Selective Protection and Relative Importance of the Carboxylic Acid Groups of Zaragozic Acid A for Squalene Synthase Inhibition

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Chemistry that allows selective modification of the carboxylic acid groups of the squalene synthase inhibitor zaragozic acid A (1) was developed and applied to the synthesis of compounds modified at the 3-, 4-, 5-, 3,4-, 3,5-, and 4,5-positions. A key step in this procedure is the selective debenzylation by transfer hydrogenolysis in the presence of other olefinic groups. These compounds were tested in the rat squalene synthase assay and *in vivo* mouse model. Modification at C3 retains significant enzyme potency and enhances oral activity, indicating that C3 is not essential for squalene synthase activity. Modification at C4 and C5 results in significant loss in enzyme activity. In contrast, substitution at C3 or C4 enhances *in vivo* activity. Furthermore, disubstitution at the C3 and C4 positions results in additive *in vivo* potency.

Introduction

Squalene synthase is the enzyme involved in the first committed step of the *de novo* cholesterol biosynthetic pathway. This enzyme catalyzes the reductive dimerization of two molecules of farnesyl pyrophosphate to form squalene.[1] The inhibition of this committed step to cholesterol should leave unhindered biosynthetic pathways to ubiquinone, dolichol, and isopentenyl t-RNA.

Inhibition of squalene synthase by pyrophosphate or pyrophosphate analogs containing isoprenoid (phosphinylmethyl)phosphonates has been reported.[2] Recently, the isolation and characterization of a potent squalene synthase inhibitor, zaragozic acid A (1), has been described.[3] In this paper, we will describe the selective synthetic manipulation of each of the three carboxyl groups of 1 and the resulting effect on inhibition of squalene synthase. The resulting compounds were also evaluated in *in vivo* models to determine if the activity of the parent molecule (1) is enhanced due to esterification at different sites.

Chemistry

The chemical methodology that permits selective manipulation of each of the three carboxyl groups of zaragozic acid A is summarized in Figures 1 and 2. The C3 carboxyl group of 1 can be selectively esterified by Fisher esterification. Stirring 1 in 3% HCl/methanol, 3% HCl/isoamyl alcohol, 3% HCl/benzyl alcohol, or 3% HCl/trimethylsilyl ethanol gave the corresponding C3 methyl, isoamyl, benzyl, and trimethylsilyl ethyl esters **2**, respectively. The C3 benzyl ester **2a** was selectively de-esterified by transfer hydrogenation with Pd–C and methylcyclohexadiene to give back zaragozic acid A (1) with no concommitant reduction of the other olefins. Other transfer hydrogenolysis conditions such as Pd–C/cyclohexene[4] yielded no reaction, while Pd–C ammonium formate[5] provided a mixture of products. The C3 benzyl ester **2a** was esterified at C4 and C5 with methyl and *O-tert*-butyl-*N,N'*-diisopropylisourea[6] to give 3-benzyl 4,5-dimethyl triester **3b** and 3-benzyl 4,5-di-*tert*-butyl triester **3a**, respectively.

Table 1. Activities of Analogs of Zaragozic Acid A

compd no.	modification site	RLSS (vs 1) IC_{50}, nM	*in vivo*, mouse mg/kg po (% inhibn)
1	none	0.50	40 (11)
2a	3	0.87 (0.50)	24 (36)
2b	3	1.80 (0.23)	24 (32)
2c	3	0.66 (0.23)	40 (62)
7	4	4.50 (0.09)	40 (43)
8	5	NA[a]	NA
4a	4,5	NA	NA
9	3,4	NA	ED_{50} = 18
10	3,5	NA	NA

[a] NA = not active at 13 nM in rat liver squalene synthase assay (RLSS) or less than 50% inhibition at 40 mg/kg in oral mouse assay.

Subsequently, the benzyl group of **3b** and **3a** was removed by transfer hydrogenation to give 4,5-dimethyl diester **4a** and 4,5-di-*tert*-butyl diester **4b**, respectively. The 3-benzyl ester **2a** was also transformed to a mixture of 3,5-dibenzyl (**5**) and 3,4-dibenzyl diesters (**6**) by stirring with DBU and benzyl chloride. The diesters **4b**, **4c**, **5**, and **6** are versatile intermediates which permit modification at the C3, C4, or C5 positions selectively by several standard procedures. Deprotection was done by using standard procedures. Deprotection of the *tert*-butyl esters was accomplished by stirring with trifluoroacetic acid (TFA) in methylene chloride, while the benzyl groups were removed by transfer hydrogenation. Esterification of **5**, **6**, and **2b** followed by deprotection gave compounds **7**, **8**, **9**, and **10**, respectively.

Results and Discussion

The *in vitro* assays were performed with rat liver homogenate squalene synthase as described in the Experimental Section. The activities of esters of the zaragozic acid A (1) are summarized in Table 1. Compounds modified at position C3 of the zaragozic acid A core including the methyl, isopentyl, or benzyl esters retain significant enzyme inhibition activity. Esterification of the C4 carboxyl group significantly reduced the enzyme inhibition, while esterification at C5 gave compounds inactive at 13 nM.

The oral *in vivo* assays were done in the mouse as shown in the Experimental Section. Zaragozic acid A (1) is not active in the *in vivo* mouse assay. Substitution at the C3 or C4 positions increases the *in vivo* activity over that of

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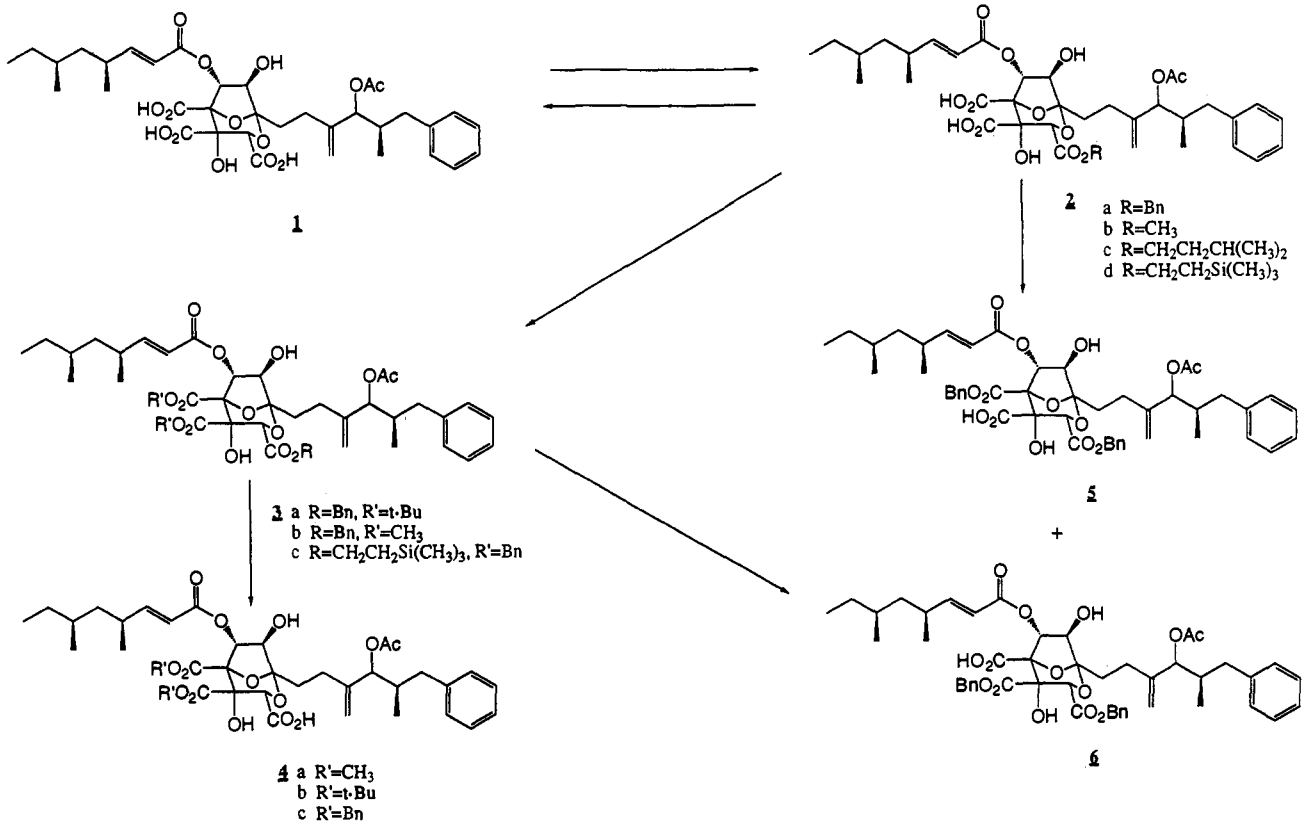

Figure 1. Synthesis of zaragozic acid A analogs.

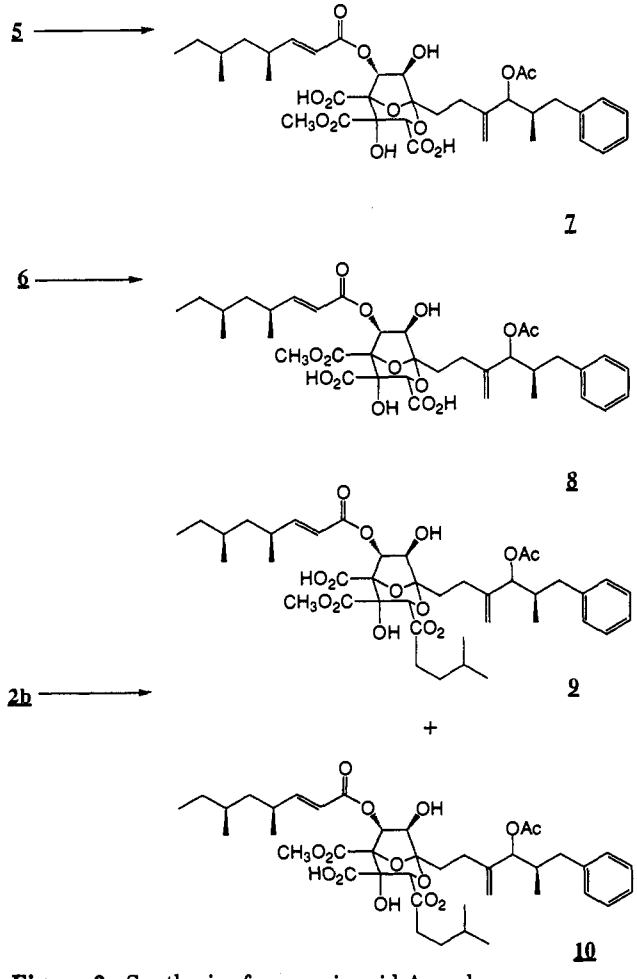

Figure 2. Synthesis of zaragozic acid A analogs.

zaragozic acid A. Bis-esterification at the C3 and C4 positions further increases *in vivo* activity in an additive manner. Since 3,4-diesters lack substantial enzyme inhibition activity as squalene synthase inhibitors, *in vivo* activity is due to enhanced oral absorption and may also result from ester hydrolysis to more enzymatically active derivatives. As expected, on the basis of the above results, other diesters such as 4,5-diesters and 3,5-diesters of 1 are inactive in the mouse *in vivo* model. The above assay results show that a C3 carboxylic acid is not essential for squalene synthase inhibition, while a C5 acid is essential for enzyme activity. It is undetermined whether the level of *in vivo* activity of any of the esters is due to their intrinsic *in vitro* activity or to their varying degree of oral absorbtion and/or esterase hydrolysis.

Experimental Section

Chemistry. General Methods. ^{1}H NMR spectra were recorded on a Varian VXR spectrometer. Chemical shifts are reported in ppm downfield from TMS at 0 ppm, and spectra are referenced with respect to the solvent peak 3.30 ppm for CHD_2-OD. Coupling constants are reported in hertz. Mass spectra were recorded on Finigan-MAT 731 mass spectrometer. High-resolution liquid secondary ion mass spectrometry (HR LSIMS, Cs^+) data were acquired on JEOL HX-110HF double-focusing mass spectrometer, operating at an accelerating voltage of 10 kV. High-resolution electron ionization mass spectrometry (HR EIMS, 90 eV) data were obtained on MAT 212 double-focusing mass spectrometer. Chromatographic purification was done with Merck silica gel (0.04–0.063 mm) or Baker C8 reverse-phase (0.04–0.063 mm) packed columns.

3-Benzyl Ester 2a. Acetyl chloride (0.4 mL) was added to benzyl alcohol (10 mL), and the reaction mixture was stirred at room temperature for 30 min. Solid 1 (1 g) was added, and the reaction mixture was stirred for an additional 6 h. The mixture was degassed, excess benzyl alcohol was removed by distillation under reduced pessure, and the resulting mixture was poured into an acetonitrile–water mixture (200 mL, 38%). The solution was filtered through a bed of C-8 reverse-phase silica (30 g) to remove unreacted benzyl alcohol. The column was then eluted with acetonitrile (400 mL). Evaporation of the acetonitrile eluate

under vacuum gave 1.13 g of 3-benzyl ester (86% pure by HPLC). Further purification was carried out by reverse phase chromatography (C-8 Baker, 58% acetonitrile in water): ^{1}H NMR (300 MHz, CD_3OD) δ 7.46–7.12 (m, 10H), 6.88 (dd, J = 8.9,18 Hz, 1H), 6.38 (br s, 1H), 5.48 (d, J = 15 Hz, 1H), 5.42 (s, 1H), 5.23 (dd, J = 14, 5.1 Hz, 2H), 5.14 (s,1H), 5.04 (s, 1H), 5.00 (s, H), 4.06 (br s, 1H), 2.71 (m, 1H), 2.54–2.00 (m, 7H), 2.12 (s, 3H), 1.50–1.1 (m, 6H), 1.07 (d, J = 6 Hz, 3H), 0.90 (m, 9H); FAB m/e 793 (M^+ + 2Li), 799 (M^+ + 3Li).

3-Methyl Ester 2b. Prepared as shown above by using methyl alcohol instead of benzyl alcohol: ^{1}H NMR (400 MHz, CD_3OD) δ 7.29–7.09 (m, 5H), 6.85 (dd, J = 15.6, 8.5 Hz, 1H), 6.31 (d, J = 1.8 Hz, 1H), 5.80 (d, J = 15.6 Hz, 1H), 5.31 (s, 1H), 5.09 (d, J = 4.9 Hz, 1H), 5.03 (s, 1H), 4.98 (s, 1H), 4.02 (d, J = 1.7 Hz, 1H), 3.72 (s, 3H), 2.70 (m, 1H), 2.45 (m 3H), 2.36–2.21 (m, 3H), 2.05 (s, 3H), 1.45–1.26 (m, 6H), 1.05 (d, J = 6.4 Hz, 3H), 0.91–0.82 (m, 10H); FAB m/e 685 (M – Na).

3-Isopentyl Ester 2c. **2c** was prepared as shown above by using isopentyl alcohol instead of benzyl alcohol: ^{1}H NMR (400 MHz, CD_3OD) δ 7.30–7.10 (m, 5H), 6.84 (dd, J = 15.6, 8 Hz, 1H), 6.31 (s, 1H), 5.78 (d, J = 15.6 Hz, 1H), 5.27 (s, 1H), 5.07 (d, J = 4.4 Hz, 1H), 5.02 and 4.96 (each s, each 1H), 4.18 (m, 2H), 4.03 (d, J = 1.9 Hz, 1H), 2.69 (m, 2H), 2.4–2.5 (m, 2H), 2.10 (s, 3H), 1.1–1.7 (m, 5H), 1.03 (d, J = 7.1 Hz), 0.86 (m, 9H).

3-Benzyl 4,5-Di-*tert*-butyl Triester 3a. A solution of **2a** (100 mg) dissolved in methylene chloride (2 mL) was treated with *O*-*tert*-butyl-*N*,*N*′-diisopropylisourea (300 mg) and heated at 40 °C for 2 days. The reaction mixture was cooled to room temperature, concentrated, and filtered through a bed of silica (25% ethyl acetate in hexane) to yield pure 3-benzyl 4,5-di-*tert*-butyl triester **3a** (101 mg): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35–7.08 (m, 10H), 6.88 (dd, J = 8.4,16 Hz, 1H), 5.97 (d, J = 1 Hz, 1H), 5.75 (d, J = 16 Hz), 1H), 5.42 (s, 1H) 5.16 (dd, J = 12, 6.4 Hz, 2H), 5.06 (br s,1H), 4.94 (br s, 2H), 4.00 (br s, 1H), 2.96 (d, J = 2 Hz, 1H), 2.66 (m, 1H), 2.5–2.2 (m, 5H).

4,5-Di-*tert*-Butyl Diester 4b. To a solution of 3-benzyl 4,5-di-*tert*-butyl triester **3a** (100 mg) in methanol (4 mL) were added 1-methyl-1,4-cyclohexadiene (200 mL) and 10% Pd/C (50 mg). The reaction mixture was stirred at 30–35 °C for 1.5 h and filtered over Celite. The filtrate was evaporated under vacuum to give 4,5-di-*tert*-butyl diester **4a** (76 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 7.30–7.10 (m, 5H), 6.89 (dd, J = 8, 16 Hz, 1H), 6.43 (d, J = 1 Hz, 1H), 5.82 (d, J = 16 Hz, 1H), 5.06 (d, J = 5 Hz, 1H) 5.04 (s,1H), 5.01 (s, 1H), 4.07 (s, 1H), 2.69 (m, 1H), 2.5–2.2 (m, 6H), 2.10 (s, 3H), 1.60 (s, 9H), 1.42 (s, 9H), 1.65–1.05 (m, 6H), 1.03 (d, J = 8.1 Hz, 3H), 0.88 (m, 10H).

4,5-Dimethyl Diester 4a. 3-Benzyl ester **2a** (30.4 mg) dissolved in ethyl acetate (1 mL) was cooled to 10 °C, treated with a solution of diazomethane in ether (0.5 mL), and stirrred for 1 h. The solvent was evaporated and residue purified by prep TLC (silica; ethyl acetate–hexane, 4:6). The purifed 3-benzyl 4,5-dimethyl triester **3b** (18.6 mg) was dissolved in methanol (1 mL) and debenzylated with methylcyclohexadiene (40 mL) and 10% Pd/C (39 mg) to yield 4,5-dimethyl diester **4a** (12.1 mg): ^{1}H NMR (CD_3OD, 400 MHz) δ 7.30–7.12 (m, 5H), 6.88 (dd, J = 9, 16 Hz, 1H), 6.28 (d, J = 1.5 Hz, 1H), 5.77 (d, J = 16 Hz, 1H), 5.27 (s, 1H), 5.06 (d, J = 4.8 Hz, 1H), 5.02 (s, 1H), 4.97 (s, 1H), 4.05 (d, J = 1.6 Hz, 1H), 3.85, 3.67 (each s, each 3H), 2.68 (m, 1H), 2.5-2.15 (m), 2.10 (s, 3H), 1.03 (d, J = 7 Hz, 3H), 0.87 (m); MS (FAB) m/e 741, 742 $(M + Na)^+$.

4,5-Dibenzyl Diester 4c. To dry TMS–ethanol (25 mL) was added thionyl chloride (0.38 mL), and the mixture was allowed to stir for 30 min. To the reaction mixture was added 1 (5.0 g), and the mixture was stirred overnight. The following day the solution was diluted with water and extracted with ether. The ether layer was dried over anhydrous magnesium sulfate, filtered, and evaporated to yield 3-(2-(trimethylsilyl)ethyl) ester **2d** which was dissolved in methylene chloride (100 mL) and *O*-benzyl-*N*,*N*′-diisopropylisourea (16.9 g). The following day methylene chloride was distilled off and the residue dissolved in hexane and filtered over Celite. The filtrate was evaporated and chromatographed on silica gel (9:1 hexane–ethyl acetate). The triester **3c** obtained as such was dissolved in THF (25 mL) and stirred with tetrabutylammonium fluoride (2.47 mL, 1.0 M in THF) for 4 h. The solution was concentrated and chromatographed on silica gel (8:1 hexane–ethyl acetate) to yield 4,5-dibenzyl diester **4c** (3.3 g): ^{1}H NMR (400 MHz, CD_3OD) δ 7.42–7.10 (m, 15H), 6.78 (dd, J = 15.61, 8.8 Hz, 1H), 2.65 (m, 1H), 2.45–2.37 (m, 3H), 2.08 (s, 3H), 1.35–1.22 (m, 4H), 1.10 (d, J = 6.55 Hz, 5H), 1.03–0.99 (m, 4H), 0.86–0.78 (m, 10H); MS (–)FAB m/e 869 (M – 1).

3,4- and 3,5-Dibenzyl Diesters 5 and 6. DBU (900 mg) was added to a solution of the 3-benzyl ester of zaragozic acid A (1) (0.90 g, 1.15 mmol) in tetrahydrofuran (25 mL), and the reaction mixture was treated with benzyl chloride (0.35 mL, 2.6 mmol). After the mixture was stirred for 3 days at room temperature, the resulting salt was filtered off through a bed of silica and then eluted with 1:1 ethyl acetate–hexane first, and the desired products were eluted with acetone containing acetic acid (1%). The acetone fraction was concentrated under vacuum, and the two isomers were separated by preparative TLC (silica; methylene chloride–acetone–acetic acid, 46:3:1) to yield 3,4-dibenzyl diester **5** (186 mg, slow moving band) [^{1}H NMR (400 MHz, CD_3OD) δ 7.4–7.15 (m, 15H), 6.85 (dd, J = 8.8,16 Hz, 1H), 6.23 (br s, 1H), 5.78 (br d, J = 16 Hz, 1H), 5.28 (s, 1H), 5.06 (d, J = 4.6 Hz, 1H), 5.0 (s, 1H), 4.95 (s, 1H), 4.06 (br s, 1H), 2.68 (m, 1H), 2.54–2.00 (m, 5H), 2.10 (s, 3H), 1.40-1.1 (m, 7H), 1.03 (d, J = 6 Hz, 3H), 0.86 (m, 9H)] and 3,-5-dibenzyl diester **6** (395 mg, fast moving band): ^{1}H NMR (400 MHz, CD3OD) δ 7.45–7.05 (m, 10H), 6.68 (dd, J = 9,16 Hz, 1H), 6.68 (dd, J = 9, 16 Hz), 6.32 (br s, 1H), 5.47 (d, J = 16 Hz, 1H), 5.37 (s, 1H), 5.4–5.1 (m, 3H), 5.06 (d, J = 4.8 Hz, 1H), 4.98 (s, 1H, 4.92 (s, 1H), 3.97 (s, 1H), 2.68 (m, 1H), 2.5–2.15 (m, 5H), 2.10 (s, 3H), 1.40–1.1 (m, 7H), 0.97 (d, J = 7 Hz, 3H), 0.87.

5-Methyl Ester 8. 3,4-Dibenzyl diester **6** (40 mg) dissolved in methylene chloride (1 mL) was treated with *O*-methyl-*N*,*N*′-diisopropylisourea, and the mixture was stirred at 40 °C overnight. The resulting triester was purified by prep TLC (silica; ethyl acetate–hexane, 3:7), and the colorless gum (23.6 mg) was dissolved in methanol (1 mL) and stirred for 3 h at room temperature with 1-methyl-1,4-cyclohexadiene (20 μL) and 10% Pd–C (15 mg). Filtration over Celite and evaporation gave the 5-methyl ester of 1 (13.8 mg): ^{1}H NMR (CD_3OD, 400 MHz) δ 7.3–7.1 (m, 5H), 6.77 (dd, J = 8, 16 Hz, 1H), 6.36 (s, 1H), 5.75 (d, J = 16 Hz, 1H), 5.33 (s, 1H), 5.06 (d, J = 5.2 Hz, 1H), 5.07 and 4.96 (each s, each 1H), 4.02 (s, 1H), 3.68 (s, 3H), 2.68 (m, 1H), 2.5–2.15 (m, 5H), 2.10 (s, 3H), 1.4-1.1 (m, 3H), 1.03 (d, J = 6 Hz, 3H), 0.86 (m, 9H).

4-Methyl Ester 7. This compound was prepared by using the above procedure and replacing 3,4-dibenzyl diester with 3,5-dibenzyl diester **5**: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.3-7.1 (m, 5H), 6.81 (dd, J = 8, 16 Hz, 1H), 6.22 (s, 1H), 5.79 (d, J = 16 Hz, 1H), 5.17 (s, 1H), 5.07 (d, J = 4.4 Hz, 1H), 5.01 (s, 1H), 4.97 (s, 1H), 4.02 (s,1H), 3.82 (s, 3H), 2.68 (m, 1H), 2.5–2.15 (m, 5H), 2.10 (s, 3H), 1.4–1.1 (m, 3H), 1.03–0.86 (m, 12H).

3-Isopentyl 4-Methyl Diester 9 and 3-Isopentyl 5-Methyl Diester 10. 3-Isopentyl ester **2c** (150 mg) in acetonitrile (2 mL) was stirred with DBU (66 mL) for 30 min. Methyl iodide (12.3 mL) was then added, and stirring continued for 16 h. The following day, purification of the two diesters by prep TLC (silica; methylene chloride–acetone–acetic acid, 46:3:1) gave 3-isopentyl 4-methyl diester **9** (29.8 mg, R_f 0.23) and 3-isopentyl 5-methyl diester **10** (36.2 mg, R_f 0.36). The above reaction when carried out in tetrahydrofuran with DBU (33 mL) gave a 4:1 ratio of 3-isopentyl 4-methyl diester **9** [^{1}H NMR (400 MHz, CD_3OD) δ 7.1-7.27 (m, 5H), 6.64 (dd, J = 15.6, 8 Hz, 1H), 6.28 (d, 1.9 Hz, 1H), 5.80 (d, J = 15.6 Hz, 1H), 5.24 (s, 1H), 5.07 (d, J = 4.4 Hz, 1H), 5.00 (s, 1H), 4.96 (s, 1H), 4.16 (m, 2H), 4.04 (d, J = 1.9 Hz, 1H), 2.67 (m, 2H), 2.4–2.5 (m, 2H), 2.10 (s, 3H), 1.1–1.7 (m, 5H), 1.03 (d, J = 7.1 Hz, 0.85–0.95 (m, 9H)] and 3-isopentyl 5-methyl diester **10**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.12–7.28 (m, 5H), 6.77 (dd, J = 15.6, 8 Hz, 1H), 6.30 (d, J = 1.9 Hz, 1H), 5.76 (d, J = 15.6 Hz, 1H), 5.29 (s, 1H), 5.07 (d, J = 4 Hz, 1H), 5.00 and 4.96 (each s, each 1H), 4.18 (m, 2H), 4.02 (d, J = 1.9 Hz, 1H), 2.67 (m, 2H), 2.4–2.5 (m, 2H), 2.10 (s, 3H), 1.1–1.7 (m, 5H), 1.03 (d, J = 7.1 Hz, 0.83–0.95 (m, 9H).

Rat Liver Squalene Synthase (RLSS) Inhibitory Activity. Inhibitory activity against squalene synthase was determined using the rat liver enzyme. Rat liver microsomes were prepared from male Charles River CD rats (120–150 g) fed a diet containing 0.1% lovastatin for 4 or 5 days. The livers from these rats were homogenized in five volumes (mL/g) of 50 mM HEPES, 5 mM EDTA pH 7.5. The 20 000g to 100 000g microsomal pellet was

isolated and resuspended in one volume (mL/g) of the homogenizing buffer. Squalene synthase activity was measured by the method of Bergstrom et al. [3b] with farnesyl diphosphate concentration of 5 μM over a drug concentration range of 0.1–13 nM.

Mouse *in Vivo* Assay. Mice, 22.5–27.5 g, were dosed orally with 150 μL of the compounds suspended in 5% emulphor with six animals per group. Thirty minutes after dosing with the test compounds, the animals were injected subcutaneously with [5-^{3}H]-mevalonolactone (0.5 μCi/animal). The animals were then sacrificed 30 min after receiving the mevalonate dose. The livers were removed, saponifed, and extracted with petroleum ether, and total dpm in the nonsaponifiables was determined. Inhibitory activity was determined by comparing [^{3}H]mevalonate incorporation into the nonsaponifiable fraction in control animals to the incorporation in the treated animals.

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