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THE SQUALESTATINS: POTENT INHIBITORS OF SQUALENE SYNTHASE. THE ROLE OF THE TRICARBOXYLIC ACID MOIETY.

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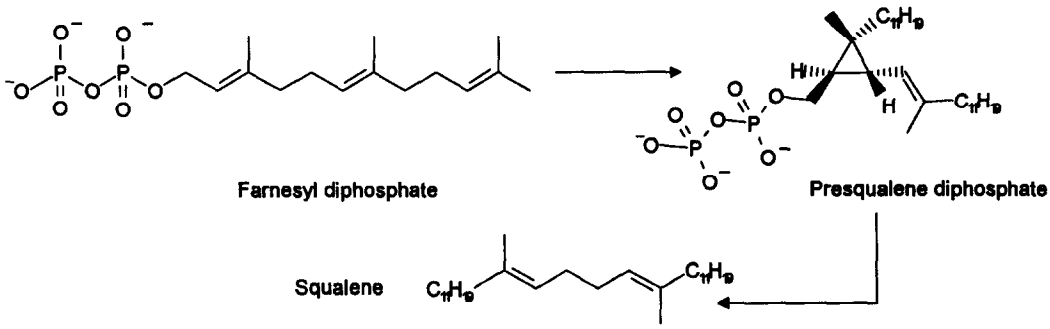
Abstract: In squalestatins possessing at C6 either a 4,6-dimethyloctenoate ester or a hydroxyl group, the 5-carboxylic acid is crucial for squalene synthase inhibitory activity. In the former series, free carboxylic acids are not required at C3 or C4 for potent enzyme inhibitory activity whereas in the latter series esterification of the carboxylic acids at C3 or C4 results in a significant reduction in enzyme inhibitory activity.

The major cause of death in Western countries is coronary heart disease and a primary risk factor in the progression of the disease is hypercholesterolemia. Currently one of the most effective approaches to lowering serum cholesterol levels is by inhibiting cholesterol biosynthesis. Two major regulatory steps in isoprenoid metabolism are catalysed by 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-R) and squalene synthase (SQS). Clinical successes in the treatment of hypercholesterolemia have been achieved with HMG-R inhibitors.[1] Major toxic effects are not commonly associated with this class of inhibitors, however, mevalonic acid, the product of the enzymic action of HMG-R, is a common precursor of all isoprenyl derivatives including ubiquinone and the dolichols. SQS is the first biosynthetic step which leads exclusively to sterols and agents which inhibit this step are particularly attractive as non-steroidal pathways should be minimally affected.

SQS catalyses the formation of squalene from farnesyl diphosphate (Fpp) in two distinct steps (Figure 1). Two molecules of Fpp are condensed head-to-head to form presqualene diphosphate (PSpp) which is then converted into squalene by a reductive rearrangement.

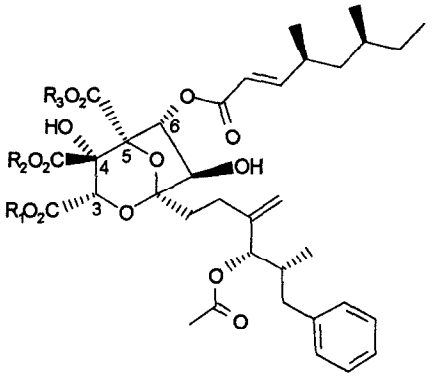
Figure 1. Conversion of Farnesyl Diphosphate into Squalene by SQS



Studies with substrate analogues of Fpp support the view that binding is strongly dependent on the presence of the diphosphate moiety or a close mimetic and, to a lesser extent, on relatively nonspecific lipophilic interactions of the hydrocarbon chain.[2] Thus in these inhibitors the labile diphosphate is essential for effective binding and the challenge has been to identify inhibitors without a phosphorus-containing diphosphate mimetic.

Recently we described the isolation[3] and structure elucidation[4] of the squalestatins, a novel group of fungal metabolites isolated from a previously unknown *Phoma* species (Coelomycetes). Squalestatin **1** (Table 1) is a potent selective inhibitor of rat and *Candida* SQS and possesses IC_{50} values of 12 nM and 5 nM respectively against these enzymes. Furthermore, **1** inhibits cholesterol biosynthesis from [^{14}C]acetate by isolated rat hepatocytes (IC_{50} = 39 nM) and by rat liver *in vivo*. More importantly, in marmosets squalestatin **1** lowers serum cholesterol by up to 75%.[5] Subsequent to our initial publications, the group at Merck have reported the isolation[6] and structure elucidation[7] of zaragozic acid A which is identical to squalestatin **1**.

Table 1. *In Vitro* SQS Inhibitory Activity for Methyl Esters of Squalestatin 1[8]

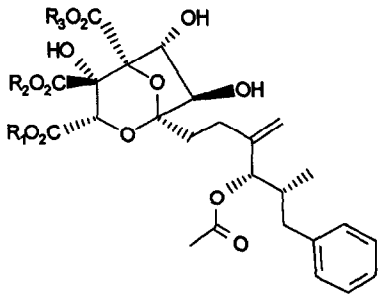


	R_1	R_2	R_3	IC_{50}(nM)
1	H	H	H	12
3	CH_3	H	H	7
7	H	CH_3	H	4
8	CH_3	CH_3	CH_3	>500
9	CH_3	CH_3	H	134
10	H	CH_3	CH_3	>500
11	H	H	CH_3	>500
12	CH_3	H	CH_3	>500

Squalestatin **1** incorporates the highly functionalised 2,8-dioxabicyclo[3.2.1]octane ring system posssessing carboxyl groups at C3, C4 and C5, tertiary and secondary hydroxyl groups at C4 and C7, respectively, and two lipophilic side-chains at C1 and C6. The related squalestatin **2** (Table 2), possessing only a

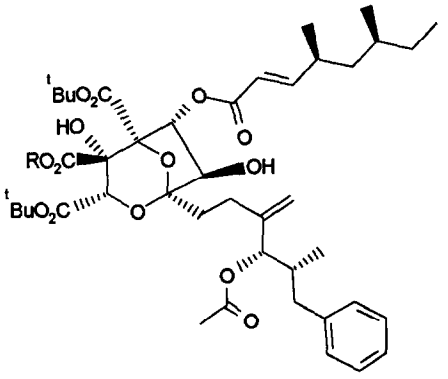
hydroxyl group at C6, also retains potent SQS inhibitory activity (IC_{50} = 6 nM).[3] A chemical programme was established to identify the key structural features which are essential for SQS inhibitory activity; this paper describes studies to establish the importance of each carboxylic acid for effective inhibition of this enzyme.

Table 2. *In Vitro* SQS Inhibitory Activity for Methyl Esters of Squalestatin 2[8]



	R_1	R_2	R_3	IC_{50}(nM)
2	H	H	H	6
13	CH_3	H	H	220
14	H	CH_3	H	355
15	H	H	CH_3	>500

Regioselective routes to the C3 and C4 monomethyl esters **3** and **7** were established. Treatment of **1** with methanol/concentrated HCl provided the 3-methyl ester **3**.[9] ^1H NMR data for the methyl groups of all methyl esters of squalestatin **1** are shown in Table 3. Reaction of **1** with *N,N*-dimethyl formamide di-t-butyl acetal in toluene at 80°C gave the tri-t-butyl ester **4**.[10] Selective removal of the 4-t-butyl ester (HCl/dioxan) followed by reaction of the derived 3,5-di-t-butyl ester **5** (*vide infra)* with methyl iodide/$NaHCO_3$ in DMF provided the related triester **6** from which the desired 4-monomethyl ester **7** was obtained readily on treatment with HCl/dioxane. The regioidentity of the derived ester **7** was established from a comparison of the ^{13}C NMR data for the carboxyl groups with those unambiguously defined in **1**[4] (Table 3); conversion of an acid to its methyl ester causes an upfield shift of 1.6 ± 0.6 ppm for the carboxyl carbon in diacids.[11]

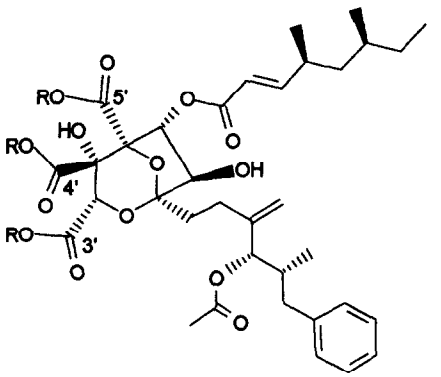


	R
4	^{t}Bu
5	H
6	Me

Regioselective routes to the 3,4- and 4,5- dimethyl esters **9** and **10** were established from the 3,4,5-trimethyl ester **8**.[4] Treatment of **8** with lithium iodide/collidine[12] provided an effective procedure for the selective removal of the 5-methyl ester to afford the 3,4-dimethyl ester **9** while reaction of **8** with 1 equiv. NaOH established an efficient route to the 4,5-dimethyl ester **10**.[13] The corresponding 5-monomethyl- and 3,5-dimethyl esters, **11** and **12**, were isolated following a reaction of **1** with 1 equiv. methyl iodide and $NaHCO_3$ in DMF followed by purification of the derived mixture by HPLC.

Selective removal of the C6 side-chain ester in **3**, **7** and **11** by *N*-methylhydroxylamine in DMF[10] provided the corresponding triols **13**, **14** and **15**.

Table 3. Key ^1H and ^{13}C Data (δ) in CD_3OD



	^1H Data (δ)			^{13}C Data (δ)		
	3'OMe	**4'OMe**	**5'OMe**	**3'**	**4'**	**5'**
1	-	-	-	170.1	172.5	168.5
3	3.73	-	-	**169.0**	172.5	168.4
7	-	3.82	-	170.0	**171.4**	168.6
8	3.73	3.83	3.68	**168.5**	**171.0**	**167.1**
9	3.71	3.84	-	**168.7**	**171.1**	168.1
10	-	3.83	3.67	170.3	**171.3**	**167.3**
11	-	-	3.68	170.1	172.5	**167.4**
12	3.72	-	3.68	nd	nd	nd

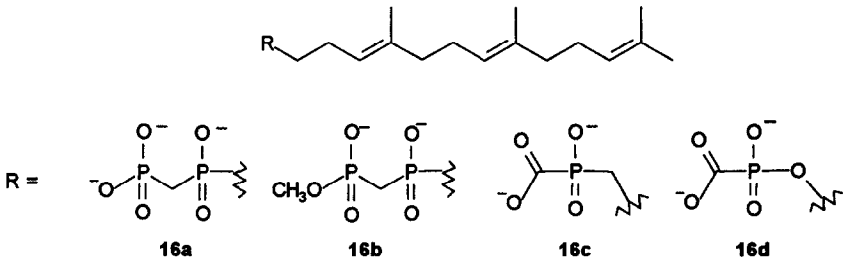
nd - not determined

The compounds shown in Table 1 were evaluated for their inhibitory activity against SQS. The enzyme preparation and assay procedures used in this study were the same as those described in our earlier publications.[5,14] Potent inhibitory activity is observed in both the 3- and 4-monomethyl esters **3** and **7** of squalestatin **1** (IC_{50} values of 7 and 4 nM, respectively); these esters were shown to be stable under the assay

conditions.[15] Furthermore, the 3,4-dimethyl ester **9** retains significant enzyme inhibitory activity (IC_{50} = 134 nM). In contrast the 5-monomethyl ester **11** is without significant activity (IC_{50} > 500 nM) as are the related 4,5- and 3,5-dimethyl esters (**10** and **12**) and the corresponding 3,4,5-trimethyl ester **8**.

The corresponding 3- and 4- monomethyl esters **13** and **14** of the squalestatin **2** (Table 2) possess a lower order of enzyme inhibitory activity (IC_{50} values of 220 and 355 nM, respectively) than the parent squalestatin **2** while the corresponding 5-monomethyl ester **15** is without significant activity (IC_{50} > 500nM).

The data for methyl esters of squalestatin **1** strongly suggest that the 5-carboxylic acid is crucial for enzyme inhibitory activity and indicate that carboxylic acid groups are not required at C3 and/or C4 for potent SQS inhibitory activity to be retained. The nature of the C6 substituent, however, critically influences SAR for modifications made to the carboxylic acids; thus, while the lack of activity shown by the 5-monomethyl ester **15** is again consistent with the 5-carboxylic acid being of critical importance, the C3 and C4 monomethyl esters (**13** and **14**) of squalestatin **2** show reduced SQS inhibitory activity when compared with that for the parent tricarboxylic acid **2** and suggests that a trianionic species may be required for maximal inhibitory activity in this series. At the outset of this programme the close structural similarities of **1** and **2** with PSpp and Fpp, respectively, were well recognised; both squalestatins possess polar trianionic head groups to which is attached either two or one lipophilic group(s), respectively. The different SAR established for esters of **1** and **2** clearly reflect different structural requirements for effective inhibition of SQS by each series. It has been reported previously that the trianionic Fpp mimetic **16a**, is a modest inhibitor of rat SQS (IC_{50} = 31.5 μM) while the related monomethyl ester **16b** is six-fold less active (IC_{50} = 177 μM).[16] Studies centred on finding replacements for the diphosphate group with reduced overall charge have yielded the dianionic C- and O-linked phosphinylformates **16c** and **16d**, which have the same overall chain length as **16a** and Fpp; **16c** was found to be equipotent with **16a** in the SQS enzyme assay and the O-linked analogue **16d** was significantly more active (IC_{50} = 8.7 μM).[17]



16a **16b** **16c** **16d**

The finding that the C3 and C4 monomethyl esters (**3** and **7**) possess potent SQS inhibitory activity closely similar to that for the parent squalestatin **1** indicates for the first time with inhibitors of this enzyme that an appropriately substituted dicarboxylic acid can act as a novel dianionic replacement for the metabolically and chemically unstable diphosphate group. The further evaluation of these esters will be the subject of a future publication.

Acknowledgement. We are indebted to Dr Philip J. Sidebottom for establishing the structural identities of the squalestatin esters by NMR techniques, to Mr John E. Farthing for detailed stability studies with methyl esters under the assay conditions and to Miss Belinda J. Fitzgerald for expert technical assistance in conducting the enzyme inhibition assay.

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8. SQS activity was measured using juvenile male rat liver microsomes as enzyme source. IC_{50} values were determined at least in duplicate at each concentration and are expressed as mean values using squalestatin **1** as a reference according to the assay procedure described in reference 14.
9. Long-range ^{1}H-^{13}C correlations for **3** obtained by the inverse method (HMBC) showed that the methyl protons (δ_H3.73) have long range coupling to the same carbonyl (δ_C 169.0) as has the 3-proton (δ_H 5.33).
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13. Long-range ^{1}H-^{13}C correlations for **9** obtained by the inverse method (HMBC) showed that the methyl protons (δ_H3.71) have long range coupling to the same carbonyl (δ_C 168.7) as has the 3-proton (δ_H 5.27); furthermore, the methyl protons (δ_H 3.84) have long range coupling to the carbonyl (δ_C 171.1) which shows an upfield shift of 1.4 ppm[11] when compared with that unambiguously assigned to the 4-carboxylic acid in **1**. Long-range ^{1}H-^{13}C correlations for **10** obtained by the inverse method (HMBC) showed that the methyl protons (δ_H 3.83 and 3.67) have long range coupling to the carbonyls (δ_C 171.3 and 167.3, respectively) but not to the same carbonyl (δ_C 170.3) which is coupled to the 3-proton (δ_H 5.22).
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15. Compounds **3**, **7** and **11** were separately incubated at 200 μg/ml with the SQS enzyme preparation (except that the microsomal preparation was 5 times the concentration used under the normal assay conditions and that ascorbate and ascorbate oxidase were omitted). While the compounds underwent partial conversion to their desacetyl derivatives (capillary zone electrophoresis) there was no evidence for significant production of the squalestatin **1** or its desacetyl analogue (HPLC). Analyses of the reaction mixtures from 5h incubations of **3** and **7** showed no evidence for transesterification either in the starting materials or in their desacetyl metabolites. Extraction efficiency was estimated at 99% thereby excluding significant quantities of other metabolic products being undetected.
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