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Biochemistry

Zaragozic acids: A family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase

(cholesterol synthesis inhibitors/fungal metabolites)

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ABSTRACT **Three closely related fungal metabolites, zaragozic acids A, B, and C, that are potent inhibitors of squalene synthase have been isolated and characterized. Zaragozic acids A, B, and C were produced from an unidentified sterile fungal culture, *Sporormiella intermedia*, and *Leptodontium elatius*, respectively. The structures of the zaragozic acids and their trimethyl esters were determined by a combination of physical and chemical techniques. The zaragozic acids are characterized by a novel 2,8-dioxobicyclo[3.2.1]octane-4,6,7-trihydroxyl-3,4,5-tricarboxylic acid core and differ from each other in the structures of the 6-acyl and 1-alkyl side chains. They were found to be potent competitive inhibitors of rat liver squalene synthase with apparent K_i values of 78 pM, 29 pM, and 45 pM, respectively. They inhibited cholesterol synthesis in Hep G2 cells, and zaragozic acid A was an inhibitor of acute hepatic cholesterol synthesis in the mouse (50% inhibitory dose of 200 μg/kg of body weight). Inhibition of squalene synthase in cells and *in vivo* was accompanied by an accumulation of label from [^{3}H]mevalonate into farnesyl diphosphate, farnesol, and organic acids. These data indicate that the zaragozic acids are a previously unreported class of therapeutic agents with potential for the treatment of hypercholesterolemia.**

Squalene synthase (farnesyl-diphosphate:farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21) is a prime target for pharmacological intervention to achieve cholesterol-lowering therapy. Cholesterol synthesis inhibitors, such as the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor lovastatin (1), are effective cholesterol-lowering agents in animals and in man. Inhibitors of squalene synthase should also serve as cholesterol-lowering agents.

The mammalian isoprenoid pathway not only produces sterols but also produces dolichol, ubiquinone, the farnesyl group of heme A, the farnesyl and geranylgeranyl groups of prenylated proteins, and the isopentenyl side chain of isopentenyl adenine. The pathways for the synthesis of these other isoprenoids diverge from the synthesis of cholesterol either at or before the farnesyl diphosphate (FPP) branch point. Thus, squalene synthase, which catalyzes the reductive dimerization of 2 mol of FPP to 1 mol of squalene (2, 3), is the first committed step in sterol synthesis. A specific inhibitor of squalene synthase should serve to inhibit cholesterol synthesis and not adversely affect the synthesis of other isoprenoids. FPP, the substrate for squalene synthase, is water soluble and may be readily metabolized (4). Thus, squalene synthase offers a potential target for the safe and specific inhibition of cholesterol synthesis.

In this report we describe the isolation, structure, physical characterization, and biological properties of three structurally similar fungal metabolites that are potent inhibitors of squalene synthase. These metabolites, zaragozic acid A (5–7), zaragozic acid B (8, 9), and zaragozic acid C (10–12), had been reported previously only in the patent literature; however, during the review process of this manuscript, three manuscripts (13–15) appeared on the squalestatins: squalestatin I is identical with zaragozic acid A, squalestatin II is des-4′-acetylzaragozic acid A, and squalestatin III is des-6-acylzaragozic acid A. This class of squalene synthase inhibitors has potential utility as cholesterol-lowering agents.

MATERIALS AND METHODS

Zaragozic Acid A, Cultures, and Media. An unidentified sterile fungal culture, ATCC 20986, isolated from a water sample taken from the Jalon river in Zaragoza, Spain (hence the name zaragozic acids), was used to produce zaragozic acid A. The culture was maintained at 25°C on medium B agar slants composed of 4 g of yeast extract, 10 g of malt extract, 4 g of dextrose, and 20 g of agar per liter at pH 7.0.

Zaragozic acid A was produced in a two-tiered fermentation process consisting of mycelial growth and development in medium A of ref. 1 and product formation in medium C. Medium C contained 5 g of malt extract, 1 g of peptone, 15 g of dextrose, 1 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter. Fermentations consisted of mycelial growth in medium A for 72 hr at 25°C with agitation, followed by inoculation (5–10%) of medium C. Maximum product was obtained from 14-day agitated fermentations at 25°C.

Isolation of Zaragozic Acid A. To isolate zaragozic acid A, 23 liters of harvested broth was filtered through Celite, and the mycelial cake was extracted twice with 7 liters of 50% aqueous methanol. The filtrate was combined with the extracts, diluted with water to a final composition of 25% methanol, and adsorbed on a 1.5-liter column of Mitsubishi HP-20 resin. After a column wash with 6 liters of 4:6 (vol/vol) methanol/water, crude zaragozic acid A was eluted from the resin with 6 liters of methanol, diluted with an equal volume of 10 mM phosphoric acid, and extracted into dichloromethane. Removal of the solvent afforded 21 g of a yellow oil that was taken up in 1 liter of 1:1 (vol/vol) methanol/20 mM potassium phosphate, pH 7, and adsorbed onto 350 ml of

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Abbreviations: FPP, farnesyl diphosphate; HMBC, heteronuclear multiple bond connectivity.

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Dowex 1 × 2 resin, chloride form. The resin was washed with 1:1 (vol/vol) methanol/3% aqueous NaCl, and the compound was eluted with 9:1 (vol/vol) methanol/3% aqueous ammonium chloride. Extraction of zaragozic acid A into dichloromethane was carried out as described above. The extract was concentrated to an oily residue, 24 mg of which was purified by preparative HPLC (Whatman Magnum 20 C_{18}, 22 mm i.d. × 25 cm) with 4:1 (vol/vol) methanol/10 mM phosphoric acid as eluting solvent. Extraction of the HPLC-rich cut into dichloromethane and concentration afforded zaragozic acid A as a pale yellow oil.

Zaragozic Acid B, Cultures, and Media. A coprophilous fungus, ATCC 20985, subsequently identified as *Sporormiella intermedia*, was isolated from cottontail rabbit dung collected near Tucson, AZ, and was used to produce zaragozic acid B. The culture was maintained on medium B agar slants as cited above.

Zaragozic acid B was produced in a two-tiered fermentation process similar to that used for zaragozic acid A production. Mycelial growth in medium A, obtained under the same conditions stated above, was used to inoculate solid-state fermentation medium D. Medium D consists of 15 g of millet per nonbaffled 250-ml Erlenmeyer flask and 10 ml of base liquid 1. Base liquid 1 contains 50 g of yeast extract, 10 g of monosodium glutamate, 10 ml of corn oil, 10 g of sodium tartrate, and 1 g of $FeSO_4 \cdot 7H_2O$ per liter. Zaragozic acid B was obtained by extraction with methanol from fungal culture incubated on this medium for 14 days at 25°C.

Zaragozic Acid C, Cultures, and Media. A strain of *Leptodontium elatius* var. *elatius*, ATCC 70411, isolated from wood in the Joyce Kilmer Forest in North Carolina, was used to produce zaragozic acid C. The culture was maintained on medium B agar slants as cited above.

Zaragozic acid C was produced in a two-stage fermentation process similar to those cited above. Mycelial growth in medium A was inoculated onto solid-state fermentation medium E. Medium E consisted of 10 g of cracked corn per nonbaffled 250-ml Erlenmeyer flask and 10 ml of base liquid 2. Base liquid 2 contained 0.2 g of ardamine PH, 0.1 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of sodium tartrate, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 0.01 g of $ZnSO_4 \cdot 7H_2O$ per liter. Zaragozic acid C was obtained from fungal culture incubated on this medium for 21 days at 25°C by extraction with methanol.

Isolation of Zaragozic Acids B and C. The isolations of zaragozic acids B and C were accomplished by using the extraction, HP-20, and Dowex 1 procedures described above for zaragozic acid A. Final purifications of the compounds were also accomplished by preparative reverse-phase HPLC with solvents of the appropriate eluting strength.

HPLC Separation and Analysis of the Zaragozic Acids. The zaragozic acids were separated on an isocratic system on a Dynamax C_8, 60A, 8-μm, 4.6 × 250 mm column with guard module. The solvent was 6:4 (vol/vol) acetonitrile/0.1% phosphoric acid in water with a flow rate of 1 ml/min run at room temperature. The retention times for zaragozic acids A, B, and C were 13.4, 23.7, and 21.7 min, respectively.

Assays of Squalene Synthase. Squalene synthase activity was monitored by the formation of [^{14}C]squalene from [4-^{14}C]*FPP* by a modification of the method of Agnew (16). One milliliter of assay mixture included 50 mM Hepes, 10 mM NADPH, 11 mM NaF, 5.5 mM $MgCl_2$, [4-^{14}C]*FPP* as specified (produced enzymatically from [4-^{14}C]isopentenyl diphosphate, geranyl diphosphate, and purified rat liver prenyl transferase), and 1 mg of squalene. The assays ran 20 min at 30°C at pH 7.5. [4-^{14}C]*FPP* was 5 μM in the standard assays and was varied from 0.2 μM to 10 μM in the kinetic studies. L-688,709—(*E*)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-ethyl-3-{[3-(5-oxazolyl)phenyl]methoxy}benzylamine, a squalene epoxidase inhibitor closely related to NB-598 (17)—was included in the assays at 1 μg/ml. [^{14}C]Squalene was extracted, isolated, and quantified essentially as described by Agnew (16). With the inclusion of the squalene epoxidase inhibitor, there was no need to degas (degassing inhibits squalene epoxidase, as O_2 is a substrate), and the reactions were run aerobically. There were no differences in squalene production or inhibition by the zaragozic acids between anaerobic assays and assays run aerobically in the presence of the squalene epoxidase inhibitors. The zaragozic acids were dissolved in dimethyl sulfoxide and added to the assay mixture to give a final concentration of 0.3% dimethyl sulfoxide. Microsomes for the assays were prepared from male Sprague–Dawley rats fed 0.1% lovastatin for 5 days, and each assay included 2.2 μg of protein.

Inhibition of Cholesterol Synthesis in Hep G2 Cells. Hep G2 cells were plated in minimal essential medium supplemented with penicillin, streptomycin, 3.6 mM glutamine, and 10% (vol/vol) fetal bovine serum at 2 × 10^5 cells per 25-cm^2 flask. After 3 days, the medium was replaced with medium containing lipoprotein-depleted fetal bovine serum (LPDM). After 2 days in LPDM, the medium was changed to serum-free medium containing the test compounds that previously had been dissolved in dimethyl sulfoxide. The final dimethyl sulfoxide concentration in the incubation was 0.5% or less. The cells were incubated with the test compounds for two hr, and then 15 μCi (1 μCi = 37 kBq) of [5-^{3}H]mevalonate (0.2 mCi/mmol) was added. One hour after the addition of the mevalonate, the medium was removed, the cells were washed three times with 2 ml of 0.9% NaCl, and then 2 ml of 1 M NaOH was added to remove the cells. After transfer to a glass screw-cap test tube, the cell extract was saponified at 90°C for 1 hr. Nonsaponifiables were extracted three times with 2 ml of petroleum ether. The basic aqueous layer was then acidified to pH 1 with HCl and extracted three times with 2 ml of petroleum ether. Found in the basic aqueous layer, *FPP* treated with acid produces nerolidol and farnesol in a ratio of 4:1 (18). Thus, the acidic petroleum ether extract contained farnesol and nerolidol that arose from *FPP* as well as the organic acids including farnesoic acid. The acidic petroleum ether extract was washed three times with 2 ml of 0.1 M NaOH. The washed petroleum ether retained the farnesol and nerolidol that arose from *FPP*, while the aqueous basic washes contained the organic acids including farnesoic acid. After addition of 10 ml of Aquasol (DuPont) to a portion of each fraction, radioactivities were measured in a Packard model 2200CA scintillation counter.

Inhibition of Hepatic Lipid Synthesis in Mice by Zaragozic Acid A. Animals were housed and cared for in keeping with the standards set forth in the NIH *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 86-23, 1985). Female Swiss Webster mice (≈25 g) were dosed subcutaneously with the trisodium salt of zaragozic acid A dissolved in 0.09% NaCl in a volume of 50 μl. Thirty minutes after receiving the dose of zaragozic acid A, the animals were dosed subcutaneously with 0.5 μCi of D,L-[5-^{3}H]mevalonolactone (35 Ci/mmol) (New England Nuclear). Fifteen minutes after receiving the mevalonolactone, the animals were euthanized, and the livers were removed and saponified in a mixture of 4 ml of 40% KOH and 2 ml of 95% ethanol overnight at 55°C. The saponified livers were extracted with petroleum ether, acidified to pH 1, and reextracted with petroleum ether to give a nonsaponifiable fraction and a *FPP*/organic acids fraction.

RESULTS

Isolation and Structure of the Zaragozic Acids. Isolation of the zaragozic acids was carried out through a four-step procedure by taking advantage of their unusual amphipathic nature. A combination of methanol and water was used for

their initial extraction. A solid-phase extraction/chromatography on HP-20 resin was then carried out, followed by acidification and extraction of the protonated acids into the easily removed solvent dichloromethane. The strongly acidic nature of the zaragozic acids was then exploited by using anion-exchange chromatography. Adsorption of the reconstituted HP-20 fraction at pH 7 onto Dowex 1 resin (chloride form), washes with increasing-ionic-strength mobile phases, and elution with a high-ionic-strength methanolic solution of ammonium chloride gave rise to material of sufficient purity for final purification by HPLC.

The structures of the three zaragozic acids (Fig. 1) were proposed on the basis of mass spectral and primarily two-dimensional NMR spectroscopic evidence. Some of their physical and spectroscopic properties are summarized in Table 1. The molecular formulae were determined from high-resolution MS and ^{13}C NMR evidence [^{13}C-decoupled, "gated-decoupled," and DEPT (Distortionless Enhancement by Polarization Transfer) data]. Comparison of the NMR data clearly identified a C_9 tricarboxylic acid moiety common to all three components whose NMR resonances are listed in Table 1. These three components readily formed a trimethyl ester derivative with diazomethane. The components clearly differed in the nature of the 6-acyloxy and 1-alkyl side chains, the structures of which followed from a consideration of correlation spectroscopy (COSY), heteronuclear two-dimensional shift correlation (HETCOR), and heteronuclear multiple bond connectivity (HMBC) data. The nature of the acyloxy side chain was independently established by MS and NMR analyses of the fatty acid methyl ester component liberated in a selective base-catalyzed transesterification reaction of each trimethyl ester.

Having accounted for the two side chains in each component, the structure of the central core moiety, $C_6H_5O_5(CO_2H)_3$ containing a secondary and tertiary hydroxyl group, remained to be determined. The carbon chemical shifts C1–C7 (Table 1) indicate they are all attached to oxygen, three of which (C3, C4, and C5) were exchange-broadened. Therefore, these were placed adjacent to the observed exchange-broadened carboxyl groups as all six carbons appeared as sharp signals in spectra of the corresponding methyl esters. This evidence, and in particular the long-range ^1H–^{13}C correlation data from HMBC and selective two-dimensional heteronuclear *J*-resolved (sel*J*res) experiments (19) (Fig. 1), established the novel 2,8-dioxobicyclo[3.2.1]-octane-4,6,7-trihydroxy-3,4,5-tricarboxylic core structure

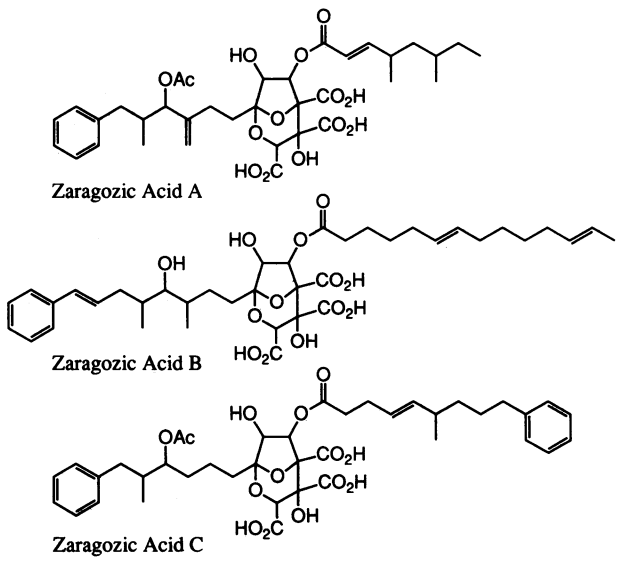

FIG. 1. Structures of the zaragozic acids.

Table 1. Physical and spectroscopic properties of zaragozic acids A, B, and C

	Zaragozic acid		
	A	B	C
M_r	690	730	754
FAB-MS, *m/z*	691 $[M+H]^+$	731 $[M+H]^+$	755 $[M+H]^+$
Formula	$C_{35}H_{46}O_{14}$	$C_{39}H_{54}O_{13}$	$C_{40}H_{50}O_{14}$
UV λ_{max}^{MeOH}, nm	214 (4.29)	205 (4.44)	209 (4.16)*
(log ε, $M^{-1}\cdot cm^{-1}$)		251 (4.32)	259 (2.81)*
$[\alpha]_D^{25}$ (MeOH)†	+37° (*c*1.29)	+5.9° (*c*0.27)	+9.6° (*c*0.29)*
^{13}C NMR (75 MHz, $C^2H_3O^2H$)‡			
C1	106.9 s	107.5 s	107.2 s
C3	76.8 d	76.7 d	76.6 d
C4	75.8 s	75.7 s	75.6 s
C5	91.3 s	91.1 s	90.9 s
C6	81.3 d	81.0 d	81.1 d
C7	82.7 d	82.0 d	82.1 d
C8	170.3 s	170.3 s	170.1 s
C9	172.7 s	172.6 s	172.4 s
C10	168.7 s	168.6 s	168.5 s
^1H NMR (300 MHz, $C^2H_3O^2H$)‡			
H-3	5.26 (s)	5.25 (s)	5.24 (s)
H-6	6.30 (d, J = 2)	6.27 (d, J = 2)	6.23 (d, J = 2)
H-7	4.03 (d, J = 2)	4.06 (d, J = 2)	4.03 (d, J = 2)

*In EtOH.
†Specific optical rotation expressed in radians, and *c* is concentration in g/100 ml.
‡NMR data were recorded on a Varian XL300 NMR spectrometer with the solvent peak at 49.0 ppm (^{13}C) and δ 3.30 (^1H) as internal reference. Multiplicities are indicated by s (singlet) and d (doublet).

common to all three components. Attachment of the alkyl and acyloxy chains at C1 and C6, respectively, followed readily from the long-range ^1H–^{13}C HMBC and heteronuclear two-dimensional shift correlation data (Fig. 2).

Inhibition of Squalene Synthase. Initial studies on inhibition of squalene synthase by the zaragozic acids with 5 μM *FPP* in assays with a protein concentration of 110 μg/ml showed that they inhibited squalene synthase with an $IC_{50} \approx 5$ nM. When the protein concentration was varied, the IC_{50} was found to be directly proportional to the protein concentration in the assay (data not shown). Inhibition by the zaragozic acids became independent of protein concentration only when the microsomal protein concentration was decreased by a factor of 50 to a value of 2.2 μg/ml. This sort of kinetic behavior would be expected with very potent reversible inhibitors when the enzyme concentration is relatively high compared with the K_i and most of the inhibitor binds to the enzyme. At reduced enzyme concentrations, most of the inhibitor would be free, a basic assumption in most forms of kinetic equations involving inhibitors. The kinetics of the inhibition of squalene synthase were studied under the conditions of reduced enzyme concentration. The three com-

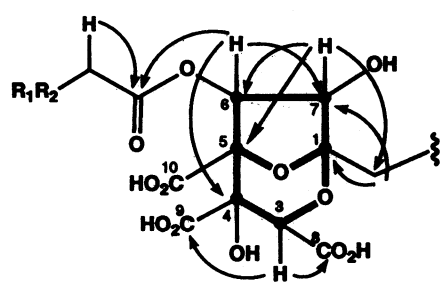

FIG. 2. Long-range ^1H–^{13}C correlations of core structure of the zaragozic acids from HMBC and selective two-dimensional heteronuclear *J*-resolved (19) experiments determined on Bruker AM-400 and Varian Unity 500 NMR spectrometers.

pounds were found to be picomolar competitive inhibitors of squalene synthase when *FPP* was the variable substrate (data not shown) and NADPH was at a saturating concentration of 1.0 mM. Under these conditions, the apparent K_m for *FPP* was 0.81 μM and the apparent K_i values were 78 pM, 29 pM, and 45 pM for zaragozic acids A, B, and C, respectively.

Inhibition of Cholesterol Synthesis in Hep G2 Cells. The zaragozic acids were shown to inhibit the incorporation of [^{3}H]mevalonate into cholesterol in Hep G2 cells (Table 2). Each zaragozic acid gave a dose-dependent decrease in cholesterol synthesis with IC_{50} values for zaragozic acids A, B, and C observed at 6 μM, 0.6 μM, and 4 μM, respectively. Concomitant with the inhibition of cholesterol synthesis was a dose-dependent increase in incorporation of label into the *FPP* and organic acid fractions (Table 2). The effects of zaragozic acid A on the incorporation of ^{3}H from mevalonate into the components of the total nonsaponifiable fraction are shown in Fig. 3. In the presence of zaragozic acid A (Fig. 3*B*), there was a dramatic decrease in labeling of cholesterol, squalene, and lanosterol, when compared with the control (Fig. 3*A*). A new labeled peak appeared that migrated with the same retention time as farnesol. The disappearance of label from the usual nonsaponifiables was indicative of inhibition of cholesterol synthesis at a step prior to the synthesis of squalene.

Activity of Zaragozic Acid A *in Vivo*. Zaragozic acid A was shown to inhibit hepatic cholesterol synthesis in the mouse with an ED_{50} of about 0.2 mg/kg (Table 3). HPLC analysis of the nonsaponifiable fraction in both control and zaragozic acid A-treated animals demonstrated that virtually all of the label in that fraction was found in cholesterol. In contrast to Hep G2 cells, labeled farnesol was not detected in the zaragozic acid A-treated animals. Label from mevalonate accumulated in the *FPP*/organic acid fraction after treatment with zaragozic acid A. Further analysis of this fraction (data not shown) showed that the accumulation was both in farnesol plus nerolidol (both from *FPP*) as well as in the organic acids.

DISCUSSION

This report summarizes studies of the isolation, physical characterization, and biochemical and biological properties of a family of natural product-derived, picomolar competitive inhibitors of squalene synthase.

Zaragozic acids, containing the 2,8-dioxobicyclo[3.2.1.]-octane-3,4,5-tricarboxylic acid core, are a novel class of natural products. Other compounds with this core structure are unknown except as cited (5–15) in the introduction.

The zaragozic acids are very potent competitive inhibitors of squalene synthase. The squalene synthase reaction is a two-step reaction catalyzed by one polypeptide chain (3, 22) with presqualene diphosphate as the intermediate between the two steps (23, 24). There is an analogy in gross structure between presqualene diphosphate and the zaragozic acids (Fig. 4). Each contains a cyclic core with acidic functions containing three negative charges and two long hydrophobic arms also attached to the cyclic core. We hypothesize that the zaragozic acids inhibit squalene synthase in part by effectively mimicking the binding of presqualene diphosphate to the enzyme.

Modestly active inhibitors of squalene synthase have been described previously, and most are analogs of *FPP* (25–29) or are ammonium analogs of proposed carbocationic intermediates in the conversion of presqualene diphosphate to

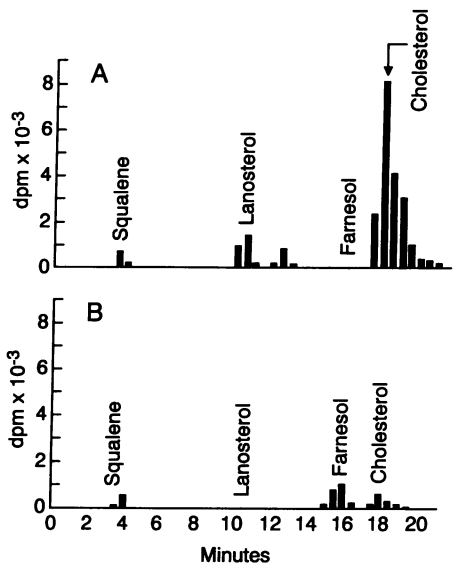

FIG. 3. HPLC analysis of the nonsaponifiables labeled with [^{3}H]mevalonate in Hep G2 cells. Hep G2 cells were incubated with no additions (*A*) or with 10 μg of zaragozic acid A per ml (*B*). Nonsaponifiables were extracted as described in text. A portion of the nonsaponifiable extract was blown to dryness with N_2, redissolved in heptane, and analyzed by HPLC by the system of Greenspan *et al.* (21). Fractions were collected every 0.5 min, and ^{3}H content of the fractions was determined. The retention times of squalene, lanosterol, farnesol, and cholesterol standards are shown.

Table 2. Effects of the zaragozic acids on [^{3}H]mevalonate metabolism in Hep G2 cells

Zaragozic acid (μg/ml)	Labeled metabolic product, dpm/mg of protein			
	Cholesterol	Farnesol	*FPP*	Acids
None	21,073	0	680	115
A (0.3)	19,257	ND	928	240
(1.0)	17,768	ND	963	386
(3.0)	13,068	ND	2931	1631
(10.0)	2,868	2058	4044	2457
B (0.3)	12,815	ND	1516	1448
(1.0)	3,822	ND	3105	1880
(3.0)	1,595	ND	3746	2692
(10.0)	1,684	1887	4033	2096
C (0.3)	19,588	ND	583	175
(1.0)	16,621	ND	1210	379
(3.0)	10,610	ND	2494	1203
(10.0)	1,697	ND	4043	3081

Hep G2 cells were incubated with the zaragozic acids and labeled with [^{3}H]mevalonate as described in text. Cells were extracted to obtain a nonsaponifiable fraction, a *FPP* fraction, and an organic acid fraction as described. Labeling of cholesterol was determined by TLC of the nonsaponifiable fraction (20). Farnesol in the nonsaponifiable fraction was determined in limited samples by HPLC analysis as in Fig. 2. ND, not determined.

Table 3. Inhibition (Inh.) of cholesterol synthesis in mice by zaragozic acid A

Zaragozic acid A, mg/kg	Cholesterol		*FPP* and acids, dpm × 10^{-3} per liver
	dpm × 10^{-3} per liver	% inh.	
0	87.6 ± 18.9	0	3.99 ± 0.78
0.1	82.2 ± 27.7	6	6.80 ± 1.10
0.3	16.0 ± 3.8	82	18.4 ± 3.2
1.0	5.9 ± 0.3	93	24.7 ± 1.3
3.0	7.0 ± 1.6	91	45.1 ± 7.0
10.0	4.7 ± 0.5	95	28.4 ± 3.3

Mice (n = 3) were treated subcutaneously with zaragozic acid A at the appropriate dose, treated with [^{3}H]mevalonate and sacrificed. Livers were removed, saponified, and extracted as described in text. The values are the means ± the SEM.

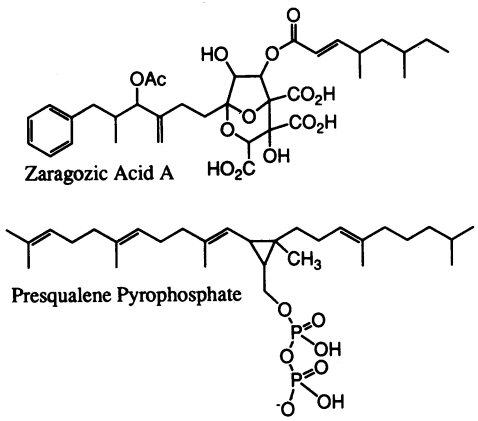

FIG. 4. Structures of zaragozic acid A (*Upper*) and of presqualene diphosphate (*Lower*).

squalene (30, 31). One potent exception to this was a farnesyl phosphinylphosphonate ether analog (29), which was a competitive inhibitor of rat liver squalene synthase with a K_i of 37 nM. Several phosphinylformate analogs of *FPP* showed micromolar activity in blocking cholesterol synthesis in freshly isolated rat hepatocytes and in human skin fibroblasts (28). The zaragozic acids are 10^3- to 10^6-fold more potent as enzyme inhibitors than these previously described compounds.

The zaragozic acids inhibit cholesterol synthesis in Hep G2 cells and *in vivo* in mice and in rats (unpublished observations). At high concentrations, this inhibition was 95% or greater in each system. This demonstrates that a squalene synthase inhibitor can serve as an effective inhibitor of cholesterol synthesis both in cells and *in vivo*.

Inhibition of squalene synthase in cells and *in vivo* was characterized by a diminishing of incorporation of mevalonate in all of the nonsaponifiables (except farnesol) and an accumulation of label in *FPP*, farnesol (observed in cell culture only), and in the organic acid fraction. Mevalonate is not normally well metabolized to organic acids as shown by the low incorporation of ^3H from mevalonate into the organic acid fraction in controls. Gonzalez-Pacanowska *et al.* (4) have shown in insect cells and in rat liver homogenates that farnesol was metabolized to farnesoic acid and then oxidized at the ω carbon to produce a 15-carbon dicarboxylic acid. Our studies have shown that the organic acid fractions from zaragozic acid-treated cells or animals contain [^3H]farnesoic acid and the 15-carbon dicarboxylic acid derivative of farnesoic acid (4). The data suggest that the diversion of mevalonate into the farnesol–farnesoic acid–dicarboxylic acid pathway described by Gonzalez-Pacanowska *et al.* (4) is one of the major metabolic consequences of inhibition of squalene synthase by the zaragozic acids. Dicarboxylic acids derived from geraniol are readily excreted in urine (32–34). It is likely that urinary excretion as the dicarboxylic acids derived from farnesol is the primary fate for the mevalonate diverted from cholesterol synthesis by a zaragozic acid.

The extraordinary potency of the zaragozic acids as inhibitors of squalene synthase and the excellent potency observed in blocking cholesterol synthesis in mice and in rats indicate the potential of the zaragozic acids as a novel class of therapeutic agents for the treatment of hypercholesterolemia.

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