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CoA esters of valproic acid and related metabolites are oxidized in peroxisomes through a pathway distinct from peroxisomal fatty and bile acyl-CoA β-oxidation

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In rat liver homogenates fortified with the appropriate cofactors (ATP and CoA), valproic acid induced H_2O_2 production rates by far lower than those recorded on the straight medium-chain fatty acid *n*-octanoic acid. Using directly the CoA esters of these carboxylic acids as substrates for the rat liver H_2O_2-generating enzyme activities, valproyl-CoA, and *n*-octanoyl-CoA were found to induce similar oxidation rates. In the rat liver homogenates, cyanide-insensitive valproyl-CoA and octanoyl-CoA oxidations occurred at rates similar to those of valproyl-CoA and octanoyl-CoA oxidase(s), respectively. Studies on fractions obtained from rat liver postnuclear supernatants by isopycnic centrifugation on a linear sucrose density gradient disclose that the density distribution of valproyl-CoA oxidase superimposes to those of catalase, fatty acyl-CoA oxidase and cyanide-insensitive fatty acyl-CoA oxidation, three peroxisomal marker activities. By contrast, the cyanide-insensitive valproyl-CoA oxidation does not adopt the typical peroxisomal distribution of these activities but rather exhibits a mitochondrial localization with, however, a minor peroxisomal component. Interestingly enough, the comparative study of rat tissue distribution, inducibility by clofibrate and sensitivity to deoxycholate indicated that valproyl-CoA oxidase is an enzyme distinct from fatty acyl-CoA oxidase and bile acyl-CoA oxidase. Taken as a whole, the results presented here support the occurrence of a peroxisomal oxidation of the CoA ester of valproic acid and its Δ4-enoic derivate which might be characterized by two major features: initiation by an acyl-CoA oxidase distinct from fatty and bile acyl-CoA oxidases, and inability to complete the β-oxidation cycle which would not proceed, at significant rates, further than the β-hydroxyacyl-CoA dehydrogenation step in peroxisomes.

Valproyl-CoA oxidase; Bile acyl-CoA oxidase; Fatty acyl-CoA oxidase; Valproate metabolism; Trihydroxycoprostanoate; Peroxisomal β-oxidation

1. INTRODUCTION

Acyl-CoA β-oxidation in mammals takes place in two distinct cellular organelles: mitochondria and peroxisomes (for a general consideration, see [1]). For a long time, β-oxidation was thought to be the prerogative of mitochondria until the discovery in 1976 by Lazarow and de Duve [2] that peroxisomes also contain the enzymic equipment for catalysis of β-oxidation. The initiating step of β-oxidation pathways is catalysed in mitochondria by various acyl-CoA dehydrogenases (3 distinct straight-chain fatty acyl-CoA dehydrogenases with overlapping chain-length specificities and 2 distinct branched-chain acyl-CoA dehydrogenases [3]; and 1 glutaryl-CoA dehydrogenase exhibiting both dehydrogenation and decarboxylation activities [4]). Specific or generalized deficiencies of these mitochondrial acyl-CoA dehydrogenases have been recognised in human patients (for a general consideration, see [5]).

In peroxisomes, it was initially thought that a single acyl-CoA oxidase existed with a broad substrate specificity, a feature not further corroborated by enzyme purification studies or by peroxisomal acyl-CoA β-oxidation activities either responsive or not to peroxisome proliferators [6–12]. In fact at least three distinct acyl-CoA oxidases have been demonstrated to occur in peroxisomes: peroxisome proliferator inducible fatty acyl-CoA oxidase [6,7,10–12], bile acyl-CoA [6,7,10] and pristanoyl-CoA oxidases [11,12], the activity of the latter two enzymes not being stimulated upon exposure to peroxisome proliferators.

Previous evidence has been provided for the occurrence of mitochondrial metabolism of valproic acid [13,14] initiated by 2-methyl-branched-chain acyl-CoA dehydrogenase [13]. Interestingly, the data presented here might argue for the occurrence of an incomplete β-oxidation of the CoA esters of valproic acid and related metabolites in peroxisomes initiated by a medium branched-chain acyl-CoA oxidase which, on the basis of two criteria, sensitivity to deoxycholic acid and either

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inducibility by fibrates or tissue distribution, is demonstrated to be distinct from fatty acyl-CoA oxidase and bile acyl-CoA oxidase.

2. EXPERIMENTAL

2.1. *Materials*

Valproic acid metabolites including 2-propyl-pent-Δ4-enoic acid, 2-propyl-4-hydroxy-pentanoic acid (2-propyl-4-methyl-γ-butyrolactone) and 2-propyl-glutaric acid were gifts from Dr. Dricot, Labaz-Sanofi, Brussels. Valproic acid (2-propyl-pentanoic acid), 2-ethyl-hexanoic acid, 2-methyl-hexanoic acid, and octanoic acid were purchased from Janssen Chimica (Beerse, Belgium). CoA, ATP and FAD were from Boehringer Pharma (Mannheim, Germany). Peroxidase type II, homovanillic acid, glycylglycine buffer, octanoyl-CoA and lauroyl-CoA were from Sigma (St. Louis, MO, USA). 4-Methylumbelliferyl derivatives were from Koch-Light Laboratories (Colnbrook, Bucks, UK). Other common chemicals were of analytical grade from Merck (Darmstadt, Germany).

2.2. *Animals*

Adult male Wistar rats were used. They were fed on either a standard laboratory animal chow (untreated animals) or a solid diet made by mixing the powdered animal chow with clofibrate (0.5% w/w) (clofibrate-treated animals).

2.3. *Chemical synthesis of CoA esters*

3α-, 7α-, 12α-Trihydroxy-5β-cholestanoic acid was purified from alligator bile [15], and its CoA ester (referred to as trihydroxycoprostanoyl-CoA in the text) was synthesized according to the method of Webster and Killenberg [16]. CoA esters of valproic acid, 2-propyl-pent-Δ4-enoic acid and 2-ethyl-hexanoic acid were synthesized essentially as previously described [17].

2.4. *Enzyme assays*

Acyl-CoA oxidase activities were measured by fluorimetric determinations of hydrogen peroxide production rates [18], using as substrates either the unesterified carboxylic acid fortified with ATP and CoA (indirect assay) or the carboxylyl-CoA ester (direct assay). For the indirect assays, the assay medium (1 ml) contained 100 mM glycylglycine buffer, pH 8.3, 5 mM homovanillic acid, 0.1 mg horseradish peroxidase type II, 8 mM ATP, 6 mM $MgCl_2$, 2 mM unesterified carboxylic acid as substrate and the homogenate proteins. For the direct assays, similar experimental conditions were utilized except that neither ATP, $MgCl_2$ nor unesterified carboxylic acids were added, and 0.2 mM acyl-CoA was included in the assay media. Cyanide-insensitive acyl-CoA oxidations were assayed using the procedure described by Lazarow [19].

Lysosomal β-hexosaminidase and mitochondrial cytochrome *c* oxidase were measured as in previous work [20]. Sulphatase C activity was determined according to the procedure described in [21].

2.5. *Rat liver homogenates*

Rat liver homogenates were routinely prepared in distilled water. In the case of cell fractionation studies (see below), the livers were homogenized in an isotonic medium buffered at pH 7.2–7.4 (see [22]).

2.6. *Cell fractionation*

Fractionation by density gradient equilibration was performed as previously described [22].

3. RESULTS

3.1. *Rat liver hydrogen peroxide-generating enzyme activities recorded on various unesterified medium-chain carboxylic acids*

In a first series of experiments, various medium-chain carboxylic acids, including valproic acid and three related metabolites (2-propyl-pent-Δ4-enoic acid 2-propyl-4-hydroxy-pentanoic acid and 2-propyl-glutaric acid), as well as 2-ethyl-hexanoic acid, 2-methyl-hexanoic acid and *n*-octanoic acid, were tested for their abilities to generate, when fortified with the appropriate cofactors, hydrogen peroxide upon incubation with rat liver homogenates. Under these conditions, optimal and minimal activities were recorded on *n*-octanoic and valproic acids, respectively (Fig. 1). The Δ4-enoic derivate of valproic acid was by far a better substrate than valproic acid, whereas other compounds displayed intermediary abilities as substrates (Fig. 1).

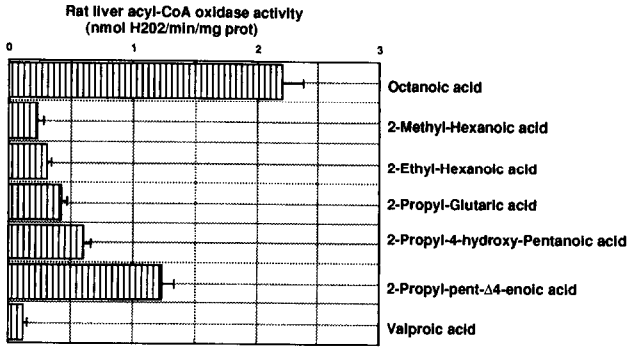

Fig. 1. Hydrogen peroxide production rates induced by various medium-chain carboxylates in rat liver postnuclear supernatants incubated with ATP and CoA. The oxidase activities are means ± S.E.M. (n = 6) expressed as nmol hydrogen peroxide generated per min per mg of rat crude liver homogenate protein after subtraction of matched blank values which correspond to spontaneous hydrogen peroxide generation upon incubation of rat liver preparations with ATP and CoA. For other details, see section 2.

3.2. *Rat liver medium-chain acyl-coA oxidase activities*

In a second series of experiments, the CoA esters of valproic acid, 2-propyl-pent-Δ4-enoic acid and 2-ethyl-hexanoic acid, as well as octanoyl-CoA and lauroyl-CoA, were tested for their abilities to be substrates of rat liver acyl-CoA oxidases. As illustrated by Fig. 2, the optimal acyl-CoA oxidase activity was obtained on 2-propyl-pent-Δ4-enoyl-CoA and, under these conditions, roughly similar rates of hydrogen peroxide production were observed for the CoA esters of *n*-octanoic and valproic acids. Cyanide-insensitive acyl-CoA oxidation rates were of the same order of magnitude as the corresponding acyl-CoA oxidase activities (data not shown).

3.3. *Subcellular distribution of rat liver medium-chain acyl-CoA oxidase activities*

In a third series of experiments, the subcellular distribution of the medium-chain acyl-CoA oxidase activities were studied (Fig. 3). The valproyl-CoA oxidase activity displayed density distributions superimposable to those of fatty acyl-CoA oxidase, cyanide-insensitive fatty acyl-CoA oxidation and catalase (peroxisomes), and

clearly distinct from those of cytochrome *c* oxidase (mitochondria), sulphatase C (endoplasmic reticulum) and *N*-acetyl-β-glucosaminidase (lysosomes). Strikingly, cyanide-insensitive valproyl-CoA oxidation adopted a density distribution distinct from the peroxisomal marker enzymes. Rather, the optimal activities were recovered in the mitochondrial fractions with only a minor peroxisomal component (Fig. 3). In perosisome-enriched fractions, but not mitochondria, the cyanide-insensitive valproyl-CoA oxidation occurred at rates lower than valproyl-CoA oxidase-dependent H_2O_2 production (Table I).

3.4. *Other characteristics of medium-chain acyl-CoA oxidases*

The occurrence of a peroxisomal valproyl-CoA oxidase activity has raised the question of its analogy with, or distinction from, the other known peroxisomal acyl-CoA oxidases, especially fatty acyl-CoA oxidase and bile acyl-CoA oxidase. Table II compares the three activities, (valproyl-CoA, fatty acyl-CoA and bile acyl-CoA oxidase) in terms of rat tissue distribution and inducibility by a peroxisome proliferator. On the one hand, bile acyl-CoA oxidase is restricted to liver whereas the valproyl-CoA and fatty acyl-CoA oxidase activities occur in all the tissues studied (Table II). On the other hand, fatty acyl-CoA oxidase activity is stimulated by clofibrate, whereas valproyl-CoA and bile acyl-CoA oxidases are not (Table II).

Fig. 4 compares the sensitivity to deoxycholate of these three activities. Each of these activities display a different sensitivity to increasing concentrations of the detergent.

4. DISCUSSION

The present work demonstrates the occurrence of an acyl-CoA oxidase active on the CoA ester of valproic acid and related metabolites. This medium branched-chain acyl-CoA oxidase appears to be located in peroxisomes and distinct from two other known peroxisomal acyl-CoA oxidases: the fatty acyl-CoA oxidase inducible by peroxisome proliferators [6,8,10] and the bile acyl-CoA oxidase [6,8,10]. It may be stressed that this medium, branched-chain acyl-CoA oxidase shares apparently several properties with the pristanoyl-CoA oxidase recently described by the group of Mannaerts [11,12]: tissue distributions are not restricted to liver and no inducibilities by peroxisome proliferators have been recorded. It is, however, too early to conclude that the medium- (valproyl-CoA) and long- (pristanoyl-CoA) branched-chain acyl-CoA oxidase activities are expressed by a single protein. Taking into account that the pristanoyl-CoA oxidase upon purification displays straight-chain acyl-CoA oxidase activity (i.e. fatty acyl-CoA oxidase not inducible by peroxisome proliferators [12]), it might be questioned whether the valproyl-CoA

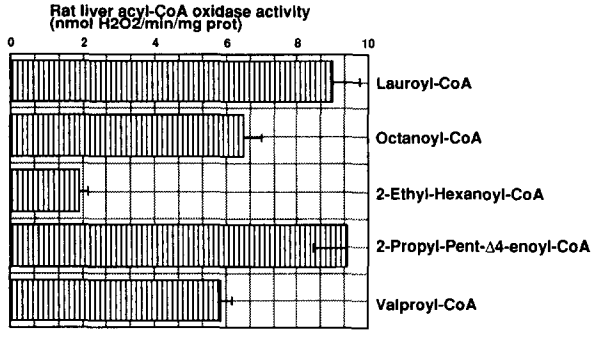

Fig. 2. Hydrogen peroxide production rates recorded upon incubation of rat liver postnuclear supernatants with various medium-chain acyl-CoAs. The oxidase activities are means ± S.E.M. (n = 6) expressed as nmol hydrogen peroxide generated per min per mg of rat crude liver homogenate protein after subtraction of matched blank values which correspond to spontaneous hydrogen peroxide generation upon incubation of rat liver preparations without added ATP and CoA.

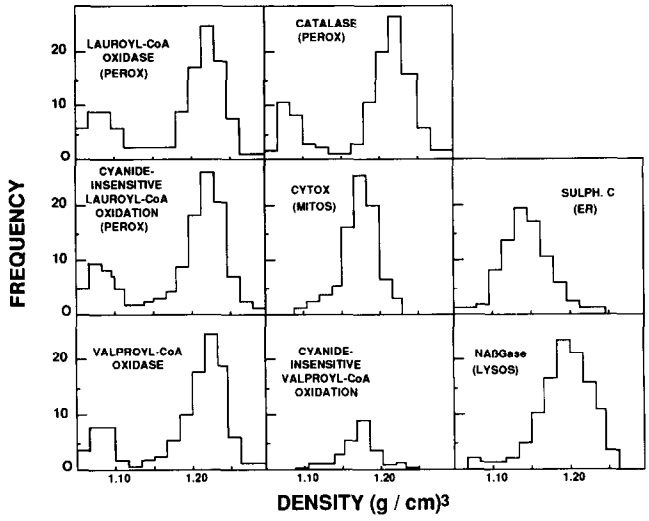

Fig. 3. Isopycnic centrifugation of rat liver postnuclear supernatants. Subcellular localizations of valproyl-CoA oxidase and cyanide-insensitive valproyl-CoA oxidation are compared with (i) three peroxisomal marker activities (fatty acyl-CoA [lauroyl-CoA] oxidase, cyanide-insensitive fatty acyl-CoA [lauroyl-CoA] oxidation and catalase, and (ii) other subcellular markers, including cytochrome *c* oxidase (CYTOX), sulphatase C (SULPH. C) and *N*-acetyl-β-glucosaminidase (NAβGase). Results are given as density distribution histograms. On the abscissa is the density span of the gradient divided into 15 sections of equal density increments (0.0167) from 1.05 to 1.30 g/cm^3. On the ordinate is the frequency of enzymic activities in each section ($Q/\Sigma Q.\Delta p$, where Q is the amount of activity equilibrating within the section, ΣQ the sum of the activities found in all subfractions, and Δp the density increment of the section). Density distributions typical of peroxisomal marker activities were found in the case of 2-propyl-pent-Δ4-enoyl-CoA and 2-ethyl-hexanoyl-CoA H_2O_2-generating oxidase activities (data not shown). Recovery of enzyme activity in the sum of fractions was between 76 and 108%, with the exception of the recovery of cyanide-insensitive valproyl-CoA oxidation which was inferior to 35%. Other comments are in the text. PEROX, peroxisomes; MITOS, mitochondria; ER, endoplasmic reticulum; LYSOS, lysosomes.

Table I

Comparison between acyl-CoA oxidase and cyanide-insensitive acyl-CoA oxidation rates in mitochondria- and peroxisome-enriched fractions obtained after isopycnic centrifugation of rat liver postnuclear supernatants

	Mitochondrial fraction	Peroxisomal fraction
Acyl-CoA oxidase rate recorded on:		
Valproyl-CoA	0.5	9.4
Lauroyl-CoA	0.8	13.7
Cyanide-insensitive oxidation rate recorded on:		
Valproyl-CoA	0.5	0.6
Lauroyl-CoA	0.9	14.1

The mitochondria- and peroxisome-enriched fractions refer to the fractions characterized by the higher content in cytochrome *c* oxidase and sedimentable catalase activities, respectively. The results of acyl-CoA oxidase and cyanide-insensitive oxidation rates are expressed as nmol hydrogen peroxide and NADH, respectively, produced per min per mg protein. The values are the mean of three determinations made on the same liver samples obtained during the course of the cell fractionation experiment, the results of which in terms of enzyme activity density distributions are illustrated in Fig. 3. Essentially comparable oxidation rates were obtained in mitochondria- and peroxisome-enriched fractions collected during the course of one other cell fractionation experiment.

oxidase activity is not a distinct enzyme, on the basis of the apparent absence of residual straight-chain acyl-CoA oxidase activity when the enzyme is assayed with concentrations of deoxycholate which completely abolish lauroyl-CoA oxidase activity (see Fig. 4). Future work is, therefore, required to establish firmly the analogous or distinct nature of valproyl-CoA and pristanoyl-CoA oxidases.

Another point is the fact that, to our knowledge, the known branched-chain acyl-CoA oxidoreductases, including mitochondrial isovaleryl-CoA and 2-methylbutyryl-CoA dehydrogenases [22,23], and peroxisomal bile acyl-CoA [6,7], pristanoyl-CoA [11] and valproyl-CoA (this work) oxidases, are not inducible by peroxisome proliferators. Interestingly enough, most of the metabolic pathways concerning these enzymes (cases of mitochondrial branched-chain acyl-CoA dehydrogenases and peroxisomal bile acyl-CoA and most likely valproyl-CoA oxidase) involve a unique modified or classic β-oxidation cycle and are not carnitine-dependent, even if the mitochondrial pathways are sensitive to carnitine, a property accounted for by the release of product inhibition of 2-oxoacid dehydrogenase, the rate limiting step in branched-chain amino acid metabolism (see [24]). In addition, these acyl-CoA oxidations occur in one compartment: isovaleryl-CoA and 2-methylbutyryl-CoA are not oxidized by peroxisomes [25], whereas bile acyl-CoA is not significantly oxidizable by mitochondria [6,26]. The mitochondrial oxidation of valproyl-CoA has been documented elsewhere [13,14]. The pathway is initiated by mitochondrial branched-chain acyl-CoA dehydrogenase and proceeds up to β-ketoacyl-CoA formation but not further [13,14]. In peroxisomes, the situation appears to be distinct, and β-oxidation concerns the acyl-CoA oxidase step, mainly. The activity is impressive since it represents half of the activity recorded on lauroyl-CoA, which is classically referred to as the best substrate for peroxisomal β-oxidation. In the case of peroxisomal handling of valproyl-CoA, one might suggest that the β-oxidation is incomplete, as may be inferred from the fact that cyanide-insensitive valproyl-CoA oxidation activity is, in peroxisomal fractions, strongly rate-limiting for peroxisomal acyl-CoA oxidase. Previous work failed to demonstrate the occurrence of a β-hydroxyvalproyl-CoA dehydrogenase activity from peroxisomal origin [14], so that the cyanide-insensitive valproyl-CoA activity recorded in the present work might be an artifact. Nevertheless, for the mitochondrial component of this

Table II

Rat tissue distribution and peroxisome proliferator inducibility of valproyl-CoA, fatty acyl-CoA and bile acyl-CoA oxidase activities

Enzyme	Substrate	H_2O_2-generating oxidase activity* in rat tissues			
		Liver	Kidney cortex	Intestinal mucosa	Heart
(A) Untreated animals					
Fatty acyl-CoA oxidase	lauroyl-CoA	8.82 ± 0.93	13.4 ± 0.8	2.15 ± 0.13	0.21 ± 0.02
Valproyl-CoA oxidase	2-propyl-pent-Δ4-enoyl-CoA	9.47 ± 1.03	13.0 ± 0.8	2.87 ± 0.16	0.42 ± 0.03
Bile acyl-CoA oxidase	trihydroxycoprostanoyl-CoA	2.51 ± 0.17	no detectable activity	no detectable activity	no detectable activity
(B) Clofibrate-treated animals					
Fatty acyl-CoA oxidase	lauroyl-CoA	95.4 ± 11.5	34.5 ± 2.3	7.51 ± 0.86	1.77 ± 0.08
Valproyl-CoA oxidase	2-propyl-pent-Δ4-enoyl-CoA	8.71 ± 0.78	12.9 ± 0.7	2.43 ± 0.19	0.35 ± 0.03
Bile acyl-CoA oxidase	trihydroxycoprostanoyl-CoA	2.11 ± 0.14	no detectable activity	no detectable activity	no detectable activity

*Expressed as nmol H_2O_2 /min/mg tissue (mean values ± S.E.M.; $n = 6$).

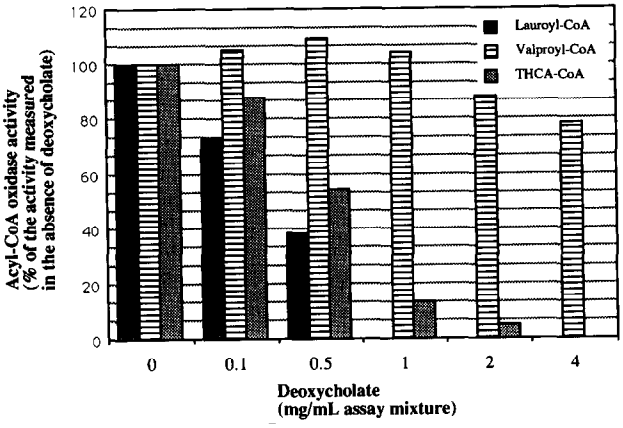

Fig. 4. Comparative sensitivities of fatty acyl-CoA, bile acyl-CoA and valproyl-CoA oxidase activities to increasing concentrations of deoxycholate. Essentially similar results were obtained in three other separate experiments. It may be stressed that results obtained with 2-propyl-pent-Δ4-enoyl-CoA or 2-ethyl-hexanoyl-CoA oxidase activities were comparable to those recorded on valproyl-CoA, whereas those monitored with octanoyl-CoA as a substrate superimposed to those of lauroyl-CoA oxidase activity (data not shown).

activity, it may be stressed that mitochondrial β-hydroxyvalproyl-CoA dehydrogenase activity is operative in the assay conditions utilized in the measurement of cyanide-insensitive valproyl-CoA oxidation rates because the mitochondrial activity is not sensitive to cyanide and is supplemented with excess oxidized NAD. Concerning the peroxisomal component of cyanide-insensitive valproyl-CoA oxidation activity, two hypotheses may be retained: either contamination by mitochondrial β-oxidation enzymes occurs, or peroxosimal β-hydroxyvalproyl-CoA dehydrogenation is operative. In the latter case, the involvement of peroxisomal membrane-linked β-hydroxyacyl-CoA dehydrogenase activity must be evoked, the peroxisomal matrix enzyme being previously shown to be inactive on β-hydroxyvalproyl-CoA [14].

On the other hand, it has previously been shown that valproate did not inhibit peroxisomal β-oxidation and could induce increased hydrogen peroxide production in rat liver [27], a feature in agreement with the idea that valproic acid and related metabolites can be oxidized by peroxisomes. Finally, the present work provides the demonstration for a sequence of events responsible for the generation from 2-propyl-pent-Δ4-enoic acid of a 2-propyl-pent-Δ2-Δ4-dienoic moiety, the toxicity of which has been the topic of other works [28,29]. The demonstrated sequence of reactions is the following: 2-propyl-pent-Δ4-enoic acid $\rightarrow$ 2-propyl-pent-Δ4-enoyl-CoA $\rightarrow$ 2-propyl-pent-Δ2-Δ4-dienoyl-CoA + H_2O_2, the first step occurring in the presence of ATP and CoA (mainly in the microsomal components, data not shown), and the second in peroxisomes. By analogy, one might mention, here, the formation of a glutaconoyl moiety in the unesterified form from glutaric acid, a pathway which proceeds according to a parallel sequence of reactions: i.e. successive involvements of microsomal dicarboxylyl-CoA synthetase [30] and peroxisomal glutaryl-CoA oxidase [20,21].

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