*,4*S*,6*S*),7*R*].

Table 3. Key NMR data[a] in DMSO-d_6 and SIMPLE results for **6**.

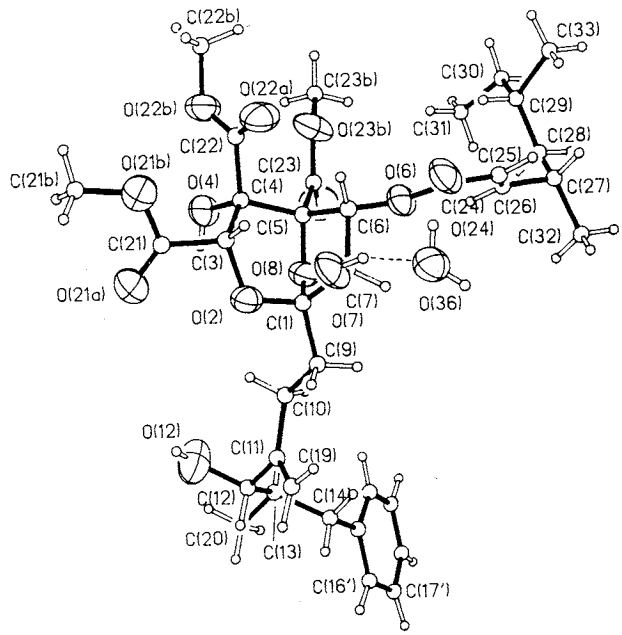
δ_C	Atom	δ_H	Isotope shifts[b] and assignments[c]	
106.3	1	—	—	—
76.6	3	5.02 (s)	26	γ_{34}
76.2	4	—	100	β_4
93.1	5	—	26+26	$\gamma_{54}+\gamma_{56}$
79.2	6	4.87 (dd, 7, 2)	105+44	$\beta_6+\gamma_{67}$
83.8	7	3.90 (dd, 5, 2)	120+64	$\beta_7+\gamma_{76}$
171.9	22	—	64	γ_{224}

[a] Chemical shifts are given in ppm referenced to CHD_2SOCD_3 (2.52 ppm) for ^1H and CD_3SOCD_3 (40.9 ppm) for ^{13}C as internal standards. Coupling constants are quoted to the nearest Hz. ^1H-^{13}C correlation data were in accord with the assignments given.

[b] In ppm × 10^{-3}, all values are negative.

[c] The notation used describes the carbon being observed by a numerical subscript and when appropriate a second numerical subscript is used for the hydroxy-group giving rise to the shift.

Fig. 4. Molecular structure of **7**.



Selective transesterification of the trimethyl ester of **4** with sodium methoxide in MeOH gave **6** which was identical with the trimethyl ester obtained by diazomethane treatment of **3**. The absolute configuration of **3** can therefore be related to that of **1**, and is [1*S*(4*S*,5*R*),3*S*,4*S*,5*R*,6*R*,7*R*].

These studies define the squalestatins as a new class of compounds which incorporate the highly functionalised bicyclic core, [1*S*-(1α,3α,4β,5α,6α,-7β)]-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]-

Table 4. Selected bond lengths (Å) and angles (°) for **7** with estimated standard deviations in parentheses.

C(1)–O(2)	1.411 (15)	O(2)–C(1)–C(7)	110.2 (10)
C(1)–C(7)	1.555 (16)	O(2)–C(1)–O(8)	109.8 (7)
C(1)–O(8)	1.403 (15)	C(7)–C(1)–O(8)	104.3 (9)
O(2)–C(3)	1.467 (13)	C(1)–O(2)–C(3)	114.8 (9)
C(3)–C(4)	1.535 (16)	O(2)–C(3)–C(4)	110.9 (9)
C(4)–C(5)	1.593 (18)	C(3)–C(4)–C(5)	108.2 (8)
C(5)–C(6)	1.521 (21)	C(4)–C(5)–C(6)	112.5 (10)
C(5)–O(8)	1.450 (14)	C(4)–C(5)–O(8)	107.3 (10)
C(6)–C(7)	1.527 (17)	C(6)–C(5)–O(8)	103.4 (9)
		C(5)–C(6)–C(7)	103.3 (11)
		C(1)–C(7)–C(6)	103.4 (10)
		C(1)–O(8)–C(5)	103.4 (9)

octane-3,4,5-tricarboxylic acid. The particularly complex substitution pattern in this family raises interesting questions of biosynthesis and a manuscript addressing this issue is in preparation.

Experimental

General Experimental Procedures

Organic solutions were dried over $MgSO_4$, and column chromatography was performed on Silica gel 60 (Merck, Art. No. 7734). MP's were determined on a Reichert apparatus and are uncorrected. Optical rotations were measured on an Optical Activity AA100 digital polarimeter. IR spectra were recorded on a Nicolet 5SXC FTIR spectrometer.

NMR spectra were recorded on a Bruker AM500, AM250 or VARIAN VXR400 spectrometers using standard pulse sequences. Homo-nuclear 2D experiments were performed by acquiring 4K × 2K data sets collected in 512 increments. The delay between scans was 2.0 seconds. The mixing time used for the NOESY experiment was 1.0 second. Normal mode hetero-nuclear 2D experiments were preformed by acquiring 4K × 512 data sets collected in 128 increments. Inverse mode hetero-nuclear 2D experiments were performed by acquiring 4K × 1K data sets collected in 256 increments. In both cases the experiments were set up for $^1J_{CH}=135$ Hz and $^{LR}J_{CH}=8$ or 10 or 12 Hz. The delay between scans was 2.0 seconds. Hetero-nuclear NOE difference spectra were recorded using a 3.0-second selective irradiation period and an acquisition time of 1.114 seconds.

Samples for SIMPLE NMR were made up in DMSO-d_6 and then examined by ^1H NMR so that the amount of D_2O required to give 50% deuteration could be calculated. The D_2O was added by syringe, the sample mixed well and then re-checked by ^1H NMR prior to recording the ^{13}C NMR spectrum.

High resolution FAB mass spectrometry was performed on a VG ZAB-2SE 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 negative ion FAB mass spectroscopy was performed on a Finnigan MAT TSQ70B spectrometer operating at a resolving power of 1,000. Xenon was used as the FAB gas and the atom gun was operated at 9 kV and 1 mA. Thioglycerol - glycerol (1 : 1) was used as the matrix. High and low resolution EI (70 eV) mass spectrometry were performed on Finnigan MAT 8400 and TSQ70B spectrometers operating at a resolving power of 10,000 and 1,000, respectively.

Elemental microanalyses were determined with a Perkin-Elmer 240C or a Carlo-Erba 1106 elemental analyser.

[1*S*-[1α(4*R**,5*S**),3α,4β,5α,6α(2*E*,4*R**,6*R**),7β]]-1-[4-Acetyloxy-5-methyl-3-methylene-6-phenylhexyl]-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyl-2-octenoate), 3,4,5-Trimethyl Ester (**4**)

A solution of **1** (940 mg, 1.36 mmol) in MeOH (15 ml) was treated with a 0.4 M solution of diazomethane in diethyl ether (16 ml). The excess diazomethane was quenched with acetic acid (0.1 ml) and the solution was concentrated under reduced pressure. The residue was chromatographed on silica gel eluting with CH_2Cl_2 and the MeOH - CH_2Cl_2 (1 : 49) to give 906 mg (91%) of **4** as a white foam: $[\alpha]_D^{23}$ +38.8° (*c* 0.96, MeOH); IR ν_{max} ($CHBr_3$) cm^{-1} 3547, 1769, 1733; ^1H and ^{13}C NMR ($CDCl_3$) Table 1; EI-MS *m/z* 732 (M, $C_{38}H_{52}O_{14}$), 581, 411, 325, 235, 170, 153, 91.

Anal Calcd for $C_{38}H_{52}O_{14}$: C 62.28, H 7.15.
Found: C 62.18, H 7.09.

[1*S*-[1α(4*R**,5*S**),3α,4β,5α,6α(2*E*,4*R**,6*R**),7β]]1-[4-Acetyloxy-5-methyl-3-methylene-6-phenylhexyl]-4,7-bis(acetyloxy)-6-hydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyl-2-octenoate), 3,4,5-Trimethyl Ester (**5**)

A solution of **4** (1.84 g, 2.5 mmol) in CH_2Cl_2 (25 ml) and triethylamine (3 ml, 21 mmol) was treated with acetic anhydride (2.5 ml, 21 mmol) and dimethylaminopyridine (122 mg, 1 mmol). The mixture was stood at 20°C for 88 hours and then poured into dilute HCl. The organic phase was washed with aq $NaHCO_3$, brine, dried and chromatographed on silica gel eluting with EtOAc - cyclohexane (1 : 4) to give

1.824 g (89%) of **5** as a clear glass: $[\alpha]_D^{24}$ +35.4° (*c* 1.17, MeOH); IR ν_{max} ($CHBr_3$) cm^{-1} 1772, 1747, 1640; ^1H NMR (500 MHz, $CDCl_3$) Table 1; EI-MS *m/z* 816 (M, $C_{42}H_{56}O_{16}$), 756, 725, 665, 495, 325, 277, 170, 153, 91; HREI-MS Calcd for $C_{42}H_{56}O_{16}$: 816.3568, Found: 816.3511.

[1*S*-[1α(4*R**,5*S**),3α,4β,5α,6α,7β]]-1-[4-Acetyloxy-5-methyl-3-methylene-6-phenylhexyl]-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 3,4,5-Trimethyl Ester (**6**)

A solution of **4** (449 mg, 0.61 mmol) in dry MeOH (6 ml) was added to a solution of sodium methoxide in MeOH (45 ml) (prepared from NaH (60% oil dispersion; 2.5 mg, 0.06 mmol)) and the mixture was stood at 20°C for 18 hours. 2 M HCl (2 ml) was added and the MeOH was removed by evaporation under reduced pressure. The residue was diluted with EtOAc and washed with 2 M HCl, aq $NaHCO_3$, brine, dried and chromatographed on silica gel eluting with CH_2Cl_2 and then with MeOH-CH_2Cl_2 (1:49) to give 231 mg (65%) of **6** as a white foam: $[\alpha]_D^{23}$ +7.4° (*c* 1.12, MeOH); IR ν_{max} ($CHBr_3$) cm^{-1} 3570, 3529, 1765, 1732; ^1H NMR (250 MHz, $CDCl_3$) δ 0.86 (3H, d, J=7 Hz, $CHCH_3$), 2.12 (3H, s, AcO), 2.69 (1H, dd, J=14 and 6 Hz, CHPh), 3.31 (1H, d, J=6 Hz, 6-OH), 3.46 (1H, d, J=4 Hz, 7-OH), 3.74, 3.80 and 3.91 each (3H, s, CO_2CH_3), 3.82 (1H, s, 4-OH), 4.15 (1H, br, 7-H), 5.00 and 5.03 each (1H, s, $C=CH_2$), 5.06 (1H, d, J=5 Hz, CHOAc), 5.12 (1H, dd, J=6 and 1.5 Hz, 6-H), 5.17 (1H, s, 3-H), 7.11~7.32 (5H, m, Ph); EI-MS *m/z* 580 (M, $C_{28}H_{36}O_{13}$), 520, 453, 429, 411, 277, 91. Ester **6** was alternatively prepared by diazomethane treatment of **3**. Its spectral characteristics were identical with those described above.

Anal Calcd for $C_{28}H_{36}O_{13}\cdot 0.5H_2O$: C 57.03, H 6.33.
Found: C 56.87, H 6.23.

[1*S*-[1α(4*R**,5*S**),3α,4β,5α,6α(2*E*,4*R**,6*R**),7β]]-1-[4-Hydroxy-5-methyl-3-methylene-6-phenylhexyl]-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyl-2-octenoate), 3,4,5-Trimethyl Ester (**7**)

A solution of **4** (4.38 mg/ml, 100 ml) in 12 M HCl-MeOH (1:100) was stirred for 68 hours at 20°C. The pH of the solution was raised to 8 by adding saturated aq $NaHCO_3$ solution. The solvent was removed by evaporation under reduced pressure, the residue was diluted with water and extracted with EtOAc. The organic solution was washed with aq $NaHCO_3$, brine, dried and chromatographed on silica gel eluting with EtOAc-cyclohexane (1:2) to give 248 mg (60%) of **7** as a white crystalline solid: MP 66~68°C (from EtOAc-hexane); $[\alpha]_D^{23}$ +34.6° (*c* 0.94, MeOH); IR ν_{max} ($CHBr_3$) cm^{-1} 3535, 1767, 1743, 1706, 1647; ^1H NMR (250 MHz, $CDCl_3$) δ 0.8~0.9 (9H, m), 1.02 (3H, d, J=7 Hz, $=CHCHCH_3$), 2.78 (1H, dd, J=14 and 6 Hz, CHPh), 3.60 (br s, 7-OH), 3.75, 3.79, 3.90 each (3H, s, CO_2CH_3), 3.99 (s, 4-OH), 4.06 (2H, m, 7-H, $=CCHOH$), 5.00, 5.13 each (1H, s, $C=CH_2$), 5.27 (1H, s, 3-H), 5.73 (1H, d, J=16 Hz, $CH=CHCO_2$), 5.85 (1H, d, J=2 Hz, 6-H), 6.82 (1H, dd, J=16 and 9 Hz, $CH=CHCO_2$), 7.12~7.30 (5H, m, Ph); EI-MS *m/z* 690 (M, $C_{36}H_{50}O_{13}$), 571, 553, 411, 343, 225, 153, 91.

Anal Calcd for $C_{36}H_{50}O_{13}\cdot H_2O$: C 61.00, H 7.40.
Found: C 60.95, H 7.46.

A portion of this ester was recrystallised from EtOAc-hexane to give crystals which were suitable for X-ray diffraction experiments.

Ester (**7**) was alternatively prepared by diazomethane treatment of **2**. Its spectral characteristics were identical with those described above.

Isolation of 4*S*(2*E*,4*R**,6*R**)-4,6-Dimethyl-2-octenoic Acid (**8**)

A solution of **1** (15 g, 21.7 mmol) in MeOH (150 ml) was treated with 0.8 M aq NaOH (220 ml) and the mixture was heated to reflux for 3 hours. The mixture was allowed to cool to 20°C, and the MeOH was removed under reduced pressure. The residue was acidified by adding 2 M HCl (200 ml) and extracted with toluene. The toluene solution was washed with brine, dried and distilled to give 3.24 g (88%) of **8** as a clear colourless liquid: BP 94~95°C/0.3 mmHg; $[\alpha]_D^{19}$ +55° (liquid, l=0.1 dm, d^{19}=0.929); IR ν_{max} ($CHBr_3$) cm^{-1} 3400~2300, 1691, 1647; ^1H NMR (500 MHz, CD_3OD) δ 0.86 (3H, d, J=7 Hz, 6-CH_3), 0.87 (3H, t, J=7 Hz, 8-H), 1.04 (3H, d, J=7 Hz, 4-CH_3), 1.08~1.21 (2H, m, 5-H, 7-H), 1.27~1.37 (2H, m, 6-H, 7-H), 1.38 (1H, m, 5-H), 2.43 (1H, m, 4-H), 5.76 (1H, d, J=15 Hz, 2-H), 6.79 (1H, dd, J=15 and 8 Hz, 3-H); ^{13}C NMR (125 MHz, CD_3OD) δ 11.6 (C-8), 19.3 (6-CH_3), 20.8 (4-CH_3), 30.8 (C-7), 33.1 (C-6),

35.3 (C-4), 44.4 (C-5), 120.8 (C-2), 156.2 (C-3), 170.1 (C-1); FAB-MS *m/z* 171 ($M+H$, $C_{10}H_{18}O_2$), 153, 123.

Anal Calcd for $C_{10}H_{18}O_2$: C 70.55, H 10.66.
Found: C 70.49, H 10.78.

2*S*(2*R**,4*R**)-2,4-Dimethylhexanoic Acid, Methyl Ester (**10**)

A solution of **8** (1.2 g, 7 mmol) in carbon tetrachloride (14 ml), acetonitrile (14 ml) and water (21 ml) was treated with sodium periodate (6.14 g, 28.7 mmol) and ruthenium trichloride trihydrate (40 mg, 0.15 mmol) at 20°C. The mixture was stirred for 2 hours, filtered through kieselgel, and the residue was washed with CH_2Cl_2. The filtrate and washings were transferred into a separating funnel, and the organic lower layer was separated. The organic solution was washed with water (10 ml), dried, evaporated and distilled under reduced pressure in a bulb-to-bulb distillation apparatus to give 400 mg (40%) of 2*S*(2*R**,4*R**)-2,4-dimethylhexanoic acid (**9**) as a colourless liquid: BP 125°C/3.8 mmHg; $[\alpha]_D^{19}$ +32.7° (*c* 0.98, EtOH); IR ν_{max} ($CHBr_3$) cm^{-1} 3400~2400, 1701; ^1H NMR (250 MHz, $CDCl_3$) δ 0.87 (3H, t, $J=7$ Hz, 6-H), 0.89 (3H, d, $J=7$ Hz, 4-CH_3), 1.19 (3H, d, $J=7$ Hz, 2-CH_3), 1.05~1.48 (4H, m, 3-H, 4-H, 5-H), 1.74 (1H, m, 3-H), 2.57 (1H, m, 2-H).

A portion of **9** (385 mg, 2.67 mmol) in MeOH (10 ml) was treated dropwise with thionyl chloride (0.4 ml, 5.5 mmol) at 0°C and then the solution was stood at room temperature overnight. The excess MeOH was removed by distillation at atmospheric pressure, and the residue was distilled to give 216 mg, (51%) of **10**: $[\alpha]_D^{19}$ +33° (liquid, $l=0.1$ dm, $d^{22}=0.871$), $[\alpha]_D^{23}$ +32.2° (liquid, $l=0.2$ dm)[9]; IR ν_{max} ($CHBr_3$) cm^{-1} 1725; ^1H NMR (400 MHz, $CDCl_3$) δ 0.87 (3H, t, $J=7$ Hz, 6-H), 0.89 (3H, $J=7$ Hz, 4-CH_3), 1.16 (3H, d, $J=7$ Hz, 2-CH_3), 1.13 (2H, m, 3-H, 5-H), 1.33 (2H, m, 4-H, 5-H), 1.73 (1H, m, 3-H), 2.57 (1H, m, 2-H), 3.67 (3H, s, CO_2CH_3) (NMR spectrum)[10].

X-Ray Crystallographic Analysis of **7**

The clear colourless data crystal, of dimensions $0.02\times0.10\times0.42$ mm, was mounted on a glass fibre. Intensities were recorded up to 115° 2θ at 295 K (ω-scan). Of the 2,956 independent intensities measured on a Nicolet R3m/V four-circle diffractometer, 1,594 were used in the solution and refinement of the structure [$I>2.0\,\sigma(I)$]. No correction was applied for X-ray absorption effects. Standard reflections -2-1-1, -1-2-1 and 121 were monitored every 97 reflections and showed some 7% decay over the 53 hours of crystal exposure to X-rays. Correction was made for crystal decay.

Crystal data: $C_{36}H_{50}O_{13}\cdot H_2O$, monoclinic, $P2_1$, $a=14.55(3)$, $b=8.185(11)$, $c=17.21(7)$Å, $\beta=100.4(3)°$, $U=2{,}016(10)$Å^3, $Z=2$, $D_{calc}=1.17$ g/cm^3, F(000)=760, Cu-K_α X-radiation (graphite monochromator), $\lambda=1.54184$Å, $\mu(CuK_\alpha)=7.1$ cm^{-1}.

The structure was solved by direct methods and was refined using full-matrix least-squares methods. Hydrogen atoms were included in calculated positions using a riding model and common thermal parameters. Anisotropic parameters were used for oxygen atoms only. Individual weights were applied according to the scheme $w=[\sigma^2(F_O)+0.0016|F_O|^2]^{-1}$, and the refinement converged at R 0.07, R_w 0.08, goodness-of-fit=1.34. The final electron density difference synthesis showed no peaks >0.2 or <−0.3 e Å^{-3}. All computations, with the exception of solution, were carried out using SHELXTL PLUS (μ-VAX II) system of programs[11]. Solution was undertaken with the MITHRIL package[12].

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References

1) DAWSON, M. J.; J. E. FARTHING, P. S. MARSHALL, R. F. MIDDLETON, M. J. O'NEILL, A. SHUTTLEWORTH, C. STYLLI, R. M. TAIT, P. M. TAYLOR, H. G. WILDMAN, A. D. BUSS, D. LANGLEY & M. V. HAYES: The squalestatins, novel inhibitors of squalene synthase produced by a species of *Phoma*. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activity. J. Antibiotics 45: 639~647, 1992

2) JACKMAN, L. M. & S. STERNHELL: Application of Nuclear Magnetic Resonance in Organic Chemistry. p. 176, Pergamon Press, 1969
3) KALINOWSKI, H-O.; S. BERGER & S. BRAUN: Carbon-13 NMR Spectroscopy. p. 215, John Wiley & Sons, 1988
4) BAX, A. & M. F. SUMMERS: ^{1}H and ^{13}C Assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093~2094, 1986
5) CHRISTOFIDES, J. C. & D. B. DAVIES: A method to assign ^{13}C NMR signals of compounds with exchangeable hydrogen atoms using two and three bond isotope effects: cellobiose. J. Chem. Soc. Chem. Commun. 1983: 324~326, 1983
6) CHRISTOFIDES, J. C. & D. B. DAVIES: Secondary isotope multiplet NMR spectroscopy of partially labeled entities. Carbon-13 SIMPLE NMR of carbohydrates. J. Am. Chem. Soc. 105: 5099~5105, 1983
7) CHRISTOFIDES, J. C. & D. B. DAVIES: Secondary isotope multiplet nuclear magnetic resonance spectroscopy of partially labeled entities (SIMPLE): ^{13}C spectra of stachyose and its subunits. J. Chem. Soc. Perkin Trans. II 1984: 481~488, 1984
8) JACKMAN, L. M. & S. STERNHELL: Application of Nuclear Magnetic Resonance in Organic Chemistry. p. 289, Pergamon Press, 1969
9) ODHAM, G.: Synthesis of (+)-methyl 2L,4L-dimethylhexanoate and (−)-methyl 2D,4L-dimethylhexanoate involving separation of diastereoisomers by preparative gas chromatography. Ark. Kemi 26: 367~377, 1967
10) CIMINO, G.; G. SODANO & A. SPINELLA: New propionate-derived metabolites from Aglaja depicta and from its prey Bulla striata (Opisthobranch molluscs), J. Org. Chem. 52: 5326~5331, 1987
11) SHELDRICK, G. M.: SHELXTL PLUS Release 4.11/V. Siemens Analytical X-Ray Instruments, 1990
12) GILMORE, C. J.: MITHRIL—integrated direct-methods computer program. J. Appl. Cryst. 17: 42~46, 1984