2,6-Dimethyloctanedioic Acid—a Metabolite of Phytanic Acid in Refsum’s Disease

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The urine of two patients with Refsum’s disease consistently contained 2,6-dimethyloctanedioic acid, a compound not normally found in human urine. In addition, their urines contained increased concentrations of 3-methylhexadecanoic acid. These two compounds may be formed from phytanic acid by an initial \( \alpha \)-oxidation and subsequent \( \beta \)-oxidations. It was calculated that this oxidation pathway may metabolize at least 30 mg of phytanic acid per day.

Additional Keyphrases: heritable disorders • urine • fatty acids • gas chromatography—mass spectrometry

Refsum’s disease is an inborn error of fatty acid metabolism, characterized by a slow accumulation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) in the lipids of several tissues (1). This slow accumulation of a minor dietary fatty acid is due to an enzyme defect in the \( \alpha \)-oxidation system (2). In healthy individuals, \( \alpha \)-oxidation of phytanic acid results in the formation of pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which is amenable to \( \beta \)-oxidation, whereas phytanic acid is not.

Another auxiliary fatty acid oxidation system is the \( \omega \)-oxidation system, which takes part in the metabolism of certain other minor dietary fatty acids such as cyclopropane fatty acids (3), acetylenic fatty acids (4), and aromatic fatty acids (unpublished results). It may be anticipated that some phytanic acid is metabolized this way, especially in patients with Refsum’s disease. After an initial \( \omega \)-oxidation, \( \beta \)-oxidation could proceed from the \( \omega \)-end without any need for \( \alpha \)-oxidation.

The presence in serum of phytanic acid metabolites incorporated into triglycerides has been reported for patients with Refsum’s disease (5).

In the present communication, we describe the occurrence of 2,6-dimethyloctanedioic acid in the urine from two patients with Refsum’s disease.

Materials and Methods

Urine was collected from two brothers with Refsum’s disease, ages 35 and 30 years. Both patients were under dietary treatment to minimize phytanic acid intake. When the diagnosis was made in 1979, phytanic acid comprised 19–22% of the esterified and free fatty acids in serum. Details on these cases will be published elsewhere.

Organic acids were extracted from urine with ethyl acetate and derivatized with methoxylamine hydrochloride, then with bis(trimethylsilyl)trifluoroacetamide (6). Alternatively, the extract was derivatized with diazomethane (4).

The derivatized urinary extracts were analyzed by packed-column gas chromatography—mass spectrometry, with an LKB-9000 instrument (LKB-Products, Solna, Sweden) operating in the electron-impact mode at an electron energy of 70 eV, an accelerating voltage of 3.5 kV, and an ion-source temperature of 270 °C. Later the samples were analyzed by capillary-column gas chromatography—mass spectrometry with a Finnigan MAT 44S quadrupole instrument and a Finnigan MAT 212 magnetic sector instrument (Finnigan MAT, Bremen, F.R.G.) under similar conditions (ion-source temperature 220 °C, accelerating voltage 3 kV). The latter two instruments were coupled to a Finnigan MAT SS200 data system.

High-resolution mass measurements on peaks eluting from the capillary column were done at a statically measured resolution of 10 000 (10% valley definition) with a scanning speed of 7.2 s per decade. The mean deviation for the mass measurements was less than 4 millimass units (mmu).

The capillary columns used were 20 to 25 m × 0.5 mm i.d. Pyrex glass columns statically coated with SE-54 (7). The temperature was programmed from 50 to 280 °C at a rate of 10 °C/min. The carrier gas was helium (MAT 44S) or hydrogen (MAT 212). Details of the injection technique used have been published (8).

The urinary metabolites were quantified by mass chromatography of the trimethylsilyl esters on the quadrupole instrument with a mean error of 7% (n = 18). The following ions were used: \( m/z \) 217, 232, and 261 for 2-methylsuccinic acid; \( m/z \) 97, 125, 155, 173, 186, 199, and 289 for 3-methyladipic acid; \( m/z \) 166, 213, 215, 231, and 331 for 2,6-dimethyloctanedioic acid; and \( m/z \) 166, 185, 215, 217, and 331 for decanedioic acid. Decanedioic acid was used instead of 2,6-dimethyloctanedioic acid for the preparation of standard curves, owing to limited availability of the latter compound. The standard curves were prepared by adding to pooled normal urine different amounts of the standard compounds.

Because not all spectra are taken precisely at the maximum of an eluting gas-chromatographic peak, the intensity of selected \( m/z \) values will vary for a given amount of substance injected if only the most-intense mass spectrum is evaluated. An obvious solution to this problem is multiple-monitoring, but for us it is more practical to use the data files routinely recorded and stored on tape within a metabolite screening program in our department. To overcome this limitation of the mass-chromatographic quantification method, we sum the intensities of selected \( m/z \) values of two to four spectra (depending on the peak shape) acquired across the eluting gas-chromatographic peak. If no correction for the number of spectra used is made (e.g., division with \( \sqrt{S \times M} \) in equation 2 below), erroneous results will be obtained.

We used the following equation to quantify the different compounds:

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C = k \times A \quad (1)
\]

where \( C \) is the concentration of the compounds, \( k \) is a constant factor derived from a standard curve for that compound, and \( A \) is the internal-standard-corrected intensity of the compound. The value of \( A \) is derived from the following expression:
The standard, acid; 2-methyl-3-hydroxybutyric ease (Fig. 1. Part of the total ion-current chromatograms of derivatized urinary extracts from a healthy individual (left) and from a patient with Refsum’s disease (right)
The quadrupole was scanned from m/z 50 to 549 (cycle time, 1.4 s). Major identified peaks are: (1) p-cresol; (2) 2-hydroxyisobutyric acid; (3) 2-hydroxyisovaleric acid; (6) 2-methyl-3-hydroxybutyric acid; (7) 3-hydroxyisovaleric acid; (8) methylmalonic acid; (9) 2-ethylhexanoic acid; (10) ursodeoxycholic acid; (11) benzoic acid + artifact; (12) phosphoric acid; (14) succinic acid; (16) 2-methylsuccinic acid; (26 and 29) 3-methylglutamic acid peaks 1 and 2; (31) adipic acid; (32) 4-hydroxybenzenecarboxylic acid; (33) 3-methyladipic acid; (35) furan-5-hydroxymethyl-2-carboxylic acid; (39) 3,4-methylenedioxycarboxylic acid + 2-oxoglutaric acid; (5) 2-hydroxy-3-methylbenzoic acid (internal standard, 50 mg/l); (41) 3-hydroxy-3-methylglutaric acid; (44) 4-hydroxyphenylacetic acid; (46) taurine acid; (47) glycyl-furanecarboxylic acid; (49) ostenodic acid; (51) suberic acid; (X) 2,6-dimethyloctanedic acid; (56) acetic acid; (59) homovanillic acid; (61) azelaic acid; (63) citric acid; (64) hippuric acid; (65) hydroxyphenyl-3-hydroxypropionic acid

where, for a given compound, I is the absolute intensity of a single m/z value, M is the number of different m/z values used, S is the number of spectra used, and A is the value obtained from the computation of A for the internal standard compound. To calculate the A value, we used the absolute intensities of the m/z 281 and 282 ions of the trimethylsilyl derivative of the internal standard (2-hydroxy-3-methyl-benzoic acid), setting the A value to one. The values obtained from the mass-chromatographic evaluation were in good agreement with control values obtained from flame-ionization capillary gas chromatography peak-height measurements. The creatinine values were determined by "high-performance" liquid chromatography (9, 10) with a mean error of 0.3% (n = 20).

Decanedioic acid and 3-methyladipic acid were obtained from Fluka, Buchs, Switzerland. 2-Methylsuccinic acid was prepared through catalytic hydrogenation (4) of itaconic acid (Fluka). All solvents were of analytical grade (Merck, Darmstadt, F.R.G., or BDH, Poole, U.K.). For the high-resolution mass measurements the 2,6-dimethyloctanedic acid was partially purified from the patients' urine by adsorption onto Porapak Q (Waters Associates, Milford, MA 01757) and elution with acetonitrile/water (1/1 by vol).

**Results**

Gas chromatography of urinary organic acids from the two patients revealed an unknown peak not normally found (peak X in Figure 1).

The mass spectrum (MAT 212 instrument) of the trimethylsilyl ester of component X (Figure 3) shows fragment ions such as M–CH₃ at m/z 331, M–H₂O–CO₂ at m/z 287, M–DMSOOCCCH at m/z 231, M–TMSOOCCCH₂ at m/z 215, M–TMSOH at m/z 215, M–2 × TMSOH at m/z 166, M–TMSOOCC–TMSOOCCCH₂–H at m/z 97, and M–TMSOOCCCH₂–TMSOOCCCH₃–H at m/z 69, all in support of a dicarboxylic acid trimethylsilyl ester with a molecular mass of 346 mass units (m.u.). This is compatible with a saturated compound with 10 carbon atoms. More unspecific but expected ions are found at m/z 73 (TMS), 117 (TMSOOC), and 147 (TMSOCDMS). The ions at m/z 69 and 97 indicate a methyl branching at position 2 or 3.

The mass spectrum (MAT 212 instrument) of the methyl ester of component X (Figure 3) suggests a molecular mass of 230 m.u. through the M–CH₃O ion at m/z 199, and strongly supports a C₁₀-dicarboxylic acid structure with fragment ions such as M–2 × CH₂OH at m/z 166, M–CH₃O–CH₂OOC at m/z 139, M–CH₂OOC–CH₂OOCCH₂–H at m/z 97, and M–CH₂OOCCH₂–CH₂OOCCH₂–H at m/z 69. Again the ions at m/z 69 and 97 support a methyl branch at position 2 or 3. Strong evidence for a methyl branch at position 2 is given by the McLafferty rearrangement fragment ion at m/z 88, which is further supported by intense ions at m/z 171 (M–CH₃OOC), 143 (M–CH₂OOCCH₂), 111 (M–CH₂OH–CH₂OOCCH₂), and 83 (M–CH₂OOC). Convincing evidence for a further methyl branch located at position 6 is given by intense ions at m/z 157 (M–CH₂OOCCH₂), 125 (M–CH₂OH–CH₂OOCCH₂), and 101 (M–CH₂OOCCH₂) and a less intense ion at m/z 129 (M–CH₂OOCCH₂CH₂). The correct assignment of a molecular mass of 230 m.u. is confirmed by a pseudomolecular ion at m/z 231 in the isobutane chemical-ionization mass spectrum (Figure 3, inset).

Derivatization with methoxylamine hydrochloride before esterification did not change the mass spectrum of either

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1 DMS = dimethylsilyl; TMS = trimethylsilyl.
derivative, which excluded keto-groups. The methyl ester spectrum was not changed by silylation, which excluded further hydroxyl groups. The mass difference of 116 m.u. between the trimethylsilyl derivative (346 m.u.) and the methyl ester derivative (230 m.u.) is twice the difference between trimethylsilyl and methyl, which further confirms the presence of two carboxyl groups.

The mass spectra obtained from the derivatives of this urinary compound are thus fully compatible with the suggested structure of 2,6-dimethyloctanedioic acid.

The structure assignment was verified by capillary gas chromatography high-resolution mass measurements of the methyl ester as shown in Table 1.

The mean concentration of 2,6-dimethyloctanedioic acid (Table 2) in the patients' urine samples was 1.4 mmol/mol of creatinine. This acid could not be detected in control urine samples (<0.1 mg/L). 3-Methylhexanedioic acid, normally present in a mean concentration of 2.9 mmol/mol of creatinine, was excreted in significantly increased amounts in most urine samples from the two patients to give a mean excretion of 5.9 mmol/mol of creatinine. 2-Methylsuccinic acid was not found to be significantly increased in the patients' urine samples.

The normal values for excretion of 2-methylsuccinic acid and the absence of 2,6-dimethyloctanedioic acid in control urine samples are in good agreement with published results (11), whereas we find that the excreted amounts of 3-methylhexanedioic acid are about half as high in our controls. This difference might be due to a better separation of the compound from interfering material in our study.

**Discussion**

In the metabolic pathway that leads to the formation of 2,6-dimethyloctanedioic acid, 3-methylhexanedioic acid, and possibly 2-methylsuccinic acid from phytanic acid, \( \alpha \)-oxidation is not involved at any point. One can calculate from our data that this pathway may oxidize 0.1 mmol (more than 30 mg) of phytanic acid per day. Obviously, that is not enough to prevent its accumulation in patients with Refsum's disease, if the dietary intake of phytanic acid is not controlled.

The low capacity of this degradation pathway is probably not due to a lack of \( \omega \)-oxidation capacity, \( \omega \)-oxidation having the capacity to oxidize at least 10 mmol of fatty acids in 24 h in (e.g.) starvation ketosis (12), whereas the estimated daily intake of phytanic acid on an unrestricted diet is only about 0.5 mmol (2). A more likely explanation is that the \( \omega \)-oxidation system has a lower affinity for long-chain fatty acids than for medium-chain fatty acids (13).

2,6-Dimethyloctanedioic acid could not be detected in control urine samples, but was constantly present in the urine of both patients. Therefore, it appears very likely that this metabolite is formed from phytanic acid by a combination of \( \omega \)- and \( \beta \)-oxidation.

The diagnosis of Refsum's disease could rely on the present metabolite, but serum phytanic acid is still a more easily detected biochemical abnormality. Occasionally, when Refsum's disease is not suspected, analysis of organic acids will give a clue to the correct diagnosis through this metabolite.

Recently, the excretion of increased amounts of urinary 3-methylhexanedioic acid in Refsum's disease has been reported by two groups (14, 15), but this could not be corroborated by another group (16). The tentative identification of 2,6-dimethyloctanedioic acid as a component of the urine of patients with Refsum's disease was also reported (14, 15), supporting our view that this metabolite regularly occurs in patients with Refsum's disease.

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**References**


