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The Isolation and Structure Elucidation of Zaragozic Acid C, a Novel Potent Squalene Synthase Inhibitor.

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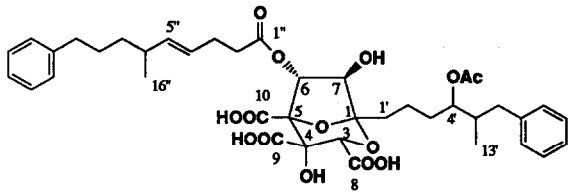
Abstract: The novel zaragozic acid C (1) has been isolated as a potent inhibitor of squalene synthase. It was found to be a competitive inhibitor of rat liver squalene synthase with an apparent K_i of 45 ± 15 pM, and a broad spectrum antifungal agent against both yeast and filamentous fungi.

The search for new pharmacological agents to treat hypercholesterolemia has been a long standing one. An elevated cholesterol level is known to be one of the prime risk factors for arteriosclerosis. Existing therapies include the use of bile acid sequestrants or cholesterol biosynthesis inhibitors. Inhibitors of the enzyme HMG-CoA reductase, such as lovastatin,[1] are effective therapeutic agents. Squalene synthase is the enzyme that catalyzes the dimerization of farnesyl pyrophosphate to squalene, the first committed step to cholesterol of the isoprenoid biosynthetic pathway. It is thus a potential target at which to potentially block cholesterol biosynthesis. Substrate analogs of farnesyl pyrophosphate have been prepared by synthesis[2] and found to be inhibitors of this enzyme. The zaragozic acids[3-6] are the first natural products found to be potent inhibitors of squalene synthase. We would like to report the isolation and structure elucidation of zaragozic acid C (**1**).

RESULTS AND DISCUSSION

A screening program designed to find inhibitors of squalene synthase uncovered an interesting culture, identified as the fungus *Leptodontium elatius*. The culture was isolated from a wood sample taken from the Joyce Kilmer Forest in North Carolina. The methanol extract of a fermentation of this culture had strong inhibitory properties in our assays.[3] The extract of a solid substrate fermentation was loaded onto an anion exchange column. Elution with a high salt solution, followed by preparative HPLC afforded pure **1**.

From the first ^{1}H NMR of **1**, it was clear that the compound was structurally related to zaragozic acids A (2) and B (3).[3] As shown in Table 1, narrow doublet resonances at δ 4.03 (H-7) and 6.23 (H-6), together with a singlet at δ 5.24 (H-3) constitute a set of signals which are very characteristic of the "core" bicyclic portion (4) of the zaragozic acids, the novel 4,6,7-trihydroxy-2,8-dioxobicyclo[3.2.1]octane-3,4,5-tricarboxylic acid. The ^{13}C NMR also showed the presence of a characteristic set of resonances for the bicyclic skeleton of the core, in particular, the two bridge-head carbon resonances at δ 107.2 and 90.9 ppm. Thus, we concluded that zaragozic acid C differed from 2 and 3 by the presence of different side chains.



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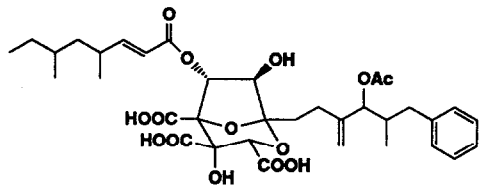

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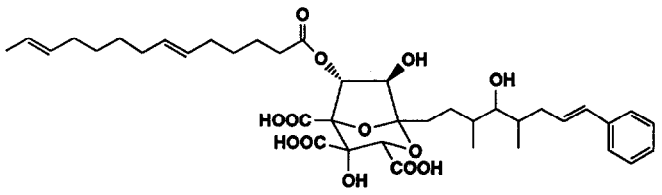

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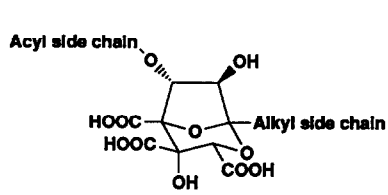

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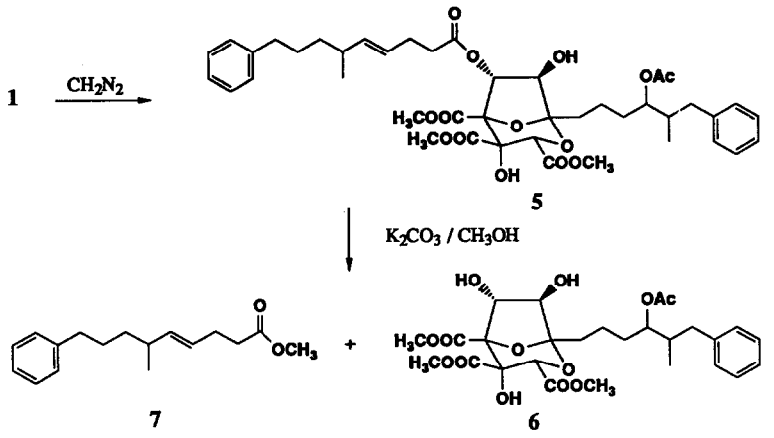
Table 1. Comparison of ^{1}H and ^{13}C NMR Shifts for 1, 2, and 3*

Carbon #	1		2		3	
1	107.2		106.9		107.5	
3	76.6	5.24, s	76.8	5.25, s	76.7	5.26 s
4	75.6		75.8		75.7	
5	90.9		91.3		91.1	
6	81.1	6.23, d, 2 Hz	81.3	6.27, d, 2 Hz	81.0	6.30, d, 2 Hz
7	82.1	4.03, d, 2 Hz	82.7	4.06, d, 2 Hz	82.0	4.03, d, 2 Hz
8	170.1		170.3		170.3	
9	172.4		172.7		172.6	
10	168.5		168.7		168.6	

*in CD_3OD, 25°C, 300 MHz

The structures of the two side chains were solved by a combination of spectroscopic and degradative studies. High resolution mass measurements (FAB) indicated a molecular formula of $C_{40}H_{50}O_{14}$ (molecular weight of 754). Both ^{1}H and ^{13}C NMR spectra (in CD_3OD) revealed the presence of two mono-substituted aromatic rings, and one olefinic double bond. This was apparent from an integral area corresponding to ten aromatic protons in the ^{1}H NMR spectrum, and from 10 aromatic / olefinic resonances (4 of which of double intensity) in the ^{13}C NMR spectrum. The olefin is further characterized by a 2 proton multiplet at δ 5.35. The only other characteristic signals are those of two methyl doublets, and of one acetate with its matching methine proton resonance at δ 4.88. Additionally, 2 methine and 9 methylene groups, are revealed by ^{13}C NMR. Because of overlapping signals in the 1.3 - 2.4 ppm region (22 protons), additional COSY data was obtained in CD_3OD / C_6D_6 mixture and in C_5D_5N. Structure **1** was proposed for zaragozic acid C.

In order to confirm the structure, the acyl side chain was cleaved. Zaragozic acid C was thus esterified to give the trimethyl ester **5**. Transesterification then led to the formation of the deacylated core **6** and the acyl side chain methyl ester **7**, which were separated by reverse phase chromatography.



^1H NMR decoupling experiments and mass spectral data allowed for direct structural elucidation of **6** and **7**. Both were fully consistent with structure **1** for zaragozic acid C. The stereochemistry of the double bond could not be defined from the CD_3OD data. However, in C_5D_5N, the two olefinic protons have distinct chemical shifts. The resonances appear at δ 5.28 as a doublet of triplets (6.6 and 15.0 Hz) and at δ 5.13 as a doublet of doublets (7.5 and 15.0 Hz); the geometry of the double bond is thus clearly *trans*. Finally, an HMBC experiment was performed at 500 MHz, allowing for a full NMR assignment (Table 2).

Table 2. Full NMR Assignments for 1.*

C #	^{13}C	^1H	mult.	C #	^{13}C	^1H	mult.
1	107.18			10'	126.91	7.14†	
3	76.62	5.24	s	13'	14.32	0.86	d, 6.5
4	75.62			15'	173.05		
5	91.00			16'	21.11	2.05	s, 3H
6	81.15	6.23	d, 2.0				
7	82.21	4.03	d, 2.0	1"	173.05		
8	170.18			2"	35.38	2.33‡	m, 2H
9	172.49			3"	28.80	2.26	m, 2H
10	168.52			4"	127.59	5.36	dt, 15.0, 6.0
1'	36.26	1.89	m, 2H	5"	138.82	5.30	dd, 15.0, 7.0
2'	20.12	1.57^	m	6"	37.83	2.07	sext, 7.0
3'	32.49	1.69	m, 2H	7"	37.61	1.28	m, 2H
4'	78.12	4.86	m	8"	30.48	1.57^	m, 2H
5'	39.67	2.02	m	9"	36.90	2.55	m, 2H
6'a	40.50	2.73	dd, 13.5, 6.0	10"	143.90		
b		2.33‡	m	11",15"	129.40 (2)	7.14†	m, 2H
7'	141.95			12",14"	129.26 (2)	7.24*	m, 2H
8',12'	130.19 (2)	7.14†	m, 2H	13"	126.62	7.14†	m
9',11'	129.28 (2)	7.24*	m, 2H	16"	21.26	0.93	d,7.0,3H

*, †, ‡, ^, overlapping signals; in CD_3OD, 25°C, 500 MHz

Zaragozic acid C was found to be a competitive inhibitor of rat liver squalene synthase with an apparent K_i of 45 ± 15 pM. The K_i value is comparable to that of the other zaragozic acids, 78 ± 15 pM for A, and 29 ± 15 pM for B. Zaragozic acid C was also effective at blocking the conversion of mevalonate to cholesterol in

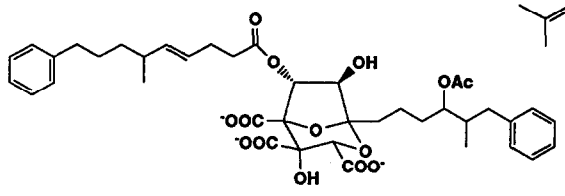
liver whole cells (HEPG-2), with an IC_{50} of 4.0 ± 0.5 μM.[3] Zaragozic acid C also inhibits yeast squalene synthase. It shows broad spectrum antifungal activity, as shown in Table 3. It is cidal against fungi species.

Table 3. Minimum Inhibitory Concentrations* of 1 in Microdilution Broth Assays.[7]

Organism	MIC (μg / mL)
Candida albicans (3)‡	8-16
Candida tropicalis	8
Candida pseudotropicalis	64
Candida parapsilosis	8
Candida guillermondii	>128
Cryptococcus neoformans (3)‡	0.25-1
Aspergillus flavus	2
Aspergillus fumigatus	2
Penicillium italicum	2

* Values are accurate within a two-fold dilution ‡ Numbers in parentheses indicate the number of strains tested.

We postulate that zaragozic acids effectively mimic the intermediate presqualene pyrophosphate (**8**) in the squalene synthase catalyzed conversion of farnesyl pyrophosphate to squalene. Both molecules possess polar triacid cores flanked by two lipophilic side chains. Because of their potency, they have the potential to become therapeutic agents against hypercholesterolemia and fungal infections.



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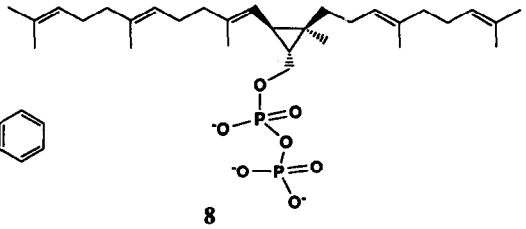

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EXPERIMENTAL

^1H NMR spectra were recorded at 300 MHz on a Varian XL-300 spectrometer, at 250 MHz on a Bruker AM-250 spectrometer, or at 500 MHz on a Varian Unity-500 spectrometer. Chemical shifts are shown in ppm relative to tetramethylsilane (TMS) at zero ppm using the solvent peak at 3.30 ppm ($C\underline{H}D_2OD$) as internal standard. ^{13}C NMR spectra were recorded at 75 MHz on a Varian XL-300 spectrometer or at 125 MHz on a Varian Unity-500 spectrometer. Chemical shifts are shown in ppm relative to TMS at zero ppm using the solvent peak at 49.0 ppm ($\underline{C}D_3OD$) as internal standard. Infrared spectra were obtained from a film on a ZnSe multiple internal reflectance (MIR) crystal, using a Perkin-Elmer Model 1750 FTIR spectrometer. Mass spectra were recorded on Finnigan-MAT Model 212 (electron impact, EI, 90 eV), MAT 90 (Fast Atom Bombardment, FAB), and TSQ70B (FAB, EI) mass spectrometers. Exact mass measurements were performed at high resolution (HR-EI) using perfluorokerosene (PFK) or perfluoropolypropylene oxide (Ultramark U1600F) as internal standards. Trimethylsilyl derivatives were prepared with a 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide-pyridine at room temperature.

Fermentation

A strain of *Leptodontium elatius* var. *elatius* (ATCC 70411), isolated from wood from the Joyce Kilmer Forest in North Carolina, was used to produce zaragozic acid C. The culture was maintained on agar slants.

Mycelium growth in liquid medium (per L: yeast extract, 4 g; malt extract, 10 g; dextrose, 4 g; agar, 20 g; pH 7) was inoculated onto a solid state fermentation medium consisting of 10 g cracked corn per non baffled 250 mL Erlenmeyer flask and 10 mL of nutrient solution (per L: ardamine PH, 0.2 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; sodium tartrate, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g). The fungal culture was incubated on this medium for 21 days at 25 °C, at which point CH_3OH (30 mL) was added to each Erlenmeyer flask.

Isolation of zaragozic acid C

The CH_3OH extracts were filtered and the solids re-extracted with 75% CH_3OH / H_2O. The extracts were filtered and combined to give 4800 mL of aqueous CH_3OH extract. This solution was loaded onto a Dowex-1X2 column (500 mL resin) at a rate of 20 mL/min. The column was washed with 50% CH_3OH / H_2O (300 mL), followed by 90% CH_3OH / H_2O (500 mL). The column was eluted with 3% ammonium chloride in 90% CH_3OH / H_2O and six fractions (500 mL) were collected. The first 3 fractions were combined, diluted with H_2O (1 L), and adjusted to pH 2.5 with concentrated HCl. The acidified eluate was extracted with CH_2Cl_2 (2 x 500 mL). Evaporation of the CH_2Cl_2 extract afforded an oily residue (402 mg). The residue was dissolved in CH_3OH (1.2 mL) and loaded on a preparative HPLC column (Dynamax 60A, 8 μm C8, 21.6 x 250 mm with guard column). The column was eluted with 72% CH_3CN / 28% dil. H_3PO_4 (0.1% in H_2O) with a 10 mL/min flow rate. Collecting 5 mL fractions, the desired compound eluted in fractions 29-34. Fractions 29-34 were combined and EtOAc (30 mL) was added. The organic layer was washed with H_2O (5 mL), and evaporated to give zaragozic acid C (**1**) as a white powder (92 mg, 77 % yield from the crude extract). $[\alpha]^{20}_D$ + 9.6 ° (EtOH, c=0.29). UV (EtOH), λ_{max}nm (log ε): 209 (4.16), 259 (2.81). IR (ZnSe film, ν cm^{-1}) : 3200 br, 2936, 1733, 1496, 1454, 1437, 1375, 1250, 1180, 1148, 1026, 972, 898, 831, 746, 700. HRMS: 754.3154, calcd for $C_{40}H_{50}O_{14}$ 754.3198.

Esterification (1 to 5)

To 5 mg of **1** in EtOAc (5 mL) was added 2 mL of freshly distilled CH_2N_2 in Et_2O (ca 0.5 M). After 15 min at 5 °C, the solvent was removed to afford **5** as an oil. $C_{43}H_{56}O_{14}$; FAB-MS : m/z 929 $[M+Cs]^{+\cdot}$ IR (ZnSe film, ν cm^{-1}) : 3200 br, 2917, 2848, 1738, 1603, 1441, 1371, 1246, 1149, 1124, 1030, 968, 748, 702 cm^{-1}. ^1H NMR (CD_3OD): 7.22 (m, 6H), 7.15 (m, 4H), 6.16 (d, 1.9), 5.32 (m, 2H), 5.24 (s), 4.9 (m), 4.00 (d, 1.9), 3.81 (s, 3H), 3.70(s, 3H), 3.68 (s, 3H), 2.73 (dd, 13.3, 5.7 Hz), 2.56 (dt, 2.5, 7.6, 2H), 2.35 (m, 3H), 2.25 (m, 2H), 2.08 (m), 2.05 (s, 3H), 2.01 (m), 1.88 (br t, 7.4, 2H), 1.68 (m, 2H), 1.56 (m, 3H), 1.29 (m, 2H), 0.94 (d, 6.8, 3H), 0.86 (d, 6.8, 3H) ppm. ^{13}C NMR (CD_3OD) : 173.04, 172.85, 171.16, 168.75, 167.39, 143.94, 141.98, 138.96, 130.21 (2x), 129.43 (2x), 129.30 (4x), 127.49, 126.96, 126.66, 107.49, 91.12, 81.89, 81.17, 78.04, 76.83, 76.20, 53.62, 53.03, 52.77, 40.52, 39.75, 37.87, 37.61, 36.93, 36.09, 35.19, 32.46, 30.53, 28.78, 21.27, 21.13, 20.13, 14.35.

Degradation (5 to 6 and 7)

To 5 mg of **5** was added 5 mL of 0.2% anhydrous K_2CO_3 in CH_3OH . After 30 min, 15 uL of 1 N HCl was added. The solution was diluted with H_2O (5 mL) and passed through a 1 mL column bed of C18 resin (LiChroprep). The column was eluted with 75 % CH_3OH / H_2O (3 mL), followed by CH_3OH (3 mL). The 75 % eluate was evaporated to afford **6**. The CH_3OH eluate was evaporated to afford **7**.

(6): $C_{27}H_{36}O_{13}$; HREIMS: 571.2264, calcd for $C_{27}H_{33}O_{13}D_3$ 571.2344. IR (ZnSe film, ν cm^{-1}) : 2928, 2855, 1741, 1604, 1497, 1454, 1437, 1363, 1250, 1170, 1031, 971, 749, 700. ^1H NMR (CD_3OD) : 7.25 (m, 2H), 7.15 (m, 3H), 5.15 (s), 5.01 (d, 2.0), 4.9 (m), 4.04 (d, 2.1), 3.84 (s, 3H), 3.72 (s, 3H), 3.70 (s, 3H), 2.75 (dd, 13.3, 5.4 Hz), 2.37 (dd, 13.2, 9.1 Hz), 2.07 (s, 3H), 2.04 (m), 1.89 (m, 2H), 1.70 (br pent, 6.7 Hz, 2H), 1.59 (m, 2H), 0.88 (d, 6.8, 3H).

(7): $C_{17}H_{24}O_2$; EIMS : m/z 260 $[M]^+$; FAB : m/z 393 $[M+Cs]^+$. IR (ZnSe film, ν cm^{-1}): 2928, 2855, 1741, 1604, 1497, 1454, 1437, 1363, 1250, 1170, 1031, 971, 749, 700. ^1H NMR (CD_3OD) : 7.25 (m, 2H), 7.15 (m, 3H), 5.38 (dt, 15.3, 6.0 Hz), 5.29 (dd, 15.3, 6.8 Hz), 3.60 (s, 3H), 2.55 (m, 2H), 2.37 (m, 2H), 2.28 (m, 2H), 2.08 (hept, 6.8 Hz), 1.57 (m, 2H), 1.30 (m, 2H), 0.95 (d, 6.8, 3H). ^{13}C NMR (CD_3OD) : 175.42, 143.96, 138.83, 129.42 (2x), 129,26 (2x), 127.79, 126.65, 51.98, 37.98, 37.70, 36.97, 35.09, 30.55, 28.99, 21.41.

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