Novel Zaragozic Acids from *Leptodontidium elatius*

Claude Dufresne,* E. Tracy Turner Jones, Mary Nallin Omstead, James D. Bergstrom, and Kenneth E. Wilson

Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065

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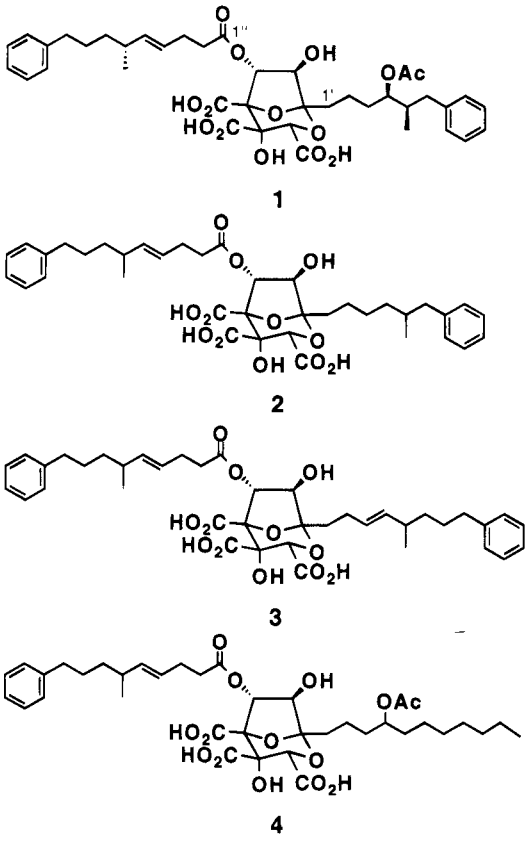
Novel zaragozic acids, potent inhibitors of squalene synthase, have been isolated from a fermentation that produces zaragozic acid C (**1**). Zaragozic acid F (**4**) represents the first zaragozic acid possessing a non-aromatic alkyl side chain (at position 1).

In our search for new cholesterol lowering drugs, we have discovered the zaragozic acids, potent inhibitors of squalene synthase.[1] Squalene synthase is the enzyme responsible for the dimerization of farnesyl pyrophosphate to squalene, the first committed step in the isoprenoid biosynthetic pathway, to the biosynthesis of cholesterol. The zaragozic acids are characterized by the novel 2,8-dioxobicyclo[3.2.1]octane-3,4,5-tricarboxylic acid ring system. They differ by variations in the side chains. For example, zaragozic acid C (**1**) competitively inhibits rat squalene synthase with an apparent K_i of 45 pM.[2,3] In order to expand our understanding of structure–activity relationships for this class of compounds, we sought novel zaragozic acids by isolating minor metabolites co-produced with zaragozic acid C, as these might include compounds unaccessible by synthetic route.[4,5]

In a process similar to that previously described,[2] the aqueous acetone extract (ca. 10 L) of the fermentation was adsorbed onto a bed of anion exchange resin (BioRad AG4-X4, formate cycle, pH 4.5). After washing the column with 60 mM HCO_2Na (pH 4.5), zaragozic acids eluted with 0.2 N H_2SO_4 in CH_3CN/H_2O (60:40). Repetitive preparative reversed-phase HPLC (Dynamax C8, CH_3CN/0.1% H_3PO_4 in H_2O, 70:30) then afforded **1** (160 mg) along with minor components: desacetoxyzaragozic acid C (**2**, 6.7 mg), zaragozic acid E (**3**, 2.6 mg), and zaragozic acid F (**4**, 1.1 mg). They inhibit squalene synthase activity with an IC_{50} of 0.3, 0.2, 4, and 2 nM, respectively.

The structure of the minor components were determined by analogy to that of **1**, using 2D NMR and mass spectra. It was clear from the first NMR spectra of each of those minors that they were structurally related to the zaragozic acids. As shown in Table 1, the chemical shifts for ^1H and ^{13}C resonances of zaragozic acids (A, B, and C) are remarkably constant, in spite of significantly different side chains, and are thus very characteristic of the bicyclic core.[2,6]

The ^1H-NMR spectrum of **2** is very similar to that of zaragozic acid C. The main difference is the absence of the acetate singlet at 2.05 ppm and of the corresponding carbinol proton at 4.88 ppm, suggesting the loss of the acetoxy group. This situation is confirmed from the ^{13}C-NMR spectrum, which shows a new methylene signal at 36.63 ppm together with the loss of the C-4′ methine signal of **1** at 78.12 ppm. Appropriate downfield shifts of the neighboring carbons account for the remaining carbons of the alkyl side chain. Negative ion fabms data for **2** gave a molecular ion at m/z 695 $[M - H]^-$ corresponding to a mol wt of 696 for 4′-desacetoxyzaragozic acid C, as concluded by NMR analysis.



The ^1H NMR of **3** at first appeared very simple. All of the resonances attributed to the acyl side chain integrate for twice the indicated number of protons, with the exception of the signals for C-2″. This suggests that both side chains are the same. The ^{13}C NMR shows distinct but very proximate resonances for each set of signals corresponding to each of the two side chains. COSY data confirmed the structure of the side chains. Negative ion FABMS data for **3** gave a MW of 722, corresponding to the structure shown.

From the ^1H NMR of **4**, it was immediately apparent that this compound was significantly different from all known zaragozic acids. Only one aromatic ring was present, along with all of the remaining signals attributed to the acyl side chain of zaragozic acid C. What remained were resonances consistent for an aliphatic alkyl chain. The ^{13}C NMR confirmed the identity of the two side chains. The placement of the acetoxy group at position 4′ was initially made through comparisons with model compounds. The structure of **4** (neg ion

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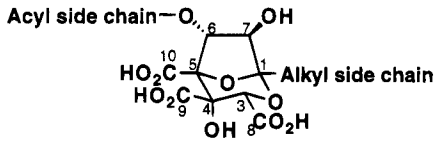
Table 1. Average ^{1}H- and ^{13}C-NMR Shifts (in CD_3OD; 25 °C) Observed for Zaragozic Acids A, B and C



carbon no.	^{13}C	^{1}H
1	107.2 ± 0.3	
3	76.7 ± 0.1	5.25 ± 0.01, s
4	75.7 ± 0.1	
5	91.1 ± 0.2	
6	81.1 ± 0.2	6.26 ± 0.04, d (2 Hz)
7	82.3 ± 0.4	4.04 ± 0.02, d (2 Hz)
8	170.2 ± 0.1	
9	172.6 ± 0.1	
10	168.6 ± 0.2	

FABMS gave a mol wt of 734) was ultimately confirmed by HMBC (optimized for 7 Hz couplings), as shown in Figure 1.

Zaragozic acid F (**4**) represents the first example of a zaragozic acid possessing a non-aromatic alkyl side chain ("alkyl" side chain refers to position 1; "acyl" side chain refers to position 6). Its discovery is significant in that the biosynthesis of zaragozic acids A and C (and presumably B) starts with benzoic acid (derived from phenylalanine.[7] The structure of **4** implies that it is biosynthesized from acetate as a starter unit. Furthermore, it shows that a terminal phenyl group is not necessary to maintain squalene synthase inhibiting activity.

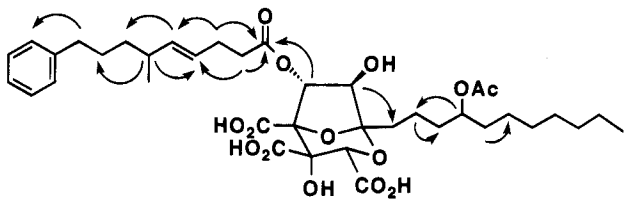


Figure 1. Selected HMBC correlations for **4**.

Experimental Section

General Experimental Procedures. ^{1}H- and ^{13}C-NMR spectra were recorded at 25 °C on a Varian Unity 500 spectrometer. ^{1}H chemical shifts are shown in ppm relative to Me_4Si at 0 ppm using the residual solvent peak at 3.30 ppm (CHD_2OD) as internal standard. ^{13}C chemical shifts are shown in ppm relative to TMS at 0 ppm using the residual solvent peak at 49.0 ppm (CD_2OD) as internal standard. IR spectra were obtained from a film on a ZnSe multiple internal reflectance crystal, using a Perkin-Elmer Model 1750 FTIR spectrometer. MS were recorded on Finnigan MAT 90 mass spectrometer. Optical rotations were measured on a Perkin-Elmer Model 241 polarimeter.

Fermentation. A strain of *Leptodontium elatius* (ATCC 70411) (Hyphomycetes) was cultured as previ-

Table 2. Full NMR assignments for **2**, **3**, and **4** (500 MHz, CD_3OD, 25 °C; δ, mult, J in Hz)

	2		**3**		**4**	
carbon no.	^{13}C	^{1}H mult	^{13}C	^{1}H mult	^{13}C	^{1}H mult
1	107.38		107.07		107.18	
3	76.63	5.24, s	76.64	5.24, s	76.73	5.24, s
4	75.65		75.63		75.65	
5	90.98		91.01		91.00	
6	81.24	6.22, d, 2.0	81.22	6.22, d, 2.0	81.22	6.22, d, 2.0
7	82.01	4.03, d, 2.0	82.14	4.03, d, 2.0	82.27	4.03, d, 2.0
8	170.24		170.22		170.38	
9	172.53		172.48		172.50	
10	168.56		168.54		168.73	
1′	36.23	1.88, m, 2H	36.75	1.94, t, 8.5, 2H	36.47	1.88, m, 2H
2′	24.04	1.5–1.6[b]	26.98	2.27,[a] m, 2H	19.74	1.58,[a] m, 2H
3′	28.42	1.5–1.6[b]	129.26	5.43, dt, 15.0, 6.5	35.31	1.55,[a] m, 2H
4′	36.63	1.5–1.6[b]	137.69	5.31, dd, 15.0, 7.5	75.69	4.88, m
5′	37.65	1.73, oct, 6.0	37.80	2.07, sext, 7.0	35.19	1.55,[a] m, 2H
6′a	44.65	2.62, dd, 13.0, 5.5	37.95	1.28,[d] m, 2H	26.48	1.3
		2.34[a]				
7′	142.74		30.55	1.57,[b] m, 2H	30.57	1.3
8′	130.21	7.12, m	36.98	2.56,[c] m, 2H	30.36	1.3
9′	129.10	7.24, m	143.98		32.92	1.3
10′	126.64	7.12, m	129.41	7.12, m	23.70	1.3
11′	129.10	7.24, m	129.25	7.24, m	14.42	0.89, t, 6.9, 3H
12′	130.21	7.12, m	126.61	7.12, m	173.18	
13′	19.82	0.83, d, 6.5	129.25	7.24, m	21.21	2.02, s, 3H
14′			129.41	7.12, m		
15′			21.41	0.94, d, 6.0, 3H		
1″	173.08		173.11		173.05	
2″	35.37	2.34,[a] m, 2H	35.37	2.33, m, 2H	35.39	2.34, m, 2H
3″	28.80	2.26, m, 2H	28.79	2.27,[a] m, 2H	28.82	2.26, m, 2H
4″	127.61	5.36, dt, 15.0, 6.5	127.62	5.36, dt, 15.0, 6.5	127.64	5.37, dt, 15.1, 6.0
5″	138.84	5.30, dt, 15.0, 7.5	138.86	5.30, dd, 15.0, 7.5	138.86	5.31, dd, 15.1, 7.5
6″	37.84	2.06, sext, 7.0	37.84	2.07, sext, 7.0	37.85	2.07, sext, 6.9
7″	37.62	1.28, m, 2H	37.62	1.28,[d] m, 2H	37.63	1.28, m, 2H
8″	30.50	1.5–1.6[b]	30.50	1.57,[b] m, 2H	30.51	1.54,[a] m, 2H
9″	36.92	2.56, m, 2H	36.92	2.56,[c] m, 2H	36.93	2.56, m, 2H
10″	143.92		143.93		143.93	
11′,15″	129.42	7.12, m, 2H	129.42	7.12, m, 2H	129.43	7.12, m, 2H
12″,14″	129.27	7.24, m, 2H	129.27	7.24, m, 2H	129.27	7.23, m, 2H
13″	126.64	7.12, m	126.63	7.12, m	126.65	7.12, m
16″	21.25	0.93, d, 7.0, 3 H	21.26	0.93, d, 7.0, 3H	21.26	0.93, d, 7.0, 3H

[a–d] Overlapping signals.

ously described, by inoculating 256 production flasks (250 mL).[2] After 22 days of static incubation at 25 °C, acetone (50 mL) was added to each flask. The growth was broken apart, and the flasks were placed on a shaker and agitated for 30 min. The contents of the flasks were pooled and filtered to yield 12.2 L of acetone extract.

Isolation. A portion of the extract (9.5 L) was concentrated under reduced pressure to 4.8 L. The concentrated aqueous extract was then loaded onto an ion exchange column (125 mL resin bed; BioRad AG4-X4; formate cycle; pH 4.5) using a flow rate of ca. 10–15 mL/min. The column was then washed with 1 L of 60 mM sodium formate in MeCN/H_2O (60:40, pH 4.5). The column was subsequently eluted with 0.2 N H_2SO_4 in CH_3CN/H_2O (60:40), collecting 250-mL fractions. Fractions 3–6 were combined and extracted with 1 liter EtOAc. The EtOAc layer was separated, dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure to yield a dark residue (784 mg). The residue was then dissolved in 4.7 mL of $CH_3OH/CH_3CN/H_2O$ (2:2:0.4) to give solution A. A 2-mL aliquot of solution A was injected on a prep. HPLC column. Using a Dynamax C8 column (8 μm; 60Å, 21.4 × 250 mm with guard column) eluting at 10 mL/min [75% CH_3CN/25% (0.1% H_3PO_4 in H_2O); pH 2.5], fractions were collected at 0.5-min intervals. Fractions 41–57 were combined. The remaining 2.7 mL of solution A were injected on the prep. HPLC, as before, and fractions 36–58 were combined. The combined fractions from both runs were combined and extracted with an equal volume of EtOAc. The EtOAc layer was separated, dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure to yield a residue. The residue was dissolved in CH_3OH (0.5 mL) and injected on a semi-prep. HPLC column. Using a Dynamax C8 column (8 μm; 60Å, 10 × 250 mm with guard column) eluting at 4 mL/min (70% CH_3CN/30% 0.1% H_3PO_4 in H_2O; pH 2.5), fractions were collected at 1-min intervals. Fraction 15 was extracted with an equal volume of EtOAc, and the EtOAc layer was separated, dried over anhydrous Na_2SO_4, and evaporated to dryness to yield desacetoxyzaragozic acid C (**2**) (6.7 mg). Fraction 20 similarly yielded zaragozic acid F (**4**) (1.1 mg), and fraction 22, zaragozic acid E (**3**) (2.6 mg).

Using analytical HPLC (Dynamax C8, 8 μm, 60Å, 4.6 × 250 mm with guard column, 26°) eluting at 1.0 mL/min with 70% CH_3CN/30% (0.1% H_3PO_4 in H_2O), the following retention times were observed for **2**, **4**, and **3**, respectively: 12.2 min, 15.5 min, and 17.2 min.

Compound **2**. Negative ion FABMS found 695 (M − H; MW 696 for $C_{38}H_{48}O_{12}$); FTIR: 2926, 2852, 1728, 1638, 1252, 1139, 975 cm^{-1}; λ_{max} (MeOH) nm (log ϵ): 208 (4.0); $[\alpha]^{25}D = +0.3$ (c = 1.5, MeOH).

Compound **3**. Negative ion FABMS found 711 (M − H; MW 712 for $C_{40}H_{40}O_{12}$); FTIR: 2925, 2853, 1733, 1640, 1250, 1144, 995 cm^{-1}; λ_{max} (MeOH) nm (log ϵ): 209 (4.1); $[\alpha]^{25}D = -2$ (c = 0.6, MeOH).

Compound **4**. Negative ion FABMS found 695 (M − H; MW 696 for $C_{38}H_{48}O_{12}$); FTIR: 2986, 1739, 1640, 1257, 1145, 978 cm^{-1}; λ_{max} (MeOH) nm (log ϵ): 208 (3.8); $[\alpha]^{25}D = +3$ (c = 0.3, MeOH).

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