Farnesol-derived Dicarboxylic Acids in the Urine of Animals Treated with Zaragozic Acid A or with Farnesol

(Received for publication, August 13, 1996, and in revised form, January 16, 1997)

Richard G. Bostedor‡, John D. Karkas‡, Byron H. Arison§, Vinay S. Bansal‡, Sanskruti Vaidya‡, John I. Germershausen‡, Marc M. Kurtz‡, and James D. Bergstrom‡¶

From the Departments of §Biochemistry and ¶Drug Metabolism, Merck Research Laboratories, Rahway, New Jersey 07065-0900

Farnesyl diphosphate, the substrate for squalene synthase, accumulates in the presence of zaragozic acid A, a squalene synthase inhibitor. A possible metabolic fate for farnesyl diphosphate is its conversion to farnesol, then to farnesoic acid, and finally to farnesol-derived dicarboxylic acids (FDDCAs) which would then be excreted in the urine. Seven dicarboxylic acids were isolated by high performance liquid chromatography (HPLC) from urine of either rats or dogs treated with zaragozic acid A or rats fed farnesol. Their structures were determined by nuclear magnetic resonance analysis. Two 12-carbon, four 10-carbon, and one 7-carbon FDDCA were identified. The profile of urinary dicarboxylic acids from rats fed farnesol was virtually identical to that produced by treating with zaragozic acid A, establishing that these dicarboxylic acids are farnesol-derived. By feeding [1-14C]farnesol and comparing the mass of the dicarboxylic acids produced with the ultraviolet absorption of the HPLC peaks, a method to quantitate the ultraviolet-absorbing FDDCAs was devised. When rats were treated with zaragozic acid A, large amounts of FDDCAs were excreted in the urine. The high level of FDDCAs that were found suggests that their synthesis is the major metabolic fate for carbon diverted from cholesterol synthesis by a squalene synthase inhibitor. A metabolic pathway is proposed to explain the production of each of these FDDCAs.

Squalene synthase is an attractive target for the development of a cholesterol synthesis inhibitor that could serve as a cholesterol lowering agent. Cholesterol synthesis inhibitors, such as lovastatin (1), a 3-hydroxy-3-methylglutaryl-coenzyme A inhibitor, are effective cholesterol-lowering agents in man and/or animals. Squalene synthase catalyzes the first committed step in cholesterol synthesis, and selective inhibition of this enzyme should result in inhibition of cholesterol synthesis without affecting the synthesis of other isoprenoids such as dolichol, ubiquinone, and the prenylated proteins. A novel class of fungal metabolites, known as zaragozic acids, has been recently discovered and characterized as potent inhibitors of squalene synthase (2–8). The zaragozic acids are subnanomolar inhibitors of squalene synthase in vitro, they inhibit cholesterol synthesis from acetate or mevalonate in cell culture and in animal models, and also have been shown to lower plasma cholesterol when administered orally in certain animal species (4, 5, 8). Other classes of squalene synthase inhibitors have also been discovered (for a review, see Ref. 9).

An important question to be answered for these compounds is the metabolic effect of inhibition of squalene synthase. The squalene synthase reaction consists of the reductive dimerization of two molecules of farnesyl diphosphate (FPP) to form a molecule of squalene (10). The primary consequence of inhibition of this reaction would be an accumulation of FPP. Thus, the metabolic fate of FPP in the presence of a squalene synthase inhibitor is of interest. Previous work in cultured cells has shown that in the presence of zaragozic acids, mevalonate metabolism is diverted into the production of farnesol and a 15-carbon dicarboxylic acid derivative of farnesolic acid (4). It has been reported that Drosophila Kc cell extracts and rat liver extracts metabolize both FPP and farnesol to farnesolic acid and two α,ω-prenyl dicarboxylic acids of 12 and 15 carbon atoms (11). It was shown in very early studies that dicarboxylic acids derived from prenyl alcohols or aldehydes fed to rabbits are readily excreted in urine (12–14). Thus it is likely that an important fate for the mevalonate diverted from cholesterol synthesis by a squalene synthase inhibitor will be urinary excretion as farnesol-derived dicarboxylic acids (FDDCAs).

As part of several studies on the in vivo effects of the zaragozic acids, the urinary production of organic acids was examined. A series of novel FDDCAs was found in the urine of dogs, rats, mice, and monkeys treated with zaragozic acid A, and these same dicarboxylic acids were not found in control animals. Furthermore, these compounds were sometimes found in massive quantities in the treated animals. In this paper, the isolation, the structural elucidation, and a method for the quantitation of some of these compounds are described. A scheme for the metabolic production of these acids is also proposed.

EXPERIMENTAL PROCEDURES
Preparation of [1-14C]Farnesol

[1-14C]Isopentenyl diphosphate (75 μCi, specific activity 60 mCi/mmol, American Radiochemicals, St. Louis, MO) was converted to [1-14C]FPP by incubation with 250 μl of 20 μM geranyl pyrophosphate and 0.175 units of purified prenyl transferase (2) in a final volume of 600 μl also containing 100 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 6 mM dithiothreitol. [1-14C]FPP precipitated as a magnesium salt, and it was collected by centrifugation. The pellet was dissolved in 1 ml of 50 mM HEPES, pH 7.5, and 5 mM EDTA. A 99% purity of [1-14C]FPP was found by HPLC. The yield of [1-14C]FPP from [1-14C]isopentenyl pyrophosphate is typically around 90% with this procedure. [1-14C]FPP was converted to [1-14C]farnesol further incubation for 2 h at 37 °C with intestinal alkaline phosphatase (Sigma, 4 mg) at pH 9.5 in the presence of...
of 5 mM MgCl₂ and 1 mM ZnCl₂. The final product was extracted by diethyl ether, and the purity was confirmed by HPLC.

**Animals**

Animals were housed and cared for in keeping with the standards set forth in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication 86-23, 1985). Beagle dogs, approximately 1 year old, weighing about 10 kg, obtained from Marshal Farm, North Rose, NY, were used in this study. The dogs were fed a certified UAR EC Lab chow (approximately 350 g daily) and were housed individually. The dogs were fasted overnight prior to bleeding for clinical chemistry, necropsy, and during urine collection. Zaragozic acid A (0.5 mg/kg/day) was solubilized in isotonic saline, sterilized by filtration through Millex-GS (0.22-μm) filters, and administered by subcutaneous injections (right and left flank, alternatively) in a volume of 0.5 ml/kg of body weight.

Female Sprague-Dawley rats obtained from Charles River and weighing between 130 and 160 g were used in this study. Animals were maintained on a standard rodent diet (Purina). Rats were dosed subcutaneously with zaragozic acid A dissolved in isotonic saline or were dosed by gavage with farnesol. For urine collection, animals were housed individually in metabolic cages throughout the period of collection.

**HPLC System**

The HPLC system used was a Varian 5020. The effluent was monitored by a Hewlett-Packard diode array detector (1040A) over a range of 190–400 nm, with 225 nm being the primary wavelength and the bandwidth set at 4 nm. A 4.5-μl flow cell with a path length of 6 mm was used. Except when otherwise indicated, the reservoirs contained (A) 0.1% formic acid and (B) acetonitrile also made 0.1% in formic acid by the addition of a small volume (1.042 ml/liter) of 96% stock formic acid (Aldrich). The gradients and the columns used will be described below.

In the cases where radioactivity of the effluent was also measured, an IN/US β-RAM flow-through monitor with a 1-ml flow cell was used, connected in series after the UV detector. Scintillation fluid (IN/US In-Flow BD) was mixed with the effluent at a ratio of 4:1.

**Analytical Scale HPLC**

The urine samples were diluted with an equal volume of methanol, and 40 μl of this dilution was injected into the column via either a Waters U6K injector or a Waters 712 WISP autosampler. A Zorbax C8-Rx column (250 × 4.6 mm) was used. A 40-min linear gradient (at 2 ml/min) from 5 to 45% acetonitrile (both containing 0.1% formic acid) was started immediately after injection, followed by a 10-min linear gradient to 45 to 100% acetonitrile (both containing 0.1% formic acid). This was followed by an isocratic step at 100% for an additional 5 min.

**Preparative Scale HPLC**

**Compounds A, B, and C**—A 5-ml sample of 24-h urine collected from a dog that had been treated with a subcutaneous dose of zaragozic acid...
A (0.5 mg/kg/day) for 28 days was taken to dryness in a Savant Speed-Vac concentrator, redissolved in 0.5 ml of acetonitrile:water (80:20 v/v), and then injected. A preparative Zorbax ODS column (21.2 × 250 mm) was used with a flow rate of 5 ml/min. Elution was performed with a 60-min linear gradient from 10 to 30% acetonitrile containing 0.03% formic acid. Selected fractions containing the peaks of compounds A, B, and C were pooled and taken to dryness in the Savant Speed-Vac concentrator. NMR analysis was performed on the samples, and structures were determined for the major components of the three isolates.

Compounds D, E, F, and G—Compounds D, E, F, and G were isolated in preparative runs on a Zorbax C8-Rx column (9.4 × 250 mm). Urine samples from rats treated with a subcutaneous dose of zaragozic acid A, 3 mg/kg for 5 days (peak D), rats treated with farnesol (500 µl, peak F), or rats treated with [1-14C]farnesol (400 µmol, peaks E and G) were taken to dryness in a Speed-Vac concentrator and redissolved in 200–500 µl of 50% acetonitrile:water and injected into the column. The elution systems and retention times for each of the peaks were: peak D, a 60-min linear gradient from 5 to 45% acetonitrile containing 0.1% formic acid at a flow rate of 4 ml/min, with a retention time of 37.5 min; compound F, a 90-min linear gradient from 10 to 40% acetonitrile containing 0.1% formic acid at a flow rate of 4 ml/min, with a retention time of 62.2 min; compounds E and G, with retention times of 88 and 106.3 min, respectively, a 90-min linear gradient from 5 to 40% acetonitrile containing 0.1% formic acid followed by a 10-min linear gradient.

![Fig. 4. Structures of the FDDCAs. Compounds isolated from the urine of dogs and/or rats treated with zaragozic acid A or farnesol were identified by NMR.](image)

![Fig. 5. HPLC analysis of rat urine. A sample of 24-h pooled urine from four rats treated with zaragozic acid A (3 mg/kg/day, subcutaneous) for 5 days was chromatographed as described under “Experimental Procedures” and compared with a sample from an untreated control pool. The absorbance at 225 nm is plotted in the area of interest (21–34 min) containing peaks B, C, D, and F. Panel a, control rats; panel b, treated rats. mAU, milliabsorbance units.](image)

### Table I

| Position | A | B | C | D | E | F | G |
|----------|---|---|---|---|---|---|---|
| 2        | 6.08 s, 1H | 5.69 s, 1H | 5.66 s, 1H | 2.20 m, 2H | 2.25 dd | 5.67 sx | 2.25 dd |
| 3        | 1.98 m, 1H | 1.91 m, 1H | 1.90 sx | 6.54 H | 6.3 H |
| 3a       | 2.22 s, 3H | 2.13 d | 2.11 d | 0.95 d, 3H | 0.93 d | 2.11 d | 0.93 d |
| 4        | 6.20 d | 2.29 t | 2.17 t | 1.46 m, 1.30 m | 1.21 m, 1.37 m | 2.44 t | 1.30 m, 1.17 m |
| 5        | 15.3 Hz, 1H | 7.4 Hz, 2H | 7.3 Hz, 2H | 2H | 2H | 7.7 Hz, 2H | 2H |
| 6        | 7.23 d | 2.37 g | 1.52 q | 2.20 m, 2H | 1.37 m, 2H | 1.42 m, 2H | 1.43 m, 2H |
| 7        | 16.0 Hz, 1H | 7.3 Hz, 2H | 7.3 Hz, 2H | 2H | 2H | 7.3 Hz, 2H | 7.7 Hz, 2H |
| 7a       | 6.69 t | 1.62 m, 1.42 m | 1.63 m, 1.37 m | 2.05 t | 2.01 t |
| 8        | 7.5, 1.5 Hz, 1H | 2H | 7.3 Hz, 2H | 2H | 7.4 Hz, 2H | 7.7 Hz, 2H |
| 9        | 2.40 t | 1.14 d | 1.80 s, 3H | 1.12 d | 1.64 s, 3H | 1.61 s, 3H |
| 10       | 7.0 Hz, 3H | 5.37 t | 5.31 t | 7.5, 1.4 Hz, 1H | 7.0 Hz, 1H |
| 11       | 7.5, 0.8 Hz, 2H | 7.0 Hz, 2H |

* Carbon position 2 is the α carbon from the far right side carboxyl group (see Fig. 4) with subsequent consecutive numbering from right to left (3A and 7A are the branched methyl carbons).
from 40 to 100% acetonitrile containing 0.1% formic acid and a 30-min isocratic step at 100% acetonitrile with a flow rate of 2 ml/min for the first 47 min and 1 ml/min for the rest of the run. The compounds isolated were analyzed by NMR.

HPLC Analysis of Urine from Rats Treated with [1-14C]Farnesol

Farnesol Treatment of Rats—Twenty-five µCi of the [1-14C]farnesol (0.417 µmol) was evaporated to dryness with a resulting 20.7% loss in radioactivity counts. The dried [1-14C]farnesol (now 0.33 µmol) was dissolved in 500 µl (2.02 nmol) of “cold” farnesol (density 0.897, Aldrich). The final specific activity of the [1-14C]farnesol was 21,565 dpm/µmol. One hundred µl (400 µmol) of this [1-14C]farnesol was given to each of four different rats by oral gavage. The rats were placed in metabolic cages, and 24-h urines were collected. Aliquots of each urine were combined to provide pooled samples for analysis.

Analytical Runs—Two-ml samples of pooled urine from the [1-14C]farnesol-treated rats were evaporated with water. For each run, 50 µl was injected onto a Zorbax C8-Rx column (9.4 × 250 mm). A flow rate of 2 ml/min was used. Elution was performed with a 60-min gradient from 5 to 40% acetonitrile (containing 0.1% formic acid), followed by a 10-min linear gradient from 40 to 100% acetonitrile (containing 0.1% formic acid) and an isocratic step at 100% acetonitrile for the next 20 min. UV absorbance and radioactivity of the effluent were monitored simultaneously, as mentioned above.

Preparation and Analysis of Methyl Esters of the Dicarboxylic Acids

One-ml samples of urine from the animals were acidified to pH 1 with HCl and extracted with petroleum ether. The acidic aqueous layer was subsequently extracted with ethyl ether. The ethyl ether extract was dried under N₂, and methyl esters were prepared with diazomethane (15). The dimethyl esters of the dicarboxylic acids were analyzed on a C18 column eluted with a gradient from 20 to 100% acetonitrile in water.

NMR Spectra

NMR spectra were obtained in CD3OD on a Varian Unity Plus 400-MHz spectrophotometer at 25 °C. Chemical shifts are in ppm relative to the CD3HOD line set at 3.30 ppm.

RESULTS

Identification of Dicarboxylic Acids in the Urine of Dogs Treated with Zaragozic Acid A—Examination of HPLC separations on an analytical C8 reverse phase column (Figs. 1 and 2) of urine samples obtained from a dog treated with zaragozic acid A and from an untreated control dog clearly indicated at least three major peaks in the treated samples which were not present in the controls. One of the peaks had an absorbance maximum at 270 nm (Fig. 1) and the other two at 225 nm (Fig. 2). A comparison of the spectra of the three peaks is shown in Fig. 3. A preparative scale HPLC isolation of those three peaks was undertaken as described under “Experimental Procedures,” and each was examined by NMR. The NMR data are summarized in Table I, and the structures of compounds A, B, and C determined from the NMR data are shown in Fig. 4. Compound A was found to be 3-methyl-2,4-hexadien-1,6-dioic acid, a 7-carbon dienedioic acid. Compound B was found to be 3,7-dimethyl-2,6-octadien-1,8-dioic acid, a 10-carbon dienedioic acid. Compound C was found to be 3,7-dimethyl-2-oct-1,8-dioic acid, a 10-carbon monoenedioic acid. All three compounds were found to be α,ω-dicarboxylic acids. The methyl groups, the double bonds, and their positions strongly suggested that these compounds were isoprenoid-derived. The 270-nm maximum of compound A was consistent with the high degree of double bond conjugation. The double bonds found α,β to the carboxyl groups in compounds B and C were the chromophores responsible for the 225-nm maxima found for these two compounds.

Identification of Dicarboxylic Acids in the Urine of Rats Treated with Zaragozic Acid A—The urine of rats treated with zaragozic acid A was examined by analytical HPLC in a number of experiments. The HPLC profile shown in Fig. 5 is typical of the pattern seen in these experiments with rats. Peaks B and C corresponded in retention time to compounds B and C isolated from dog urine. Spiking the rat urine samples with compounds B and C isolated from dog urine confirmed that the...
Farnesol were administered orally to rats, and urine was collected. The identification of the peaks observed in the chromatograms is given in the second column. The flow-corrected cpm (see Results) recorded for each peak is shown in the third column. From these cpm, the specific activity of the farnesol and the efficiency of the counter the number of nmol in each dicarboxylic acid peak were calculated (fourth column). The UV absorbance of each peak in A̅225 nm area counts (see Results) is recorded in the fifth column. Division of the values in the fifth column by the corresponding values in the fourth column provides the specific absorbance in A̅225 nm area counts/nmol for each dicarboxylic acid (sixth column). Finally, the specific absorbance values obtained for each animal/peak are averaged in the seventh column.

| Rat no. | Peak | cpm | nmol | A̅225 nm area counts | A̅225 nm area counts/nmol | Average |
|--------|------|-----|------|---------------------|--------------------------|---------|
| 1      | B    | 2,963 | 179 | 48,995 | 273 | 247 |
| 2      | B    | 3,883 | 235 | 51,758 | 220 | 150 |
| 1      | C    | 2,637 | 160 | 23,734 | 149 | 150 |
| 2      | C    | 2,810 | 170 | 25,653 | 151 | 146 |
| 1      | D    | 3,540 | 214 | 32,053 | 150 | 134 |
| 2      | D    | 5,197 | 315 | 44,898 | 143 | 109 |
| 1      | F    | 3,590 | 217 | 27,553 | 127 | 114 |
| 2      | F    | 2,700 | 164 | 23,041 | 141 | 93 |

* Using determined factors for our HPLC system for the conversion of area counts to absorbance units, we were able to calculate molar absorption coefficients for the four dicarboxylic acids. They are: A, 17,500; B, 17,300; C, 10,700; D, 10,400; F, 9,500.

### Table III

*Dicarboxylic acids in the urine of rats treated with zaragozic acid A*

Four rats were treated with zaragozic acid A225 nm (3 mg/kg/day) for 5 days. A 15-μl sample of the pooled 24-h urine from the 5th day was analyzed by HPLC at A̅225 nm. The total absorbance (area counts) for each dicarboxylic acid peak (B, C, D, and F; first line) are recorded in the second column. To obtain the values in the third column (area counts/day/kg) the average urine volume (14 ml) and average weight of the animals (133 g) were taken into account. The specific absorbance values in the fourth column were obtained as detailed in Table II. The numbers in the third column were divided by the corresponding specific absorbance values given in the fourth column to provide the amount of each dicarboxylic acid in mmol/kg/day (fifth column); the sixth column presents the amounts in mg/kg/day, computed by the use of the molecular weight of each acid.

| Peak | Area counts/15 μl urine | Area counts/kg/day × 10^-6 | Specific Absorbance (from Table II) | mmol/kg/day | mg/kg/day |
|------|-------------------------|-----------------------------|-------------------------------------|-------------|-----------|
| B    | 10,174                  | 71.4                        | 247                                 | 0.289       | 57        |
| C    | 6,051                   | 42.5                        | 150                                 | 0.283       | 57        |
| D    | 8,467                   | 59.4                        | 146                                 | 0.407       | 81        |
| F    | 7,866                   | 55.2                        | 134                                 | 0.412       | 93        |

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 Peaks comigrated. Dimethyl esters of these acids from rat urine and of compounds B and C isolated from dog urine were prepared and separated by HPLC as described under “Experimental Procedures.” Two peaks having retention times identical to those of the dimethyl esters of compounds B and C from dog urine were found in rat urine. The two peaks from rat urine also had UV spectra identical to those of compounds B and C. Thus it was concluded that peaks B and C in Fig. 5 were the same two 10-carbon isoprenoid-derived dicarboxylic acids, compounds B and C, found in dog urine. The compounds from the two unidentified peaks, D and F in Fig. 5, were isolated by preparative HPLC runs (described under “Experimental Procedures”). This demonstrates that the dicarboxylic acids observed upon inhibition of squalene synthase are farnesol-derived.

### Identification of Additional Dicarboxylic Acids in the Urine of Rats Treated with Radioactive Farnesol

Urine collected from rats dosed with the [1-14C]farnesol was analyzed by reverse phase HPLC as before with simultaneous monitoring of UV absorbance and radioactivity (Fig. 7). The peaks corresponding to compounds B, C, D, and F were observed as before, but two new peaks, E and G, were observed, which were radio-labeled and thus were derived from farnesol, but were lacking in significant UV absorbance. A preparative HPLC isolation was undertaken as described under “Experimental Procedures” to purify and identify the components of the two peaks. NMR analysis of the isolated free acids (Table I) led to the determination of the structures for compounds B and C, found in dog urine. The compounds from the two unidentified peaks, D and F in Fig. 5, were isolated by preparative HPLC runs (described under “Experimental Procedures”). This demonstrates that the dicarboxylic acids observed upon inhibition of squalene synthase are farnesol-derived.

Additional Dicarboxylic Acids in the Urine of Rats Treated with Radioactive Farnesol—Urine collected from rats dosed with the [1-14C]farnesol was analyzed by reverse phase HPLC as before with simultaneous monitoring of UV absorbance and radioactivity (Fig. 7). The peaks corresponding to compounds B, C, D, and F were observed as before, but two new peaks, E and G, were observed, which were radio-labeled and thus were derived from farnesol, but were lacking in significant UV absorbance. A preparative HPLC isolation was undertaken as described under “Experimental Procedures” to purify and identify the components of the two peaks. NMR analysis of the isolated free acids (Table I) led to the determination of the structures for compounds B and C, found in dog urine. The compounds from the two unidentified peaks, D and F in Fig. 5, were isolated by preparative HPLC runs (described under “Experimental Procedures”). This demonstrates that the dicarboxylic acids observed upon inhibition of squalene synthase are farnesol-derived.
farnesol that is dosed. Dual monitoring of the HPLC effluent for radioactivity and for absorbance at 225 nm should enable the determination of the mass under each peak (from the dpm and the specific activity) and thus allow the determination of absorption coefficients for each of the UV-absorbing dicarboxylic acids.

For the quantitation aspects of the experiment described above, both detectors had integration programs: the diode array detector presented the results for each peak as $A_{225\text{ nm}}$ area counts (absorbance at the specified wavelength multiplied by a time factor), whereas the radioactivity flow detector presented the flow-corrected cpm for each peak (computed from the counts recorded, the flow rates of the effluent and scintillation fluid, the size of the cell, and the frequency of reading). The counting efficiency of the flow detector (76.5%) was determined from the peak area flow-corrected cpm of an HPLC run with a known amount of $^{14}$C-octanedioic acid (Sigma) which was injected under the same HPLC conditions.

Using the specific activity of the farnesol, the counting efficiency and the integration results from the flow counter, it was possible to calculate the amount of dicarboxylic acid present in each peak. This, in turn, was related to the absorbance of the same peak to provide the final coefficient, in $A_{225\text{ nm}}$ area counts/nmol, which could now be used to calculate the amount of dicarboxylic acid in a sample when only UV data were available. The results of an experiment using two rats treated with radioactive farnesol in which these coefficients were determined are summarized in Table II.

This technique for the quantitation of these acids applies only to the dicarboxylic acids B, C, D, and F and not to A, which was not seen in the rat, or to G and F, which have minimal absorption in the UV. Using the absorption coefficients determined in Table II, we were able to quantitate the production of these four dicarboxylic acids in rats treated with zaragozic acid A. In Table III, the amounts of these four farnesol-derived dicarboxylic acids present in the 24-h urine during the 5th day of dosing with zaragozic acid A are shown.

DISCUSSION

The administration to an animal of a squalene synthase inhibitor, such as zaragozic acid A, will block the major pathway for the utilization of FPP, the synthesis of sterols. In liver, the utilization of FPP by other pathways such as the synthesis of dolichol and the prenylation of proteins is extremely limited when compared with the pathways for both the production of FPP and its utilization for sterol synthesis. In labeling experiments with mevalonic acid, typically greater than 95% of the label incorporated into the nonsaponifiable lipids is found in squalene, oxidosqualene, and the sterol peaks (4). Thus, the metabolic fate of FPP is a major issue in the development of a squalene synthase inhibitor as a cholesterol-lowering agent. FPP can be readily dephosphorylated to farnesol by a specific FPP pyrophosphatase (16). This enzyme has a high $K_m$ of 7–30 $\mu$M for FPP compared with the $K_m$ of squalene synthase for FPP of 0.8–1.0 $\mu$M, and it should become more active as the FPP concentration rises in the presence of a squalene synthase inhibitor (16). Previous studies (11–14) suggested that the urinary production of FDDCAs might be a likely consequence of inhibition of hepatic squalene synthase. Therefore, we collected the urine of animals treated with squalene synthase inhibitors and examined it for the presence of FDDCAs.

Seven isoprenoid dicarboxylic acids were found in the urine of dogs or rats treated with a squalene synthase inhibitor or fed farnesol (Fig. 4). Compound B and compound D are apparently identical with Hildebrandt’s acid and dihydro-Hildebrandt’s acid, respectively, described previously (12–14), in the urine of...
rabbis fed citral, a 10-carbon isoprenoid aldehyde. Two new 10-carbon dicarboxylic acids are described here, compounds C and E. Two 12-carbon dicarboxylic acids (F and G), were also found. These two compounds are distinct from the C-12 dicarboxylic acid described by Gonzalez-Paeonowska et al. (11) in that both have a Δ\(^7\) double bond, whereas the C-12 dicarboxylic acid described previously (11) has a Δ\(^6\) double bond. The presence of the Δ\(^7\) double bond, rather than the Δ\(^6\) double bond found in farnesol, suggests further metabolism of these acids (see below) from that seen in vitro (11). The seventh compound that has been found and characterized, compound A, is a C-7 dicarboxylic acid, and so far it has only been detected in dog urine.

The profile of dicarboxylic acids produced by the in vivo inhibition of squalene synthase with the zaragozic acids was very similar to the profile produced by feeding farnesol (Fig. 6). This demonstrates that these dicarboxylic acids are farnesol-derived. Oxidation of farnesol can lead to the formation of farnesoic acid (17). The dicarboxylic acid derivative of farnesoic acid can be generated by the process of ω-oxidation (11). Once the ω-carboxyl is formed, β-oxidation becomes possible from the ω-end. A plausible scheme for the metabolic formation of these compounds starting with the CoA ester of the ω-carboxyl of the dicarboxylic acid of farnesol is illustrated in Fig. 8. In this scheme, the CoA esters of compounds B, C, and F are produced in an unbranched pathway by the action of the normal enzymes of β-oxidation and a dienyl-CoA reductase\(^2\) (18). Subsequent metabolism through β-oxidation could produce the CoA ester of compound A found in dog urine (reactions not shown in Fig. 8). Action by an acyl-CoA hydrolase would produce the free dicarboxylic acids, A, B, C, and F. It is apparent that reduction of the Δ\(^2\) double bond (reaction 8 in Fig. 8) can occur at some point in the metabolism of these compounds leading to the formation of compounds D, E, and G, but it is not clear to us by what process or enzyme this reduction is accomplished or at what point (variable) in the scheme it takes place. This reduction apparently occurs in rats and mice\(^3\) but not in dogs or rhesus monkeys\(^4\) because only acids with the Δ\(^2\) double bond were found in these species. It is suggested by the results that the pathway for the metabolism of these dicarboxylic acids may be very similar in all of the above species and may only differ in how far the β-oxidation proceeds before the removal of the CoA and whether or not there is reduction of the Δ\(^2\) double bond.

The FDDCAs were not detectable in the urine of control animals. Their presence in the animals treated with a squalene synthase inhibitor is a demonstration of the in vivo efficacy of the zaragozic acids. Should a squalene synthase inhibitor ever reach clinical trials, the urinary production of these or related dicarboxylic acids might be of use in demonstrating and monitoring the in vivo efficacy of potential drugs even in single dose experiments.

The presence of the FDDCAs in the urine of animals treated

\(^2\) The idea of these two steps being the most plausible enzymatic reactions to introduce the Δ\(^2\) double bond was suggested to us by Dr. Horst Schulze.

\(^3\) V. Bansal and S. Vaidya, unpublished observations.

\(^4\) J. D. Bergstrom and R. Bostedor, unpublished results.

with a squalene synthase inhibitor was expected; however, what was not expected was the level at which they were found. The urine from the first dog examined had levels of compounds A, B, and C in concentrations of about 0.1 mg/ml each. In rats treated with zaragozic acid A for 5 days (Table III) the total of compounds B, C, D, and F produced exceeded 250 mg/kg/day. This underestimated the total production of FDDCAs since compounds E and G were not determined because of their lack of UV absorbance. These results suggest that production of FDDCAs can be a major metabolic consequence of inhibition of cholesterol biosynthesis by a squalene synthase inhibitor. Further reports from our laboratory will show that there are striking variations in the urinary levels of these dicarboxylic acids found in various species treated with the zaragozic acids and that in some species a mechanism-based toxicity is observed. This toxicity is an acidosis that results from the overproduction of these FDDCAs.\(^5\)

Acknowledgments—We acknowledge gratefully the help and assistance of Dr. George Lankas and Dr. Christine Bourquis-Leroux in planning and performing the dosing of dogs with zaragozic acid A and in providing the collected urine samples from that experiment. We also acknowledge gratefully the help of Dr. David B. R. Johnston in discussions of the NMR results.

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J. D. Bergstrom, R. Bostedor, G. Lankus, V. Bansal, and J. Karkas, manuscript in preparation.
Farnesol-derived Dicarboxylic Acids in the Urine of Animals Treated with Zaragozic Acid A or with Farnesol

Richard G. Bostedor, John D. Karkas, Byron H. Arison, Vinay S. Bansal, Sanskruti Vaidya, John I. Germershausen, Marc M. Kurtz and James D. Bergstrom

J. Biol. Chem. 1997, 272:9197-9203.

doi: 10.1074/jbc.272.14.9197

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