THE PREPARATION OF ZARAGOZIC ACID A ANALOGUES BY DIRECTED BIOSYNTHESIS

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Zaragozic acid A analogues are produced by an unidentified sterile fungus when it is exogenously supplied with 2-thiophenecarboxylic acid, 3-thiophenecarboxylic acid, 2-furoic acid, 2-fluorobenzoic acid, 3-fluorobenzoic acid, or 4-fluorobenzoic acid. The analogues carry 2-thiophenyl, 3-thiophenyl, 2-furyl, *o*-fluorophenyl, *m*-fluorophenyl, or *p*-fluorophenyl group, respectively, at C-6′ of the C-1 alkyl side chain replacing the phenyl group of natural zaragozic acid A. All the new analogues of zaragozic acid A possess picomolar inhibitory activity against squalene synthase *in vitro*.

Zaragozic acid A[1~3], a novel secondary metabolite with potent squalene synthase inhibitory activity, is produced by an unidentified fungus ATCC 20986. Zaragozic acid A contains a highly functionalized bicyclic core with the 6-*O*-acyl and 1-alkyl side chains. The squalestatins, which correspond to zaragozic acid A, 4′-desacetyl-zaragozic acid A and 6-*O*-desacyl-zaragozic acid A, were recently reported to be produced by a strain of the fungus *Phoma*[4,5]. Zaragozic acid A (squalestatin) is formed from 10 acetates, 4 methyls of methionines, 1 succinate, and 1 benzoic acid *via* the polyketide pathway[6,7]. The compound appears to be derived from two polyketide chains. Benzoic acid is presumably the more immediate precursor to the starter unit of the major polyketide chain containing the aromatic ring. This paper reports on production of zaragozic acid A analogs by ATCC 20986 by supplementation of producing cultures with 2-thiophene-carboxylic acid, 3-thiophene-carboxylic acid, 2-fluorobenzoic acid, 3-fluorobenzoic acid, 4-fluorobenzoic acid and 2-furoic acid. Two independent reports were recently published on production of fluorinated, 2-thienyl and 3-thienyl squalestatins by directed biosynthesis[9,10].

Materials and Methods

All organic solvents (EM Science; Gibbstown, NJ) were HPLC grade. H_2O was purified in a Millipore Milli-Q system (Bedford, MA). Solvent for NMR analysis (CD_3OD) was purchased from Merck Sharp and Dohme Isotopes. Aromatic acid analogues were purchased from Aldrich (Milwaukee, WI). ^1H NMR spectra were recorded in CD_3OD at 400 MHz at room temperature on a Varian Unity 400 NMR spectrometer, and chemical shifts were referenced to the CD_2HOD solvent peak ($\delta_H = 3.30$). FABMS measurements were obtained on a Finnigan Mat TSQ 70 instrument with glycerol as matrix.

Fermentation

The unidentified fungus ATCC 20986 was stored and maintained as frozen vegetative mycelia in seed medium containing 10% glycerol. The vegetative mycelia (2 ml) were used to inoculate a 250-ml Erlenmeyer flask containing 50 ml of autoclaved seed medium consisting of (in g/liter) tomato paste 40, corn steep liquor 5, D-glucose 10, oat flour 10, and 10 ml/liter of a trace elements solution. The pH of the seed medium was adjusted to 6.8 before autoclaving. The trace element solution is composed of (in g/liter) $FeSO_4 \cdot 7H_2O$ 1.0, $MnSO_4 \cdot 4H_2O$ 1.0, $CuCl_2 \cdot 2H_2O$ 0.025, $CaCl_2 \cdot 2H_2O$ 0.1, H_3BO_3 0.056, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.019,

and $ZnSO_4 \cdot 7H_2O$ 0.2, dissolved in 0.6 N HCl. The culture was grown on a rotary shaker for 3 days at 25°C and 225 rpm. Cells were harvested by centrifugation, washed twice with distilled water and finally resuspended in 50 ml of 20 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES) at pH 6.1 containing 3% sucrose. Five ml aliquots of this suspension were transferred to 50-ml Erlenmeyer flasks and incubated at 25°C and 225 rpm. After 24 hours incubation, benzoic acid analogues were added individually to a final concentration of 0.25, 0.5 and 1.0 mM: 2-thiophenecarboxylic acid, 3-thiophenecarboxylic acid, 2-furoic acid, 2-fluorobenzoic acid, 3-fluorobenzoic acid and 4-fluorobenzoic acid. After an additional 96 hours incubation, biosynthesis was terminated by addition of two volumes of methanol; the broths were then clarified and then examined by HPLC. Stock solutions of 2-thiophenecarboxylic acid, 3-thiophenecarboxylic acid, 2-furoic acid, 2-fluorobenzoic acid, 3-fluorobenzoic acid, and 4-fluorobenzoic acid were prepared in distilled water at pH 7.0, and sterilized by using 0.22 μm Millipore filters.

HPLC Analysis

HPLC analysis was performed using a Beckman Ultrasphere ODS column or an ES Industries Chromega-bond FD column (both 5 μm particle size; 4.6 mm inside diameter by 25 cm length) at room temperature. UV detection was at 215 nm. The solvent system was a thirty minute gradient from 30% to 90% acetonitrile in water containing 0.1% phosphoric acid (v/v); the 90% acetonitrile was held an additional 5 minutes, followed by a 9 minute equilibration in starting solvent. The chromatograms from the control (no added compounds) and the experimental broth extracts were overlaid on the same time scale to compare results; it was here that new peaks, subsequently identified as the biosynthetic analogues of zaragozic acid A, were first detected.

Isolation and Purification

Two hundred ml of whole broth from a feeding experiment with 2-thiophenecarboxylic acid was combined with an equal volume of methanol. The crude extract was filtered to remove cells, diluted with 400 ml of distilled water and applied to a water-equilibrated 15 mm × 220 mm column of HP-20 resin (Mitsubishi Chemical). After washing with water, the column was eluted with 200 ml methanol. The eluate was reduced to dryness, then dissolved in twenty ml of 60% aqueous methanol. Ten two-ml injections were made onto a Beckman preparative HPLC (9.6 mm × 250 mm) ODS column. The column was developed at 3.0 ml/minute using a 35 minute linear gradient from 40% to 80% acetonitrile in water containing 0.1% H_3PO_4 (v/v). Detection was at 215 nm. Peaks eluting at 28.9 minutes were collected and pooled. The pooled materials were diluted with four volumes of distilled water, then applied to water-equilibrated C_{18} SPE columns. After washing with five volumes of distilled water, the columns were eluted with methanol. The eluates were evaporated to dryness to yield 300 μg of the 2-thienyl analogue of zaragozic acid A (**IV**).

The whole broth (1 liter) from a feeding experiment with 3-thiophenecarboxylic acid was processed as described above to yield 500 μg of the 3-thienyl analogue of zaragozic acid A (**V**).

The whole broth (600 ml) from a feeding experiment with 2-furoic acid was processed as described above to yield 300 μg of the 2-furyl analogue of zaragozic acid A (**VI**).

Twenty ml of whole broth from a feeding experiment with 3-fluorobenzoic acid were combined with twenty ml methanol. The crude extract was filtered to remove cells, diluted with 40 ml of water and applied to a water equilibrated C_{18} Spe-ed (Applied Separations, Bethlehem, PA) cartridge. After washing with water, the cartridge was eluted with 20 ml methanol. The eluate was reduced to dryness, then dissolved in two ml of 70% MeOH - H_2O. Two one-ml injections were made onto a Chromegabond FD column (4.6 mm × 250 mm). The column was developed isocratically at 1.0 ml/minute using 43% acetonitrile in water containing 0.1% H_3PO_4 (v/v). Detection was at 215 nm. Peaks with an elution time at 29.1 minutes were collected. Each fraction was diluted with four volumes of water, then applied to water-equilibrated C_{18} SPE columns. After washing with five volumes of water, the columns were then eluted with methanol. The eluates were evaporated to dryness to yield 665 μg of the 3-fluorophenyl-zaragozic acid A (**II**).

Six hundred ml of whole broth from feeding experiments with 2- and 4-fluorobenzoic acid was processed as described above to yield 500 μg of 2-fluorophenyl-zaragozic acid A (**I**) and 4-fluoro-zaragozic acid A (**III**). The retention times of 3- and 4-fluoro-zaragozic acid A are 29.1 minutes. The retention time of 2-fluorophenyl-zaragozic acid was 26.2 minutes.

Assays of Squalene Synthase

[4-^{14}C]Farnesyl pyrophosphate was enzymatically synthesized as follows. The solvent (EtOH - 0.15 N NH_4OH, 1 : 1) was removed from 55 μCi of [4-^{14}C]isopentenyl pyrophosphate (47.9 mCi/mmol) by rotary evaporation. Six hundred microliters of 100 mM Tris, 10 mM $MgCl_2$, 4 mM dithiothreitol pH 7.5 was added and the solution was transferred to a 1.5-ml Eppendorf centrifuge tube. Geranyl pyrophosphate, 250 μl of a 20 mM solution, and 50 μl of the ammonium sulfate suspension of prenyl transferase were added to initiate the reaction. This incubation contained 5 μmol of geranyl pyrophosphate, 1.15 μmol of isopentenyl pyrophosphate, 6μmol of $MgCl_2$ and 0.18 units of prenyl transferase in a volume of 900 μl. The incubation was conducted at 37°C. During the incubation, the mix turned cloudy white as the newly formed magnesium complex of farnesyl pyrophosphate precipitated out of solution. The [4-^{14}C]farnesyl pyrophosphate was collected by centrifugation for 3 minutes at 14,000 rpm in an Eppendorf centrifuge tube, the supernatant removed, and the pellet was dissolved in 1.0 ml of 50 mM HEPES, 5 mM EDTA, pH 7.5. The yield was 50.7 μCi (92%) of [4-^{14}C] farnesyl pyrophosphate. The [4-^{14}C]farnesyl pyrophosphate was stored in aliquots at -70°C.

Inhibition of squalene synthase by zaragozic acid A analogues was evaluated as previously described[1]. Reactions were performed in 16 × 125 mm screw cap test tubes. A batch assay mix was prepared from the following solution:

		μl per assay
1.	250 mM HEPES, pH 7.5	20
2.	NaF, 110 mM	10
3.	$MgCl_2$, 55 mM	10
4.	Dithiothreitol, 30 mM	10
5.	NADPH, 10 mM	10
6.	[4-^{14}C]Farnesylpyrophosphate 47.9 μCi/μmol, and 0.025 μCi/3.0 μl	3.0
7.	H_2O	24

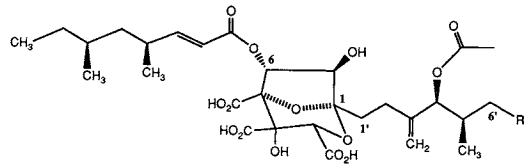
This assay mix was degassed under vacuum and flushed with nitrogen. Solutions of the squalene synthase inhibitors were prepared either in DMSO or MeOH and a 1 : 120 dilution of the microsomal protein was made with the original homogenizing buffer. For each reaction, 87 μl of the assay mix was taken with 3 μl of an inhibitor solution (DMSO or MeOH in the controls), warmed to 30°C in a water bath and then the reaction was initiated by the addition of 10 μl of the 1 : 120 dilution of microsomal protein (0.6 μg protein total in the assay). The reactions were stopped after 20 minutes by the addition of 100 μl of a 1 : 1 mix of 40% KOH with 95% EtOH. The stopped mix was heated at 65°C for 30 minutes, and cooled. Ten ml of heptane was added and the mix was vortexed. Two g of activated alumina was then added, the mix vortexed again, the alumina allowed to settle and 5 ml of the heptane layer was removed. Ten ml of scintillation fluid was added to the heptane solution and radioactivity was determined by liquid scintillation counting. Percent inhibition is calculated by the formula: $\{1\text{-}[\text{Sample} - \text{Blank}]/[\text{Control} - \text{Blank}]\} \times 100$.

Results and Discussion

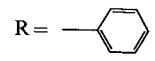
Zaragozic acid A is derived from two polyketide chains[6,7]. The six aromatic ring carbon atoms along with the adjacent benzylic carbon (C-6'), presumably the starter molecule of the major polyketide chain, are derived from benzoic acid. In this study, we have investigated the feasibility of incorporating 44 commercially available aromatic acid analogues into the zaragozic acid A molecule using a resting cell system. Six analogues were incorporated by the biosynthetic machinery of the producing culture ATCC 20986 to give zaragozic acid A analogues I through VI (Fig. 1). Detection of 2-thienyl (**IV**), 3-thienyl (**V**) and 2-furyl (**VI**) analogues was easily achieved on a C-18 reversed-phase HPLC column because of their distinctive, more polar elution characteristics compared to natural zaragozic acid A. Separation of fluoro analogues (**I**~**III**) from the natural zaragozic acid A could be achieved only by using a fluorodecyl bonded-phase column.

Negative ion FABMS of compounds **I**~**III** gave $(M-H)^-$ signals at *m/z* 707 which corresponds to an increase of 18 mass units from zaragozic acid A, consistent with the presence of a fluorine atom in

Fig. 1. Chemical structures of zaragozic acid A and directed biosynthetic analogues.



Zaragozic acid A R=



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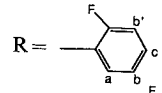

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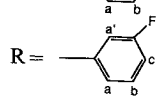

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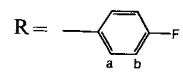

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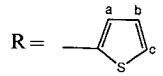

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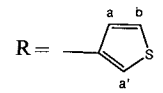

VI R=

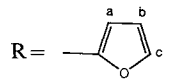

Table 1. ^{1}H NMR spectra differences between zaragozic acid A and its analogues **I**~**VI**.

Proton	I	II	III
a	7.28 (1H, td, 8.1, 6.2)	7.03 (1H, dt, 6.5, 1.0)	7.36 (1H, dd, 8.7, 5.6)
a′		6.93 (1H, ddd, 10.1, 2.5, 1.6)	
b	7.08 (1H, td, 7.5, 1.2)	7.26 (1H, td, 8.1, 6.2)	6.98 (2H, t, 8.8)
b′	6.95 (1H, ddd, 10.3, 8.2, 1.3)		
c	7.17 (1H, m)	6.85 (1H, tdd, 9.0, 2.5, 1.0)	

Proton	IV	V	VI
a	6.83 (1H, dd, 3.4, 1.2)	6.99 (1H, dd, 5.0, 1.3)	6.05 (1H, dd, 3.3, 0.9)
a′		7.04 (1H, dd, 3.0, 1.3)	6.26 (1H, dd, 3.3, 2.0)
b	6.90 (1H, dd, 5.0, 3.4)	7.29 (1H, dd, 5.0, 3.0)	6.30 (1H, dd, 2.0, 0.9)
b′			
c	7.15 (1H, dd, 5.0, 1.2)		

Only protons showing differences are presented: a, a′, b, b′, and c aromatic protons on the aromatic group at C6′ of the side chain at C_1. ^{1}H NMR was recorded at 400 MHz in CD_3OD; δ_H (multiplicity, J=Hz).

each of the molecules. The final structure identifications were based on ^{1}H NMR analysis. The key features (Table 1) were the four aromatic proton signals which could be readily assigned from their chemical shifts and very characteristic ^{19}F-^{1}H coupling constants, especially, $J_{mF} = \sim 6$ Hz[10]. All other signals in the spectra of analogues **I**~**III** closely resembled those of zaragozic acid A.

The key observations in ^{1}H NMR spectra for compounds **IV** and **V** are the absence of the typical monosubstituted phenyl proton resonances of zaragozic acid A and the presence of three novel low field protons between δ 6.8~7.3 whose chemical shifts and relatively small J_{ortho} coupling constants ($J_{ortho} < 5.0$ Hz)[10] are typical for a 2- or 3-alkylated thiophene. In other respects, the spectra closely resembled that of zaragozic acid A. The negative ion FABMS spectra of **IV** and **V** exhibit a $(M-H)^-$ signal of 695, in agreement with thiophene substitution at C-6′ of the C-1 alkyl side chain.

Appearance of an $(M-H)^-$ ion of 679 in a negative FABMS spectrum of compound **VI** signaled incorporation of the 2-furoic acid. Compound **VI** was identified as a furan substituted zaragozic acid A analogue at C-6′ based on ^{1}H NMR analysis. The key features of the ^{1}H NMR spectrum were an absence of the typical monosubstituted phenyl resonances and the presence of three novel low field protons with chemical shifts and coupling constants characteristic of a 2-furan moiety[10]. All

other signals were virtually unaffected. Unlike thiophene carboxylic acids, 3-furoic acid was not utilized by the organism; only inhibition of natural zaragozic acid A production was found.

The squalene synthase inhibition data of the directed biosynthetic analogues **I**~**VI** are summarized in Table 2. They all possess picomolar inhibitory activity against squalene synthase *in vitro*, demonstrating that the zaragozic acid A phenyl moiety can be replaced by other aromatic rings without significant loss in biological activity.

Table 2. Squalene synthase (rat liver) inhibitory activity of zaragozic acid A and its biosynthetic analogues **I**~**VI**.

Compound	IC_{50} (ng/ml)
Zaragozic acid A	0.2
I	0.4
II	0.3
III	0.2
IV	0.4
V	0.2
VI	0.1

The potential value of directed biosynthesis as a powerful tool in drug development work is illustrated in this paper. Obtaining six novel natural products by using the producing microorganism to incorporate aromatic acid analogues shows that directed biosynthesis is a useful adjunct to organic synthesis for producing novel derivatives to evaluate as therapeutic agents.

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