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Isolation, Structure Determination and Squalene Synthase Activity of L-731,120 and L-731,128, Alkyl Citrate Analogs of Zaragozic Acids A and B

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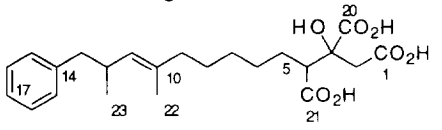
Abstract: Two new alkyl citrates, L-731,120 and L-731,128, with alkyl chains corresponding to those of zaragozic acids A and B, were isolated as minor components of large scale fungal fermentations producing zaragozic acid A and B . They are submicromolar inhibitors of squalene synthase *in vitro*.

The zaragozic acids (squalestatins) are a family of fungal natural products characterized by a bicyclic tricarboxylic acid containing core with two lipophilic side chains.[1] They are picomolar inhibitors of squalene synthase and thus have therapeutic potential as antihypercholesteremic and antifungal agents.[2] Numerous related minor components co-produced with squalestatin I have been isolated and characterized.[3] We report here the isolation, structure elucidation and biological activity of two new alkyl citrates, L-731,120 (**1**) and L-731,128 (**2**), co-produced with zaragozic acids A and B.



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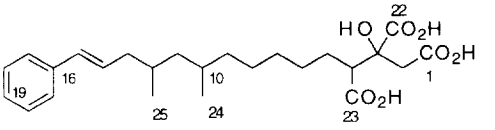

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Results and Discussion

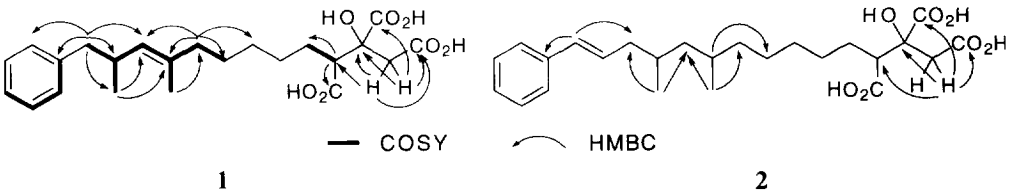
Isolation and Structure Determination of L-731,120 - L-731,120 was isolated from mycelia of a fermentation of culture MF5453 (ATCC 20986) which produced 980 mg / L of zaragozic acid A (ZA-A).[4] The collected mycelia were extracted with aqueous methanol and crude **1** obtained from the filtrate by solid phase extraction using Mitsubishi SP-207 resin. Anion exchange chromatography of the eluate on DOWEX 1x2 (Cl^-) and BioRad AG4x4 (formate) yielded a fraction highly enriched in tricarboxylic acids from which **1** was purified using repeated reverse phase HPLC.[5]

The molecular formula for **1** of $C_{23}H_{32}O_7$ was determined from HREIMS data acquired on the tetra-TMS derivative (found 708.3730, calculated 708.3729 for $C_{23}H_{32}O_7 + (C_3H_8Si)_4$) and was supported by the observation of 23 resonances in the ^{13}C NMR spectrum. ^1H and ^{13}C NMR spectra of **1** suggest the presence of a monosubstituted phenyl ring, one trisubstituted double bond and three carbonyls between 172 and 175 ppm to account for the 8 unsaturation equivalents required by the molecular formula.[6] In addition to the above structural features, the APT spectrum of **1** indicates 2 methyl groups, 7 methylenes, 2 methines and one oxygenated quaternary carbon for a total of 28 non-exchangable protons. The four exchangable protons can thus be assigned

to the tertiary alcohol and three carboxyl acids. The above data are consistent with an acyclic, tricarboxylic acid structure for **1**.

Application of ^{1}H-^{1}H COSY, HMQC, and HMBC techniques allowed assignment of the ^{1}H and ^{13}C NMR spectra of **1** (Table 1). Selected HMBC ($^nJ_{CH}$ = 7 Hz) and COSY correlations for **1** are shown in Figure 1. The resonances in the ^{1}H NMR spectrum for the isolated methylene protons on C-2 appearing at 2.65 and 3.02 ppm are characteristic of the compound and an alkyl citrate stucture. Both protons show two-bond coupling to C-1 (172.1 ppm) and C-3 (76.5 ppm). Three-bond coupling for H-2a (2.65 ppm) is observed only to C-4 (53.9 ppm) and for H-2b (3.02 ppm) only to C-20 (174.6 ppm). Extensive long range ^{1}H-^{13}C correlations, observed in HMBC spectra, from the C-13 methylene protons to C-11, 12 and 23 locates the trisubstituted double bond between C-10 and C-11.

Figure 1. Selected ^{1}H - ^{1}H COSY and HMBC Correlations for **1** and **2**.



1 **2**

Isolation and Structure Determination of L-731,128 - L-731,128 was isolated as a minor component of *Sporormiella intermedia* (MF 5447, ATCC 20985) fermentations which produced 48 mg/L zaragozic acid B (ZA-B).[7] Mycelia were collected after a 187 h liquid fermentation and extracted with methanol. The filtrate was collected and chromatographed on DOWEX 1 (Cl^-) to yield a crude carboxylic acid fraction. This material was desalted by adsorption onto Dianon HP-20 and **2** was purified from the eluant using repeated reverse phase HPLC.[8]

FAB MS analysis of **2** indicated a molecular weight of 448. It yielded a tetra-TMS derivative and esterification (CH_2N_2) gave a trimethyl ester (HREIMS found 490.2937, calc. for $C_{28}H_{42}O_7$ 490.2931). From this information the molecular formula of **2** was determined to be $C_{25}H_{36}O_7$ and was supported by the observation of 25 resonances in the ^{13}C NMR spectrum. ^{1}H and ^{13}C NMR spectra of **2** indicate a styrene moiety, common to zaragozic acid B derivatives, consistent with the adsorption maximum at 250 nm in the ultraviolet spectrum.[9] The styrene and three carboxylic acids provide the eight unsaturation equivalents required for the molecular formula and therefore the remaining portion of **2** must have an acyclic structure. The complete structure of **2** was deduced from ^{1}H-^{1}H COSY, HMQC, and HMBC data and similarity of the alkyl side chain to that of ZA-B. ^{1}H and ^{13}C NMR assignments for **2** are shown in Table 1 and selected HMBC ($^nJ_{CH}$ = 7 Hz) correlations are shown in Figure 1.

A second alkyl citrate, L-731,127, (**3**) was isolated in small quantities with **2**.[10] Partial NMR and MS characterization suggest it to be an analog of **2**, hydroxylated at C-4.

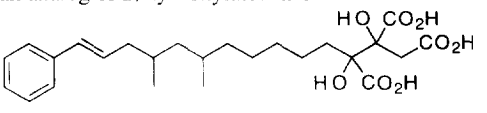


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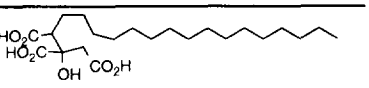
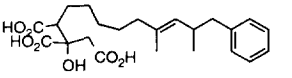
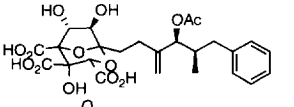
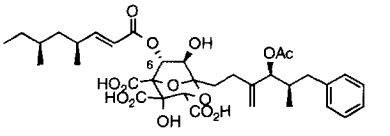
Table 1. ^{1}H and ^{13}C NMR Assignments for **1** and **2.**

	1		**2**	
Position	^{13}C δ (ppm)	^{1}H δ (mult., J in Hz)	^{13}C δ (ppm)	^{1}H δ (mult., J in Hz)
1	172.1	-	173.9	-
2	41.8	2.65 (d, 16.5)	42.3	2.68 (brd, 16.5)
		3.02 (d, 16.5)		3.07 (brd, 16.5)
3	76.5	-	76.7	-
4	53.9	2.60 (m)	54.8	2.63 (brd, 10.0)
5	27.9	1.40 (m)	28.1	1.46 (m)
		1.66 (m)		1.79 (brq, 10.0)
6	28.1	1.22 (m), 2H	30.8	1.30 (m)
				1.35 (m)
7	29.3	1.22 (m), 2H	28.8	1.29 (m), 2H
8	28.2	1.22 (m), 2H	27.7	1.30 (m), 2H
9	40.0	1.86 (brt, 7), 2H	37.8	1.07 (m)
				1.30 (m)
10	134.9	-	31.2	1.54 (m)
11	131.3	4.94 (dq, 1.2, 9.0)	45.8	1.30 (m)
12	35.3	2.64 (m)	31.9	1.70 (oct, 7.0)
13	44.5	2.46 (dd, 8.1, 12.8)	41.4	2.00 (ddt, 15.0, 1.0, 7.0)
		2.57 (dd, 6.2, 12.3)		2.21 (m)
14	142.3	-	130.2	6.21 (dt, 15.5, 7.0)
15	130.2	7.13 (m)	132.4	6.36 (d, 15.5)
16	126.5	7.22 (m)	139.3	-
17	128.9	7.13 (m)	126.9	7.33 (dd, 8.5, 1.5)
18	126.5	7.22 (m)	129.5	7.25 (t, 8.5)
19	130.2	7.13 (m)	127.8	7.15 (brtt, 8.5, 1.5)
20	174.6	-	129.5	7.25 (t, 8.5)
21	174.1	-	126.9	7.33 (dd, 8.5, 1.5)
22	16.0	1.36 (d, 1.9), 3H	176.7	-
23	21.3	0.92 (d, 6.4), 3H	176.2	-
24			20.6	0.88 (d, 6.5), 3H
25			20.7	0.91 (d, 7.0), 3H

Table 2. Biological Activities of L-731,120 and L-731,128

Compound	Rat Squalene Synthase IC_{50} (nM)	Hepatic Cholesterol Synthesis (Mouse) SC ED_{50} (mpk)	Hepatic Cholesterol Synthesis (Mouse) Oral % Inhibition	Structure
agaricic acid	9000	-	0% at 48 mpk	
1	260	15	0% at 48 mpk	
2	767	-	-	-
L-731,078 (6-deacyl ZA-A)	30	-	34% at 24 mpk	
zaragozic acid A	0.29	0.2	20% at 40 mpk	

Biological Activity of L-731,120 and L-731,128 - Both **1** and **2** were found to be potent, submicromolar inhibitors of squalene synthase (Table 2); however they are 1000-fold or more less active than ZA-A.[11] Because **1** is a minor component isolated from a broth extract that contained large amounts of ZA-A, the concern existed that this inhibitory activity was a result of a 0.1% contamination of **1** by ZA-A. To further substantiate this inhibitory activity of **1**, the purified compound was subjected to reverse phase HPLC and 15 s fractions collected.[12] The inhibitory activity of the fractions correlated well with the content of **1** in the fractions across the peak. As ZA-A was well separated from **1** in this chromatography system, this data shows that the inhibitory activity of **1** was not due to contamination by ZA-A.

When the compounds presented in Table 2 are examined, an interesting SAR is apparent. Agaricic acid is a weak inhibitor of squalene synthase.[13] The changes between agaricic acid and **1**, termination of a shorter alkyl chain in a phenyl ring and addition of two methyl branches and the unsaturation, confer about a 35-fold enhancement in inhibitory activity. Altering **1** by oxidations, cyclizations and acetylation to produce L-731,078, enhance the enzyme activity by only 9-fold.[14] However, adding the 6-acyl arm to L-731,078, to produce ZA-A, enhances the enyme inhibitory activity a further 100-fold. These data suggest that the potent squalene synthase inhibition of ZA-A is largely due to the citrate moiety, phenyl terminated arm and the 6-acyl side chain. Thus addition of an acyl or alkyl side chain to **1** at a position equivalent to the 6-position of ZA-A (carbon 5 of **1**) might be expected to produce a very potent low nanomolar inhibitor of squalene synthase.

L-731,120 was found to inhibit cholesterol synthesis in the liver of mice when it was administered subcutaneously (Table 2). *In vivo*, **1** was more active than expected based upon the *in vitro* squalene synthase inhibition and was only 75-fold less active than ZA-A.

L-731,120 and L-731,128 are alkyl citrates with alkyl chains corresponding to the C-1 side chain of zaragozic acids A and B respectively. Alkyl citrate biosynthesis generally involves condensation of a fatty acid and oxaloacetic acid which is consistent with that reported for zaragozic acid A.[15,16] The bicyclic core of the zaragozic acids could then arise by further oxidation of **1** and **2**. L-731,127 may represent a further biosynthetic intermediate.

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4. Fermentation and Extraction of MF 5453 - Culture 5453 was grown through four seed stages at 25 °C in a medium of the following composition per liter: corn steep liquor, 5 g; glucose, 10 g; tomato paste, 40 g; oat flour, 10 g. A 400 L sample from the fourth stage was used to inoculate 11,000 L of production medium of the following composition per liter: malt extract, 15 g; glucose, 55 g; Hysoy peptone, 10 g; KH_2PO_4, 3 g; $MgSO_4.7H_2O$, 1.5 g. The fermentation was run at 25 °C, pressure 0.7 kg/cm^2, air flow 5000 L / min and agitator speed of 50 rpm. Agitator speed was increased during the fermentation to maintain a dissolved oxygen concentration of 40% of atmospheric saturation. A total of 9300 L of broth was harvested after 385 hr. The whole broth was adjusted to pH 4.1 with H_2SO_4, extracted with MeOH (9300 L) and the solids removed using a Westfalia decanter. The solids were further extracted with 79% aq. MeOH, the solids removed as above and the MeOH extracts combined.

5. Isolation of L-731,120 - The methanol mycelial extract was adjusted to 50% MeOH/50% H_2O and loaded onto a column of Mitsubishi SP-207 resin (bed volume 500 L) at 15 L/min. The column was washed with 60% MeOH/40% H_2O (2000 L) at 15 L/min. followed by elution with 90% MeOH/H_2O at 15 L/min. collecting 189 L fractions. The ZA-A containing fractions were combined, adjusted to pH 6.5 and 80% MeOH and loaded onto DOWEX 1x4 (V_b = 500 L). The column was washed with MeOH/acetone/H_2O (35/35/30, 1500 L) and eluted with MeOH/acetone/0.1 M NH_4-citrate pH 3.0 (35/35/30, 4500 L). The ZA-A containing fractions from DOWEX 1 were pooled and adjusted to 40% H_2O. This solution was desalted in two equal portions. Each portion was loaded onto a column of Diaion HP-20 resin (bed volume 100 L) at 2.3 L/min. The column was washed with 60% MeOH/H_2O (400 L) and then eluted with MeOH at 2.3 L/min. collecting 57 L fractions. Fractions 2 - 4 from each run were pooled and concentrated in vacuo to 57 L.

   2.78 L from the above volume of 57 L were adjusted to 66% MeOH and 60 mM sodium formate pH 4.5. This solution was loaded onto a column of BioRad AG4x4 (100 - 200 mesh, formate cycle, bed volume 440 mL) which had been equilibrated with 4 column volumes of 60% MeOH/40% 0.1 M formic acid adjusted to pH 4.4 with NaOH. After loading, the column was washed with 1800 mL of this solution followed by 60% CH_3CN/40% H_2O (2000 mL) at 40 mL/min. The column was eluted with 60% CH_3CN/40% 0.5N H_2SO_4 collecting 500 mL fractions. Fractions 5 - 11 were pooled (3500 mL, pH 1.77) and H_2O (1000 mL) added. This solution was extracted with CH_2Cl_2 (4000 mL), the CH_2Cl_2 layer washed with H_2O, dried over andhydrous Na_2SO_4 and concentrated in vacuo to a brown oil (81 g). A portion of this oil (50 g) was chromatographed on Amicon C_{18} (20 μ, 10.0 x 50 cm, volume = 4 L) eluted with a gradient of 35 - 90% CH_3CN in aqueous 0.1% H_3PO_4 over 80 min at a flow rate of 250 mL/min. The fractions containing crude **1** were combined, diluted with an equal volume of 0.1N HCl containing 10% NaCl and the mixture extracted with an equal volume of CH_2Cl_2. The CH_2Cl_2 layer was washed with H_2O, brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo to yield 12 g of oily residue. This residue was chromatographed as described above using a 35 - 60 % CH_3CN gradient over 120 min. with a flow rate of 200 mL/min. Fractions of 800 mL were collected and fraction 33 contained **1**. This fraction was desalted as described above to yield 200 mg of oily residue which was further purified by chromatography on Rainin Instrruments Microsorb C_{18} (5 μ, 21.4 x 250 mm). The column was eluted with CH_3CN : 0.02 M sodium phosphate pH 7 (58.5 : 41.5) at 10 mL/min, 40 °C. Compound **1** eluted at 7.0 min and was desalted as described above yield 27.4 mg pure **1**. Compound **1** eluted at 5.15 min on Rainin Instrruments Microsorb C_{18} (5 μ, 21.4 x 250 mm) with a mobile phase of CH_3CN : H_2O : H_3PO_4 (65 : 35 : 0.1) at a flow rate of 1.0 mL/min and temperature of 40 °C. ZA-A eluted at 6.65 min under the same conditions.

6. ^1H and ^{13}C NMR of **1** were recorded in CD_3CN at 500/125 MHz, referenced to solvent peak. IR: 3452, 2927, 2857, 1713, 1212 cm^{-1}. UV: λ_{max} (CH_3OH) = 208 nm (ε = 108). $[\alpha]^{20}_D$ = - 48 ° (*c* 0.55, CH_3OH).

7. Fermentation and Extraction of *Sporormiella intermedia* (MF5520) - Frozen vials of *Sporormiella intermedia* were grown through three seed stages at 25 °C. Five 500 L production fermentors were used, each containing 400 L of CPP medium (cerelose, Pharmamedia (cottonseed flour), phosphate) of the following composition per liter: glucose monohydrate, 50 g; Pharmamedia, 20 g; KH_2PO_4, 9 g; P-2000, 4 mL. The production fermentors were each inoculated with 50 L of broth from the seed fermentor and intially operated at a temperature of 23.5 °C, an air flow of 300 L/min., a pressure of 0.7 kg / cm^2, and an

agitator speed of 80 rpm. The air flow rate and agitator speed were increased during the fermentation to maintain a minimum dissolved oxygen content of 25% of atmospheric saturation. The five fermentors were harvested after 187 hr., combined (2000 L) and centrifuged to collect the mycelia. The wet mycelia were extracted twice with MeOH and solids removed to yield a MeOH extract (combined volume 3000 L).

8. Isolation of L-731,127 / L-731,128 - The MeOH extract above was adsorbed in two 1500 L portions onto 2 columns of DOWEX 1 (Cl^-, bed volume 56 L each). After adsorption the columns were washed with 80% MeOH/H_2O (110 L), 90% MeOH/H_2O (110 L) and then eluted with 3% NH_4Cl in 90% MeOH/H_2O collecting 56 L fractions. Fractions 3 - 6 were combined, diluted with 225 L H_2O (final volume 680 L) and adsorbed onto HP-20 resin (bed volume 56 L). The column was eluted consecutively with 56 L each of 50% MeOH/H_2O, 60% MeOH/H_2O, 70% MeOH/H_2O, 80% MeOH/H_2O and 90% MeOH/H_2O (75 L). A portion (4 L) of the 90% MeOH/H_2O eluate was concentrated *in vacuo* to 500 mL, adjusted to pH 2.5 with 2 N HCl and extracted with EtOAc. The EtOAc layer was separated and concentrated to an oily residue. The residue was chromatographed on DYNAMAX C_8 (8 μ, 21.4 x 250 mm) eluted with CH_3CN : 0.1% aq. H_3PO_4 (70 : 30) at 10 mL/min. Fractions were collected at 0.5 min intervals. Fractions 33 - 38 were combined an equal volume of H_2O added and the solution extracted wih EtOAc. The EtOAc layer was separated, concentrated to dryness and chromatographed on DYNAMAX C_8 (8 μ, 10 x 250 mm) eluted with CH_3CN : 0.1% aq. H_3PO_4 (65 : 35) at 4 mL/min. Fractions were collected at 0.5 min intervals. Fraction 28 was desalted as above to yield **3** (0.9 mg). Fractions 29 and 30 were combined and desalted as above to yield **2** (1.3 mg).

9. ^1H and ^{13}C NMR of **2** were recorded in CD_3OD at 500/125 MHz, referenced to solvent peak. IR: 2925, 1715, 1224 cm^{-1}. UV: λ_{max} (CH_3OH) = 205, 249 nm.

10. Physical Properties of **3** - ^1H NMR: (500 MHz, CD_3OD, partial) δ 0.87 (d, 6.5, 3H), 0.91 (d, 7.0, 3H), 0.97 (m), 2.00 (m), 2.21 (m), 2.75 (br d, 17.5), 3.14 (br d, 17.0), 6.21 (dt, 15.5, 7.0), 6.35 (d, 15.5), 7.15 (br tt, 8.5, 1.5), 7.25 (t, 8.5, 2H), 7.32 (dd, 8.5, 1.5, 2H). ^{13}C NMR: (125 MHz, CD_3OD, partial) δ 20.6, 20.7, 31.1, 31.6, 37.6, 41.4, 45.7, 81.6, 93.1, 126.9(2), 127.8, 129.5(2), 130.2, 132.4, 139.3, 173.9, 175.4, 176.6. UV: λ_{max} (CH_3OH) = 205, 249 nm. A molecular ion was not observed by EI or FAB MS due to facile loss of H_2O. **3** formed a trimethyl ester upon treatment with CH_2N_2; HREIMS: calcd for $C_{27}H_{38}O_7$ (M - CH_3OH) , 474.2618; found 474.2622. **3** formed a pentasilyl derivative upon treatment with pyridine-BSTFA (Regis); EIMS: 824 m/z (M^+), 809 m/z (M-15).

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12. Purified **1** (0.5 mg) was chromatographed on Rainin Microsorb C18, 5μ, 10 x 250 mm, with a mobile phase of 0.1% aq. H_3PO_4 : CH_3CN (1:1) at a flow rate of 4.0 mL/min and temperature of 40 °C. **1** eluted at 19.2 min and 1.0 mL (15 sec.) fractions were collected across the peak.

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