The Squalestatins: Novel Inhibitors of Squalene Synthase. Enzyme Inhibitory Activities and *in Vivo* Evaluation of C1-Modified Analogues

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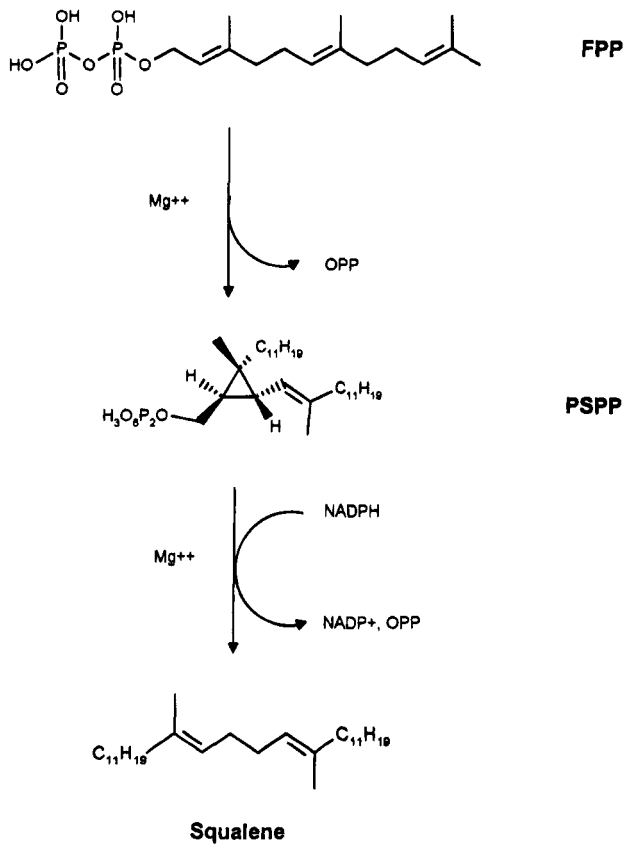
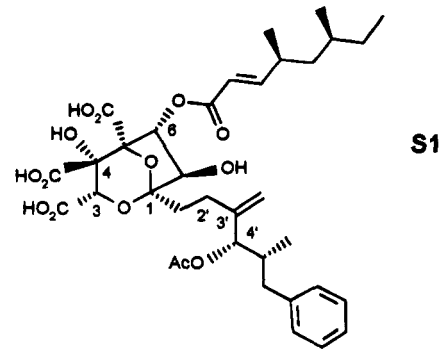
Squalestatin analogues modified in the C1 side chain were prepared and evaluated for their ability to inhibit rat liver microsomal and *Candida* squalene synthase (SQS) *in vitro*. While maintaining the 4,6-dimethyloctenoate or 4,6-dimethyloctanoate ester groups at C6, a number of modifications to the C1 side chain were well tolerated. However, in the absence of the C6 ester group, similar modifications to the C1 side chain caused substantial loss of activity. Compounds were also evaluated for their ability to inhibit cholesterol biosynthesis *in vivo* in rats and to reduce serum cholesterol levels in marmosets. These studies revealed that compounds with similar SQS inhibitory activities can possess different *in vivo* durations of action and lipid-lowering abilities.

High levels of blood cholesterol and blood lipids are conditions which are implicated in the onset of vessel wall disease. Methods for effective reduction of serum cholesterol levels are therefore of major interest. Currently, the most effective approach to lowering serum cholesterol concentrations is by inhibiting sterol biosynthesis, and a number of therapeutic agents are available which work by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme which catalyzes the biosynthesis of mevalonic acid. Mevalonic acid, however, is a common precursor of all isoprenyl derivatives, including in animals the ubiquinones, heme A, the dolichols, isopentenyl tRNA, and isoprenylated proteins (Scheme 1). A more selective inhibition of cholesterol biosynthesis may be achieved by inhibiting steps beyond the branch in the pathway. The first biosynthetic step which leads exclusively to sterols, the head-to-head condensation of two farnesyl diphosphate (FPP) molecules to give squalene *via* the intermediate presqualene diphosphate (PSPP), is catalyzed by squalene synthase[1] (SQS) (farnesyl-diphosphate:farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21). Agents which inhibit this enzyme are therefore potential drugs for the regulation of cholesterogenesis.



Conversion of farnesyl diphosphate into squalene by SQS

We have recently described the isolation[2] and structure elucidation[3] of the squalestatins, a novel group of fungal metabolites isolated from a previously unknown *Phoma* species (*coelomycetes*). Squalestatin S1 (**1a**) is a potent and selective inhibitor of both rat and *Candida* SQS; 50% inhibition of rat liver microsomal SQS activity is observed *in vitro* at a concentration of 12 nM.



Furthermore when **1a** is administered orally to marmosets, a species with a similar lipoprotein profile to

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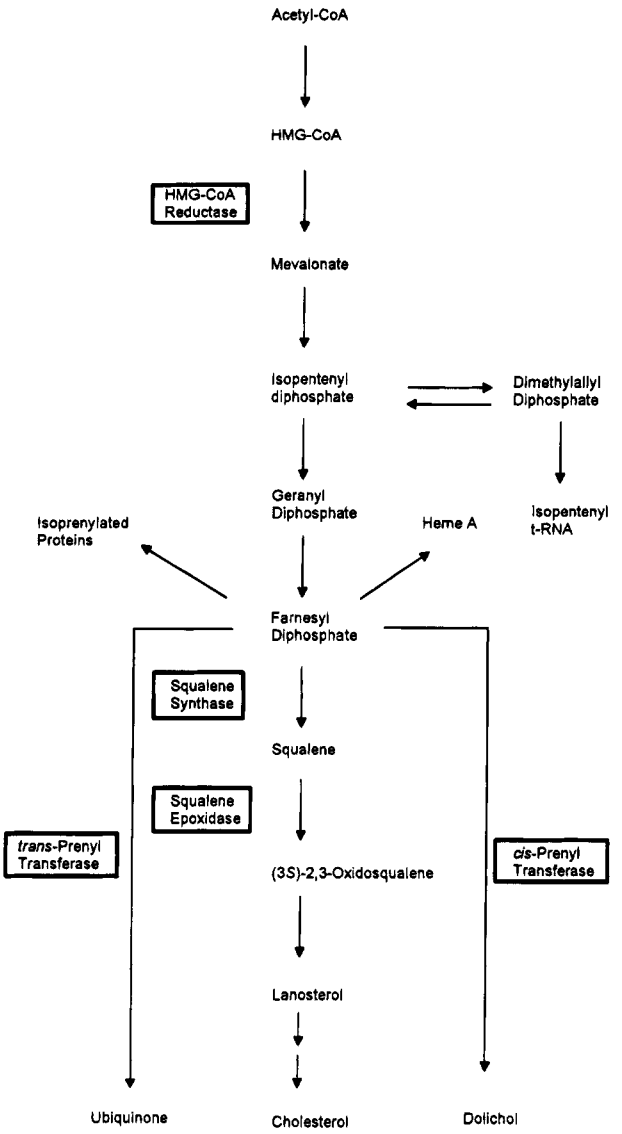
Scheme 1

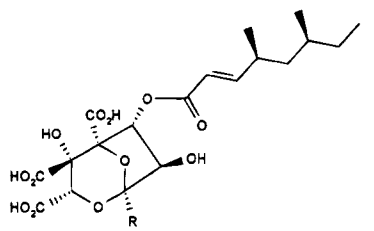


that of humans, 50% reduction in serum cholesterol levels is observed at a dose of 10 mg/kg/day for 7 days. Moreover only apolipoprotein-B levels are reduced, whereas apo-A1 levels (indicative of a high-density lipoprotein fraction) are unchanged following administration of **1a** to marmosets.[4] Squalestatin **1a** incorporates the highly substituted 2,8-dioxabicyclo[3.2.1]-octane system possessing carboxylic acid groups at C3, C4, and C5, hydroxyl groups at C4 and C7, a lipophilic side chain at C1, and an α,β-unsaturated ester chain at C6. Subsequent to our publications, the group at Merck has published the isolation of zaragozic acids, the structure of zaragozic acid A[5,6] being identical with that of squalestatin **1a**. More recently, the group at Tokyo Noko University - Mitsubishi[7] have also isolated squalestatin **1a** from *Setosphaeria khartoumensis*.

As a part of our chemical program aimed at the modification of the complex squalestatin structure and the identification of the key structural features responsible for the biological activity, we have reported on the C1 chain-length requirements,[8] on the role of the tricarboxylic acid moiety,[9] on C6 and C7 modifications,[10] and on the C6,C7-dideoxy,[11] C3-decarboxy,[12] monocyclic,[13] and acyclic[14] analogues. In this paper, we report on SAR in the C1 side chain of the squalestatins and on their lipid-lowering properties.

Chemistry

The natural product S2 (**1b**)[2,3,15] isolated as a minor metabolite in fermentation broths producing S1 was alternatively produced in large quantities by selective acid-catalyzed aqueous acetone hydrolysis of S1. Partial hydrogenation of **1a** over 10% Pd–C in ethyl acetate for 1 h provided three products: the C6 4,6-dimethyloctanoate ester **2a**, which is also a natural product,[16] together with the isomerized C2′,3′ olefin **2c** and the tetrahydro analogue **2d** isolated as a 1:1 mixture of diastereoisomers at C3′. The *E* geometry of the double bond in **2c** was established by a NOE experiment between the 2′-H and the 4′-H. Prolonged hydrogenation of **1a** over 10% Pd–C in ethanol provided two products: **2d** and the hydrogenolyzed derivative **2e**. Furthermore, hydrogenation of **1a** over PtO_2 in acetic acid provided the cyclohexyl derivative **2f**. Prolonged hydrogenation of S2 over 10% Pd–C in ethyl acetate provided two products: the tetrahydro analogue **2g** and the ketone[8] **2h** as a 1:1 diastereoisomeric mixture at C3′. The unexpected formation of the ketone **2h** is explained by the isomerization of the C3′ methylene to the C3′,4′ olefin (enol form) which tautomerizes to the keto form.



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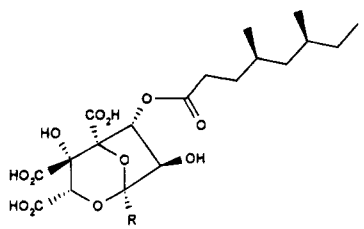

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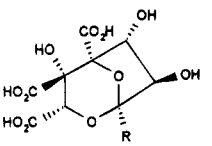

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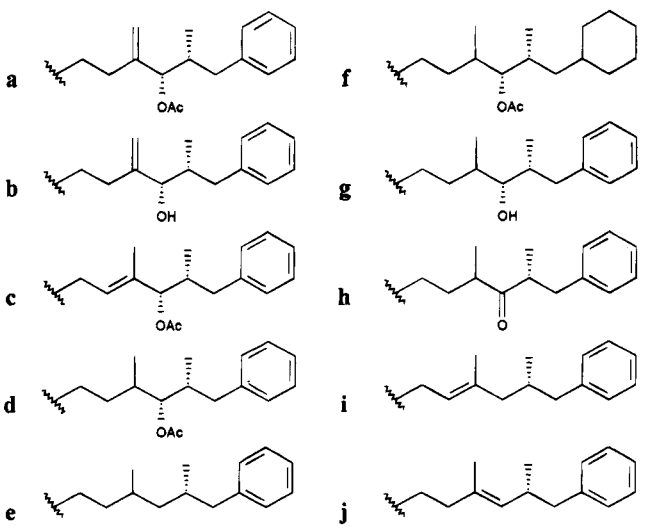
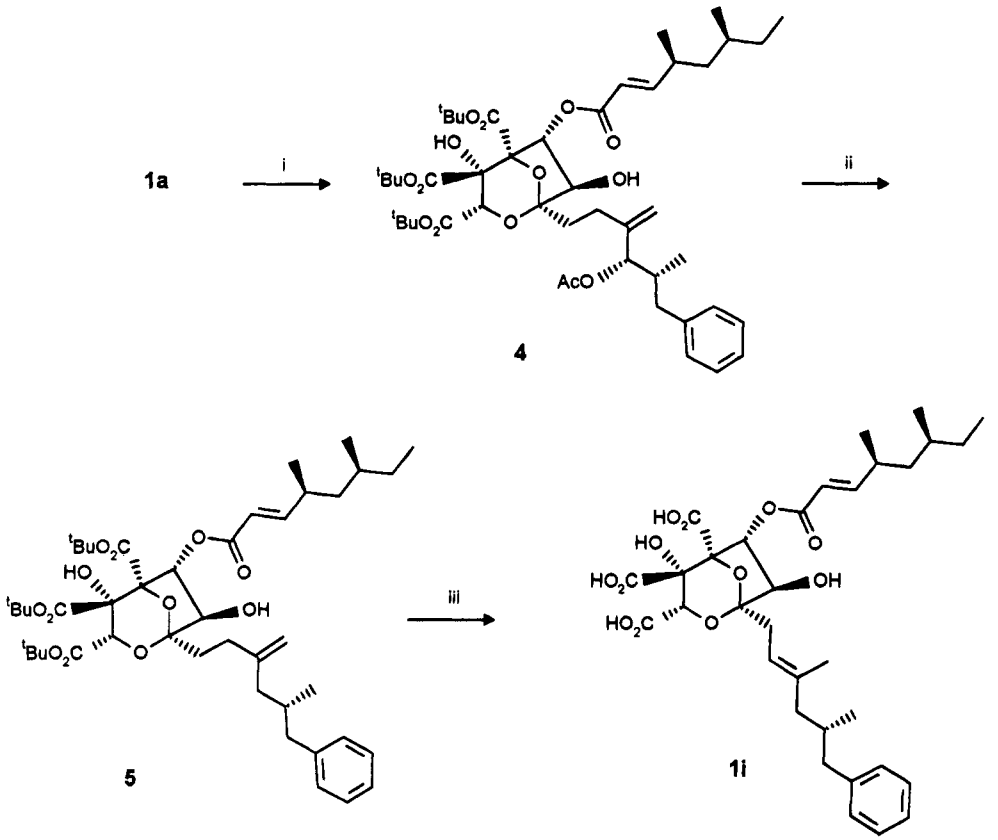


Squalestatin H1 (**3a**) is another natural product isolated[2,3] as a minor metabolite in fermentation broths producing **1a** and was alternatively produced in large

Scheme 2[a]



[a] Reagents and conditions: (i) $Me_2NCH(OBu^t)_2$, PhMe; (ii) $(Ph_3P)_2PdCl_2$, NH_4HCO_2, dioxan; (iii) 6.4 M HCl/dioxan.

quantities by selective removal of the C6 ester in S1 in the presence of the allylic acetate with *N*-methylhydroxylamine[17] in DMF. Squalestatin **3b** was obtained by hydrolysis of S1 with aqueous NaOH in refluxing MeOH. Prolonged catalytic hydrogenation of **3a** over 10% Pd–C in ethanol provided **3d,e**.

Removal of the acetoxy group of S1 was achieved by the procedure outlined in Scheme 2. Treatment of **1a** with *N,N*-dimethylformamide di-*tert*-butyl acetal in dry toluene provided the tri-*tert*-butyl ester **4**, reduction of which with bis(triphenylphosphine)palladium(II) chloride and ammonium formate in refluxing dioxan[18] gave olefin **5**. Deprotection of the tri-*tert*-butyl ester **5** with 6.4 M hydrogen chloride in dioxan was accompanied by olefin isomerization to provide **1i** as a single isomer; the geometry of the C2′,3′ double bond has not been established. Compounds **1j** and **3j** are natural products reported elsewhere.[16]

Biological Results and Discussion

The compounds listed in Table 1 were evaluated for their inhibitory activity against juvenile male rat liver microsomal SQS and *Candida albicans* 2005E microsomal SQS. In addition, compounds were evaluated in an antifungal whole cell assay to obtain minimum inhibitory concentrations (MIC) against *Candida albicans* C316, *Aspergillus niger* 48238, and *Cryptococcus neoformans* 2867E. The rat and *Candida* enzyme assay procedures measured the conversion of [2-^{14}C]FPP to [^{14}C]squalene. The assay methodology is described in detail in our earlier publication.[19] The data in Table 1 clearly indicate that potent SQS activity is retained in the C6 4,6-dimethyloctanoate series (**2a**). While retaining this ester group at C6, further modifications to the C1 side chain such as conversion of the methylene at C3′ into a methyl group (**2d,g**) with or without the presence of the acetoxy/hydroxy group at C4′ (**2e**) are well tolerated as is isomerization of the methylene at C3′ toward C2′,3′ (**2c**). In compounds retaining the 4,6-dimethyloctenoate ester at C6, isomerization of the methylene at C3′ toward C2′,3′ or C3′,4′ with loss of the acetoxy group at C4′ causes a small reduction in inhibitory activity (**1i,j**). However in the absence of the C6 ester group, similar modifications to the C1 side chain cause substantial loss of activity (**3d,e,j**). Furthermore the natural products **1a,b** possessing the C6 4,6-dimethyloctenoate ester group exhibit similar inhibitory activities; however, in the C6 hydroxy series,

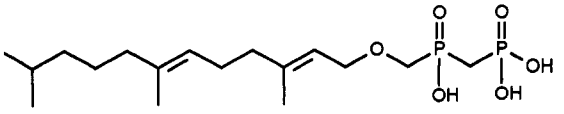
Table 1. *In Vitro* SQS Inhibitory Activity[a]

		IC_{50} (nM)		MIC[b] (μg/mL)		
compd no.	formula	rat	*Cand*	*Cand*	*Asper*	*Crypt*
1a	$C_{35}H_{46}O_{14}$	12	5	8	16	0.5
1b	$C_{33}H_{44}O_{13}$	5	NT	125	16	1
1i	$C_{33}H_{44}O_{12}$	65	NT	31	31	2
1j	$C_{33}H_{48}O_{12}$	32	NT	31	16	4
2a	$C_{35}H_{48}O_{14}$	13	NT	8	1	0.1
2c	$C_{35}H_{48}O_{14}$	22	NT	125	31	8
2d	$C_{35}H_{50}O_{14}$	3	7	16	8	0.5
2e	$C_{33}H_{48}O_{12}$	13	14	31	16	1
2f	$C_{35}H_{56}O_{14}$	165	93	125	125	4
2g	$C_{33}H_{48}O_{13}$	8	9	125	31	8
3a	$C_{25}H_{30}O_{13}$	26	NT	>125	>125	>125
3b	$C_{23}H_{28}O_{12}$	252	NT	62	62	62
3d	$C_{25}H_{32}O_{13}$	143	200	125	125	125
3e	$C_{23}H_{30}O_{11}$	415	200	125	125	125
3j	$C_{23}H_{28}O_{11}$	200	208	NT	NT	NT

[a] IC_{50} values were determined at least on two different occasions with a minimum of five and a maximum of eight dose levels of each inhibitor at least in duplicate and are expressed as mean values, using S1 as a reference. [b] MIC's were determined by microtiter broth dilution testing in yeast nitrogen base glucose (YNBG). See the Experimental Section.

the loss of the acetyl group causes a 10-fold reduction in inhibitory activity (cf. **3a,b**). These findings demonstrate differences in SAR for C1 side-chain modifications between squalestatins possessing a C6 dimethyloctenoate/dimethyloctanoate ester and those possessing C6 hydroxyl groups and are in line with our previous results showing that the nature of the C6 side chain critically influences SAR for modifications made elsewhere in the molecule.[9] We are proposing that squalestatins possessing the above mentioned C6 ester groups are presqualene diphosphate mimetics, whereas those possessing only a hydroxyl group at C6 are mimetics of farnesyl diphosphate.

Finally a significant loss of activity is observed when the phenyl ring is replaced with a cyclohexyl group (**2f**). No investigations have appeared in the literature concerning the SAR of presqualene diphosphate mimetics. However, SAR for farnesyl diphosphate mimetics have indicated the critical dependence of inhibitory activity on the presence of the double bonds in the farnesyl chain.[20] The loss of inhibitory activity in the cyclohexyl analogue (**2f**) may be analogous to the 10-fold reduction in SQS inhibitory activity observed for [[[[(10,11-dihydrofarnesyl)oxy]methyl]hydroxyphosphinyl]-methyl]phosphonic acid (**6**) compared with [[[(farnesyl-oxy)methyl]hydroxyphosphinyl]methyl]phosphonic acid (**7**).[21] Thus the terminal aryl ring of the squalestatins appears to be of critical importance for effective binding to the enzyme.



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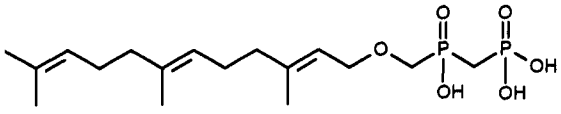


7

Significant homology exists between the rat and fungal enzymes,[22] and the inhibitory activities of the squalestatin analogues listed in Table 1 against the fungal enzyme closely resemble those for the rat enzyme. The squalestatin H1 analogues tested are devoid of significant whole cell antifungal activity, whereas the analogues possessing the C6 dimethyloctenoate/dimethyloctanoate ester groups are good antifungal agents. A possible explanation for this difference is the increased hydrophilicity of H1 analogues, resulting in reduced ability to penetrate the fungal cell membrane.

Having established the *in vitro* profiles of the above squalestatins, our attention was focused on their *in vivo* evaluation. We have reported previously[4] that when S1 is administered orally to marmosets for 7 days, 50% reduction in serum cholesterol levels is observed at a dose of 10 mg/kg/day. In order to eliminate any potential differences in oral absorption of a series of S1 analogues, the squalestatins listed in Table 2 were tested in adult marmosets for their effect on serum cholesterol levels after 7 days intravenous (iv) administration (1 mg/kg/day) according to our previously published[4] methodology. All four compounds showed excellent serum cholesterol level reduction *in vivo*

Table 2. Effect of Squalestatins (1 mg/kg/day) on Serum Cholesterol Levels in Marmosets after 7 Days iv Administration

compd no.	no. of animals	cholesterol reduction (%)
1a	8	86 ± 2
1b	6	59 ± 6[a]
2a	6	72 ± 4
3a	6	56 ± 3

[a] Three animals only were dosed all 7 days due to venous collapse.

Table 3. Effect of S1 on Serum Cholesterol Levels in Marmosets (n = 6) after a Single iv Dose

	cholesterol reduction (%)		
dose (mg/kg)	day 2	day 3	day 7
1.00	47 ± 3	53 ± 3	60 ± 5
0.33	53 ± 4	63 ± 3	62 ± 8
0.11	39 ± 4	37 ± 4	41 ± 3

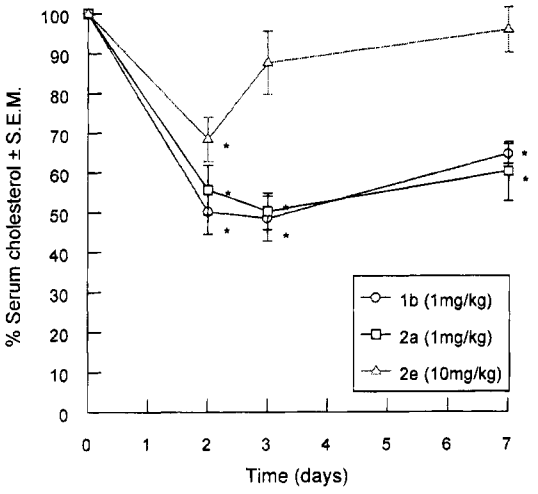


Figure 1. Effect of **1b** and **2a,e** on serum cholesterol levels in marmosets (n = 6) after a single iv dose.

ranging from 56% to 86%. It became apparent during the course of these studies that lipid lowering could be achieved within 24 h of dosing with S1. Furthermore, following a single iv dose of 1 mg/kg of S1, a profound and extended lipid-lowering effect was observed. Dose–response studies with S1 in marmosets revealed that excellent reduction of serum cholesterol concentrations was achieved in a dose-related manner which was maintained over a period of 7 days (Table 3). Lipid lowering studies with squalestatins **1b** and **2a,e** in marmosets following a single iv dose (1 mg/kg) revealed good lipid-lowering abilities for **1b** and **2a** but only a marginal effect for **2e** even at the higher dose of 10 mg/kg (Figure 1).

We have shown[4] previously that squalestatin **1a** inhibits cholesterol biosynthesis from [1-^{14}C]acetate when administered to rats, with an ED_{50} of 0.1 mg/kg iv. The potent SQS inhibitors **2d** and **3a** were selected for further evaluation in rats, and their relative potencies against **1a** at 1 h postadministration are shown in Figures 2 and 3, respectively. The potency of **2d** was found to be the same as that of **1a**, whereas that of **3a** was found to be 0.3 that of **1a**. The duration of action of **2d** and **3a** relative to **1a** was examined at the equipotent doses of 1 and 4 mg/kg, respectively, and are shown in Figure 4. All three compounds show maximal inhibition of hepatic cholesterol biosynthesis *in vivo* in

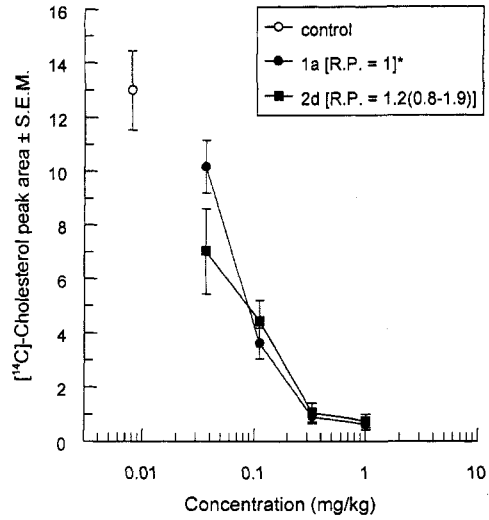


Figure 2. Effect of S1 and tetrahydro S1 (**2d**) on cholesterol biosynthesis in rats at 1 h after iv administration. *R.P. = relative potency compared to S1. Figures in brackets are fiducial ranges at $p = 0.95$.

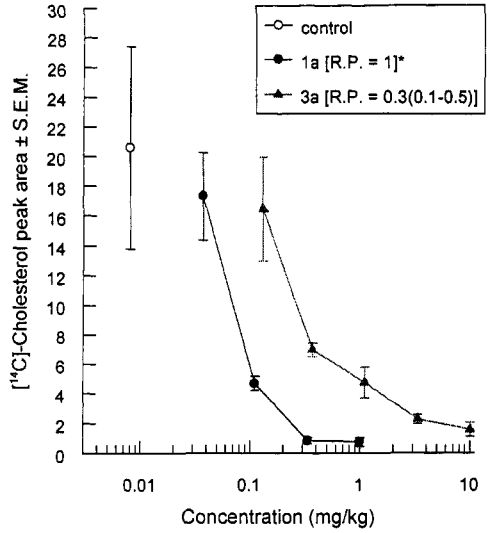


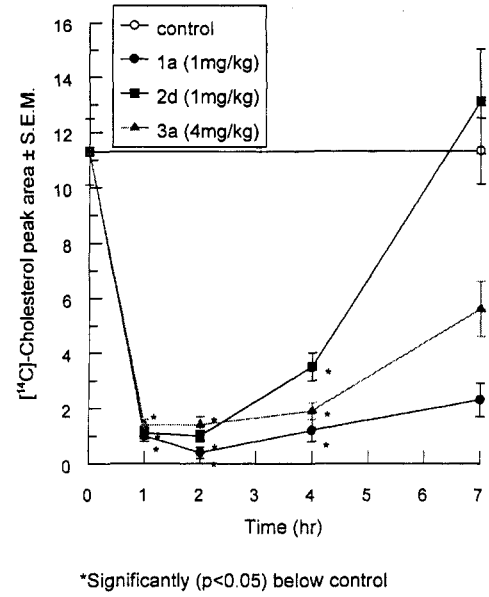
Figure 3. Effect of S1 and H1 (**3a**) on cholesterol biosynthesis in rats at 1 h after iv administration. *R.P. = relative potency compared to S1. Figures in brackets are fiducial ranges at $p = 0.95$.

rats at 1 h postdose. Significant inhibition is observed for at least 7 h with **1a** and **3a** and only up to 4 h with **2d**.

Finally single-dose lipid-lowering studies in marmosets at doses of 0.33, 0.33, and 1.0 mg/kg for **1a**, **2d**, and **3a**, respectively, were performed, and the data are presented in Figure 5. Squalestatin **1a** has a profound and extended cholesterol lowering effect in marmosets and **2d** retains good lipid-lowering ability while **3a** has a reduced ability to lower lipids.

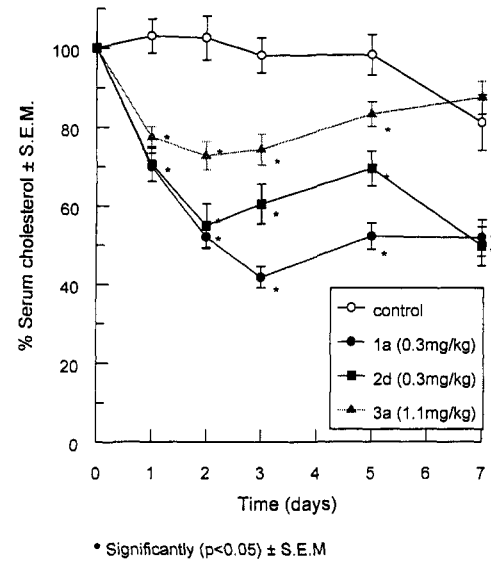
Conclusion

Squalestatin analogues modified in the C1 side chain were prepared and evaluated for their ability to inhibit rat and fungal SQS *in vitro*. A number of modifications to the C1 side chain were well tolerated while maintaining the C6 ester group found in the naturally occurring squalestatins. In the absence of the C6 ester group, however, similar modifications to the C1 side chain caused substantial reduction of inhibitory activity. We have shown that when **1a** is administered iv to marmosets for 7 days excellent reduction in serum cholesterol levels (86%) is observed; analogues of **1a** modified in either the C6 (**2a**, **3a**) or C1 (**1b**) side chains can retain similar lipid-lowering abilities. Following a single iv dose of squalestatin **1a** to marmosets, a profound and extended lipid-lowering effect is observed; analogues without the allylic system in the C1 side chain and/or the C6 α,β-unsaturated ester retain good lipid-lowering activities as evidenced by the data generated for the dihydro and tetrahydro S1 analogues, **2a**,**d**. However removal of the acetoxy group of **2d** to provide **2e** resulted in a major reduction of its serum cholesterol-lowering ability. The more hydrophilic analogue **3a** possesses significant but reduced lipid-lowering ability in these single-dose studies.



*Significantly (p<0.05) below control

Figure 4. Duration of effect of S1, tetrahydro S1 (**2d**), and H1 (**3a**) on cholesterol biosynthesis in rats after iv administration.



* Significantly (p<0.05) ± S.E.M

Figure 5. Effect of **1a**, **2d**, and **3a** on serum cholesterol levels in marmosets after a single iv dose.

Experimental Section

Organic solutions were dried over $MgSO_4$, and column chromatography was performed on silica gel 60 (Merck, Art. No. 9385). Analytical HPLC was performed on a Spherisorb 5 ODS-2 column (25 cm × 0.46 cm) using CH_3CN/H_2O containing 0.15 mL/L concentrated H_2SO_4 as eluent at a flow rate of 1.5 mL/min (column A) and detection at 210 nm or on

a 15 cm × 0.46 cm column using CH_3CN/H_2O containing 0.15 mL/L concentrated H_2SO_4 as eluent at a flow rate of 2 mL/min (column B) and detection at 210 nm. Preparative HPLC was conducted on a Spherisorb 5 ODS-2 column (25 cm × 2 cm i.d.) using CH_3CN/H_2O containing 0.15 mL/L concentrated H_2SO_4 as eluent at a flow rate of 15 mL/min and detection at 210 nm. The appropriate fractions from each run were combined, the CH_3CN removed *in vacuo* (bath temperature < 40 °C), and the remainder extracted with EtOAc. The combined extracts were washed with brine and evaporated, and the residue was dissolved in H_2O/dioxan and freeze-dried. IR spectra were recorded on a Nicolet 5SXC FTIR spectrometer. NMR spectra were recorded on a Bruker AM 500 or AM 250 or Varian VXR 400 spectrometer using standard pulse sequences. Chemical shifts are expressed as parts per million downfield from internal tetramethylsilane. Negative ion FAB mass spectrometry [MS (FAB-ve)] was performed on a Finnigan MAT TSQ70B spectrometer, and high-resolution negative ion LSI mass spectrometry [HRMS (LSI-ve)] was performed on a VG Autospec spectrometer. Elemental analyses were determined with a Perkin-Elmer 240C or Carlo-Erba 1106 elemental analyzer.

[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) (1b).** The natural product **1a** (50 g, 72.4 mmol) in acetone (500 mL) was treated with H_2SO_4 (3.6 M, 500 mL), and the solution stood at 20 °C for 10 days. The solution was diluted with H_2O (500 mL), concentrated under reduced pressure to remove the acetone, and extracted with EtOAc. The organic solution was washed with H_2O and brine, dried, and evaporated to dryness to give **1b** (40.38 g, 86%) as a white foam: NMR (CD_3OD) δ 0.80–0.90 (9H, m, CH_3), 1.03 (3H, d, J = 7 Hz, =CHCHCH_3), 2.76 (1H, dd, J = 13 and 5 Hz, CH_2Ph), 3.92 (1H, d, J = 5 Hz, =CCHOH), 4.08 (1H, d, J = 2 Hz, 7-H), 5.00 and 5.10 (2H, 2s, =CH_2), 5.27 (1H, s, 3-H), 5.78 (1H, d, J = 16 Hz, OCOCH=CH), 6.31 (1H, d, J = 2 Hz, 6-H), 6.84 (1H, dd, J = 16 and 8 Hz, OCOCH=CH), 7.1–7.3 (5H, m, Ph); MS (FAB-ve) m/z 647 (M − H)$^-$.

[1*S*-[1α(4*R,5*S**),3α,4β,5α,6α(4*S**,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-Dimethyloctanoate) (2a), [1*S*-[1α(2*E*,4*R**,5*S**),3α,4β,5α,6α(4*S**,6*R**),7β]]-1-[4-(Acetyloxy)-3,5-dimethyl-6-phenyl-2-hexenyl]-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyloctanoate) (2c), and [1*S*-[1α(3*R**,4*S**,5*S**),3α,4β,5α,6α(4*S**,6*R**),7β]]-1-[4-(Acetyloxy)-3,5-dimethyl-6-phenylhexyl]-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyloctanoate) (2d).** A solution of **1a** (1 g, 1.45 mmol) in EtOAc (100 mL) was hydrogenated for 1 h using 10% Pd–C (120 mg) as catalyst. The catalyst was removed by filtration through Kieselguhr, and the filtrate was evaporated to dryness to give a gum. The mixture was purified by preparative HPLC to give in order of elution **2c** (70 mg, 7%): analytical HPLC (column B) eluting with 55% $CH_3CN/H_2O/H_2SO_4$, t_R = 5.88 min, 97% pure; NMR (CD_3OD) δ 0.80–0.90 (12H, m, CH_3), 1.70 (3H, s, C(CH_3)=CH), 2.06 (3H, s, AcO), 4.07 (1H, br s, 7-H), 5.25 (1H, s, 3-H), 5.61 (1H, t, J = 8 Hz, CH_2CH=), 6.25 (1H, br s, 6-H), 7.10–7.30 (5H, m, Ph); MS (FAB-ve) m/z 691 (M − H)$^-$, 647 (M − CO_2H)$^-$, 633 (M − AcO)$^-$; HRMS (LSI-ve) found 691.2980, calcd for $C_{35}H_{47}O_{14}$ 691.2966. **2a** (532 mg, 53%): analytical HPLC (column A) eluting with 60% $CH_3CN/H_2O/H_2SO_4$, t_R = 9.29 min, 100% pure; NMR (CD_3OD) δ 0.80–0.90 (12H, m, CH_3), 2.10 (3H, s, AcO), 4.01 (1H, br s, 7-H), 4.99 and 5.02 (2H, 2s, =CH_2), 5.08 (1H, d, J = 5 Hz, CHOAc), 5.25 (1H, s, 3-H), 6.25 (1H, br s, 6-H), 7.10–7.30 (5H, m, Ph); MS (FAB-ve) m/z 691 (M − H)$^-$, 647 (M − CO_2H)$^-$. Anal. ($C_{35}H_{48}O_{14}$·0.5H_2O) C, H. **2d** (312 mg, 31%): analytical HPLC (column B) eluting with 55% $CH_3CN/H_2O/H_2SO_4$, t_R = 7.10 min, 99.5% pure; IR ($CHBr_3$) 3451, 3191, 1728 cm^{-1}; NMR (CD_3OD) δ 0.80–0.98 (15H, m, CH_3), 2.10 and 2.12 (3H, 2s, AcO), 2.31 (2H, br t, J = 8 Hz, $OCOCH_2$), 3.99 and 4.01 (1H, 2 br s, 7-H), 5.22 and 5.23 (1H, 2s, 3-H), 6.25 (1H, m, 6-H), 7.10–7.35 (5H, m, Ph); MS (FAB-ve) m/z 693 (M − H)$^-$, 649 (M − CO_2H)$^-$. Anal. ($C_{35}H_{50}O_{14}$·H_2O) C, H.

[1*S*-[1α(3*R,5*S**),3α,4β,5α,6α(4*S**,6*R**),7β]]-1-(3,5-Dimethyl-6-phenylhexyl)-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyloctanoate) (2e).** A solution of **1a** (6.3 g, 9.1 mmol) in EtOH (500 mL) was hydrogenated for 6 days using 10% Pd–C (2.87 g) as catalyst. The catalyst was removed by filtration through Kieselguhr, and the filtrate was evaporated to give a gum. The mixture was purified by preparative HPLC to give in order of elution **2d** (1.2 g, 20%) and **2e** (2.79 g, 48%): analytical HPLC (column B) eluting with 55% $CH_3CN/H_2O/H_2SO_4$, t_R = 11 min, 99.9% pure; IR ($CHBr_3$) 3528, 3458, 1733 cm^{-1}; NMR (CD_3OD) δ 0.80–0.90 (15H, m, CH_3), 4.04 (1H, m, 7-H), 5.23 (1H, s, 3-H), 6.25 (1H, m, 6-H), 7.10–7.40 (5H, m, Ph); MS (FAB-ve) m/z 635 (M − H)$^-$, 591 (M − CO_2H)$^-$. Anal. ($C_{33}H_{48}O_{12}$·2H_2O) C, H.

[1*S*-[1α(3*R,4*S**,5*S**),3α,4β,5α,6α(4*S**,6*R**),7β]]-1-[4-(Acetyloxy)-3,5-dimethyl-6-cyclohexylhexyl]-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyloctanoate) (2f).** A solution of **1a** (1 g, 1.45 mmol) in acetic acid (50 mL) and CH_2Cl_2 (0.5 mL) was hydrogenated using PtO_2 (100 mg) as catalyst. The catalyst was collected by filtration through Kieselguhr, and the filtrate was evaporated and purified by preparative HPLC to give **2f** (1 g, 98%): IR ($CHBr_3$) 3472, 1724 cm^{-1}; NMR (CD_3OD) δ 0.80–0.95 (15H, m, CH_3), 2.08 and 2.09 (3H, 2s, AcO), 2.30 (2H, t, J = 8 Hz, $OCOCH_2$), 4.03 (1H, br s, 7-H), 5.25 (1H, s, 3-H), 6.25 (1H, br s, 6-H); MS (FAB-ve) m/z 699 (M − H)$^-$, 655 (M − CO_2H)$^-$. Anal. ($C_{35}H_{56}O_{14}$·1.5H_2O) C, H.

[1*S*-[1α(3*R,4*S**,5*S**),3α,4β,5α,6α(4*S**,6*R**),7β]]-1-(3,5-Dimethyl-4-hydroxy-6-phenylhexyl)-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyloctanoate) (2g) and [1*S*-[1α(3*R**,5*S**),3α,4β,5α,6α(4*S**,6*R**),7β]]-1-(3,5-dimethyl-4-oxo-6-phenylhexyl)-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyloctanoate) (2h).** A solution of **1b** (250 mg, 0.38 mmol) in EtOAc (30 mL) was hydrogenated for 3 days using 10% Pd–C (150 mg) as catalyst. The catalyst was removed by filtration through Kieselguhr, and the filtrate was evaporated to dryness and purified by HPLC to give **2g** (158 mg, 64%) as a white foam: analytical HPLC (column B) eluting with 50% $CH_3CN/H_2O/H_2SO_4$, t_R = 4.93 min, 97.7% pure; NMR (CD_3OD) δ 0.8–0.9 (9H, m, CH_3), 0.98 (3H, d, J = 7 Hz, CH_3), 1.83–1.94 (2H, m), 1.95–2.06 (1H, m), 2.2–2.4 (3H, m), 2.77 (1H, dd, J = 13 and 5 Hz, CH_2Ph), 3.20 (1H, t, J = 3 Hz), 4.0 (1H, s, 7-H), 5.23 (1H, s, 3-H), 6.25 (1H, s, 6-H), 7.1–7.3 (5H, m, Ph); MS (FAB-ve) m/z 651 (M − H)$^-$, 607 (M − CO_2H)$^-$; HRMS (LSI-ve) found 651.3019, calcd for $C_{33}H_{47}O_{13}$ 651.3017. **2h** (12 mg, 5%): white foam; analytical HPLC (column B) eluting with 45% $CH_3CN/H_2O/H_2SO_4$, t_R = 11.89 min, 46% and 13.01 min, 45% pure; IR ($CHBr_3$) 3600–2200, 1741 cm^{-1}; NMR (CD_3OD) δ 1.04 and 1.08 (3H, 2d, J = 7 Hz, =CHCHCH_3), 3.96 and 4.02 (1H, 2d, J = 2 Hz, 7-H), 5.22 (1H, s, 3-H), 6.24 (1H, m, 6-H), 7.1–7.3 (5H, m, Ph); ^{13}C NMR (CD_3OD) δ 220.5; MS (FAB-ve) m/z 649 (M − H)$^-$, 605 (M − CO_2H)$^-$. Anal. ($C_{33}H_{46}O_{13}$·H_2O) C, H.

[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 (3a).** A solution of **1a** (100.2 g, 145 mmol) in DMF (700 mL) was treated with triethylamine (101 mL, 725 mmol) and *N*-methylhydroxylamine hydrochloride (24.2 g, 290 mmol) with ice cooling. After 24 h, further quantities of triethylamine (20.2 mL, 135 mmol) and *N*-methylhydroxylamine hydrochloride (12.1 g, 145 mmol) were added, and the mixture was stirred for a total of 43 h. The reaction mixture was then diluted with H_2O (120 mL), acidified with HCl (11 M, 88 mL) to pH 3, and filtered on a weakly basic ion-exchange column (IRA-68, 2L). The product was eluted with aqueous acidic acetone (4.3% HCl–30.7% H_2O–65% acetone) and finally purified by HPLC, eluting with 36% CH_3CN/H_2O containing trifluoroacetic acid (1 mL/L) to give **3a** (18.1 g, 23%) as a white solid: analytical HPLC on a Chromasil C8 (25 cm × 0.46 cm) column eluting with 36% CH_3CN/H_2O containing CF_3CO_2H (1 mL/L) with a flow rate of 2 mL/min and detection at 210 nm,

t_R = 6.35 min, 100% pure; NMR (CD_3OD) δ 0.85 (3H, d, J = 7 Hz, CH_3CH), 2.11 (3H, s, AcO), 2.41 (1H, dd, J = 13 and 9 Hz, CH_2Ph), 2.71 (1H, dd, J = 13 and 6 Hz, CH_2Ph), 4.07 (1H, d, J = 2 Hz, 7-H), 4.98 and 5.03 (2H, 2s, $=CH_2$), 5.10 (1H, d, J = 5 Hz, $CHOAc$), 5.14 (1H, d, J = 2 Hz, 6-H), 5.16 (1H, s, 3-H), 7.10–7.30 (5H, m, Ph).

[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, Tripotassium Salt (tripotassium salt of 3a).** A solution of $KHCO_3$ (7.89 g, 0.789 mol) in H_2O (100 mL) was added to a solution of **3a** (14.15 g, 0.263 mol) in H_2O (300 mL) over 10 min. The resulting clear solution was freeze-dried to give the tripotassium salt of **3a** (17 g, 99%) as a white solid: NMR (D_2O) δ 0.9 (3H, d, J = 7 Hz, CH_3CH), 2.15 (3H, s, AcO), 4.05 (1H, d, J < 2 Hz, 7-H), 4.80 (1H, d, J = 5 Hz, $CHOAc$), 4.90 (1H, d, J < 2 Hz, 6-H), 4.96 and 5.00 (2H, 2s, $=CH_2$), 5.27 (1H, s, 3-H), 7.2–7.4 (5H, m, Ph). Anal. ($C_{25}H_{27}K_3O_{13}$·2.5H_2O) C, H, K.

[1*S*-[1α(4*R,5*S**)3α,4β,5α,6α,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 (3b).** A solution of **1a** (1.38 g, 2 mmol) in MeOH (15 mL) was treated with NaOH (0.8 M, 22 mL), and the mixtrure was heated to reflux for 18 h. The mixture was allowed to cool to room temperature, and the MeOH was removed under reduced pressure. The residue was acidified by adding HCl (2 M, 30 mL) and extracted with toluene. The aqueous solution was saturated with NaCl and extracted with EtOAc (6×). The organic phase was dried and purifed by HPLC to give **3b** (405 mg, 41%) as a white foam: analytical HPLC (column B) eluting with 20% $CH_3CN/H_2O/H_2SO_4$ t_R = 5.72 min, 92.5% pure; IR (Nujol) 3408, 1737 cm^{-1}; NMR (CD_3OD) δ 0.82 (3H, d, J = 7 Hz, $CHCH_3$), 2.78 (1H, dd, J = 13 and 5 Hz, CH_2Ph), 3.94 (1H, d, J = 5 Hz, $=CCHOH$), 4.09 (1H, d, J = 2 Hz, 7-H), 5.01 and 5.11 (2H, 2s, $=CH_2$), 5.14 (1H, d, J = 2 Hz, 6-H), 5.16 (1H, s, 3-H), 7.10–7.30 (5H, m, Ph); MS (FAB-ve) m/z 495 $(M - H)^-$. Anal. ($C_{23}H_{28}O_{12}$·2H_2O) C, H.

[1*S*-[1α(3*R,5*S**),3α,4β,5α,6α,7β]]-1-[4-(Acetyloxy)-3,5-dimethyl-6-phenylhexyl]-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]octane-3,4,5-tricarboxylic Acid (3d) and [1*S*-[1α(3*R**,5*S**),3α,4β,5α,6α,7β]]-1-(3,5-Dimethyl-6-phenylhexyl)-4,6,7-trihydroxy-2,8-dioxabicyclo[3.2.1]-octane-3,4,5-tricarboxylic Acid (3e).** A solution of **3a** (134 mg, 0.25 mmol) in ethanol (20 mL) was hydrogenated for 5 days using 10% Pd–C as catalyst. The catalyst was removed by filtration through Kieselguhr; the filtrate was evaporated to dryness and purified by HPLC to give in order of elution **3d** (27 mg, 20%): analytical HPLC (column B) eluting with 30% CH_3CN/H_2O, t_R = 5.76 min, 94.7% pure; NMR (CD_3OD) δ 0.79–0.85 (3H, m, CH_3), 0.91 and 0.94 (3H, 2d, J = 7 Hz, CH_3), 2.10 and 2.13 (3H, 2s, AcO), 4.02 and 4.04 (1H, 2d, J = 2 Hz, 7-H), 4.78 and 4.80 (1H, d, J = 6 Hz, $CHOAc$), 5.12 (1H, br s, 3-H), 7.10–7.30 (5H, m, Ph); MS (FAB-ve) m/z 539 $(M - H)^-$. **3e** (21 mg, 17%): analytical HPLC (column B) eluting with 30% $CH_3CN/H_2O/H_2SO_4$, t_R = 10.17 min, 96.7% pure; NMR (CD_3OD) δ 0.80–0.90 (4.5H, m, CH_3), 0.96 (1.5H, d, J = 7.5 Hz, CH_3 of one diastereoisomer), 4.07 (1H, d, J = 2 Hz, 7-H), 5.13 (1H, s, 3-H), 7.10–7.28 (5H, m, Ph); MS (FAB-ve) m/z 481 $(M - H)^-$; HRMS (LSI-ve) found 481.1692, calcd for $C_{23}H_{29}O_{11}$ 481.1710.

[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-Tris(1,1-dimethylethyl) Ester (4).** A solution of **1a** (14.7 g, 21.28 mmol) in dry toluene (50 mL) was treated dropwise with *N,N*-dimethylformamide di-*tert*-butyl acetal (61 mL, 255 mmol) over 0.5 h at 80 °C. The mixture was heated for a further 2.5 h and then allowed to cool to room temperature, diluted with diethyl ether, and washed with brine. The organic phase was dried, evaporated, and chromatographed eluting with EtOAc–cyclohexane (1:6) to give **4** (10.6 g, 58%) as a foam: IR ($CHBr_3$) 3564, 3451, 1755, 1730, 1648 cm^{-1}; NMR ($CDCl_3$) δ 0.78–0.9 (9H, m, CH_3), 1.04 (3H, d, J = 7 Hz, $=CHCHCH_3$), 1.43, 1.48, 1.60 (27H, 3s, t-BuOCO), 2.10 (3H, s, AcO), 2.70 (1H, dd, J = 14 and 6 Hz, CH_2Ph), 2.91 (1H, br d, 7-OH), 4.00 (1H, br s, 7-H), 4.08 (1H, s, 4-OH), 4.95 (2H, br s, $=CH_2$), 5.05 (1H, s, 3-H), 5.11 (1H, d, J = 5 Hz, CHOAc), 5.78 (1H, d, J = 16 Hz, $OCOCH=CH$), 6.01 (1H, d, J = 2 Hz, 6-H), 6.91 (1H, dd, J = 16 and 7 Hz, $OCOCH=CH$), 7.10–7.30 (5H, m, Ph). Anal. ($C_{47}H_{70}O_{14}$) C, H.

[1*S*-[1α(5*S),3α,4β,5α,6α(2*E*,4*R**,6*R**),7β]]-1-(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-Tris(1,1-dimethylethyl) Ester (5).** A solution of **4** (655 mg, 0.76 mmol) in dioxan (9 mL) was treated with ammonium formate (199 mg, 312 mmol) and bis(triphenylphosphine)palladium(II) chloride (105 mg, 0.15 mmol), and the mixture was heated under reflux for 22 h under nitrogen. The mixture was allowed to cool to room temperature, poured into water, and extracted with EtOAc. The organic phase was dried, evaporated, and chromatographed eluting with EtOAc–cyclohexane (1:4) to give **5** (558 mg, 91%) as a foam: IR ($CHBr_3$) 3568, 3456, 1755, 1733, 1647 cm^{-1}; NMR ($CDCl_3$) δ 0.75–0.88 (9H, m, CH_3), 1.03 (3H, d, J = 7.5 Hz, $=CHCHCH_3$), 1.44, 1.48 and 1.60 (27H, 3s, t-BuOCO), 2.90 (1H, d, J =2.5 Hz, 7-OH), 4.03 (1H, m, 7-H), 4.07 (1H, s, 4-OH), 4.74 and 4.81 (2H, s, $=CH_2$), 5.06 (1H, s, 3-H), 5.76 (1H, d, J = 16 Hz, $OCOCH=CH$), 6.90 (1H, dd, J = 16 and 8 Hz, $OCOCH=CH$), 7.08–7.30 (5H, m, Ph). Anal. ($C_{45}H_{68}O_{12}$) C, H.

1*S*-[1α(5*R),3α,4β,5α,6α(2*E*,4*R**,6*R**),7β]]-1-(3,5-Dimethyl-6-phenyl-2-hexenyl)-4,6,7-trihydroxy-2,8-dioxabicyclo-[3.2.1]octane-3,4,5-tricarboxylic Acid, 6-(4,6-Dimethyl-2-octenoate) (1i).** A solution of **5** (543 mg, 0.68 mmol) in 6.4 M HCl in dioxan (3 mL) stood at room temperature for 24 h. The solvent was removed under reduced pressure, and the residue was purified by HPLC to give **1i** (25 mg, 6%) as a solid: analytical HPLC (column A) eluting with 55% $CH_3CN/H_2O/H_2SO_4$, t_R = 16.22 min, 94% pure; NMR (CD_3OD) δ 0.76 (3H, d, J = 6 Hz, CH_3), 0.80–0.90 (6H, m, CH_3), 0.94 (3H, d, J = 7 Hz, $=CHCHCH_3$), 1.64 (3H, s, $CH=C(CH_3)$), 4.12 (1H, br s, 7-H), 5.26 (1H, s, 3-H), 5.39 (1H, br t, J = 6 Hz, $CH_2CH=C(CH_3)$), 5.55 (1H, d, J = 16 Hz, $OCOCH=CH$), 6.30 (1H, br s, 6-H), 6.73 (1H, dd, J = 16 and 8 Hz, $OCOCH=CH$), 7.09–7.29 (5H, m, Ph); MS (FAB-ve) m/z 631 $(M - H)^-$; HRMS (LSI-ve) found 631.2742, calcd for $C_{33}H_{43}O_{12}$ 631.2754.

Animals. The rats used in these studies were juvenile, male CD rats (Charles River) weighing ~150 g, fed on standard laboratory chow, and allowed access to water *ad libitum*. The marmosets used were adults between 15 and 28 months of age and fed a standard primate diet supplemented with fruit.

Growth Conditions for *Candida albicans* 2005E and Microsomal Enzyme Preparation. *Candida albicans* 2005E from a fresh plate of YNGA was inoculated into 50 mL of YNGB and grown overnight at 37 °C with shaking; 15 mL of an overnight culture was used per liter to inoculate fresh YNGB which was grown to stationary phase before harvesting. After harvesting with a 16 min spin at 8000*g* in a Beckman JA-10 rotor at 4 °C, the cells were resuspended in 100 mM MOPS, pH 7.4, and broken by three passes through an X-press (A. B. Biox, Sweden). Debris was removed with a 8000*g* spin in a Beckman JA-20 rotor for 10 min. The resulting supernatant was spun at 100000*g* for 1 h at 4 °C in a 45ti rotor. The resulting pellet was resuspended in 100 mM MOPS, pH 7.4, and homogenized with 30 strokes in a hand-held homogenizer on ice. Protein content of the preparations were in the region of 18–25 mg/mL. Enzyme dilution curves on each preparation were performed to obtain linear response.

***Candida albicans* SQS Assay.** The SQS assay was adapted from the mammalian assay.[19] Assays were carried out in microtiter plates. Inclusions in 50 μL were 50 mM MOPS, pH 7.4, 10 mM KF, 10 mM $MgCl_2$, 0.5 mM NADPH, 50 mM ascorbic acid, 20 units/mL ascorbate oxidase, 20 μL of [2-^{14}C]FPP (1.9 KBQ), 10 μL of H_2O, DMSO or compound, and 20 μL of microsomal enzyme preparation. Incubation was at 25 °C for 30 min. The assay was terminated with 50 μL of propan-2-ol; 25 μL was spotted out using positive displacement pipettes onto silica TLC plates divided into 2 cm squares. After air drying for 10 min, the plates were washed in 0.1 M ethanolamine 1% SDS, pH 11. The plates were dried in a microwave oven and then cut up and counted in 10 mL of optiscint.

Antifungal Activity. Minimum inhibitory concentrations were determined by microtiter broth dilution testing in yeast nitrogen base (Difco) containing 0.05% glucose. Frozen spore suspensions of filamentous fungi or growth from an overnight plate culture (yeast) were prepared in YNBG, and wells were inoculated to a final concentration of 5×10^4 cfu/mL. Plates were incubated at 35 °C for 24–48 h and the MIC's defined as the lowest concentration of drug completely inhibiting visible growth.

Measurement of Cholesterol Biosynthesis *in Vivo* in Rats. Squalestatins were dosed iv to rats ($n = 8$ per group) followed immediately by intraperitoneal administration of [1-^{14}C]acetate (250 μCi/kg). The rats were killed after 1 h, the livers were removed, and a sample of 0.5 g was saponified in alcoholic KOH at 80 °C for 1 h. After extraction, [^{14}C]cholesterol was separated by HPLC using a Spherisorb ODS-2 column eluted with methanol–isopropyl alcohol (4:1). The [1-^{14}C]cholesterol was quantified using the procedure described previously.[4]

Time Course Studies in Rats. Compounds were administered iv at time 0, and the rats were sacrificed 1, 2, 4, and 7 h later. One hour prior to sacrifice, [1-^{14}C]acetate (250 μCi/kg) was administered ip. Rats were killed by CO_2 asphyxiation and the livers dissected free. A 0.5 g sample of liver was saponified and the [1-^{14}C]cholesterol separated and quantified as above.

Effect on Serum Cholesterol Levels in Marmosets. Marmosets of mixed sex were fasted overnight, and blood samples (500 μL) were taken from the femoral vein and collected in microtainers (Becton Dickinson). Serum was obtained by centrifugation for 4 min at 10000*g* in a Beckman microfuge. Serum cholesterol concentrations were determined using a standard assay kit (Boehringer 237574) on a Kone Plus autoanalyzer. Animals ($n = 6$) were allocated to treatment groups on the basis of their fasting serum cholesterol levels such that the mean and distribution of serum cholesterol levels were similar for each group. Squalestatins were converted to their tripotassium salts and dissolved in water before administration. The control group animals ($n = 6$) were dosed with vehicle. Compounds were either administered daily for 7 days and serum cholesterol levels determined at the end of this dosing regime (Table 2) or dosed once and serum cholesterol levels determined at days 2, 3, 5, and 7 postdosing (Table 3 and Figures 1 and 5). All results are expressed as the mean of the change in serum cholesterol levels from predose values (± SEM).

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