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ZARAGOZIC ACIDS D AND D_2: POTENT INHIBITORS OF SQUALENE SYNTHASE AND OF RAS FARNESYL-PROTEIN TRANSFERASE

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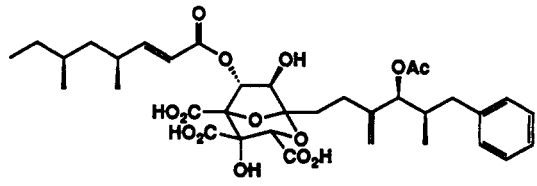
ABSTRACT.—Two new zaragozic acids, D and D_2, have been isolated from the keratinophilic fungus *Amauroascus niger*. Zaragozic acids D [**4**] and D_2 [**5**] are related to the previously described zaragozic acids A [**1**], B [**2**], and C [**3**] and are potent inhibitors of squalene synthase. Furthermore, all the zaragozic acids (A, B, C, D, and D_2) are also active against farnesyl transferase. Zaragozic acids D and D_2 inhibit farnesyl transferase with IC_{50} values of 100 nM, while zaragozic acids A and B are less potent.

The treatment of hypercholesterolemia with pharmacological agents reduces the risk of developing arteriosclerosis. Several therapies such as bile acid sequestrants or cholesterol biosynthesis inhibitors are available. Lovastatin is one such agent that represses cholesterol biosynthesis by inhibiting the enzyme HMG-CoA reductase (1). Other enzymes involved in cholesterol biosynthesis can also be targeted for inhibition. In the isoprenoid biosynthetic pathway, the first committed step to the biosynthesis of cholesterol involves the dimerization of farnesyl pyrophosphate to squalene. This step is catalyzed by squalene synthase and is a potential drug target. Substrate analogues of farnesyl pyrophosphate have been prepared by synthesis (2–5) and found to be inhibitors of this enzyme. The search for natural product inhibitors of squalene synthase has resulted in the discovery of the zaragozic acids (6–11). Zaragozic acids A [**1**] (6, 8, 10, 11), B [**2**] (6, 9, 10), and C [**3**] (6, 7) are potent inhibitors of squalene synthase and most likely act by mimicking presqualene pyrophosphate (6,7). Squalestatin, a compound identical to **1**, has also recently been reported (12–15).

The *ras* oncogene has been implicated in a variety of human cancers (particularly colon and pancreatic cancer). Extensive research has focused on the biochemistry and biology of the *ras* oncogene and its protein product, Ras (16, 17). The Ras protein is normally localized in the plasma membrane, and extensive post-translational processing must occur for this localization and for the biological activity of Ras to be expressed. The first step in this process is the farnesylation of Ras by a farnesyl-protein transferase (FPTase). Farnesylation of Ha-Ras occurs in vivo on a cysteine residue at the C-terminal sequence Cys-Val-Leu-Ser (termed a CAAX box) and appears to be required for Ras membrane localization and cell transforming activity. Forms of Ras having a deletion of the CAAX box, or a Cys to Ser substitution within this sequence, are not substrates for farnesylation and do not transform cells. It is possible that interfering with Ras farnesylation might lead to the development of a useful anticancer drug (16,17). Zaragozic acid A was previously found to be an inhibitor of Ras FPTase (18).

This paper describes the discovery of zaragozic acids D and D_2, as potent inhibitors of human squalene synthase and Ras FPTase.



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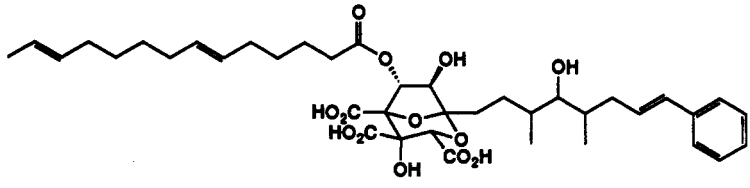

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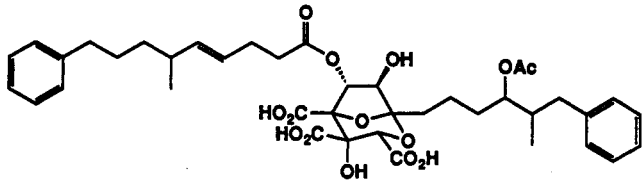

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RESULTS AND DISCUSSION

During screening for inhibitors of squalene synthase, an extract of a culture of *Amauroascus niger* Schroeter (Ascomycotina, Onygenales) was found to be active. *A. niger* has been only rarely isolated, and there are few published accounts of this fungus (19, 20). The culture was isolated from a soil sample from Spain using the technique of hair baiting (21). When sterilized hair is incubated on the surface of moistened soil samples in Petri dishes, keratinophilic fungi preferentially colonize the hair. Fungal hyphae or spores are then transferred to a secondary isolation medium and purified.

Fermentation broth extracts were purified using bioassay (squalene synthase and farnesyl transferase) guided fractionations. The extract was passed through an anion exchange column equilibrated at pH 4.5 which results in the retention of strong acids. The column was then eluted with a low pH solution. Active eluate fractions were then fractionated by reversed-phase hplc to afford zaragozic acids D and D_2.

The ^{1}H-nmr spectra of these compounds indicate that they are structurally related to the zaragozic acids. The presence of 2 Hz doublets at 4.03 and 6.24 ppm and of a singlet at 5.25 ppm is characteristic of H-7, H-6, and H-3, respectively, of the zaragozic acid core (7, 10), as shown in Figure 1.

The ^{1}H-nmr spectrum of zaragozic acid D is similar to that of zaragozic acid B, in that the presence of a styrene unit is immediately apparent. This is in agreement with

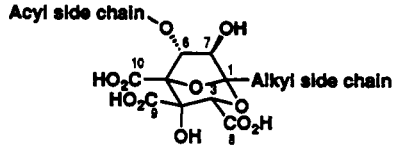
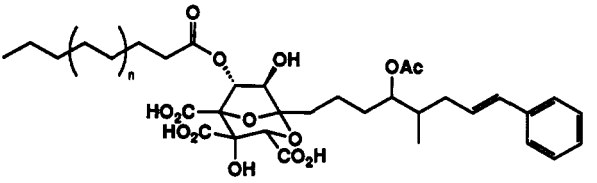


FIGURE 1. Zaragozic acid core.

the observed absorption maximum at 250 nm in the uv spectrum. The presence of an acetate group (singlet at 2.05 ppm and the corresponding methine proton at 4.88 ppm) is also evident. Additionally, only one methyl doublet is observed, as opposed to two in zaragozic acid B. By analogy, it was proposed that the alkyl side chain of zaragozic acid D was essentially the same as that of zaragozic acid B but lacking the methyl at C-3′ and with the hydroxyl group at C-4′ acetylated (as in zaragozic acids A and C). The remaining resonances in the ^{13}C nmr spectrum, not accounted for by the proposed alkyl side chain or by the core unit, are then consistent with octanoate as the acyl side chain as shown by structure **4**. The structure is consistent with the observed mol wt of 678 ($C_{34}H_{46}O_{14}$, hreims). The mass spectrum (eims) of the trimethyl ester (CH_2N_2, *m/z* 720) shows fragment ions at *m/z* 660 and 517 corresponding to $[M-HOAc]^+$ and $[M-HOAc-C_8H_{15}O_2]^+$, respectively. Loss of the acyl side chain is a characteristic mass spectral fragmentation of zaragozic acid trimethyl esters (unpublished data). The structure was further confirmed with the aid of HMQC and HMBC (7 Hz) experiments. Selected HMBC correlations are shown in Figure 2. A full nmr assignment is presented in Table 1. Assignment of C-9 and C-10 is based on comparison with other zaragozic acids. Carbon multiplicities were consistent with the assignments.



4 n=1
5 n=2

The ^1H-nmr spectrum of zaragozic acid D_2 is almost identical to that of zaragozic acid D. The only change is the larger integral area for the aliphatic protons at 1.3 ppm, suggesting two additional methylene groups. The ^{13}C-nmr spectrum is also identical to that of zaragozic acid D, with the exception of two additional resonances in the 30.0–30.5 ppm range. Since the ^{13}C resonances of the alkyl side chain of zaragozic acid D are present in the spectrum of D_2, the additional methylenes must be present in the acyl side chain. Thus a decanoate acyl side chain was proposed, as shown in **5**. This was confirmed by mass spectral analysis. A mol wt of 706 ($C_{36}H_{50}O_{14}$, hreims), corresponding to two extra methylene groups, was observed. The mass spectrum (eims) or the trimethyl ester (CH_2N_2, *m/z* 748) shows fragment ions at *m/z* 688 and 517 corresponding to $[M-HOAc]^+$ and $[M-HOAc-C_{10}H_{19}O_2]^+$, respectively. Both **4** and **5** give rise to the same fragment ion at *m/z* 517.

Since these new compounds are closely related to the previously described zaragozic acids, it was expected that they should exhibit similar biological properties. This was

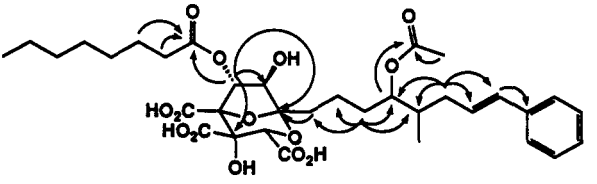


FIGURE 2. Selected HMBC correlations for zaragozic acid D [**4**].

TABLE 1. Nmr Assignments (^{1}H, 500 MHz; ^{13}C, 125 MHz) for Zaragozic Acid D [4] (CD_3OD, 25°).

Position	^{13}C	^{1}H	mult., J in Hz
Core			
1	107.21		
3	76.63	5.23	s
4	75.67		
5	90.97		
6	80.99	6.24	d, 2.0
7	82.20	4.02	d, 2.0
8	170.22		
9	172.52		
10	168.48		
Alkyl side chain			
1′	36.31	1.92[a]	m, 2H
2′	20.12	1.59[b]	m, 2H
3′	32.86	1.69	m, 2H
4′	78.03	4.95	m
5′	37.97	1.89[a]	m
6′a	38.02	2.32[c]	m
b		2.08	m
7′	129.61	6.23	dt, 16.0, 7.0
8′	132.87	6.38	d, 16.0
9′	139.12		
10′, 14′	127.04 (2)	7.34	br d, 7.5, 2H
11′, 13′	129.51 (2)	7.25	t, 7.5, 2H
12′	127.95	7.15	br t, 7.5
15′	14.64	0.97	d, 7.0, 3H
16′	173.08		
17′	21.16	2.05	s, 3H
Acyl side chain			
1″	173.64		
2″	35.04	2.28[c]	m, 2H
3″	25.86	1.59[b]	m, 2H
4″	30.15[d]	1.3	m, 2H
5″	30.07[d]	1.3	m, 2H
6″	32.68	1.3	m, 2H
7″	23.70	1.3	m, 2H
8″	14.44	0.88	t, 7.0, 3H

[a–c]Overlapping signals.
[d]Interchangeable.

examined by determining the effect of zaragozic acids D and D_2 on squalene synthase and Ras FPTase. The results are presented in Table 2.

Relative to zaragozic acids A, B, and C, zaragozic acids D and D_2 are less active against squalene synthase. In contrast, zaragozic acids C, D, and D_2 are more active against Ras FPTase than zaragozic acids A and B. Zaragozic acids D and D_2 are equally active against bovine Ras FPTase, exhibiting IC_{50} values of 100 nM. These two new compounds are approximately twofold more active than zaragozic acid A, and 10-fold more active than zaragozic acid B, which inhibit Ras FPTase with IC_{50} values of 250 nM and 1000 nM, respectively. We speculate that zaragozic acids may inhibit Ras FPTase by mimicking farnesyl pyrophosphate. Indeed, zaragozic acid A inhibits FPTase in a manner that is competitive with respect to farnesyl pyrophosphate and noncompetitive with respect to Ras (18). The tricarboxylic acid core of the zaragozic acids may mimic

TABLE 2. Effect of Zaragozic Acids A [**1**], B [**2**], C [**3**], D [**4**], and D_2 [**5**] on Squalene Synthase and Farnesyl Transferase (IC_{50} values in nM).

Assay	Compound				
	1	**2**	**3**	**4**	**5**
Rat liver squalene synthase	0.5	0.2	0.4	6	2
Bovine brain FPTase	250	1000	150	100	100

pyrophosphate while one or both of the extended hydrophobic chains may replace the isoprenoid moieties.

The zaragozic acids are much more potent inhibitors toward squalene synthase than toward Ras FPTase, even more so than indicated by the IC_{50} values. The IC_{50}values for squalene synthase were determined using a high farnesyl pyrophosphate concentration, 3 uM, while the IC_{50}'s for Ras FPTase were determined with a low farnesyl pyrophosphate concentration, 0.1 uM. The K_m of squalene synthase for farnesyl pyrophosphate is 0.8 uM, while the K_m of Ras FPTase for farnesyl pyrophosphate is about 0.05 uM (24). If they had both been tested under K_m conditions, it is apparent that zaragozic acid D, the most selective zaragozic acid toward Ras FPTase, would probably be at least 100 times more potent against squalene synthase than against Ras FPTase.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H-nmr spectra were recorded at 500 MHz on a Varian unity-500 spectrometer. Chemical shifts are shown in ppm relative to TMS at 0 ppm using the solvent peak at 3.30 ppm (CHD_2OD) as internal standard. ^{13}C-nmr spectra were recorded at 125 MHz on a Varian Unity-500 spectrometer. Chemical shifts are shown in ppm relative to TMS at 0 ppm using the solvent peak at 49.0 ppm (CD_3OD) as internal standard. Ir 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, 90 eV) mass spectrometer. Exact mass measurements were performed at high resolution using perfluorokerosene (PFK) as internal standard.

PRODUCING ORGANISM.—*A. niger*, MF 5683=ATCC 74156, was isolated from forest soil, Los Montes de Poblet, Tarragona, Spain; a representative culture has been deposited at the American Type Culture Collection, Rockville MD 20852–1776. This strain was compared with the neotype strain of *A. niger* from the American Type Culture Collection (ATCC 22339=Orr 0-315), and except for some minor differences in colony pigmentation, they were essentially identical in morphology. Both strains, MF 5683 and ATCC 22339, agreed well with published descriptions of *A. niger* (19,20).

FERMENTATION.—A 250-ml unbaffled Erlenmeyer flask with 54 ml of medium A (see Table 3) was inoculated by transferring culture growth from the preserved culture in a soil tube using a sterile cotton swab which was prewetted with sterilized H_2O. The flask was then incubated under aerobic conditions at 25° for 3 days at 220 rpm and 85% relative humidity. This seed culture was then used as an inoculum for the

TABLE 3. Fermentation Media.

Medium A	(per/liter)	Trace Element Mixture	(g/liter)	Medium B	(g/liter)
Corn steep liquor	5 g	$FeSO_4 \cdot 7H_2O$	1.0	Glycerol	75
Tomato paste	40 g	$MnSO_4 \cdot 4H_2O$	1.0	Dextrose	10
Oat flour	10 g	$CuCl_2 \cdot 2H_2O$	0.025	Ardamine pH	5
Glucose	10 g	$CaCl_2 \cdot 2H_2O$	0.1	Soybean meal	5
Trace element mixture	10 ml	H_3BO_3	0.056	Tomato paste	5
Agar	4 g	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.019	Sodium citrate	2
		$ZnSO_4 \cdot 7H_2O$	0.2	$(NH_4)_2SO_4$	2
adjust to pH 6.8		adjust to pH 7.0		adjust to pH 7.0	

production fermentation. Thirteen flasks, each containing 45 ml of medium B, were inoculated with 2 ml of the above seed culture. Flasks were incubated at 25° for 7 days at 220 rpm and 85% relative humidity. The contents of the flasks were then pooled.

ISOLATION.—A portion of the pooled broth (250 ml) was extracted with 2-butanone (250 ml). The 2-butanone extract was evaporated to dryness, and MeCN (30 ml) was added to the residue. After sonication, the MeCN solution was diluted with H_2O (20 ml) and loaded onto an ion exchange column (5 ml resin bed; BioRad AG4-X4; formate cycle; pH 4.5) using a flow rate of ca 0.5 ml/min. The column was washed with 25 ml of 60 mM sodium formate in MeCN-H_2O (60:40), pH 4.5. The column was subsequently eluted with 25 ml of 0.2 N H_2SO_4 in MeCN-H_2O (60:40). The eluate was diluted with EtOAc (50 ml) and the partitioned aqueous layer discarded. The organic layer was washed with H_2O (5 ml) and evaporated to dryness under reduced pressure. The residue was dissolved in MeOH (0.25 ml) and injected on a semi-preparative hplc column. Using a Dynamax 60 A, C8 column (8 um; 10×250 mm with guard column) eluting at 4 ml/min (65% MeCN/35% 0.1% H_3PO_4 in H_2O; pH 2.5), fractions were collected at 1.0 min intervals. Fractions 10–12 were combined and extracted with an equal volume of EtOAc. The EtOAc layer was then evaporated to dryness to yield zaragozic acid D [**4**] (5.6 mg). Fractions 17–20 similarly yielded zaragozic acid D_2 [**5**] (2.2 mg). Zaragozic acid D [**4**]: hreims found 678.2904 (678.2886 calcd for $C_{34}H_{46}O_{14}$); ir (ZnSe film) ν cm^{-1} 3450 (br), 2928, 1730, 1249, 1149, 967, 747; λ max (MeOH) 206 (4.14), 251 (4.07) nm; $[\alpha]^{25}D$ +13.7° (c=1.8, MeOH). Zaragozic acid D_2 [**5**]: hreims found 706.3194 (706.3198 calcd for $C_{36}H_{50}O_{14}$); ir (ZnSe film) ν cm^{-1} 3430 (br), 2929, 1730, 1250, 1149, 1000, 741; λ max (MeOH) 206 (4.23), 251 (4.10) nm; $[\alpha]^{25}D$ +8.3° (c=0.58, MeOH).

SQUALENE SYNTHASE ACTIVITY MEASUREMENTS.—Squalene synthase assays were performed using microsomes prepared from rat liver as described by Bergstrom *et al.* (6). The reactions were performed in 1.2 ml polypropylene tube strips. The assay mixtures consisted of 150 mM HEPES, 11 mM KF, 3 mM dithiothreitol, 5.5 mM $MgCl_2$, 0.1 μg per ml of a squalene epoxidase inhibitor (Banyu FW-439H), and microsomes at 1.2 to 2.5 μg protein per ml. After 10 min of preincubation at room temperature, the enzyme reaction was initiated with 3 μM ^{3}H-farnesyl pyrophosphate and 1 mM NADPH. The assay mixtures were incubated for 20 min at 30°. The reactions were stopped by the addition of 95% EtOH (100 μl). A suspension of Bio-Rad AG 1×8 resin (400 mesh, Cl; 1 g/ml; 100 μl) was added. Heptane (800 μl) was added to each tube strip, and the strips were vortexed for 10 minutes. An aliquot (400 μl) of the heptane layer was removed and mixed with 2.5 ml of scintillation fluid. The radioactivity was determined by liquid scintillation counting. IC_{50} values were determined by plotting the log of the concentration of the test compound versus the percentage inhibition.

FARNESYL TRANSFERASE ACTIVITY MEASUREMENTS.—Bovine farnesyl transferase was purified as described by Schaber *et al.* (22). Ras acceptor peptides (Ras-CVLS) were prepared as described by Moores *et al.* (23). Assays were performed in a final volume of 100μl containing 100 mM hepes pH 7.4, 5 mM $MgCl_2$, 5 mM dithiothreitol, 20 μM $ZnCl_2$, 100 nM ^{3}H-farnesyl diphosphate (NEN, 20 Ci/mmol), 100 nM Ras-CVLS peptide, and 2 nM farnesyl transferase at 31° for 30 min. Reactions were initiated by the addition of farnesyl transferase and were stopped with one ml of acidic EtOH (1.0 N HCl in 100% EtOH). Tubes were incubated at 37° for 30 min to facilitate hydrolysis of the ^{3}H-farnesyl pyrophosphate. Protein precipitates were collected onto filtermats using a TomTec Mach II cell harvester and counted in a LKB β-plate counter.

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