Stereochemistry of the peroxisomal branched-chain fatty acid α- and β-oxidation systems in patients suffering from different peroxisomal disorders

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Abstract Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid derived from dietary sources and broken down in the peroxisome to pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) via α-oxidation. Pristanic acid then undergoes β-oxidation in peroxisomes. Phytanic acid naturally occurs as a mixture of (3S,7R,11R)- and (3R,7R,11R)-diastereomers. In contrast to the α-oxidation system, peroxisomal β-oxidation is stereospecific and only accepts (2S)-isomers. Therefore, a racemase called α-methyl-acyl-CoA racemase is required to convert (2R)-pristanic acid into its (2S)-isomer. To further investigate the stereochemistry of the peroxisomal oxidation systems and their substrates, we have developed a method using gas-liquid chromatography-mass spectrometry to analyze the isomers of phytanic, pristanic, and trimethylundecanoic acid in plasma from patients with various peroxisomal fatty acid oxidation defects. In this study, we show that in plasma of patients with a peroxisomal β-oxidation deficiency, the relative amounts of the two diastereomers of pristanic acid are almost equal, whereas in patients with a defect of α-methyl-acyl-CoA racemase, (2R)-pristanic acid is the predominant isomer. Furthermore, we show that in α-methylacyl-CoA racemase deficiency, not only pristanic acid accumulates, but also one of the metabolites of pristanic acid, (2,6,10)-trimethylundecanoic acid, providing direct in vivo evidence for the requirement of this racemase for the complete degradation of pristanic acid.—Ferdinandusse, S., H. Rusch, A. E. M. van Lint, G. Dacremont, R. J. A. Wanders, and P. Vreken. **Stereochemistry of the peroxisomal branched-chain fatty acid α- and β-oxidation systems in patients suffering from different peroxisomal disorders.** *J. Lipid Res.* 2002. **43:** 438–444.

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Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid derived from the chlorophyll component phytol and is degraded in the peroxi-some. Phytanic acid first undergoes α-oxidation, which leads to shortening of the chain by one carbon atom yielding pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and carbon dioxide (1). Pristanic acid is then further degraded in the peroxisome via three cycles of β-oxidation, followed by transport of the pristanic acid metabolite, 4,8-dimethylnonanoic acid, to the mitochondrion, where it is β-oxidized to completion (2). The β-oxidation process in peroxisomes consists of four sequential enzymatic steps. After activation of pristanic acid to its CoA-ester, pristanoyl-CoA is converted into 2,3-pristenoyl-CoA by branched-chain acyl-CoA oxidase (BCOX). This compound is then hydrated to 3-hydroxy-pristanoyl-CoA and subsequently dehydrogenated to 3-keto-pristanoyl-CoA. These reactions are catalyzed by D-bifunctional protein (D-BP), which harbors both enoyl-CoA hydratase and 3-hydroxy-acyl-CoA dehydrogenase activity. Finally, thiolytic cleavage occurs via sterol carrier protein X (SCPx), yielding propionyl-CoA and (4,8,12)-trimethyltridecanoyl-CoA, which re-enters the β-oxidation spiral (1, 3).

In 1967, Ackman and Hansen studied the stereochemical composition of phytanic and pristanic acid in ruminant fats and fish oils. They found that there are two diastereomers of these fatty acids present, namely the (S,R,R)- and (R,R,R)-isomers. Phytanic acid synthesized from phytol of plant origin also consists of these two isomers (4). Recent studies have shown that peroxisomal α-oxidation is not a stereoselective process (5), so that after α-oxidation of phytanic acid both (2R,6R,10R,14)- and (2S,6R,10R,14)-pristanic acid are formed. In contrast to the α-oxidation
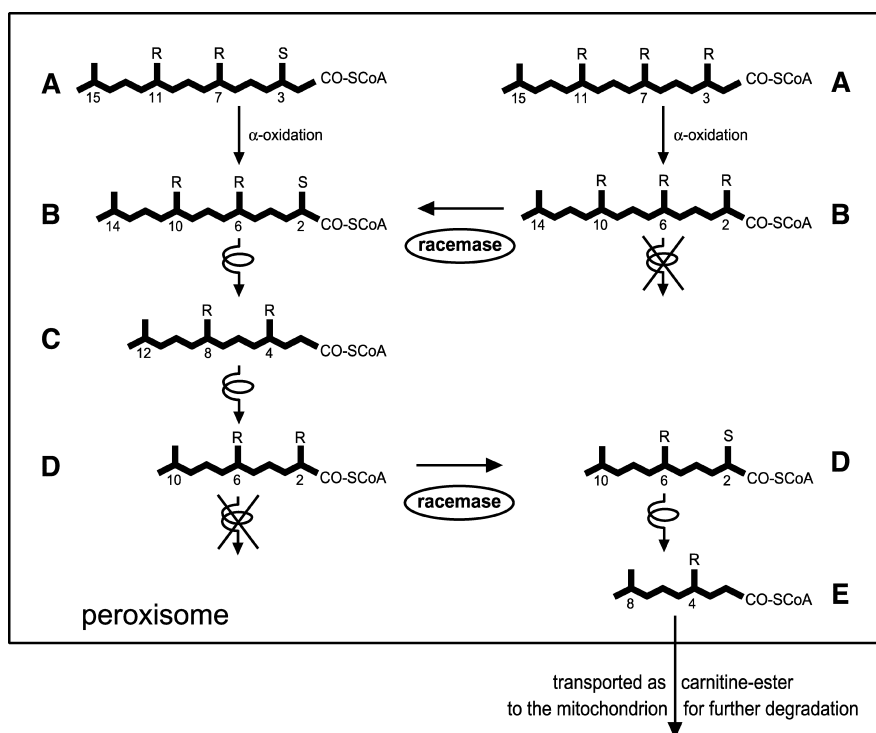

Fig. 1. Schematic representation of the oxidation of phytanic and pristanic acid in the peroxisome. Both (3*R*,7*R*,11*R*,15)- and (3*S*,7*R*,11*R*,15)-phytanoyl-CoA (A) undergo one round of α-oxidation, producing a mixture of (2*R*,6*R*,10*R*,14)- and (2*S*,6*R*,10*R*,14)-pristanoyl-CoA (B). The (2*R*)-isomer needs to be converted to its (*S*)-isomer before it can enter the β-oxidation spiral, because the peroxisomal oxidase, the first enzyme of the β-oxidation system, can only handle (*S*)-isomers. The resulting product, (4*R*,8*R*,12)-trimethyltride-canoyl-CoA (C), can be β-oxidized without any problem, but the next intermediate in the breakdown process of pristanic acid is again a 2-methyl-branched fatty acyl-CoA [(2*R*,6*R*,10)-trimethylundecanoyl-CoA (D)] with the (*R*)-configuration and therefore requires a racemase to convert it to its (*S*)-isomer. After another cycle of β-oxidation, (4*R*,8)-dimethylnonanoyl-CoA (E) is transported from the peroxisome to the mitochondrion as carnitine-ester for further oxidation.

system, however, the peroxisomal β-oxidation system is stereoselective because only (2*S*)-pristanoyl-CoA is accepted as substrate by BCOX, the first enzyme of the β-oxidation system (6–8). For (2*R*)-pristanoyl-CoA to be degraded, it first needs to be converted to its (2*S*)-isomer by the enzyme α-methylacyl-CoA racemase (9, 10) (**Fig. 1**). After two cycles of β-oxidation (2*R*,6*R*,10)-trimethylundecanoyl-CoA is formed. Before this substrate can be degraded further, it needs to be converted to the (2*S*)-isomer. α-Methylacyl-CoA racemase is most likely the enzyme responsible for this racemization, although this has not yet been demonstrated experimentally.

A variety of different genetic diseases in humans have been identified in which there is a defect in the peroxisomal α- and/or β-oxidation of fatty acids, resulting in the accumulation of certain fatty acids in the plasma of these patients. Two groups can be distinguished. In the first group, both fatty acid α- and β-oxidation are impaired. These patients, who suffer from a peroxisome biogenesis disorder, lack functional peroxisomes and, as a consequence, are deficient for many processes taking place in the peroxisome, including the degradation of very long-chain fatty acids and branched-chain fatty acids via β-oxidation. In the second group, either the fatty acid α-oxidation or β-oxidation is deficient, because in this group

of patients only a single enzyme is deficient. Patients suffering from Refsum disease have a deficiency of the first enzyme of the α-oxidation system (phytanoyl-CoA hydroxylase) (11, 12) and, as a consequence, accumulate phytanic acid in their plasma. Patients with a deficiency of D-BP are deficient in peroxisomal β-oxidation of both very long-chain fatty acids and the branched-chain fatty acids, including the bile acid intermediates (13–16). Recently, we identified a new disorder, α-methylacyl-CoA racemase deficiency, which affects the peroxisomal oxidation of 2-methyl branched-chain fatty acids and the bile acid intermediates (17).

To obtain more insight in the stereochemistry of the peroxisomal oxidation systems and their substrates, we developed a method to determine the relative amounts of phytanic, pristanic, and trimethylundecanoic acid diastereomers in plasma samples of patients suffering from the various peroxisomal disorders described above.

MATERIALS AND METHODS

Patient material

All samples used in this study were obtained from patients with a confirmed deficiency of α-methylacyl-CoA racemase (17),

D-BP (13–16), or phytanoyl-CoA hydroxylase (11, 12) due to mutations in the encoding genes, or from patients affected by a peroxisome biogenesis disorder, as demonstrated by biochemical studies performed in fibroblasts (18). Informed consent was obtained from parents or guardians of the patients whose plasma was studied in this article, and the studies were approved by the Institutional Review Board of the Academic Medical Center, University of Amsterdam.

Synthesis of (2,6,10)-trimethylundecanoic acid

(2,6,10)-Trimethylundecanoic acid was synthesized from (2,6,10)-trimethyl-5,9-undecadien-1-ol (Acros Organics, Geel, Belgium). First, (2,6,10)-trimethyl-5,9-undecadien-1-ol (a mixture of diastereomers) was hydrogenated in ethanol in the presence of 5 mol% PtO_2 as a catalyst, under a H_2 pressure of three bars for 16 h to (2,6,10)-trimethylundecan-1-ol with a yield of 95%. To form the corresponding aldehyde, (2,6,10)-trimethylundecan-1-ol was dissolved in dichloromethane-acetonitrile (9:1, v/v, 2 ml/mmol) and reacted with 1.5 molar equivalents of *N*-methylmorpholine-*N*-oxide in the presence of 500 mg/mmol 4A molecular sieve and 5 mol% tetrapropylammonium perruthenate at room temperature for 2 h. The aldehyde was purified on a silica gel column using dichloromethane-ethyl acetate (8:2, v/v) as eluent. Subsequently, the eluent was taken to dryness with a rotary evaporator and the residue was purified by silica gel chromatography using hexane-ethyl acetate (95:5, v/v) as eluent. The yield of the aldehyde was 85%. For oxidation of (2,6,10)-trimethylundecan-1-al to the corresponding carboxylic acid, the aldehyde was dissolved in acetonitrile (1 ml/mmol) and mixed with 5 M equivalents of H_2O_2 (added as a 35% aqueous solution) in the presence of a 0.66 M NaH_2PO_4 buffer, pH 2. Subsequently, 1.4 M equivalents of 1 M aqueous $NaClO_2$ was slowly added over a period of 1 h at 10°C. The reaction was allowed to continue for 4 h at room temperature. After addition of a small amount of Na_2SO_3 to destroy unreacted HOCl and H_2O_2, the product was extracted from the reaction mixture with hexane. The hexane was removed under a stream of N_2 and the residue purified by chromatography on a silica gel column with a discontinuous gradient of hexane-ethyl acetate (98:2–95:5, v/v) as solvent. The yield of (2,6,10)-trimethylundecanoic acid was 80%. Gas-liquid chromatography-mass spectrometry (GC-MS) of the methyl ester showed one homogeneous peak with a molecular ion at m/z 242. Mass spectrometry of the unesterified acid gave the following results: m/z 228 (M^+ 2.5%), 152 (9.2%), 115 (5.7%), 97 (20.7%), 87 (31.4%), 74 (100%), 55 (50%), and 41 (65%). The overall yield from (2,6,10)-trimethyl-5,9-undecadien-1-ol to (2,6,10)-trimethylundecanoic acid was 60%.

Quantification and analysis of plasma phytanic, pristanic, and trimethylundecanoic acid diastereomers

Fatty acids were extracted from plasma as described (19). After extraction, the hexane phase was used for both quantification of the branched-chain fatty acids and analysis of the diastereomers of the branched-chain fatty acids. Phytanic and pristanic acid were quantified as described (19). Trimethylundecanoic acid was quantified using standard gas chromatography-analysis of methylated essential fatty acids, essentially as described (20). The isomers of the different branched-chain fatty acids were separated essentially as described by Schmitz et al. (9). One hundred microliters of the hexane-phase, containing the branched-chain fatty acids, were evaporated under a stream of N_2. Five hundred microliters of 30 mM carbonyldiimidazole (Sigma, St. Louis, MO) dissolved in toluene were added and, after 10 min at room temperature, the sample was acidified with 10 µl glacial acetic acid. Subsequently, 50 µl (*R*)-1-phenylethylamine (Sigma, St. Louis, MO) was added. After 30 min at room temperature, the sample was mixed with 5 ml 50 mM

sodium/potassium phosphate pH 7.5. The reaction products were extracted with 1 ml ethyl acetate, which was dried under a stream of N_2, and the residue dissolved in 75 µl hexane. Finally, the different branched-chain fatty acids were analyzed by GC-MS. Capillary column: 25 m × 0.25 mm i.d. CP-sil 19 CB (Chrompack, Middelburg, The Netherlands); column temperature: 50°C for 2 min; 50–240°C at a rate of 30°C/min; 240°C for 5 min; 240–285°C at a rate of 2.5°C/min; 285°C for 2 min; injection port and GC-MS interface at 250°C and 300°C, respectively; ionization energy: 70 eV; 2 µl splitless injection; carrier gas helium, pressure 0.7 bar, 1.5 ml/min constant flow. Single ion monitoring was used for the respective M^+ ions (m/z 331, 401, and 415; masses of the molecular ions of the phenylethylamide derivatives of trimethylundecanoic, pristanic, and phytanic acid, respectively). Racemic mixtures of trimethylundecanoic acid (synthesized as described above), pristanic acid (purchased from Dr. H. ten Brink, Amsterdam, The Netherlands), and phytanic acid (Sigma, St. Louis, MO) were used to set up the analysis.

Identification of the (2*R*)- and (2*S*)-isomers of pristanic and trimethylundecanoic acid

To assess the configuration of the isomers of pristanic and trimethylundecanoic acid, the racemic mixtures of these fatty acids were converted into their CoA-esters as described by Rasmussen et al. (21) and incubated with purified long-chain acyl-CoA dehydrogenase (LCAD), which was a generous gift from Prof. Dr. T. Hashimoto (Shinshu University School of Medicine, Matsumoto, Japan) (22). Because only (2*S*)-isomers are substrate for LCAD (23), incubation with and without LCAD allows discrimination between the (2*S*)- and (2*R*)-isomers (**Fig. 2**). The incubation mixture consisted of purified LCAD (2.6 µU, determined with C8-CoA as substrate), 100 mM sodium phosphate/0.1 mM EDTA (pH 7.2), 0.4 mM hexafluorophosphate, 20 µM FAD, and 50 µM trimethylundecanoyl-CoA or pristanoyl-CoA, with a final volume of 100 µl. Reactions were allowed to proceed for 60 min at 37°C.
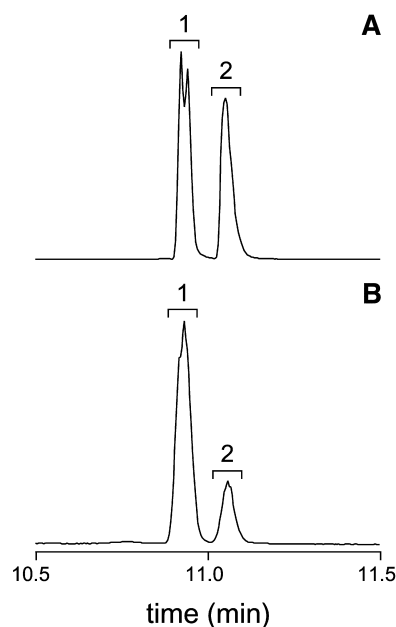

Fig. 2. GC-MS analysis of the (*R*)-1-phenylethylamine derivatives of a chemically synthesized racemic mixture of trimethylundecanoic acid before (A) and after (B) incubation of the corresponding CoA-esters with LCAD. Cluster 1 corresponds to the (2*R*)-isomers and cluster 2 to the (2*S*)-isomers.
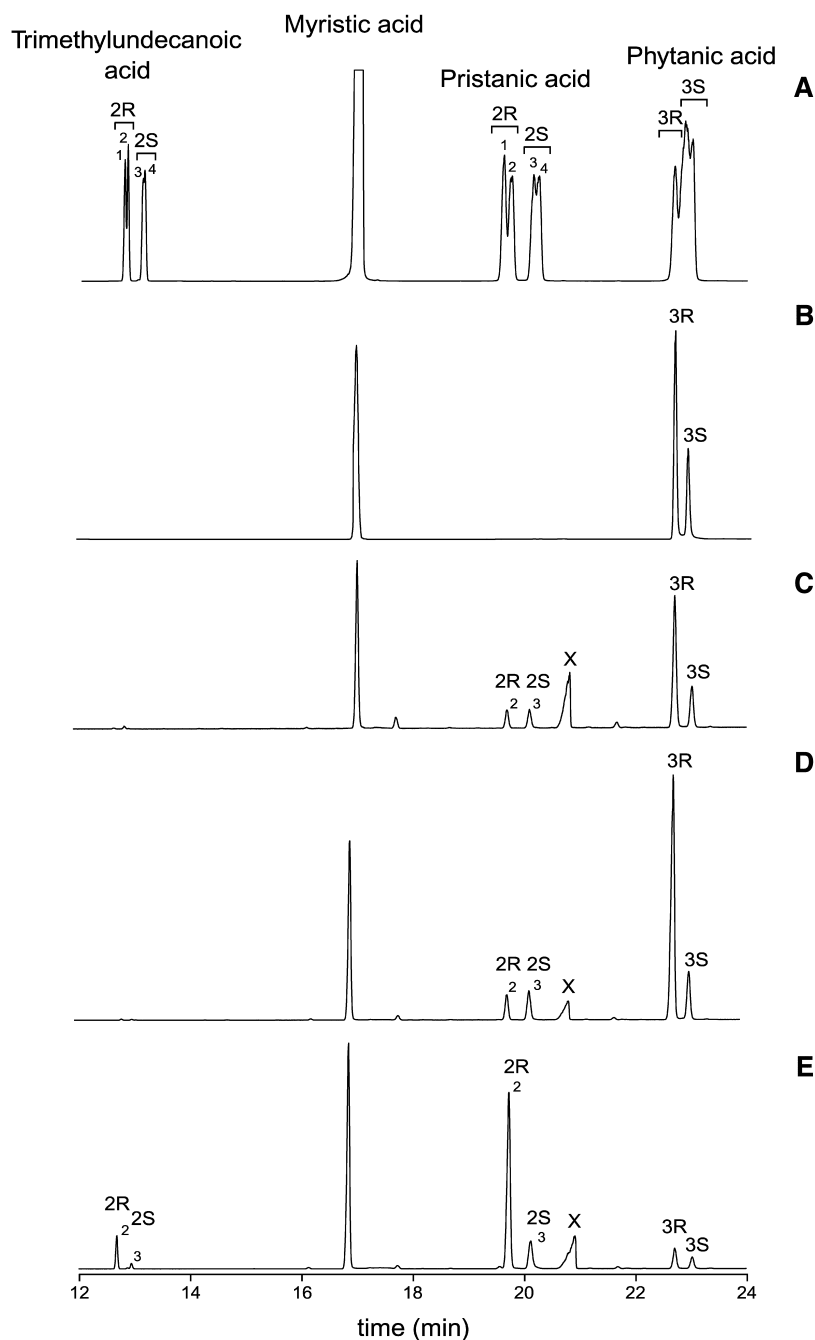
Fig. 3. Representative chromatograms of the GC-MS analysis of phytanic, pristanic, and trimethylundecanoic acid isomers in a chemically synthesized standard mixture (A), and in plasma from a patient with Refsum disease (B), Zellweger syndrome (C), D-BP deficiency (D), and α-methylacyl-CoA racemase deficiency (E). The stereochemical configuration of the first methyl group (R-S) is indicated for all the branched-chain fatty acids. GC-MS analysis of (R)-1-phenylethylamine derivatives of both pristanic and trimethylundecanoic acid revealed four peaks for each fatty acid (numbered 1–4). In plasma from patients with a deficiency of the peroxisomal β-oxidation, however, only peaks 2 and 3 were present. This strongly suggests that peaks 2 and 3 correspond to (2R,6R,10R,14)- and (2S,6R,10R,14)-pristanic acid, respectively, and in case of trimethylundecoanoic acid, to the (2R,6R,10)- and (2S,6R,10)-isomer, respectively. The identity of peak X is unknown.

After termination of the reaction, the CoA-esters were hydrolyzed by the addition of 10 μl 5 N NaOH followed by an incubation period of 2 h at 60°C. The isomers of the branched-chain fatty acids were then analyzed as described above (Fig. 2).

RESULTS

GC-MS analysis of chemically synthesized standards of phytanic, pristanic, and trimethylundecanoic acid as their (R)-1-phenylethylamine derivatives resulted in two clusters of peaks for all three branched-chain fatty acids (see **Fig. 3A**). For both pristanic and trimethylundecanoic acid, the clusters clearly consisted of two peaks each (numbered 1 through 4 in Fig. 3A). As described in the Materials and Methods section, we incubated the CoA-esters of these fatty acids with purified LCAD to discriminate between the (2S)- and the (2R)-isomers because LCAD dehydrogenates (2S)- but not (2R)-isomers (23). After an incubation period of 60 min, there was a strong reduction of the abundance of the second peak cluster for both trimethylundecanoyl-CoA (Fig. 2) and pristanoyl-CoA (data not shown), while the abundance of the first peak cluster remained unchanged, indicating that the second cluster consists of the (2S)-isomers. These results are in agreement with the results obtained for the (R)-1-phenylethylamine derivatives of 2-methylmyristic acid (9) and for 2-methylpentadecanoic acid (5), where the second cluster corresponded to the (2S)-isomers. Figure 3B–E shows representative chromatograms of the GC-MS analysis of phytanic, pristanic, and trimethylundecanoic acid in

TABLE 1. Concentrations and relative amounts of branched-chain fatty acid isomers
in different peroxisomal disorders

	Trimethylundecanoic Acid		Pristanic Acid		Phytanic Acid	
	(μmol/l)	(S/R)-isomer[a]	(μmol/l)	(S/R)-isomer[a]	(μmol/l)	(S/R)-isomer[a]
Controls [n = 50]	ND	—	0–1.2[b]	—	0–10.2[b]	—
Refsum 1	ND	—	ND	—	230	0.41
Refsum 2	ND	—	0.5	—	377	0.35
Refsum 3	ND	—	0.1	—	216	0.59
Refsum 4	ND	—	0.3	—	361	0.43
Zellweger 1	ND	—	16.6	1.38	43.8	0.43
Zellweger 2	4.8	0.35	91.2	0.79	554	0.35
Zellweger 3	ND	—	14.2	1.04	43.6	0.32
Zellweger 4	ND	—	11.8	1.04	51.4	0.32
D-BP 1	ND	—	21.6	1.04	74	0.79
D-BP 2	ND	—	56.6	1.27	192	0.18
D-BP 3	ND	—	81.0	0.37	21.8	0.54
Racemase 1	31.4	0.19	326	0.20	8.6	0.37
Racemase 2	80.4	0.25	207	0.25	26.2	0.56
Racemase 3	28.0	0.15	114	0.20	16.8	0.58
Racemase 4	18.4	0.15	32	0.33	22.6	0.75

ND, not detected; Refsum, patients with Refsum disease; Zellweger, patients with Zellweger syndrome; D-BP, patients with a deficiency of D-BP; Racemase, patients with a deficiency of α-methylacyl-CoA racemase.

[a] Ratio.
[b] Range.

plasma from a patient with Refsum disease (Fig. 3B), Zellweger syndrome (Fig. 3C), D-BP deficiency (Fig. 3D), and α-methylacyl-CoA racemase deficiency (Fig. 3E). In plasma from all patients affected by a peroxisomal disorder only peak 2 and 3 could be detected (Fig. 3C–E).

Plasma analysis in all the patients studied showed that the ratio $(3S,3R)$-phytanic acid did not differ significantly between the different peroxisomal disorders (see **Table 1**). The mean value $(\pm SD)$ for the $(3S,3R)$-phytanic acid ratio was 0.46 (± 0.10), which is similar to the ratio found by Ackman and Hansen (0.39) in patients suffering from Refsum disease (4). In plasma samples from patients with a deficiency of α-methylacyl-CoA racemase (17), the ratio $(2S,2R)$-pristanic acid was significantly lower than in plasma from patients with Zellweger syndrome $(P < 0.005$, t-test) and in most patients with a deficiency of D-BP (Table 1). The mean value $(\pm SD)$ for the $(2S,2R)$-pristanic acid ratio was 1.06 (± 0.24) in patients with Zellweger syndrome and 0.89 (± 0.47) in patients with a D-BP deficiency, whereas the mean value $(\pm SD)$ in patients with a deficiency of α-methylacyl-CoA racemase was 0.24 (± 0.06).

Next to phytanic and pristanic acid, trimethylundecanoic acid accumulated in plasma from patients with a deficiency of α-methylacyl-CoA racemase (Table 1, Fig. 3E). Although the $(2R)$-isomer was the predominant isomer, a small amount of $(2S)$-isomer was also found. In addition, one patient suffering from Zellweger syndrome accumulated this fatty acid in his plasma (Table 1).

DISCUSSION

GC-MS analysis of (R)-1-phenylethylamine derivatives of both pristanic and trimethylundecanoic acid revealed four peaks for each fatty acid. In plasma from a patient with a defi-

ciency of the peroxisomal β-oxidation, however, only peaks 2 and 3 were present. In combination with the fact that the sixth and tenth carbon atom of naturally occurring pristanic acid have the (R)-configuration (4), this strongly suggests that peaks 2 and 3 correspond to $(2R,6R,10R)$- and $(2S,6R,10R)$-pristanic acid, respectively, and in the case of trimethylundecanoic acid, to the $(2R,6R)$- and $(2S,6R)$-isomer, respectively. When these branched-chain fatty acids are synthesized chemically, they can exist in several different stereoisomeric configurations. Only four different stereoisomers of trimethylundecanoic acid exist, suggesting that peaks 1 and 4 are the $(2R,6S)$- and $(2S,6S)$-isomer, respectively. Pristanic acid, however, when synthesized chemically, can exist in eight different stereoisomeric configurations. Therefore, the exact identity of peaks 1 and 4 of pristanic acid is not known.

The mean value (0.46) for the $(3S,3R)$-phytanic acid ratio in all patients studied probably reflects the ratio of these isomers in a normal diet. Both isomers occur in animals, but in terrestrial mammals the $(3R,7R,11R)$-isomer predominates, whereas in marine life the $(3S,7R,11R)$-isomer is predominant (4). Different ratios of these isomers in the diet might explain why the values in the patients ranged between 0.18 and 0.79, although in most patients the ratio was close to the mean.

In plasma samples from patients with a deficiency of α-methylacyl-CoA racemase (17), the ratio $(2S,2R)$-pristanic acid was significantly lower than in plasma from patients with Zellweger syndrome and in most patients with a deficiency of D-BP. In patients with a deficiency of α-methylacyl-CoA racemase, $(2S)$-pristanic acid can be degraded normally via peroxisomal β-oxidation, but $(2R)$-pristanic acid cannot be converted to its $(2S)$-isomer and therefore accumulates. In contrast to the exclusive accumulation of $(25R)$-DHCA and $(25R)$-THCA in plasma of patients with an α-methylacyl-CoA racemase deficiency (24), no exclusive accumulation of

(2R)-pristanic acid was found. This can be explained by the fact that DHCA and THCA are only synthesized in the liver as (R)-isomer, whereas pristanic and phytanic acid are derived from the diet, consisting of a mixture of isomers. Accumulation of both pristanic acid and the bile acid intermediates probably inhibits β-oxidation of branched-chain fatty acids in the peroxisome, which causes some dietary (2S)-pristanic acid to accumulate. For this reason, the method described in this article for the analysis of the isomers of pristanic acid cannot be used for the unequivocal diagnosis of patients with a deficiency of α-methylacyl-CoA racemase. Identification of α-methylacyl-CoA racemase deficiency can best be done using our previously described method in which LC-MS-MS is used to analyze the isomers of DHCA and THCA in plasma (24).

In patients with a deficiency of α-methylacyl-CoA racemase, half of the pristanic acid can enter the β-oxidation spiral. The degradation can, however, not proceed beyond (2,6,10)-trimethylundecanoyl-CoA, of which all methyl groups have the (R)-configuration. For this compound to be β-oxidized further, the (2R)-methyl group needs to be converted to the (S)-configuration by a racemase. In case of a deficiency of α-methylacyl-CoA racemase, trimethyl-undecanoic acid accumulates in plasma, confirming that this is the racemase responsible for the conversion of this compound. Unexpectedly, however, the (2R)-isomer was not the only isomer that accumulated, as a small amount of (2S)-isomer was also found. This might be explained by the presence of some racemic trimethylundecanoic acid derived from the diet. In this respect it is important to mention that all racemase patients were adults when their plasma was analyzed and could have accumulated some (2S)-tri-methylundecanoic acid over the years. A problem with this interpretation remains that (2S)-trimethylundecanoic acid would be expected to undergo normal β-oxidation. In analogy to the situation with (2S)-pristanic acid, we hypothesize that the peroxisomal β-oxidation of both (2S)-pristanic acid and (2S)-trimethylundecanoic acid is compromised due to inhibition by the competing (2R)-compounds.

Because all the patients with a peroxisomal β-oxidation deficiency were still very young when their plasma was analyzed, no accumulation of dietary trimethylundecanoic acid was expected. One patient suffering from Zellweger syndrome, however, did accumulate this fatty acid in his plasma. In addition, the patient had extremely elevated levels of both phytanic and pristanic acid. The high levels of phytanic and pristanic acid, in combination with a low residual pristanic acid β-oxidation activity in fibroblasts from this patient [56 pmol/h/mg vs. 898 ± 223 pmol/h/mg (n = 50) in control fibroblasts], suggest that there might have been some endogeneous production of tri-methylundecanoic acid. Probably, the (2S)-trimethylunde-canoic acid in the plasma of this patient is formed by the mitochondrial α-methylacyl-CoA racemase, which is the same enzyme as the peroxisomal but is unaffected in Zellweger syndrome (25).

In conclusion, we have shown that in plasma from patients with a peroxisomal fatty acid oxidation defect, only two diastereomers of phytanic and/or pristanic acid accumulate, most likely the (S,R,R)- and (R,R,R)-isomers. In patients with a defect of α-methylacyl-CoA racemase, almost all pristanic acid was the (2R)-isomer, whereas in patients with a peroxisomal β-oxidation deficiency, the relative amounts of the two diastereomers of pristanic acid were almost equal. Furthermore, we have shown that in α-methyl-acyl-CoA racemase deficiency, not only pristanic acid accumulates, but also one of the metabolites of pristanic acid, (2,6,10)-trimethylundecanoic acid, providing direct in vivo evidence for the requirement of this racemase for the complete degradation of pristanic acid.

REFERENCES

1. Wanders, R. J., P. Vreken, S. Ferdinandusse, G. A. Jansen, H. R. Waterham, C. W. Van Roermund, and E. G. Van Grunsven. 2001. Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. *Biochem. Soc. Trans.* **29:** 250–267.
2. Verhoeven, N. M., D. S. Roe, R. M. Kok, R. J. Wanders, C. Jakobs, and C. R. Roe. 1998. Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. *J. Lipid Res.* **39:** 66–74.
3. van Veldhoven, P. P., M. Casteels, G. P. Mannaerts, and M. Baes. 2001. Further insights into peroxisomal lipid breakdown via alpha- and beta-oxidation. *Biochem. Soc. Trans.* **29:** 292–298.
4. Ackman, R. G., and R. P. Hansen. 1967. The occurrence of diastereomers of phytanic and pristanic acids and their determination by gas-liquid chromatography. *Lipids.* **2:** 357–362.
5. Croes, K., M. Casteels, M. Dieuaide-Noubhani, G. P. Mannaerts, and P. P. Van Veldhoven. 1999. Stereochemistry of the alpha-oxidation of 3-methyl-branched fatty acids in rat liver. *J. Lipid Res.* **40:** 601–609.
6. Pedersen, J. I., T. Veggan, and I. Bjorkhem. 1996. Substrate stereospecificity in oxidation of (25S)-3 alpha, 7 alpha, 12 alpha-trihydroxy-5 beta-cholestanoyl-CoA by peroxisomal trihydroxy-5 beta-cholestanoyl-CoA oxidase. *Biochem. Biophys. Res. Commun.* **224:** 37–42.
7. van Veldhoven, P. P., K. Croes, S. Asselberghs, P. Herdewijn, and G. P. Mannaerts. 1996. Peroxisomal beta-oxidation of 2-methyl-branched acyl-CoA esters: stereospecific recognition of the 2S-methyl compounds by trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase. *FEBS Lett.* **388:** 80–84.
8. Schmitz, W., and E. Conzelmann. 1997. Stereochemistry of peroxisomal and mitochondrial beta-oxidation of alpha-methylacyl-CoAs. *Eur. J. Biochem.* **244:** 434–440.
9. Schmitz, W., R. Fingerhut, and E. Conzelmann. 1994. Purification and properties of an alpha-methylacyl-CoA racemase from rat liver. *Eur. J. Biochem.* **222:** 313–323.
10. Schmitz, W., C. Albers, R. Fingerhut, and E. Conzelmann. 1995. Purification and characterization of an alpha-methylacyl-CoA racemase from human liver. *Eur. J. Biochem.* **231:** 815–822.
11. Jansen, G. A., R. Ofman, S. Ferdinandusse, L. IJlst, A. O. Muijsers, O. H. Skjeldal, O. Stokke, C. Jakobs, G. T. Besley, J. E. Wraith, and R. J. Wanders. 1997. Refsum disease is caused by mutations in the phytanoyl-CoA hydroxylase gene. *Nat. Genet.* **17:** 190–193.
12. Mihalik, S. J., J. C. Morrell, D. Kim, K. A. Sacksteder, P. A. Watkins, and S. J. Gould. 1997. Identification of PAHX, a Refsum disease gene. *Nat. Genet.* **17:** 185–189.
13. Suzuki, Y., L. L. Jiang, M. Souri, S. Miyazawa, S. Fukuda, Z. Zhang, M. Une, N. Shimozawa, N. Kondo, T. Orii, and T. Hashimoto. 1997. D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA de-

hydrogenase bifunctional protein deficiency: a newly identified peroxisomal disorder. *Am. J. Hum. Genet.* **61:** 1153–1162.

14. van Grunsven, E. G., E. van Berkel, L. IJlst, P. Vreken, J. B. de Klerk, J. Adamski, H. Lemonde, P. T. Clayton, D. A. Cuebas, and R. J. Wanders. 1998. Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency: resolution of the enzyme defect and its molecular basis in bifunctional protein deficiency. *Proc. Natl. Acad. Sci. USA.* **95:** 2128–2133.

15. van Grunsven, E. G., E. van Berkel, P. A. Mooijer, P. A. Watkins, H. W. Moser, Y. Suzuki, L. L. Jiang, T. Hashimoto, G. Hoefler, J. Adamski, and R. J. Wanders. 1999. Peroxisomal bifunctional protein deficiency revisited: resolution of its true enzymatic and molecular basis. *Am. J. Hum. Genet.* **64:** 99–107.

16. van Grunsven, E. G., P. A. Mooijer, P. Aubourg, and R. J. Wanders. 1999. Enoyl-CoA hydratase deficiency: identification of a new type of D-bifunctional protein deficiency. *Hum. Mol. Genet.* **8:** 1509–1516.

17. Ferdinandusse, S., S. Denis, P. T. Clayton, A. Graham, J. E. Rees, J. T. Allen, B. N. McLean, A. Y. Brown, P. Vreken, H. R. Waterham, and R. J. Wanders. 2000. Mutations in the gene encoding peroxisomal alpha-methylacyl-CoA racemase cause adult-onset sensory motor neuropathy. *Nat. Genet.* **24:** 188–191.

18. Wanders, R. J., R. B. Schutgens, and P. G. Barth. 1995. Peroxisomal disorders: a review. *J. Neuropathol. Exp. Neurol.* **54:** 726–739.

19. Vreken, P., A. E. van Lint, A. H. Bootsma, H. Overmars, R. J. Wanders, and A. H. van Gennip. 1998. Rapid stable isotope dilution analysis of very-long-chain fatty acids, pristanic acid and phytanic acid using gas chromatography-electron impact mass spectrometry. *J. Chromatogr. B Biomed. Sci. Appl.* **713:** 281–287.

20. Dacremont, G., and G. Vincent. 1995. Assay of plasmalogens and polyunsaturated fatty acids (PUFA) in erythrocytes and fibroblasts. *J. Inherit. Metab. Dis.* **18:** 84–89.

21. Rasmussen, J. T., T. Borchers, and J. Knudsen. 1990. Comparison of the binding affinities of acyl-CoA-binding protein and fatty-acid-binding protein for long-chain acyl-CoA esters. *Biochem. J.* **265:** 849–855.

22. Furuta, S., S. Miyazawa, and T. Hashimoto. 1981. Purification and properties of rat liver acyl-CoA dehydrogenases and electron transfer flavoprotein. *J. Biochem. (Tokyo).* **90:** 1739–1750.

23. Battaile, K. P., M. McBurney, P. P. Van Veldhoven, and J. Vockley. 1998. Human long chain, very long chain and medium chain acyl-CoA dehydrogenases are specific for the S-enantiomer of 2-methylpentadecanoyl-CoA. *Biochim. Biophys. Acta.* **1390:** 333–338.

24. Ferdinandusse, S., H. Overmars, S. Denis, H. R. Waterham, R. J. A. Wanders, and P. Vreken. 2001. Plasma analysis of di- and trihydroxycholestanoic acid diastereoisomers in peroxisomal alpha-methylacyl-CoA racemase deficiency. *J. Lipid Res.* **42:** 137–141.

25. Ferdinandusse, S., S. Denis, L. IJlst, G. Dacremont, H. R. Waterham, and R. J. Wanders. 2000. Subcellular localization and physiological role of alpha-methylacyl-CoA racemase. *J. Lipid Res.* **41:** 1890–1896.