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

V. MINOR METABOLITES

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The isolation and structure determination by ^{1}H and ^{13}C NMR and MS of 24 novel squalestatins from cultures of *Phoma* sp. C2932 is described.

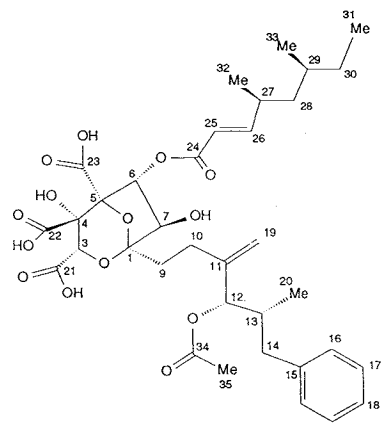
The isolation[1] and structure determination[2] of three squalestatins from *Phoma* sp. C2932, a biosynthetic investigation[3] of one of these squalestatins and the directed biosynthesis of novel squalestatins[4] have already been reported. We now wish to report the isolation and structure determination of related metabolites produced by *Phoma* sp. C2932.

Results and Discussion

The three squalestatins which have been described previously[1,2], we here name as squalestatins S1, S2 and H1. The nomenclature applied to these materials and other squalestatins reported in this paper is illustrated in Table 1 which shows a generalised structure for a squalestatin (**1**) which is named squalestatin R_1R_2. Substitution of a letter for group R_1 followed by a number for group R_2, both drawn from Table 1, then describes a particular squalestatin.

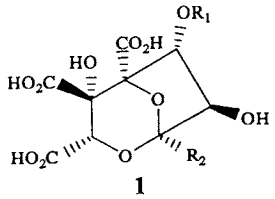
The extraction procedure outlined in Fig. 1 was applied to 500- and 5,000-litre fermentations of *Phoma* sp. C2932 to give crude calcium salts. Analytical HPLC (Fig. 2) of acidified extracts of these salts indicated that the major components in the chromatogram were squalestatins S1 (**2**), S2 and H1. However, many minor components could also be seen.

Large scale reverse-phase LC of acidified extracts of the calcium salts gave fractions from which squalestatins S1 and S2 were isolated as crystalline tripotassium salts. Side fractions from this large scale chromatography, or the mother liquors after crystallisation of squalestatin S2 tripotassium salt, after further chromatography yielded the squalestatins listed in Table 2. The retention times for these materials in a gradient HPLC system are recorded in this table.

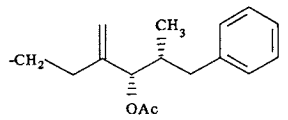
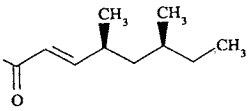
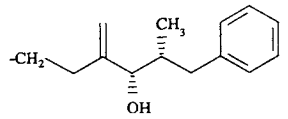
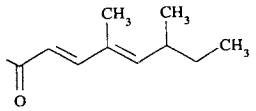
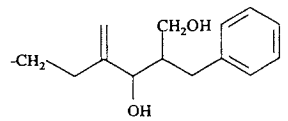
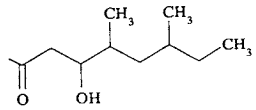
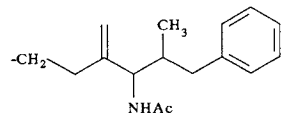
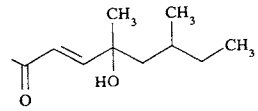
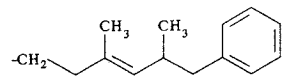
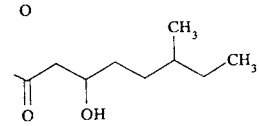
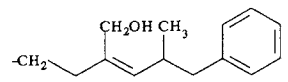
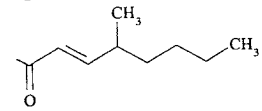
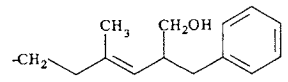
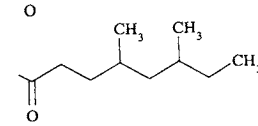
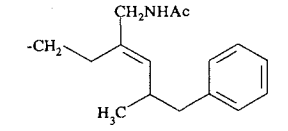
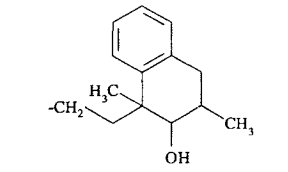


Squalestatin S1 (**2**)

Table 1. Notation for different groups R_1 and R_2 found in squalestatins.



1

R_1	Letter	R_2	Number
H	H		1
	S		2
	T		3
	U		4
	V		5
	W		6
	X		7
	Y		8
			9

Structure Elucidation

Examination of the negative ion fast atom bombardment (FAB) mass spectra recorded on each of these compounds (Table 3) showed that seven afforded fragment ions at *m/z* 475 and *m/z* 537 as seen for S1 (Fig. 3). These compounds therefore appeared to differ from S1 only by virtue of having different ester

Fig. 1. Isolation of crude calcium salt from fermentations of *Phoma* sp. C2932.

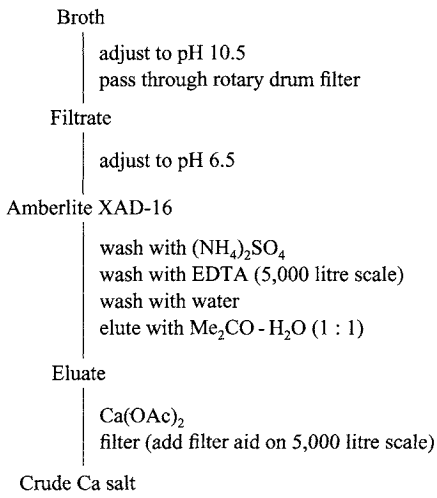
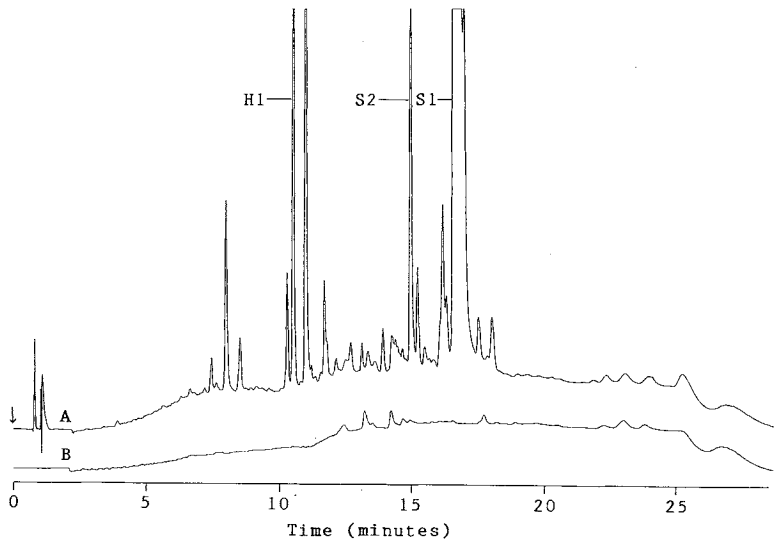


Table 2. Squalestatins isolated in the course of this work and their retention times in a gradient HPLC system.

Squalestatin	Rt (minutes)	Squalestatin	Rt (minutes)
H7	5.8	V1 isomer a	13.6
H9	5.9	V1 isomer b	13.7
H2	6.4	S4	13.7
H6	6.9	W1	14.0
H1	9.4	S8	14.2
H5	9.8	S2	14.3
6-Deoxy H1	10.2	U1	14.5
7-Deoxy H5	11.2	X1	15.4
6-Deoxy H5	11.2	T1	15.6
V2	11.3	S1	15.8
W2	11.4	Y1	16.1
6,7-Dideoxy H5	11.8	S5	16.7
S3	12.6	7-Deoxy S1	16.8
U2	12.9		

HPLC conditions as for Fig. 2 but flow 3 ml/minute.

Fig. 2. Typical trace after HPLC of an acidified extract of crude calcium salt.



Column: Phase Separations Ltd Spherisorb C6, 5 μm (15 × 0.46 cm). Mobile Phases: A, H_2O-H_2SO_4, 1,000 : 0.15. B, MeCN - H_2O - H_2SO_4, 500 : 500 : 0.15. Linear gradient 0 to 100% B in 15 minutes, hold 10 minutes, 100 to 0% B in 1.5 minutes. Flow: 2 ml/minute. Detection: λ 210 nm. Range: 0.1 AUFS. Chart speed: 1 cm/minute. Trace B is of a blank gradient.

side chains. Three of the compounds afforded fragment ions at *m/z* 433 and 495 as seen for S2 and thus appeared to differ from S2 only by virtue of having different ester side chains. The ions associated with the ester side chain were not observed for nine of the compounds suggesting that this side chain was missing in these derivatives. A further five compounds appeared to contain the normal ester side chain (d ion at *m/z* 169) while differing elsewhere in the molecule. Ten compounds showed a shift in mass for ion d suggesting deviation from the normal ester side chain. Examination of the proton and carbon NMR

Table 3. High and low resolution-ve ion FAB data for squalestatins.

Squalestatin	$(M-H)^-$	a	b	c	d
T1	687.2652[e]	643	537	475	167
U1	707.2943[f]	663	537	475	187
V1 isomer a	705	661	537	475	185
V1 isomer b	705	661	537	475	185
W1	693	649	537	475	173
X1	675.2679[g]	631	537	475	155
Y1	691	647	537	475	171
U2	665	621	495	433	187
V2	663	619	495	433	185
W2	651	607	495	433	173
H2	495[h]	451	—	—	—
H5	479[h]	435	—	—	—
H6	495[h]	451	—	—	—
H7	495[h]	451	—	—	—
H9	495[h]	451	—	—	—
S3	663	619	511	449	169
S4	688	644	536	474	169
S5	631	587	479	417	169
S8	688	644	536	474	169
7-Deoxy S1	673	629	521	459	169
6-Deoxy H1	521	477	—	—	—
6-Deoxy H5	463	419	—	—	—
7-Deoxy H5	463	419	—	—	—
6,7-Dideoxy H5	447	403	—	—	—

[e] Δ 0.08 mmu of calculated for $C_{35}H_{43}O_{14}$.
[f] Δ −2.81 mmu of calculated for $C_{35}H_{47}O_{15}$.
[g] Δ −2.62 mmu of calculated for $C_{34}H_{43}O_{14}$.
[h] Δ $(M-H)^-$ for H series ≡ b ions.

Fig. 3. Negative ion FAB mass spectrum of squalestatin S1.

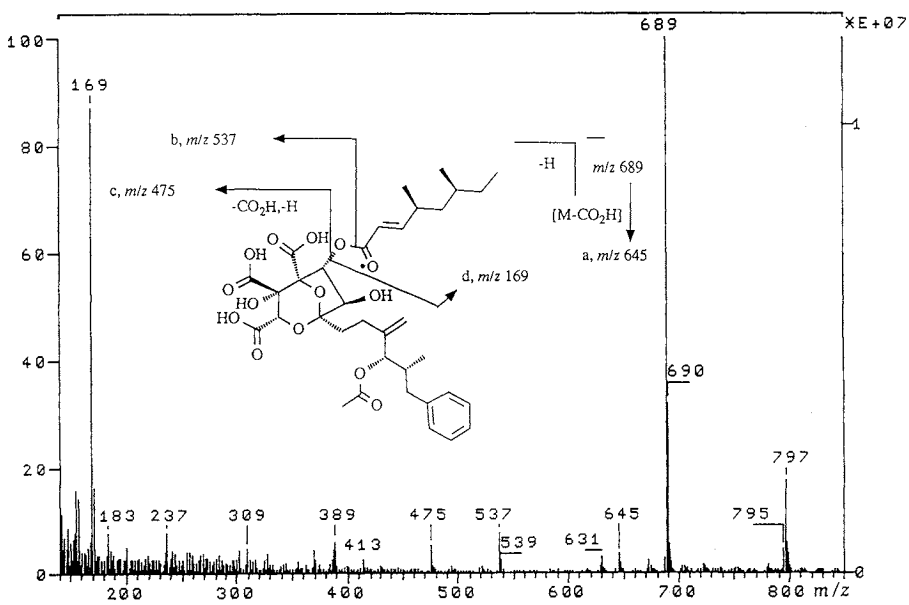


Table 4. ^{13}C NMR data[a] for the minor components differing from S1 only in the ester side chain.

Carbon No.[b]	Squalestatins							
	S1	T1	U1	V1a	V1b	W1	X1	Y1
6	81.0	81.0	81.3	81.3	81.3	81.5	81.2	80.8
12	80.1	80.2	80.4	80.3	80.3	80.5	80.2	80.3
24	166.5	167.2	172.1	166.8	166.8	171.9	166.7	173.8
25	119.8	115.6	40.7	118.0	118.0	43.3	119.9	32.6
26	157.6	152.3	71.7	158.3	158.4	69.2	157.4	32.6
27	35.6	133.0	36.7	73.8	73.8	33.3	37.8	30.8
28	44.4	150.4	41.4	49.8	49.8	35.5	36.7	45.4
29	33.1	36.1	32.6	31.3	31.3	35.6	30.5	32.7
30	30.8	31.1	29.7	32.2	32.1	30.5	23.6	30.1
31	11.4	12.2	11.3	11.5	11.5	11.6	14.2	11.4
32	20.5	12.4	14.6	28.3	28.3	—	19.6	20.1[c]
33	19.2	20.4	20.2	21.4	21.4	19.4	—	20.0[c]

[a] In CD_3OD.
[b] The chemical shifts of the carbons not listed here are within ± 0.2 ppm of those of S1[2].
[c] Assignments may be reversed.

spectra of the compounds (See Tables 4 to 11) confirmed these observations. These spectra in conjunction, in some cases, with additional NMR experiments enabled the structures to be identified as outlined below.

Compounds Differing from Squalestatin S1 Only in the Ester Side Chain (Tables 4 and 5)

Squalestatin T1: The molecular weight, at two mass units lower than S1, and the presence of extra olefinic signals in the NMR spectra indicated an extra double bond. Loss of coupling on the signal for the 26-proton and changes to the chemical shift and coupling observed for the methyl group at position 32 established that the double bond was located between carbons 27 and 28. The stereochemistry of the double bond was determined by NOE difference. Irradiation of the methyl group (position 32) enhanced the signal for the 29-proton.

Squalestatin U1: The molecular weight, at 18 mass units higher than S1, suggested the addition of H_2O. This was confirmed by the NMR data which showed the replacement of the ester side chain double bond by a $-CH_2-CH-O$ group. The proton proton coupling observed for the new CH–O signal served to establish that it was at position 26.

Squalestatin V1 Isomers a and b: Two compounds having essentially the same mass spectra and very similar NMR spectra were identified as a pair of diastereoisomers. The increase in mass of 16 over S1 suggested hydroxylation. This was confirmed by the replacement, in the carbon spectrum, of a CH signal by one from a quaternary C–O. Changes in the chemical shifts and couplings observed for positions 26 and 32 indicated that the hydroxylation was at carbon 27.

Squalestatin W1: Comparison of the NMR spectra with those of U1 established that the ester side chain double bond had been replaced in the same way and that there was one less methyl group. The complex multiplet seen for proton 26 indicated that it was between two CH_2 groups and thus that the 32-methyl group was the one missing.

Squalestatin X1: The mass spectrum of this compound showed that the ester side chain contained 14 mass units less than that of S1. Inspection of the NMR spectra revealed that there was one less methyl group. Full analysis of phase sensitive double quantum filtered COSY (PS DQF COSY) and ^1H-^{13}C

Table 5. ^{1}H NMR data[a] for the minor components differing from S1 only in the ester side chain.

Position No.[b]	Squalestatins			
	S1	T1	U1	V1a
6	6.31 (d, 2)	6.33 (d, 2)	6.24 (d, 2)	6.31 (d, 2)
25	5.80 (d, 16)	5.80 (d, 16)	2.41 (m)[e], 2.51 (dd, 16, 4)	5.99 (d, 16)
26	6.85 (dd, 16, 8)	7.34 (d, 16)	3.93 (dt, 8, 4)	7.03 (d, 16)
27	2.40~2.51 (m)	—	1.61 (m)	—
28	1.09~1.19 (m), 1.39 (m)	5.73 (d, 10)	0.96 (m), 1.36~1.46 (m)	1.38 (dd, 16, 6), 1.60 (dd, 14, 4)
29	1.27~1.37 (m)	2.48 (m)[c,d]	1.46 (m)	1.52 (m)
30	1.09~1.19 (m), 1.27~1.37 (m)	1.31 (m), 1.43 (m)	1.08 (m), 1.36~1.46 (m)	1.16 (m), 1.31~1.42 (m)
31	0.86 (t, 7)	0.86 (t, 7)	0.88 (t, 7)	0.85 (t, 7)
32	1.03 (d, 7)	1.80 (d, 1)	0.87 (d, 7)	1.28 (s)
33	0.87 (d, 7)	0.99 (d, 7)	0.88 (d, 7)	0.88 (d, 7)

Position No.[b]	Squalestatins			
	V1b	W1	X1	Y1
6	6.32 (d, 2)	6.26 (d, 2)	6.31 (d, 2)	6.27 (d, 2)
25	6.01 (d, 16)	2.40 (m)[e], 2.51 (dd, 15, 5)	5.78 (dd, 16, 1)	2.25~2.37 (m)[c]
26	7.01 (d, 16)	3.96 (m)	6.89 (dd, 16, 8)	1.28~1.40 (m), 1.66 (m)
27	—	1.41~1.52 (m)	2.31 (m)	1.55 (m)
28	1.41 (dd, 16, 6), 1.60 (dd, 14, 4)	0.85~0.92 (m), 1.23~1.40 (m)	1.34~1.41 (m)	0.96 (m), 1.24 (m)
29	1.48 (m)	1.23~1.40 (m)	1.23~1.34 (m)	1.43 (m)
30	1.14 (m), 1.34 (m)	1.16 (m), 1.23~1.40 (m)	1.23~1.34 (m)	1.09 (m), 1.28~1.40 (m)
31	0.84 (t, 7)	0.88 (t, 7)	0.89 (t, 7)	0.87 (t, 7)
32	1.28 (s)	—	1.04 (d, 7)	0.86 (d, 7)[e]
33	0.93 (d, 7)	0.87 (d, 7)	—	0.87 (d, 7)[e]

[a] In CD_3OD.
[b] The chemical shifts of the protons not listed here are within ±0.03 ppm of those of S1[2].
[c] Overlaps with other signals not given in this table.
[d] Located by ^{13}C-^1H correlation.
[e] Assignments may be reversed.

correlation data confirmed that the 33-methyl was absent.

Squalestatin Y1: The molecular weight, at 2 mass units higher than S1, suggested the addition of H_2. This was confirmed by the NMR data which showed the replacement of the ester side chain double bond by a CH_2–CH_2 group.

Compounds Differing from S2 Only in the Ester Side Chain (Tables 6 and 7)

Squalestatins U2, V2 and W2: These compounds were identified by comparison of their mass spectral and NMR data with those of S2 and the corresponding 1 series compounds: U1, V1 and W1.

Compounds Differing from H1 Only in the Alkyl Side Chain (Tables 8 and 9)

Squalestatin H2: This compound was identified by the comparison of its mass spectral and NMR

Table 6. ^{13}C NMR data[a] for the minor components differing from S2 only in the ester side chain.

Carbon No.[b]	Squalestatins				Carbon No.[b]	Squalestatins			
	S2	U2	V2[c]	W2[c]		S2	U2	V2[c]	W2[c]
6	81.0	81.5			28	44.4	41.4		
12	78.6	78.9			29	33.1	32.6		
24	166.5	172.2			30	30.8	29.7		
25	119.8	40.7			31	11.4	11.3		
26	157.6	71.7			32	20.5	14.7		
27	35.6	36.7			33	19.2	20.2		

[a] In CD_3OD.
[b] The chemical shifts of the carbons not listed here are within ± 0.3 ppm of those of S2[2].
[c] Not measured.

Table 7. ^{1}H NMR data[a] for the minor components differing from S2 only in the ester side chain.

Position No.[b]	Squalestatins			
	S2	U2	V2	W2
6	6.31 (d, 2)	6.24 (d, 2)	6.31 (d, 2)	6.26 (d, 2)
12	3.92 (d, 5)	3.93 (m)[c]	3.93 (d, 5)	3.93 (d, 5)
25	5.78 (d, 16)	2.40 (m)[c], 2.50 (dd, 16, 4)	5.98 (d, 16)	2.39 (dd, 15, 9), 2.51 (dd, 15, 5)
26	6.84 (dd, 16, 8)	3.93 (m)[c]	7.02 (d, 16)	3.96 (m)
27	2.39~2.51 (m)	1.61 (m)	—	1.41~1.52 (m)
28	1.09~1.19 (m), 1.39 (m)	0.96 (m), 1.36~1.46 (m)	1.30~1.41 (m), 1.60 (dd, 14, 4)	0.92 (m), 1.23~1.40 (m)
29	1.26~1.36 (m)	1.46 (m)	1.52 (m)	1.23~1.40 (m)
30	1.09~1.19 (m), 1.26~1.36 (m)	1.08 (m), 1.36~1.46 (m)	1.16 (m), 1.30~1.41 (m)	1.16 (m), 1.23~1.40 (m)
31	0.86 (t, 7)	0.88 (t, 7)	0.85 (t, 7)	0.88 (t, 7)
32	1.03 (d, 7)	0.87 (d, 7)	1.28 (s)	—
33	0.85 (d, 7)	0.88 (d, 7)	0.88 (d, 7)	0.87 (d, 7)

[a] In CD_3OD.
[b] The chemical shifts of the protons not listed here are within ± 0.03 ppm of those of S2[2].
[c] Overlaps with other signals not given in this table.

data with those of S2 and H1.

Squalestatin H5: Examination of the ^{13}C NMR data for this compound revealed that the alkyl side chain contained no oxygen bearing carbons, acetate group or terminal double bond. Signals for a tri-substituted double bond and an extra methyl group were observed. The ^{1}H chemical shift and couplings observed for this methyl group indicated that it was attached to the double bond. Further analysis of the ^{1}H NMR data yielded the structure. The stereochemistry of the double bond was determined from the ^{13}C chemical shift of the 19-methyl group[5].

Squalestatin H6: Comparison of the mass spectral and NMR data with that for H5 showed that the 19-methyl group had been replaced by a CH_2–OH. The stereochemistry of the double bond was determined by NOE difference. Irradiation of one of the 19-protons enhanced the signal for the 13-proton.

Squalestatin H7: Comparison of the mass spectral and NMR data with that for H5 showed that the 20-methyl group had been replaced by a CH_2–OH.

Squalestatin H9: From the mass spectral data this compound was isomeric with H2. Initial inspection of the NMR data revealed that the aromatic ring was 1,2-disubstituted and that the double bond had

Table 8. ^{13}C NMR data[a] for the minor components modified in the alkyl side chain.

Carbon No.[b]	Squalestatins									
	S1	H2	S3	S4	S5	H5	H6	H7	S8	H9
1	106.8	106.8	107.0	107.0	107.1	106.9	106.9	106.8	106.9	107.3
9	34.9	35.3	35.0	35.1	35.3	35.4	35.7	35.2	35.2	30.5
10	26.5	25.9	25.7	26.4	33.5	33.5	28.8	33.7	22.7	31.4
11	147.7	152.2	151.6	149.3	135.0	135.1	138.6	138.4	135.6	44.7
12	80.1	79.0	77.0	59.8	131.4	131.2	134.4	126.3	134.4	76.1
13	37.7	39.0	46.0	37.7	35.9	35.9	35.6	44.4	35.5	31.8
14	40.9	41.3	35.3	41.4	45.0	45.0	45.0	39.2	44.9	39.7
15	141.6	142.4	142.0	142.0	142.3	142.3	141.9	141.9	142.0	137.1
16	130.2	130.2	130.3	130.2	130.3	130.3	130.3	130.4	130.5	129.2
16′	130.2	130.2	130.3	130.2	130.3	130.3	130.3	130.4	130.5	144.2
17	129.3	129.1	129.2	129.2	128.9	128.9	129.0	128.9	129.1	126.4
17′	129.3	129.1	129.2	129.2	128.9	128.9	129.0	128.9	129.1	128.3
18	126.9	126.6	126.7	126.8	126.5	126.5	126.7	126.6	126.5	127.1
19	111.5	110.4	111.3	111.9	16.1	16.2	59.9	16.5	45.5	27.1
20	14.1	13.9	62.0	15.0	21.2	21.2	21.5	66.5	21.3	19.1
34	172.1	—	—	169.9	—	—	—	—	173.1	—
35	20.9	—	—	22.5	—	—	—	—	22.5	—

[a] In CD_3OD.

[b] The chemical shifts of the carbons not listed here are within ±0.4 ppm of those of S1[2] for compounds in the S series and within ±0.4 ppm of those of H1[2] for compounds in the H series.

been replaced by a CH_3–C group. This suggested the structure which was confirmed by detailed analysis of the proton and ^1H-^{13}C correlation data.

Compounds Differing from S1 Only in the Alkyl Side Chain (Tables 8 and 9)

Squalestatin S3: Scrutiny of the NMR data showed that there was no acetate group and that the 20-methyl had been replaced by a CH_2–O group.

Squalestatin S4: The molecular weight, at 1 mass unit lower than S1, suggested the replacement of an oxygen atom by NH. This was confirmed by the NMR data, with the chemical shift changes observed for the 12, 34 and 35 positions locating the point of modification.

Squalestatin S5: This compound was identified by comparison of its mass spectral and NMR data with those of S1 and H5.

Squalestatin S8: From the mass spectral data this compound was isomeric with S4. Examination of the NMR data, including ^1H-^{13}C correlation, revealed that the double bond was now tri-substituted and that the CH–NHAc group had been replaced by a CH_2–NHAc. Analogous changes having been seen between H2 and H6 these observations led directly to the structure. The stereochemistry of the double bond was determined by NOE difference. Irradiation of the CH_2 group (position 19) enhanced the signal for the 12-proton.

Compounds Differing from S1 Only in the Core (Tables 10 and 11)

7-Deoxysqualestatin S1: The molecular weight, at 16 mass units lower than S1, suggested the loss of an oxygen atom. This was confirmed by the NMR data which clearly showed the replacement of the CH–O group at the 7-position by a CH_2. Assignments were confirmed by ^1H-^{13}C correlation. Note the shift to high field of the 3-proton (-0.40δ) and to low field of the 6-proton ($+0.14\delta$) and 1-carbon ($+2.3\delta$)

Table 9. ^{1}H NMR data[a] for the minor components modified in the alkyl side chain.

Position No.[b]	Squalestatins				
	S1	H2	S3	S4	S5
7	4.04 (d, 2)	4.09 (d, 2)	4.05 (d, 2)	4.06 (d, 2)	4.10 (d, 2)
9	2.00 (m), 2.05 (m)	1.98~2.16 (m)	1.98 (m), 2.08 (m)	1.98~2.10 (m)	1.90~2.00 (m)
10	2.34 (m), 2.40~2.51 (m)[c]	2.28 (m), 2.47 (m)	2.28 (m), 2.49 (m)	2.35 (m), 2.40~2.53 (m)[c]	2.21 (m), 2.26 (m)
12	5.08 (d, 5)	3.94 (d, 5)	4.12 (d, 6)	4.30 (d, 7)	5.06 (m)
13	2.24 (m)	1.98~2.16 (m)	2.01 (m)	2.15 (m)	2.64 (m)
14	2.44 (dd, 13, 9), 2.68 (dd, 13, 6)	2.36 (dd, 13, 9), 2.78 (dd, 13, 5)	2.67 (dd, 13, 7), 2.72 (dd, 13, 8)	2.26 (dd, 13, 9), 2.76 (dd, 13, 5)	2.49 (dd, 13, 8), 2.56 (dd, 13, 6)
16	7.19 (d, 7)	7.21 (d, 7)	7.23~7.28 (m)	7.19 (d, 7)	7.12 (d, 7)
16′	7.19 (d, 7)	7.21 (d, 7)	7.23~7.28 (m)	7.19 (d, 7)	7.12 (d, 7)
17	7.26 (t, 7)	7.25 (t, 7)	7.23~7.28 (m)	7.25 (t, 7)	7.22 (t, 7)
17′	7.26 (t, 7)	7.25 (t, 7)	7.23~7.28 (m)	7.25 (t, 7)	7.22 (t, 7)
18	7.14 (t, 7)	7.14 (t, 7)	7.13 (m)	7.13 (t, 7)	7.11 (t, 7)
19	4.97 (s), 5.02 (s)	5.01 (s), 5.11 (s)	5.04 (s), 5.15 (s)	5.00 (s), 5.01 (s)	1.41 (d, 1)
20	0.87 (d, 7)	0.82 (d, 7)	3.54 (dd, 11, 4), 3.69 (dd, 11, 5)	0.83 (d, 7)	0.95 (d, 7)
35	2.10 (s)	—	—	2.01 (s)	—

Position No.[b]	Squalestatins				
	H5	H6	H7	S8	H9
7	4.08 (d, 2)	4.09 (d, 2)	4.07 (d, 2)	3.93 (d, 2)	3.97 (d, 2)
9	1.92 (m), 1.96 (m)	1.98 (m)	1.94 (m)	1.27~1.43 (m)[c], 1.81 (m)	2.09 (m), 2.30 (m)
10	2.20 (m), 2.29 (m)	2.38 (m)	2.23 (m), 2.31 (m)	2.22 (m), 2.26 (m)	1.51 (m), 1.65 (m)
12	5.06 (m)	5.20 (d, 10)	5.05 (m)	5.18 (d, 10)	3.62 (d, 12)
13	2.64 (m)	2.75 (m)	2.71 (m)	2.85 (m)	2.04 (m)
14	2.49 (dd, 13, 8), 2.57 (dd, 13, 6)	2.51 (dd, 13, 8), 2.59 (dd, 13, 6)	2.39 (dd, 13, 9), 2.88 (dd, 13, 5)	2.51 (dd, 13, 8), 2.59 (dd, 13, 6)	2.52 (dd, 16, 10), 2.83 (dd, 16, 5)
16	7.13 (d, 7)	7.13 (d, 7)	7.15 (d, 7)	7.18 (dd, 7, 1)	6.99 (br d, 7)
16′	7.13 (d, 7)	7.13 (d, 7)	7.15 (d, 7)	7.18 (dd, 7, 1)	—
17	7.22 (t, 7)	7.24 (t, 7)	7.22 (t, 7)	7.22 (t, 7)	7.03 (dt, 1, 7)
17′	7.22 (t, 7)	7.24 (t, 7)	7.22 (t, 7)	7.22 (t, 7)	7.34 (br d, 7)
18	7.12 (t, 7)	7.14 (t, 7)	7.12 (t, 7)	7.08 (tt, 7, 1)	7.13 (br t, 7)
19	1.42 (d, 1)	3.67 (d, 12.5), 3.93 (d, 12.5)	1.39 (d, 1)	3.71 (m)	1.19 (s)
20	0.96 (d, 7)	1.00 (d, 7)	3.46 (m)	0.98 (d, 7)	1.16 (d, 7)
35	—	—	—	1.96 (s)	—

[a] In CD_3OD.
[b] The chemical shifts of the protons not listed here are within ±0.03 ppm of those of S1[2] for compounds in the S series and within ±0.05 ppm of those of H1[2] for compounds in the H series.
[c] Overlaps with other signals not given in this table.

which occur on the loss of the 7-hydroxyl group from S1[2].

Compounds Differing From H1 in the Core and/or the Alkyl Side Chain (Tables 10 and 11)

6-Deoxysqualestatin H1: The molecular weight, at 16 mass units lower than H1, suggested the loss of an oxygen atom. Replacement of a CH–O group by a CH_2 was confirmed by the NMR data. The shifts to low field of the 3-proton (+0.30δ) and the 7-proton (+0.16δ) and the essentially unchanged position of the 1-carbon when compared to H1[2] served to establish that the modification was at the 6-

Table 10. ^{13}C NMR data[a] for the minor components modified in the core.

Carbon No.[b]	Squalestatins				
	7-Deoxy S1	6-Deoxy H1	6-Deoxy H5	7-Deoxy H5	6,7-Dideoxy H5
1	109.1	106.8	107.1	109.2	109.8
3	76.2	76.3	76.2	76.1	75.7
4	75.3	76.0	76.0	75.5	76.0
5	92.8	87.9	87.7	95.0	89.3
6	74.5	39.7	39.7	72.1	30.3
7	42.4	73.8	73.3	43.6	32.0
9	36.1	34.8	35.0	36.6	36.3
10	27.2	26.5	33.6	34.6	34.6
12	79.7	80.3	131.3	131.7	131.7
21	169.9	170.9	170.9	170.6	170.8
22	172.5	173.1	173.0	173.2	173.1
23	168.5	172.0	171.9	169.7	172.1

[a] In CD_3OD.
[b] The chemical shifts of the carbons not listed here are within ± 0.4 ppm of those of the parent compounds.

Table 11. ^{1}H NMR data[a] for the minor components modified in the core.

Position No.[b]	Squalestatins				
	7-Deoxy S1	6-Deoxy H1	6-Deoxy H5	7-Deoxy H5	6,7-Dideoxy H5
3	4.87 (s)	5.46 (s)	5.44 (s)	4.66 (s)	4.87 (s)
6	6.45 (dd, 7, 2)	2.51 (dd, 14, 11), 3.05 (dd, 14, 3)	2.48 (dd, 14, 11)[c], 3.03 (dd, 14, 4)	5.35 (dd, 7, 2)	1.98~2.08 (m), 3.18 (m)
7	1.96 (dd, 15, 2), 2.85 (dd, 15, 7)	4.24 (dd, 11, 3)	4.28 (dd, 11, 4)	1.87 (dd, 15, 2), 2.44~2.52 (m)[c]	1.98~2.08 (m)
9	2.00~2.18 (m)[c]	1.97 (m), 2.04 (m)	1.85~1.97 (m)	1.94 (m), 2.09 (m)	1.87 (m), 1.98~2.08 (m)
10	2.22~2.38 (m)	2.31 (m), 2.40~2.46 (m)[c]	2.18 (m), 2.26 (m)	2.12~2.23 (m)	2.09~2.23 (m)

[a] In CD_3OD.
[b] The chemical shifts of the protons not listed here are within ± 0.4 ppm of those of the parent compounds.
[c] Overlaps with other signals not given in this table.

position.

6-Deoxysqualestatin H5: This compound was identified by comparison of its mass spectral and NMR data with those of 6-deoxy H1 and H5.

7-Deoxysqualestatin H5: From the mass spectral data this compound was isomeric with 6-deoxy H5. The chemical shift changes observed for this compound when compared to H5 were in accord with the replacement of the CH–O group at the 7-position by a CH_2.

6,7-Dideoxysqualestatin H5: The molecular weight, at 32 mass units lower than H5, suggested the loss of two oxygen atoms. The replacement of both the 6- and 7-CH–O groups by a CH_2 was confirmed by the NMR data. Assignments were confirmed by ^{1}H-^{13}C correlation.

Experimental

General

NMR spectra were recorded on a Bruker AM500 using standard pulse sequences. Chemical shifts are reported in ppm referenced to CHD_2OD (3.31 ppm) for ^{1}H and CD_3OD (48.9) for ^{13}C as internal

standards. PS DQF COSY measurements were performed by acquiring 4K × 2K data sets collected in 512 increments. The delay between scans was 2 seconds. ^{1}H-^{13}C correlation measurements were performed in the normal mode by acquiring 4K × 512 data sets collected in 128 increments. The delay between scans was 2 seconds. NOE difference experiments were recorded using a 5 second irradiation period and an acquistion time of 1.262 seconds. High resolution negative and positive ion FAB mass spectrometry was performed on a VG ZAB-2SE mass spectrometer operating at a resolving power of 10,000. A caesium 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 spectrometry was performed on a Finnigan MAT TSQ70B mass 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 a matrix. High resolution EI mass spectrometry was performed on a Finnigan MAT 8400 mass spectrometer operating at a resolving power of 10,000. Low resolution EI mass spectrometry was performed on a Finnigan MAT TSQ70B operating at a resolution of 1,000. Column chromatography was on Partisil Prep 40 ODS-3 (Whatman Biosystems Inc cat no 8510~1250) abbreviated to P40 and on Amberlite XAD16 (Rohm and Haas). Preparative HPLC was on columns (25 × 2.1 cm) of 5 μm Spherisorb ODS2 (C18) or C6 (Phase Separations Ltd). Conditions for preparative HPLC are described in the abbreviated form: column; solvent system; flow rate; number (*n*) of preparative runs; elution time for component and weight isolated. The solvents were in the proportions stated for mixtures of:

Solvent A; MeCN - H_2O and 2 ml H_2SO_4 added to 1 litre

Solvent B; MeCN - H_2O and 0.4 ml H_2SO_4 added to 1 litre

Solvent C; MeCN - 0.1 M $NH_4H_2PO_4$ adjusted to pH 6.5

Solvent D; MeCN - H_2O and 0.15 ml H_2SO_4 added to 1 litre

Solvent E; MeCN - H_2O with 0.15 ml H_2SO_4 and 20 ml THF added to 1 litre

Detection of components was at λ 210 nm. Separated components were recovered from bulked chromatographic eluates by dilution of these eluates with an equal volume of water and then re-adsorbing them onto the cleaned water-equilibrated chromatographic column. The absorbent was washed with water until the effluent was free of acid or salt. Elution was with MeCN - H_2O, 9 : 1. The component was recovered from this eluate, after evaporation of the MeCN, by lyophilisation.

Fermentation

Seed medium of *Phoma* sp. C2932 (200 ml) prepared as before[1] was used to inoculate 4 litres of the same medium in a 7-litre fermentor. The medium was maintained at 25° for 48 hours (aeration 4 litres/minute, agitation with three 6-bladed turbine impellers at 500 rpm and silicone antifoam addition as required) and then 1.2 litres was used to inoculate 40 litres of the same medium. This was maintained at 25° for 48 hours (head pressure 0.2 bar, aeration 40 litres/minute, agitation with three 6-bladed turbine impellers at 500 rpm and silicone antifoam addition as required) and 15 litres was used to inoculate 500 litres of production medium (glycerol 50 g, soyabean oil 30 g, cotton seed flour 10 g and distilled water to 1 litre, pre-autoclave pH 6.2~6.3). The inoculated production medium was maintained at 25° for 17 days (head pressure 0.5 bar, aeration 500 litres/minute, agitation with three 6-bladed turbine impellers at 200 rpm and silicone antifoam addition as required) before harvest.

For 5,000-litre fermentations the final seed stage volume was 200 litres.

Extraction

Preparation of Crude Calcium Salt

From 500-litre fermentations. Harvest broth was removed from the fermenter with a water wash (40 litres) and adjusted to pH 10.5 with ammonia. Cells were removed by filtration, suspended in water (450 litres) adjusted to pH 10.5 with ammonia and removed by filtration and discarded. The combined filtrates were adjusted to pH 6.8 (H_2SO_4) and passed through a column (40 litres) of Amberlite XAD16 which was then washed with $(NH_4)_2SO_4$ (1% w/v) until clear and then water (40 litres). Elution was with Me_2CO - H_2O, 1 : 1. A fraction (87 litres) was collected after a forerun (32 litres). Calcium acetate (200 g in 1.2 litres water) was added to the stirred fraction and the ppt was removed by filtration after 16 hours, washed with Me_2CO - H_2O (1 : 1, 1.2 litres), Me_2CO (1 litre) and dried *in vacuo* to give the crude calcium salt (153 g).

From 5,000-litre fermentations. The extraction procedure was essentially the same as for 500 litres scale except that the Amberlite XAD16 column after the adsorbtion stage was washed with solutions of ammonium acetate 3% w/v, tetrasodium EDTA 1% w/v (one column volume of each) followed by a water wash and that the precipitated calcium salt was removed with a filter aid.

Open Column Chromatography on Whatman Partisil Prep 40 C18 (P40)

Column 1: Calcium salt (60 g) from a 500-litre fermentation was stirred in MeCN - H_2O (1 : 1, 880 ml) and H_2SO_4 (20 ml) and the precipitate was removed by centrifugation. The supernatant was applied to a column (82 × 9.6 cm) of P40 in Solvent A (1 : 1) and elution was with the same solvent. After a forerun of 8.33 litres fraction 1 (1.07 litres) was collected.

Column 2: The procedure for column 1 was repeated but after a forerun of 8.89 litres fraction 1 (1.5 litres) and fraction 2 (1.22 litres) were collected.

Column 3: The procedure for column 1 was repeated but with 750 g calcium salt containing filter aid from a 5,000-litre fermentation and on a column 90 × 18 cm. After an initial volume of 100 litres fraction 1 (10 litres) was collected.

Column 4: The procedure for column 3 was repeated but with 500 g calcium salt. After an initial volume of 42 litres fraction 1 (10 litres) was collected. Three more chromatographic runs were performed to give pooled fraction 1 (44 litres).

Column 5: The procedure for column 3 was repeated but with 550 g calcium salt. After fraction 1 (10 litres), 2 (35 litres) and 3 (42 litres) had been collected, the developing solvent was changed to MeCN - 38 mM H_2SO_4 (6 : 4) and fractions 6 (11.25 litres) and 7 (6 litres) were collected.

Column 6: Fraction 2 from column 5 (35 litres) was stirred with P40 (1.5 kg), water (35 litres) and H_2SO_4 (0.2 litre) and then filtered. The adsorbent was washed with water (10 litres) and adsorbed components were eluted with MeCN (3 litres). Evaporation of MeCN followed by lyophilisation gave a solid which was dissolved in Solvent A (1 : 1, 100 ml). Chromatography of this solution on a column (5.8 litres) of P40 in the same solvent gave after a forerun of 2.44 litres fraction 1 (4.1 litres), 2 (1.86 litres), 3 (2.5 litres), 4 (3.35 litres), 5 (2.52 litres), 6 (2.64 litres), 7 (5.7 litres) and 8 (8.24 litres).

Crystallisation of Squalestatin S2 Tripotassium Salt

Pooled fraction 1 from column 4 (44 litres) was stirred with P40 (4 kg) and water (45 litres). The adsorbent was removed by filtration and washed free of H_2SO_4 with water. Elution was with MeCN (6 litres). The solvent composition of the eluate was adjusted to MeCN - H_2O, 1 : 1 (8 litres) and this solution at 50°C was titrated to pH 9.1 with 1 M KOH. Potassium acetate (38 g) in water (400 ml) was added followed by hot Me_2CO (32 litres). Crystalline squalestatin S2 tripotassium salt was removed by filtration and dried (103 g). The mother liquors were retained.

Isolation of Squalestatin Y1

Fraction 7 from column 5 (6 litres) was stirred with P40 (500 g), water (14 litres) and H_2SO_4 (28 ml) and then filtered. The adsorbent was washed with water. Elution of adsorbed components with MeCN (2 litres) followed by evaporation of the MeCN gave a concentrate (30 ml) which after addition of H_2SO_4 (0.06 ml) was subjected to preparative HPLC:

C18; A 52.5 : 47.5; 25 ml/minute; $n = 10$; 15.2 ~ 17 minutes, crude squalestatin Y1.

Crude squalestatin Y1 was subjected to further preparative HPLC:

C18; A 52.5 : 47.5; 25 ml/minute; $n = 1$; 17.0 ~ 18.6 minutes, squalestatin Y1, 10 mg.

Isolation of Squalestatins H7, H9, H2 and H6

Fraction 1 from column 6 was stirred with P40 (1 litre) and water (16 litres) and then filtered. The adsorbent was washed with water. Elution of adsorbed components with MeCN (2 litres) followed by evaporation of the MeCN gave a residue which was subjected to preparative HPLC:

C18; B 25 : 75; 15 ml/minute; $n = 10$; 8.6 ~ 9.3 minutes, squalestatin H7, 6.5 mg; 9.7 ~ 10.4 minutes, squalestatin H9, 12.1 mg; 10.5 ~ 12.4 minutes, squalestatin H2, 100 mg; 13.6 ~ 15.5 minutes, squalestatin H6, 149 mg.

Isolation of Squalestatin H5 and 6-Deoxysqualestatin H1

Fraction 2 from column 6 was stirred with P40 (1 litre) and water (12 litres) and then filtered. The adsorbent was washed with water. Elution of adsorbed components with MeCN (1.5 litres) followed by evaporation of the MeCN gave a residue which was subjected to preparative HPLC:

C18; B 25 : 75; 20 ml/minute; $n=10$; 20.8 ~ 25.2 minutes, squalestatin H5, 302 mg; 25.4 ~ 27.5 minutes, 6-deoxysqualestatin H1, 13.5 mg.

Isolation of 7-Deoxy and 6-Deoxysqualestatin H5

Fraction 3 from column 6 was stirred with P40 (1.1 litres) and water (14 litres) and then filtered. The adsorbent was washed with water. Elution of adsorbed components with MeCN (2.5 litres) followed by evaporation of the MeCN gave a residue which was subjected to preparative HPLC:

C18; B 30 : 70; 20 ml/minute; $n=10$; 12.6 ~ 13.5 minutes, 7-deoxysqualestatin H5, 9 mg; 13.5 ~ 14.8 minutes, 6-deoxysqualestatin H5, 23.7 mg.

Isolation of Squalestatin V2

Fraction 4 from column 6 was stirred with P40 (1 : 1 litres) and water (10 litres) and then filtered. The adsorbent was washed with water. Elution of the adsorbed components with MeCN followed by evaporation of the MeCN gave a residue which was subjected to preparative HPLC:

C18; B 35 : 65; 20 ml/minute; $n=10$; 23.6 ~ 25.5 minutes, squalestatin V2, 11.3 mg.

Isolation of Squalestatin W2

Fraction 5 from column 6 was stirred with P40 (1 litre) and water (12 litres) and filtered. The adsorbent was washed with water. Elution of the adsorbed components with MeCN (2 litres) followed by evaporation of the MeCN gave a residue which was subjected to preparative HPLC:

C18; B 35 : 65; 20 ml/minute; $n=10$; 31 ~ 32.1 minutes, squalestatin W2, 1.5 mg.

Isolation of 6,7-Dideoxysqualestatin H5

Fraction 6 from column 6 was stirred with P40 (1 litre) and water (10 litres) and filtered. The adsorbent was washed with water. Elution of the adsorbed components with MeCN (2.5 litres) followed by evaporation of the MeCN gave a residue which was subjected to preparative HPLC:

C18; B 2 : 3; 20 ml/minute; $n=10$; 13.7 ~ 15.1 minutes, 6,7-dideoxysqualestatin H5, 12.1 mg.

Isolation of Squalestatin S4

Fraction 8 from column 6 was stirred with P40 (0.25 kg) and water (9 litres) and filtered. The adsorbent was washed with water. Elution of the adsorbed components with MeCN followed by concentration of the eluate to 100 ml gave a solution which was subjected to preparative HPLC:

C18; A 45 : 55; 20 ml/minute; $n=20$; 14.9 ~ 15.9 minutes, crude squalestatin S4.

Crude squalestatin S4 was subjected to further preparative HPLC:

C6; A 45 : 55; 20 ml/minute, $n=8$; 16.1 ~ 16.8 minutes, squalestatin S4, 8.7 mg.

Isolation of Squalestatins T1, X1 and U1

Fraction 1 from column 1 was reduced in volume to 500 ml by evaporation and this solution was then pumped onto a cleaned water-equilibrated preparative HPLC column (C18). The column was washed with water. Elution was with MeCN. The eluate was concd to 20 ml and water (10 ml) and H_2SO_4 (75 μl) were added and this solution was subjected to preparative HPLC:

C18; A 1 : 1; 18 ml/minute; $n=15$; 17 ~ 19 minutes, crude squalestatin U1, 112 mg; 28 ~ 30 minutes, crude squalestatin X1, 10 mg; 31.4 ~ 33.4 minutes, squalestatin T1, 12 mg.

Crude squalestatin X1 (8 mg) was subjected to further preparative HPLC:

C18; A 1 : 1; 25 ml/minute; $n=1$; 24 ~ 27.4 minutes, squalestatin X1, 5 mg.

Crude squalestatin U1 (100 mg) was subjected to further preparative HPLC:

C6; A 1 : 1; 25 ml/minute; $n=2$; 9.8 ~ 11.6 minutes, crude squalestatin U1, 80 mg.

Crude squalestatin U1 (60 mg) was subjected to further preparative HPLC:

C18; C 3 : 7; 25 ml/minute; $n=2$; 26 ~ 35 minutes, crude squalestatin U1, 46 mg.

Crude squalestatin U1 (46 mg) was subjected to further preparative HPLC:
C6; A 1 : 1; 25 ml/minute; $n=2$; 7.2~13.8 minutes, squalestatin U1, 24 mg.

Isolation of Squalestatins S3 and U2

Fraction 1 from column 2 was stirred with P40 (1 litre) and water (8.5 litres) and filtered. The adsorbent was washed with water. Elution was with MeCN. The eluate was concd to 20 ml, MeCN (15 ml) was added and the solution was subjected to preparative HPLC:
C18; A 35 : 65; 25 ml/minute; $n=10$; 29.6~45.6 minutes, crude squalestatin S3; 45.6~62.4 minutes, crude squalestatin U2.
Crude squalestatin S3 was subjected to further preparative HPLC:
C18; A 2 : 3; 25 ml/minute; $n=1$; 16.6~18.8 minutes, crude squalestatin S3, 20 mg.
Crude squalestatin S3 was subjected to further preparative HPLC:
C18; D 2 : 3; 25 ml/minute; $n=1$; 12.4~13.8 minutes, squalestatin S3, 6 mg.
Crude squalestatin U2 was subjected to further preparative HPLC:
C18; A 2 : 3; 25 ml/minute; $n=1$; 22~25.4 minutes, crude squalestatin U2, 16 mg.
Crude squalestatin U2 was subjected to further preparative HPLC:
C18; D 2 : 3; 25 ml/minute; $n=1$; 15.8~16.8 minutes, squalestatin U2, 7.5 mg.

Isolation of Squalestatins V1 Isomer a, V1 Isomer b and W1

Fraction 2 from column 2 was concd to 700 ml, MeCN (80 ml) followed by P40 (0.5 litres) and water (10 litres) were added and then the adsorbent was removed by filtration and washed with water. Elution was with MeCN (1.3 litres). The eluate was concd to 20 ml, H_2SO_4 (60 μl) was added and the clarified solution was subjected to preparative HPLC:
C18; A 2 : 3; 25 ml/minute; $n=11$; 25~27.8 minutes, squalestatin V1 isomer a, 23 mg; 27.8~30 minutes, squalestatin V1 isomer b, 27 mg; 31.2~33 minutes, crude squalestatin W1, 14.5 mg; 33~34.4 minutes, crude squalestatin W1, 14 mg.
Crude squalestatin W1 (31.2~33 minutes) was subjected to further preparative HPLC:
C18; E 3 : 2+2% w/v; 25 ml/minute; $n=2$; 27~28.6 minutes, squalestatin W1.
Crude squalestatin W1 (33~34.4 minutes) was subjected to further preparative HPLC:
C18; A 2 : 3; 25 ml/minute; $n=1$; 38~40 minutes, crude squalestatin W1.
Crude squalestatin W1 (38~40 minutes) was subjected to further preparative HPLC:
C18; E 2 : 3+2% w/v; 25 ml/minute; $n=1$; 37.6~40 minutes, squalestatin W1, 3 mg when combined with squalestatin W1 above.

Isolation of 7-Deoxysqualestatin S1 and Squalestatin S5

Fraction 1 from column 3 was stirred with P40 (1 litre) and water (14 litres) and filtered. The adsorbent was washed with water. Elution was with MeCN (2 litres). The eluate was concd to 30 ml, H_2SO_4 (60 μl) was added and the clarified solution was subjected to preparative HPLC:
C18; A 52.5 : 47.5; 25 ml/minute; $n=10$; 23.2~28.0 minutes, 7-deoxysqualestatin S1, 177 mg; 18.6~22.8 minutes, crude squalestatin S5, 307 mg.
Crude squalestatin S5 (80 mg) was subjected to further preparative HPLC:
C18; A 45 : 55 with THF 4% v/v; 25 ml/minute; $n=4$; 58.5~61.5 minutes, squalestatin S5, 11.3 mg.

Isolation of Squalestatin S8

To the mother liquors after crystallisation of squalestatin S2 tripotassium salt was added P40 (0.5 kg) water (75 litres) and H_2SO_4 (0.1 litre). The adsorbent was removed by filtration and washed with water. Elution was with MeCN (2 litres). The eluate was concd to 90 ml, 10 ml of this was diluted with mobile phase A 45 : 55 (10 ml) and this solution was subjected to preparative HPLC:
C18; A 45 : 55; 20 ml/minute; $n=8$; 17.6~18.8 minutes, squalestatin S8, 10.5 mg.

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