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CATABOLISM OF 4-HYDROXYACIDS AND 4-HYDROXYNONENAL *VIA* 4-HYDROXY-4-PHOSPHOACYL-COAS

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Running title: 4-Phosphoacyl-CoAs in 4-hydroxyacid metabolism

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4-Hydroxyacids are products of ubiquitously occurring lipid peroxidation (C_9, C_6) or drugs of abuse (C_4, C_5). We investigated the catabolism of these compounds using a combination of metabolomics and mass isotopomer analysis. Livers were perfused with various concentrations of unlabeled and labeled saturated 4-hydroxyacids (C_4 to C_{11}) or 4-hydroxynonenal. All the compounds tested form a new class of acyl-CoA esters, 4-hydroxy-4-phosphoacyl-CoAs, characterized by LC-MS/MS, accurate mass spectrometry and ^{31}P-NMR. All 4-hydroxyacids with 5 or more carbons are metabolized by two new pathways. The first and major pathway, which involves 4-hydroxy-4-phosphoacyl-CoAs, leads in 6 steps to the isomerization of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoAs. The latter are intermediates of physiological β-oxidation. The second and minor pathway involves a sequence of β-oxidation, α-oxidation and β-oxidation steps. In mice deficient in succinic semialdehyde dehydrogenase, high plasma concentrations of 4-hydroxybutyrate result in high concentrations of 4-hydroxy-4-phospho-butyryl-CoA in brain and liver. The high concentration of 4-hydroxy-4-phospho-butyryl-CoA may be related to the cerebral dysfunction of subjects ingesting 4-hydroxybutyrate, and to the mental retardation of patients with 4-hydroxybutyric aciduria. Our data illustrate the potential of the combination of metabolomics and mass isotopomer analysis for pathway discovery.

4-Hydroxy-*n*-acids are involved in different areas of mammalian metabolism. Some unsaturated 4-hydroxyacids are derived from 4-hydroxynonenal and 4-hydroxyhexenal which are products of lipid peroxidation (1). The metabolism of 4-hydroxynonenal has been extensively studied, especially its conjugation with glutathione (2), covalent modification of proteins (3;4), conversion to 4-hydroxynonenoate, 4-hydroxynonanoate and 1,4-dihydroxynonene, as well as its role in inflammatory processes (1;5-11). However, the catabolism of its carbon skeleton has not been unraveled.

The four-carbon 4-hydroxybutyrate is a physiological neurotransmitter derived from γ-aminobutyrate. Humans with inborn disorder of succinic semialdehyde dehydrogenase have high 4-hydroxybutyrate concentrations in body fluids, mental retardation and seizures (12). 4-Hydroxybutyrate is also a drug of abuse which impairs the capacity to exercise judgment for unknown reasons. 4-Hydroxybutyrate is used for the treatment of narcolepsy (13). Its known metabolism (14;15) proceeds *via* oxidation to succinic semialdehyde then to succinate, an intermediate of the citric acid cycle. The five-carbon 4-hydroxypentanoate is also a drug of abuse (16). The calcium salt of a compound closely related to 4-hydroxypentanoate, levulinate (4-ketopentanoate, 4-ketovalerate) is used as an oral or intravenous

source of calcium in humans.

We conducted a study on the catabolism of C_4 to C_{11} 4-hydroxyacids in perfused rat livers using a combination of metabolomics (17;18) and mass isotopomer analysis[1] (19). Our metabolomic strategy concentrated on the identification of carboxylic acids and acyl-CoA esters derived from 4-hydroxyacids. The filiation between 4-hydroxyacids and metabolites was demonstrated by conducting experiments with unlabeled and multiply ^{13}C- or ^{2}H-labeled substrates. We demonstrated that the 4-hydroxyacids are degraded by two parallel pathways. The first pathway, which involves 4-hydroxy-4-phosphoacyl-CoAs (4-P-acyl-CoAs) leads to the formation of 3-hydroxyacyl-CoAs which are physiological β-oxidation intermediates. The second pathway is a sequence of β-oxidation, α-oxidation (20;21), and β-oxidation steps. Via the two pathways, 4-hydroxyacids with 5 or more carbons are degraded to acetyl-CoA, propionyl-CoA and formate.

EXPERIMENTAL PROCEDURES

Materials- Sigma-Aldrich supplied most chemicals, and the following isotopically labeled compounds: $^{2}H_2O$ (99%), $^{13}CO_2$ gas, 4-hydroxy[$^{13}C_4$]butyrolactone, 4-hydroxy-[$^{2}H_6$]butyrolactone, [$^{2}H_5$]propionic acid, [$^{13}C_6$]glucose, $^{15}NH_4Cl$, sodium [$^{13}C_2$]acetate and sodium [^{13}C]formate. [3,3,5,5,5-$^{2}H_5$]Levulinate (4-ketopentanoate) was prepared by isotopic exchange between unlabeled levulinate, $^{2}H_2O$ and $NaO^{2}H$ (22). 4-hydroxy-[3,3,4,5,5,5-$^{2}H_6$]pentanoate was prepared by reducing [3,3,5,5,5-$^{2}H_5$]levulinate with $NaB^{2}H_4$ (22). 4-Hydroxy-[$^{2}H_{11}$]nonenal was prepared as in (23). 4-Hydroxy-[3-^{13}C]nonanoate, 4-hydroxy-[3,4-$^{13}C_2$]nonanoate and 4-hydroxy-[3-^{13}C]hexanoate were prepared by methods to be reported elsewhere. The purity of synthesized compounds was verified by gas chromatography-mass spectrometry and NMR. The lactones of all 4-hydroxyacids were hydrolyzed with 10% excess NaOH at 60°C for 1 hr. [$^{2}H_5$]Propionyl-CoA was prepared from the acid as described in ref (24).

Liver perfusions- Livers from male Sprague-Dawley rats were perfused (25) with bicarbonate buffer containing 4 mM glucose and either 4% dialyzed, fatty acid-free, bovine serum albumin (recirculating perfusions) or no albumin (non-recirculating perfusions). After equilibration, 0 to 2 mM of various 4-hydroxyacids, labeled or unlabeled, was added to the perfusate. In some experiments, albuminated perfusate was prepared in 100% $^{2}H_2O$. Livers were quick-frozen at the end of the experiments.

Analytical Procedures- The concentrations and mass isotopomer distributions of the various acids and 4-hydroxyacids were assayed by gas chromatography-mass spectrometry of trimethylsilyl or *tert*-butyldimethylsilyl derivatives, using analog unlabeled or labeled compounds as internal standards.

For the concentration and labeling pattern of acyl-CoA esters, powdered frozen liver (≈ 200 mg), spiked with 10 nmol [$^{2}H_5$]propionyl-CoA internal standard was extracted for 1 min with 4 ml of (methanol/water 1:1 containing 5% acetic acid) using a Polytron homogenizer. The supernatant was run on an 3 ml ion exchange cartridge packed with 300 mg of 2-2(pyridyl)ethyl silica gel (Sigma). The cartridge had been pre-activated with 3 ml methanol, then with 3 ml of extraction buffer. The acyl-CoAs trapped on the silical gel cartridge were released with (i) 3 ml of a 1:1 mixture of ammonium formate 50 mM pH 6.3, and methanol (to release the short- and medium-chain acyl-CoAs), then (ii) 3 ml of a 1:3 mixture of ammonium formate 50 mM pH 6.3, and (iii) 3 ml of methanol (to release the medium- and long-chain acyl-CoAs (26)).The combined effluent was dried with nitrogen gas and stored at -80°C until LC-MS analysis.

After dissolving the acyl-CoAs in 100 µl of buffer A (5% acetonitrile in ammonium formate 100 mM, pH 5.0), 40 µl were injected on a Thermo Electron Hypersil GOLD column (150 x 2.1 mm) protected by a guard column (Hypersil Gold 5 µm, 10 x 2.1 mm), in an Agilent 1100 liquid chromatograph. The chromatogram was developed at 0.2 ml/min (i) for 3 min with 98% buffer A and 2% buffer B (95% acetonitrile in ammonium

formate 5 mM, pH 6.3) , (ii) from 3 to 25 min with a 2 to 60% gradient of buffer B in buffer A, (iii) from 26 to 31 min with 10% buffer A/90% buffer B, (iv) from 32 to 41 min with a 90% to 2% gradient buffer B in buffer A, and (v) 10 min stabilization with 98% buffer A before the next injection.

The liquid chromatograph was coupled to an 4000 QTrap mass spectrometer (Applied Biosystems, Foster City, CA) operated under positive ionization mode with the following source settings: turbo-ion-spray source at 600 ˚C under N_2 nebulization at 65 psi, N_2 heater gas at 55 psi, curtain gas at 30 psi, collision-activated dissociation gas pressure was held at high, turbo ion-spray voltage at 5,500 V, declustering potential at 90 V, entrance potential at 10 V, collision energy at 50 V, collision cell exit potential at 10 V. The Analyst software (version 1.4.2; Applied Biosystems) was used for data registration.

The concentration of 4-hydroxybutyrate in mouse blood (27), the labeling of the acetyl moiety of citrate (28), a proxy i.e., an indicator of mitochondrial acetyl-CoA, β-hydroxybutyrate (BHB) (22) and of free acetate (29) and formate (29) were assayed as described previously. Exact mass analysis of 4-P-butyryl-CoA and 4-P-pentanoyl-CoA was run on a Thermo-Finnigan Fourier Transform LTQ mass spectrometer. ^{31}P-NMR spectra of 4-P-pentanoyl-CoA and of malonyl-CoA standard were run on a 600 MHz Varian Inova equipped with a Broad Band probe. Spectra were acquired for 26 min with a 45 degree pulse width and acquisition time of 1.5 sec with proton decoupling.

Calculations- Correction of measured mass isotopomer distributions for natural enrichment was performed using the CORMAT software (30). The labeling of the C-1+2 acetyl of BHB, a proxy of mitochondrial acetyl-CoA, was calculated using the mass isotopomer distributions of the whole BHB molecule, and of the C-3+4 fragment of BHB. When mitochondrial acetyl-CoA was only M1 labeled, as from 4-hydroxy-[3-^{13}C]nonanoate, the m1 enrichment of the C-1+2 acetyl of BHB was calculated as:

m1 of C-1+2 = [(2 m2 + m1) of C-1➜4] - (m1 of C-3+4) **(Eq. 1)**

When mitochondrial acetyl-CoA was M1 and M2 labeled, as from 4-hydroxy-[3,4-$^{13}C_2$]nonanoate, the m1 and m2 enrichments of the C-1+2 acetyl of BHB were calculated assuming that the m2 and m1 labeling ratios of the two acetyls of BHB are identical (Eq. 2)

R = (m2 of C-3+4)/(m2 of C-1+2) = (m1 of C-3+4)/(m1 of C-1+2) **(Eq. 2)**

The total m2 labeling of BHB is expressed as a function of the labeling of its C-3+4 moiety:

(m2 of C-1➜4) = (m2 of C-3+4)[1 - (m2 of C-3+4)/R] + [(m2 of C-3+4)/R][1 - (m2 of C-3+4)] + (m1 of C-3+4)^2/R **(Eq. 3)**

Equation 3 is solved for R. R is introduced in Eq. 2 to solve for the m1 and m2 labeling of C-1+2 of BHB.

Data presentation and statistics- We present data from 123 liver perfusion experiments. For a number of conditions, we ran 6 perfusions in the presence of selected unlabeled or ^{13}C-labeled substrate(s) with the concentration parameters being allowed to vary. The data points shown in the figures represent means of duplicate gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry injections, which differed by <2%. The statistical differences between some profiles was tested using a paired *t* test (Graph Pad Prism Software, version 3).

RESULTS

Identification of 4-P-acyl-CoAs- In extracts from rat livers perfused with C_4 to C_{11} 4-hydroxyacids, LC-MS/MS analysis identified the expected CoA esters of the substrates. These, and all subsequently identified CoA esters, showed the typical transitions (31) from the mass of the parent molecular ion to the product ions at m/z = 428 and 261. These correspond to the nucleoside and pantetheine (minus OH) fragments of CoA (Suppl. Fig 1S, ions B and

E). In addition, we found unexpected CoA esters that migrate faster than the expected esters on the C18 column. This suggested the presence of additional polar group(s) compared to usual CoA esters. In perfusions with 4-hydroxybutyrate, the new CoA ester had a parent mass of 934 *vs* 854 for 4-hydroxybutyryl-CoA. When unlabeled 4-hydroxybutyrate was replaced by the [$^{13}C_4$] or [$^{2}H_6$]substrate, the mass of the new CoA ester increased by 4 and 6 Da, respectively (not shown). In perfusions with 4-hydroxypentanoate, the new CoA ester had a parent *m/z* of 948 *vs* 868 for 4-hydroxypentanoyl-CoA. When unlabeled 4-hydroxypentanoate was replaced by the [$^{2}H_6$]substrate, the *m/z* of the new CoA ester increased by 6 (not shown). This demonstrated that the two new CoA esters had (i) all the carbon and hydrogen atoms of the corresponding 4-hydroxyacids, and (ii) that their masses were 80 Da greater than the corresponding 4-hydroxyacyl-CoAs. The mass of the new CoA ester of 4-hydroxybutyrate was not affected when (i) unlabeled glucose in the perfusate was replaced by [$^{13}C_6$]glucose, or (ii) when 10 mM [$^{13}C_2$]acetate or 5 mM $^{15}NH_4Cl$ was added to the perfusate. This suggested that the additional polar group did not contain carbon or nitrogen atoms derived from intermediary metabolism.

When we compared the fragmentation patterns of 4-hydroxypentanoyl-CoA and of the corresponding new CoA ester, we hypothesized that the unknown ester was 4-hydroxy-4-phospho-pentanoyl-CoA (4-P-pentanoyl-CoA) because the singly charged ion transitions A ➜ C and D ➜ F (Suppl. Fig 1S) were accompanied by the loss of *m/z* 98 which is equivalent to phosphoric acid. Corresponding transitions were observed for the unknown CoA ester derived from 4-hydroxybutyrate, identified as 4-P-butyryl-CoA. Also, in experiments with 4-hydroxy-[$^{13}C_4$]butyrate, 4-hydroxy-[$^{2}H_6$]butyrate, and 4-hydroxy-[$^{2}H_6$]pentanoate, the masses of fragments A, C, D, F increased by the same *m/z* as the substrates.

To confirm the identity of 4-P-butyryl-CoA and 4-P-pentanoyl-CoA, we isolated these compounds from 10 rat livers perfused with 10 mM 4-hydroxybutyrate or 4-hydroxypentanoate, using semi-preparative HPLC. The isolated esters were analyzed by accurate mass spectrometry which yielded very good matches between the theoretical and measured masses (Supplementary Table 1S). In addition, the presumed 4-P-pentanoyl-CoA was analyzed by ^{31}P-NMR (Fig 1). Comparison between the ^{31}P-NMR spectra of the presumed 4-P-pentanoyl-CoA and of a standard of malonyl-CoA showed that it has one extra P atom. This confirmed the identity of 4-P-pentanoyl-CoA.

All the saturated C_4 to C_{11} 4-hydroxyacids tested formed 4-P-acyl-CoA esters (Fig 2) which accumulated to very different concentrations in the liver. Also, in livers perfused with unlabeled 4-hydroxynonenal or 4-hydroxy-[$^{2}H_{11}$]nonenal (labeled on the terminal 5 carbons), we identified the corresponding saturated 4-P-acyl-CoAs (Table 1, to be discussed below).

To test whether phospho-acyl-CoAs are generated from other hydroxyacids, we perfused livers, each with 2 mM of 2-hydroxyacid (C_3), 3-hydroxyacids (C_3 to C_5), or 5-hydroxyacid (C_5). None of these compounds formed a detectable phospho-acyl-CoA.

To gather data that might lead to a hypothesis on the role of 4-P-acyl-CoAs in the metabolism of 4-hydroxyacids, we conducted (i) non-targeted metabolomics analyses on the various liver perfusates, and (ii) detailed assays of acyl-CoA profiles in livers perfused with unlabeled and labeled 4-hydroxyacids.

Experiments with 4-hydroxynonenal- We perfused one liver with recirculating perfusate to which 2 mM [$^{2}H_{11}$]HNE (labeled on the last 5 carbons) was added at zero time. In the liver tissue and perfusate collected after 2 hr, we identified (Table 1) eight labeled compounds: 4-P-nonanoyl-CoA, four acyl-CoA esters (C_7, C_6, C_5, C_3), 4-hydroxynonanoate, 4-hydroxynonenoate, and 2-hydroxyheptanoate. Table 1 shows the mass isotopomer distributions of these compounds. The same but unlabeled compounds were identified in one liver perfusion with unlabeled 4-hydroxynonenal. The data of Table 1 show that

the catabolism of 4-hydroxynonenal leads to compounds containing 7, 6, 5 and 3 carbons. This points to more than one mechanism of degradation because β-oxidation of 4-hydroxynonanoate or 4-hydroxynonenoate would not form the six-carbon hexanoyl-CoA, nor 2-hydroxyheptanoate.

Table 1 shows that the mass isotopomer distribution of C_9 metabolites of 4-hydroxy-[$^2H_{11}$]nonenal (4-P-nonanoyl-CoA, 4-hydroxynonanoate and 4-hydroxynonenoate) included an unlabeled component (M). The latter was not present in the labeled substrate. Also, in control livers perfused without 4-hydroxynonenal, we could not detect these compounds. It is likely that the metabolism of 4-hydroxy-[$^2H_{11}$]nonenal induced the peroxidation of endogenous lipids resulting in the formation of unlabeled 4-hydroxynonenal. A similar finding has been reported by Cadenas *et al* who observed the stimulation of the production of ethane and *n*-pentane by hepatocytes incubated with 2 mM 4-hydroxynonenal (32).

Metabolomics of metabolites of saturated 4-hydroxyacids released by the perfused rat liver- To expand on the data of perfusions with 4-hydroxynonenal, we did a metabolomic study of carboxylic acids released by livers perfused with saturated 4-hydroxyacids of various chain length. We identified a pattern of metabolite production which is exemplified in Fig 3 with the products of the metabolism of 4-hydroxyacids with 6, 9 and 11 carbons. With each of these 4-hydroxyacids, we observed the progressive accumulation of (i) a 3-hydroxyacid with the same number of carbons, (ii) a 2-hydroxyacid with 2 carbons less than the starting substrate, and (iii) acids with 2 and 3 carbons less than the starting substrate (not shown on Fig 3). This suggested the existence of two mechanisms of degradation of 4-hydroxyacids with at least 5 carbons. These mechanisms are outlined in Fig 4 using 4-hydroxynonanoate (compound *1*) as the starting substrate. The first mechanism (Fig 4, pathway A) is the isomerization of 4-hydroxyacyl-CoA (compound *3*) to 3-hydroxyacyl-CoA (compound *10*), the normal intermediate of β-oxidation, *via* 4-P-acyl-CoA and other CoA esters to be described below. This is followed by regular β-oxidation cycles producing (i) acetyl-CoA and propionyl-CoA (in the case of odd-chain 4-hydroxyacids), or (ii) acetyl-CoA (in the case of even-chain 4-hydroxyacids). The second mechanism (Fig 4 , pathway B), starting at 4-hydroxyacyl-CoA (compound *3*), involves one cycle of β-oxidation, followed by one α-oxidation step, and cycles of β-oxidation. Pathway B leads to formate (compound *13*, *via* α-oxidation of 2-hydroxyacyl-CoA and formyl-CoA hydrolysis (21;33)), and either (i) acetyl-CoA + propionyl-CoA (in the case of even-chain 4-OH-fatty acids), or (ii) acetyl-CoA (in the case of odd-chain 4-hydroxyacids with at least 5 carbons). This double mechanism was confirmed by the identification and mass isotopomer distribution of acyl-CoAs and of carboxylic acids formed during the degradation of 4-hydroxyacids labeled with ^{13}C or ^2H. The evidence is described in the following paragraphs.

Experiments with 4-hydroxy-[3-^{13}C]nonanoate and 4-hydroxy-[3,4-$^{13}C_2$]nonanoate- We synthesized these labeled compounds because both would lead to the formation of [^{13}C]formate *via* pathway B (Fig 4, follow the fate of C-3 of 4-hydroxynonanoate shown in red color). 4-Hydroxy-[3-^{13}C]nonanoate would lead to the formation of M1 acetyl-CoA *via* pathway B. 4-Hydroxy-[3,4-$^{13}C_2$]nonanoate would lead to the formation of M1 acetyl-CoA *via* pathway B and M2 acetyl-CoA *via* pathway A (Fig 4, follow the fates of C-3 and C-4 of 4-OH-nonanoate shown in red and green color, respectively). In livers perfused with recirculating buffer containing 2 mM of 4-hydroxynonanoate that was unlabeled, [3-^{13}C]-labeled or [3,4-$^{13}C_2$]-labeled, we observed the M0, M1 or/and M2 compounds listed in Supplementary Table 2S, and the time-dependent accumulation of [^{13}C]formate in perfusions with labeled substrates (Fig 5). The mass isotopomer distribution of these compounds is compatible with the scheme presented in Fig 4. We also observed the formation of the unlabeled and labeled 4-ketoacids corresponding to the 4-hydroxyacids (Supplementary Table 2S and Fig 4, compound *2*). Evidence for the reversible

interconversion of 4-hydroxyacids and 4-ketoacids, as well as their CoA esters, is presented below.

We also perfused livers with non-recirculating buffer containing increasing concentrations of 4-hydroxy-[3-^{13}C]nonanoate or 4-hydroxy-[3,4-$^{13}C_2$]nonanoate. Careful examination of the LC-MS/MS spectra of the CoA esters eluting in the vicinity of the 4-phospho-acyl-CoAs, identified a number of additional CoA esters of interest. Fig 6 shows, for a perfusion with 4-hydroxy-[3,4-$^{13}C_2$]nonanoate, 10 acyl-CoA esters identified by (i) by the *m/z* of the molecular ion, (ii) the corresponding ion A at *m/z* = 428 (Fig 1S), and (iii) the retention time. The first 9 strips of Fig 6 correspond each to one multiple reaction monitoring (MRM) transition; the 10th strip (acetyl-CoA) corresponds to two transitions. The acyl-CoAs listed in Fig 6 are presented in the order that corresponds to the metabolic scheme we had hypothesized (Fig 4). The labeling of these acyl-CoAs, i.e., M2 of all C_9 and C_7, M1 of C_6, M1 and M2 of C_2 confirms the hypothesis. The acyl-CoA profiles assayed in perfusions with unlabeled or 4-hydroxy-[3-^{13}C]nonanoate (not shown) further confirm the hypothesis.

The six panels of Fig 7 show the concentrations of unlabeled or labeled acyl-CoA esters assayed in livers perfused with increasing concentrations of 4-hydroxy-[3-^{13}C]nonanoate (left side) or 4-hydroxy-[3,4-$^{13}C_2$]nonanoate (right side). For each labeled substrate, the acyl-CoA metabolites are presented in 3 groups: (i) CoA esters formed before the bifurcation of 4-hydroxynonanoate metabolism (4-hydroxynonanoyl-CoA and 4-ketononanoyl-CoA, upper level), (ii) CoA esters in pathway A (4-P-nonanoyl-CoA, 3-hydroxy-4-P-nonanoyl-CoA, heptanoyl-CoA and pentanoyl-CoA, middle level), and (iii) CoA esters in pathway B (dihydroxynonanoyl-CoA, 2-hydroxyheptanoyl-CoA and hexanoyl-CoA, lower level). The concentration profiles of these unlabeled and labeled CoA esters are also compatible with the scheme presented in Fig 4. In the perfusates of these livers we also observed the accumulation of all the free acids corresponding to the acyl-CoAs, except for 4-P-nonanoyl-CoA (not shown).

Figs 8A and 8B show the labeling of acetyl-CoA and of 3 proxies of acetyl-CoA (acetyl moiety of citrate (28;34), C-1+2 acetyl of BHB (35) and free acetate (35;36)) in livers perfused with 4-hydroxy-[3-^{13}C]nonanoate (Fig 8A) or 4-hydroxy-[3,4-$^{13}C_2$]nonanoate (Fig 8B). In the presence of 4-hydroxy-[3-^{13}C]nonanoate which forms M1 acetyl-CoA *via* pathway A (Fig 4), the M1 labeling of acetyl-CoA and its 3 proxies were similar. In the presence of 4-hydroxy-[3,4-$^{13}C_2$]nonanoate which forms M2 acetyl-CoA *via* pathway A and M1 acetyl-CoA *via* pathway B (Fig 4), the M2 labeling of acetyl-CoA and its 3 proxies was 5-6 times higher than the M1 labeling.

The mass isotopomer distribution of acetyl-CoA labeled from 4-hydroxy-[3,4-$^{13}C_2$]nonanoate allows to calculate the contributions of pathways A and B to the production of acetyl-CoA from this substrate. Pathways A and B yield 3 and 4 acetyl-CoA, respectively, with one acetyl-CoA being labeled in each pathway. Thus, the contribution of pathway A to acetyl-CoA production is 3 times the m2 enrichment of acetyl-CoA (Fig 8C, middle curve). Likewise, the contribution of pathway B to acetyl-CoA is 4 times the m1 enrichment of acetyl-CoA (Fig 8C, lower curve). The total contribution of 4-hydroxy-[3,4-$^{13}C_2$]nonanoate to acetyl-CoA plateaued at 55 to 60% (Fig 8C, upper curve). Thus, pathway A is the predominant pathway of 4-hydroxy-[3,4-$^{13}C_2$]nonanoate metabolism. Therefore, 4-hydroxy-[3,4-$^{13}C_2$]nonanoate can provide a substantial fraction of the energy generated *via* the citric acid cycle. This fraction is similar to what would be generated by [^{13}C]oleate (Bian, F. and Brunengraber, H. *unpublished*). Note that 4-hydroxynonanoate is rapidly taken up by the perfused rat liver: 0.32 ± 0.04 μmol·min^{-1}·g^{-1} in 3 recirculating perfusions with an initial substrate concentration of 2 mM. This rate is similar to the uptake of oleate by the perfused rat liver (37). Note that part of the carbon derived from the catabolism of 4-hydroxy-[3,4-$^{13}C_2$]nonanoate is not converted to acetyl-CoA, but is released as shorter carboxylic acids into the perfusate (Supplementary Table 2S).

We also conducted one recirculating liver perfusion experiment with 2 mM 4-hydroxy-[3-^{13}C]hexanoate. This compound also led to the accumulation in the perfusate of M1 formate (Fig 5), 4-ketohexanoate, 3-hydroxyhexanoate, dihydroxyhexanoate (presumably 3,4) and 2-hydroxybutyrate. In the liver, we identified M1 4-P-hexanoyl-CoA, 3-hydroxy-4-P-hexanoyl-CoA and butyryl-CoA. This spectrum of labeled compounds is compatible with the scheme shown in Fig 4, if one starts with 4-hydroxyhexanoate as compound *1*.

Experiments with 4-hydroxy-[3,3,4,5,5,5-$^{2}H_6$]pentanoate or [3,3,5,5,5-$^{2}H_5$]levulinate- In orientation perfusion experiments, we found that unlabeled 4-hydroxypentanoate is partly converted to the 4 keto analog levulinate (4-ketopentanoate). Likewise, in perfusions with unlabeled levulinate, we observed the formation of 4-hydroxypentanoate. Such keto-hydroxy interconversion should impact on the labeling pattern of 4-hydroxypentanoate deuterated in positions 3, 4 and 5, as well as on the labeling pattern of its metabolites. This is why we conducted liver perfusion experiments with 4-hydroxy-[3,3,4,5,5,5- $^{2}H_6$]pentanoate or [3,3,5,5,5-$^{2}H_5$]levulinate. Fig 9 shows the mass isotopomer distribution of the 4-hydroxy-[3,3,4,5,5,5-$^{2}H_6$]pentanoate substrate and of three metabolites (3-hydroxypentanoate, propionyl-CoA and lactate). Note that 4-hydroxy-[3,3,4,5,5,5- $^{2}H_6$]pentanoate was prepared by NaB$^{2}H_4$ reduction of [3,3,5,5,5-$^{2}H_5$]levulinate which itself was prepared by equilibrating unlabeled levulinate with a solution of NaO^{2}H in 100% $^{2}H_2O$. Since the isotopic exchange was not complete, the 4-hydroxy-[3,3,4,5,5,5-$^{2}H_6$]pentanoate contained not only M6, but also some M5, M4 and M3 mass isotopomers (Fig 9). The fates of the M6 isotopomer is schematized in Fig 9B. Evidence for pathway A includes the identification of 3-hydroxy[^{2}H]pentanoate in the perfusate and of [^{2}H]propionyl-CoA in the liver (Fig 9A). Evidence for pathway B is the presence in the perfusate of [$^{2}H_4$]lactate resulting, presumably, from the hydrolysis of liver [$^{2}H_4$]lactyl-CoA. The latter could not be identified in the liver. Note that the main mass isotopomer of 3-hydroxypentanoate (compound *3* on Fig 9B) was not M5, but M4 because of additional loss of label in the interconversion of 3-hydroxypentanoate and 3-ketopentanoate (compounds *3* and *4* on Fig 9B).

The corresponding mass isotopomer profile of metabolites derived from M5 [3,3,5,5,5-$^{2}H_5$]levulinate, shown in Supplementary Fig 2S, confirms the dual degradation pathway of the substrate, as well as the interconversion of levulinate and 4-hydroxypentanoate. Because 3-hydroxypentanoate and levulinate were ^{2}H-labeled, it was not possible to assess whether the lactyl-CoA undergoes α-oxidation to formate in addition to hydrolysis to lactate. This will be investigated with [^{13}C]substrates.

Experiments with 4-hydroxybutyrate- In livers perfused with 4-hydroxybutyrate, we detected 4-P-butyryl-CoA which was present at much lower concentrations than in perfusions with other 4-hydroxyacids (Fig 2, expanded insert). We did not detect any additional CoA ester which would be part of pathway A or B (Fig 4). In perfusions with 4-hydroxy-[$^{13}C_4$]butyrate, we did not identify 3-hydroxy-[$^{13}C_4$]butyrate which would be formed *via* pathway A. Also, we could not identify M2 glycolate which would be formed by β-oxidation of the substrate as was hypothesized by Vamecq *et al* (38) . Lastly we could not detect label in acetyl-CoA. In contrast, we identified M4 succinate and other multi-labeled citric acid cycle intermediates. These are formed by the oxidation of 4-hydroxybutyrate to succinate *via* succinate semialdehyde, as shown by Kaufman's group (14;39;40). This is the only known pathway of 4-hydroxybutyrate catabolism. We will present separately a study of the hepatic metabolism of 4-hydroxybutyrate *via* the citric acid cycle.

Perfusions of 4-hydroxyacids in 100% $^{2}H_2O$ buffer- To gain additional information on the metabolism of 4-hydroxyacids, we perfused 2 livers for 2 hr with recirculating buffer made with 100% $^{2}H_2O$, and containing 2 mM of unlabeled 4-hydroxyacid with 5 or 9 carbons. The data reveal incorporation of ^{2}H at multiple levels of 4-hydroxyacid metabolism (Table 2). First, analysis of the recirculating

perfusate sampled just before freeze-clamping the liver at 120 min revealed (i) the presence of mass isotopomers of the substrate with 1 to 3 ^2H atoms (Table 2), and (ii) the 4-keto counterparts of the 4-hydroxyacid substrate (not shown). This demonstrates that the 4-hydroxyacids equilibrate with their keto counterparts. The equilibration is accompanied by the incorporation of ^2H from the perfusate *via* keto-enol tautomerism. The maximal ^2H incorporation of ^2H (5 atoms/molecule of 4-hydroxyacid) was not achieved in 2 hr. Second, in the liver tissue, LC-MS/MS analysis revealed the presence of up to 4 ^2H atoms in free CoA (Table 2). This opens the possibility to investigate the turnover of CoA and its moieties with ^2H-enriched water. Third, the tissue 4-hydroxyacyl-CoAs had more ^2H atoms (up to 5/molecule) than the free 4-hydroxyacids, after correction for the ^2H incorporated in the CoA moiety. This shows that the 4-hydroxyacyl-CoAs picked up ^2H atoms from water in their equilibration with 4-ketoacyl-CoAs. Fourth, the 4-P-acyl-CoAs had more ^2H atoms incorporated than their 4-hydroxyacyl-CoA precursors. It is thus likely that the 4-phosphoacyl-CoAs are involved in some reversible reaction(s) which result in the incorporation of ^2H atoms from the perfusate.

Trapping of CoA during the metabolism of 4-hydroxyacids- The very different accumulations of 4-P-acyl-CoAs in livers perfused with 2 mM of 4-hydroxyacids (Fig 2) hinted that, in some cases, the metabolism of these compounds could lead to substantial trapping of CoA. To gain more insight on this question, we assayed the concentrations of all newly identified CoA esters in livers perfused with increasing concentrations of 4-hydroxyacids. Fig 3S shows the concentrations of a number of CoA esters in livers perfused with increasing concentrations of 4-hydroxyacids with 4 to 9 carbons. For substrates with 5 to 9 carbons, we observed (i) large accumulations of CoA esters formed in the initial metabolism of the substrates (Fig 3S), (ii) large decreases in free CoA (Fig 10A), and (iii) large decreases in acetyl-CoA and malonyl-CoA (Figs 10B and 10C). Thus, the metabolism of high concentrations of 4-hydroxyacids can lead to substantial CoA trapping which, in some cases, has been linked to metabolic perturbations (41).

Experiments with mice deficient in succinic semialdehyde dehydrogenase- Mice deficient in succinic semialdehyde dehydrogenase (ssadh-/-) were developed (42) as a model to study the biochemical perturbations found in humans with 4-hydroxybutyric aciduria (12). We assayed the concentrations of 4-P-butyryl-CoA in quick-frozen livers and brains from control, heterozygote and homozygote mice. The concentration of 4-P-butyryl-CoA was 9 and 40 times greater in livers and brains of ssadh deficient mice than in livers and brains of control and heterozygote mice (Fig 11). The high concentration of 4-P-butyryl-CoA in brains of ssadh-/- mice is clearly related to the high concentration of 4-hydroxybutyrate in their plasma (935 ± 97 μM, SE, n = 20; range 264-1683 μM). The concentration of 4-hydroxybutyrate in the plasma of control and heterozygote mice was undetectable.

DISCUSSION

Our findings demonstrate the existence of the previously unknown phospho-acyl-CoAs which appear to be derived only from 4-hydroxyacids. These 4-P-acyl-CoAs are intermediates in the catabolism of 4-hydroxyacids with at least 5 carbons. The combination of metabolomics and mass isotopomer analysis reveal that the 4-hydroxyacids are metabolized by two pathways. The main pathway (pathway A, Fig 4) involves the isomerization of 4-hydroxyacyl-CoAs to 3-hydroxyacyl-CoA. We propose that this isomerization proceeds *via* the scheme shown in Fig 4. According to this scheme, the phosphorylation of 4-hydroxyacyl-CoA is followed by dehydrogenation and hydration forming a 3-hydroxy-4-P-acyl-CoA. The latter would be dephosphorylated to the *enol* form of 3-ketoacyl-CoA which undergoes thiolytic cleavage to a ß-cleaved acyl-CoA. Pathway A (isomerization followed by β-oxidation) is supported by the finding that, in perfusions with 4-hydroxy-[3,4-$^{13}C_2$]nonanoate (Fig 6), the following intermediates are doubly-labeled: 4-P-nonanoyl-

CoA, 3-hydroxy-4-P-nonanoyl-CoA, 3-hydroxynonanoate, heptanoyl-CoA, heptanoate, the C-1+2 acetyl unit of BHB, and acetyl-CoA.

The accumulation of intermediates in pathway A especially of 4-P-acyl-CoAs (Fig 3S) and the extensive ^{2}H-labeling of 4-P-acyl-CoAs in perfusions conducted in 100% $^{2}H_2O$ (Table 2) suggest that (i) one of the distal steps of pathway A (Fig 4) is limiting, and that (ii) the dehydrogenation and hydration steps are reversible. Thus, our data are compatible with the following sequence of pathway A: 4-hydroxyacyl-CoA ➜ 4-P-acyl-CoA ⟷ 4-P-2-*enoyl*-CoA ⟷ 3-hydroxy-4-P-acyl-CoA ➜ 3-*enol*-acyl-CoA ⟷ 3-ketoacyl-CoA ⟷ 3-hydroxyacyl-CoA. The latter undergoes β-oxidation *via* 3-ketoacyl-CoA.

Pathway B (β-oxidation followed by α-oxidation and β-oxidation) is supported by the finding that, in perfusions with 4-hydroxy-[3,4-$^{13}C_2$]nonanoate (Fig 6), (i) the following metabolites are doubly labeled: dihydroxynonanoyl-CoA, dihydroxynonanoate, 2-hydroxyheptanoyl-CoA, 2-hydroxyheptanoate, while (ii) the subsequent metabolites are singly labeled: hexanoyl-CoA, hexanoate, the C-1+2 acetyl unit of BHB and acetyl-CoA. Through our careful review of the literature, this seems the only example of an α-oxidation step inserted between two β-oxidation cycles. Pathway B is also supported by the release of [^{13}C]formate in livers perfused with 4-hydroxy-[3-^{13}C]nonanoate and 4-hydroxy-[3-^{13}C]hexanoate (Fig 5).

4-Hydroxynonanoate and related compounds are derived from the lipid peroxidation product 4-hydroxynonenal (1), the concentration of which in microsomal membranes can reach mM levels during bursts of lipid peroxidation (43). Although mM concentrations of 4-hydroxynonenal, as used in our study, can have cytotoxic effects (11), our study concentrated on the degradative pathways of the 4-hydroxynonenal carbon skeleton. 4-Hydroxynonenal is converted to 4-hydroxynonenoate 4-hydroxynonanoate and 4-hydroxynonene (for reviews, see (6;8;11)). The release of [^{3}H]water after injection of 4-hydroxynonenal ^{3}H-labeled on C-4 or C-2 has been interpreted as demonstrating that part of the substrate undergoes β-oxidation (8). Actually, ^{3}H on C-4 of 4-hydroxynonenal must be released to water at the interconversions of (i) 4-hydroxy- and 4-ketononanoate, and (ii) 4-hydroxy- and 4-ketononanoyl-CoA (Fig 4). ^{3}H on C-2 of 4-hydroxynonenal must be released to water in the first β-oxidation cycle of pathway B, and in the reversible dehydrogenation and hydration steps of pathway A (4-P-acyl-CoA ⟷ 4-P-2-*enoyl*-CoA ⟷ 3-hydroxy-4-P-acyl-CoA). Our data demonstrate the complete oxidation of 4-hydroxynonenal by following the label from 4-hydroxy-[$^{2}H_{11}$]nonenal down to propionyl-CoA (Table 1). Also, our experiments with 4-hydroxy-[3,4-$^{13}C_2$]nonanoate (Figs 4-8) demonstrate the complete oxidation of the carbon skeleton of 4-hydroxynonenal to acetyl-CoA, propionyl-CoA and formate.

The trapping of CoA in intermediates of the degradation of 4-hydroxyacids with 5 or more carbons (Fig 3S) results in the decrease in the liver concentration of free CoA, acetyl-CoA and malonyl-CoA (Fig 11). Trapping of CoA by the metabolism of some drugs has been implicated in the deleterious effects of the drugs (44). Also, in some inborn errors of metabolism, the accumulation of CoA esters has been implicated in the physiopathology of the diseases (41). However, in the case of 4-hydroxynonanoate, this trapping of CoA does not prevent the substrate (i) to be taken up at a rate similar to that of a long-chain fatty acid like palmitate, and (ii) to contribute 55 to 60% of acetyl-CoA produced by the liver. However, in some situations the trapping of CoA by the metabolism of 4-hydroxyacids could result in metabolic perturbations.

Note that some of the reactions of 4-hydroxynonanoate metabolism probably occur in peroxisomes. This is the site of fatty acid α-oxidation (20) which, in the present case, forms [^{13}C]formate from 4-hydroxynonanoate labeled on C-3. Given the low labeling of acetyl-CoA and its proxies formed from 4-hydroxy[3-^{13}C]nonanoate (Fig 8A), it is likely that pathway B is peroxisomal, and pathway A is mitochondrial.

Our data on the accumulation of 4-P-butyryl-CoA in the brain and liver of mice unable to convert

4-hydroxybutyrate to succinate (Fig 10) suggest that 4-P-butyryl-CoA may contribute to the perturbation of brain metabolism in these mice who experience severe epileptic seizures (42). Also, 4-P-butyryl-CoA may contribute to the severe mental retardation of patients with 4-hydroxybutyric aciduria (12). Lastly, 4-P-butyryl-CoA may be implicated in acute mental dysfunction of subjects who ingested 4-hydroxybutyrate. Similar mental dysfunction is likely to be caused by the accumulation of 4-P-pentanoyl-CoA in subjects ingesting 4-hydroxypentanoate as an alternate drug of abuse to 4-hydroxybutyrate (16).

Unlike 4-hydroxyacids with five or more carbons, 4-hydroxybutyrate is not degraded to acetyl-CoA, as demonstrated by the absence of M2 acetyl-CoA and M2 + M4 BHB in livers perfused with 4-hydroxy-[$^{13}C_4$]butyrate. Other metabolites, not yet identified, may contribute to the brain toxicity of 4-hydroxybutyrate, in addition to 4-P-butyryl-CoA.

The present report illustrates the potential of the association of metabolomics and mass isotopomer analysis for pathway discovery. Our data open the way to extensive studies that will identify and characterize the enzymes which catalyze the reactions involved in the isomerization of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoAs (Fig 4, Pathway A). Our identification of new acyl-CoAs derived from 4-hydroxyacids was based on mass, mass fragmentation, chromatographic properties and isotopomer distribution. Confirmation of the identity of these acyl-CoAs will require designing synthetic techniques to prepare them. The difficulty of this endeavor is illustrated by the failure to chemically synthesize 4-hydroxybutyryl-CoA because of the propensity of 4-hydroxyacids to form lactones (45). However, once these difficulties are resolved, future studies will provide a clear picture of the catabolism of 4-hydroxyacids, especially those derived from the lipid peroxidation products 4-hydroxynonenal and 4-hydroxyhexenal. The catabolism of these compounds may modulate their cytotoxic effects.

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FOOTNOTES

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1. Mass isotopomers are designated as M, M1, M2,...Mn where n is the number of heavy atoms in the molecule. The designation m1, m2,...mn refers to the isotopic enrichment of each mass isotopomer expressed as a mol percent.

Abbreviations: BHB, β-hydroxybutyrate; HNE, 4-hydroxynonenal.

FIGURE LEGENDS

Fig 1. ^{31}P-NMR of a malonyl-CoA standard (panel A) and of 4-phosphopentanoyl-CoA isolated from a rat liver perfused with 4-hydroxypentanoate (panel B). Note the additional peak at chemical shift value of 85.815 in the spectrum of 4-phosphopentanoyl-CoA.

Fig 2. Retention times and relative abundances of 4-phosphoacyl-CoAs assayed under identical LC-MS/MS conditions in extracts of rat livers perfused with 2 mM of C_4 to C_{11} 4-hydroxy-*n*-acids (composite chromatogram).

Fig 3. Conversion of C_6, C_9 and C_{11} 4-hydroxyacids to (i) 3-hydroxyacids with same number of carbons, and (ii) 2-hydroxyacids with two carbons less than the original substrate. Each color corresponds to the two products of a given 4-hydroxyacid. Not shown on the figure are the accumulations of free carboxylic acids with 2 and 3 carbons less than the original substrate.

Fig 4. Proposed scheme of the catabolism of 4-hydroxyacids using 4-hydroxynonanoate as an example. Carbons 3 and 4 of the substrate are colored in red and green, respectively, to facilitate the tracing of their fates through pathways A and B. Note that the doubly-labeled substrate forms acetyl-CoA, part of which is doubly-labeled (M2) *via* pathway A and singly-labeled (M1) *via* pathway B. Formate, derived from carbon 3 of the substrate, is formed *via* pathway B. The compounds shown are 4-hydroxynonanoate *(1)*, 4-ketononanoate *(2)*, 4-hydroxynonanoyl-CoA *(3)*, 4-ketononanoyl-CoA *(4)*, 4-phosphononanoyl-CoA *(5)*, 4-phospho-2-*ene*-nonanoyl-CoA *(6)*, 3-hydroxy-4-phosphononanoyl-CoA *(7)*, *enol* form of 3-ketononanoyl-CoA *(8)*, 3-ketononanoyl-CoA *(9)*, 3-hydroxynonanoyl-CoA *(10)*, 2-hydroxyheptanoyl-CoA *(11)*, hexanoyl-CoA *(12)*, formate *(13)*, unlabeled acetyl-CoA *(14)*, singly-labeled (M1) acetyl-CoA *(15)*, doubly-labeled

(M2) acetyl-CoA *(16)*, propionyl-CoA *(17)*. Note that for even-chain 4-hydroxyacids with at least six carbons, pathway A leads to acetyl-CoA, while pathway B leads to acetyl-CoA, propionyl-CoA and formate.

Fig 5. Release of [^{13}C]formate by livers perfused with non-recirculating buffer containing 2 mM of 4-OH-[3-^{13}C]nonanoate (■), 4-OH-[3,4-$^{13}C_2$]nonanoate (▲) or 4-OH-[3-^{13}C]hexanoate (◆). The three profiles are not statistically different.

Fig 6. Identification of singly-labeled (M1) and doubly-labeled (M2) acyl-CoAs in a liver perfused with 2 mM of doubly-labeled 4-OH-[3,4-$^{13}C_2$]nonanoate. Numbers in red italics to the right of the strips refer to intermediates shown in Fig 4. *: M2 heptanoyl-CoA is an intermediate of one of the β-oxidation cycles between compounds 9 and 14 (Fig 4). **: M2 dihydroxynonanoyl-CoA is an intermediate of the β-oxidation cycle between compounds 3 and 11 (Fig 4). ***: M2 diketononanoyl-CoA is an intermediate of the β-oxidation of compound 4 to compound 11, without passing by compound 3 (Fig 4).

Fig 7. Accumulation of acyl-CoAs in livers perfused with increasing concentrations of 4-hydroxy-[3-^{13}C]nonanoate (panels A, B, C) and 4-hydroxy-[3,4-$^{13}C_2$]nonanoate (panels D, E, F). Unlabeled, singly-labeled and doubly-labeled compounds are designated as M, M1 and M2. The 3 levels of panels groups acyl-CoAs formed (i) before the bifurcation of the pathways outlined in Fig 5 (panels A, D), (ii) in pathway A (panels B, E), and (iii) in pathway B (panels C, F).

Fig 8. Labeling of acetyl-CoA and its proxies in livers perfused with increasing concentrations of 4-hydroxy-[3-^{13}C]nonanoate (panel A) or 4-hydroxy-[3,4-$^{13}C_2$]nonanoate (panel B). The acetyl-CoA proxies are the acetyl moiety of citrate, free acetate, and C-1+2 of β-hydroxybutyrate (BHB). Panel C: contribution of 4-hydroxy-[3,4-$^{13}C_2$]nonanoate to liver acetyl-CoA *via* pathway A of Fig 4 (middle curve), pathway B (lower curve). The upper curve is the total contribution of the substrate to acetyl-CoA.

Fig 9. A. Mass isotopomer distribution of synthetic 4-hydroxy-[3,3,4,5,5,5-$^{2}H_6$]pentanoate and its metabolites assayed in liver perfusate (3-hydroxypentanoate, lactate) and in liver tissue (propionyl-CoA). B. Scheme of the metabolism of M6 4-hydroxy-[3,3,4,5,5,5-$^{2}H_6$]pentanoate in liver. ^{2}H atoms are designated as D. The compounds shown are: 4-hydroxypentanoate M6 *(1)*, 4-hydroxypentanoyl-CoA M6 *(2)*, 3-hydroxypentanoyl-CoA M5 *(3)* relased as the free acid, 3-ketopentanoyl-CoA M4 *(4)*, propionyl-CoA M4 *(5)*, lactyl-CoA M4 *(6)* and lactate M4 *(7)*.

Fig 10. Evidence for CoA trapping in livers perfused with increasing concentrations of 4-hydroxyacids with 4 to 8 carbons. Concentrations of free CoA (panelA), acetyl-CoA (panel B) and malonyl-CoA (panel C).

Fig 11. Concentrations of 4-phosphobutyryl-CoA in the brains (panel A) and liver (panel B) of wild-type (W), heterozygote (T) and of mutant (M) mice deficient in succinic semialdehyde dehydrogenase.

Table 1. **Mass isotopomer distribution of metabolites derived from 4-hydroxy-[$^{2}H_{11}$]nonenal.**

	M	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11
4-P-nonanoyl-CoA	14.6	0.5	0.2	1.0	0.0	0.0	0.0	0.0	0.0	18.7	9.4	55.5
Heptanoyl-CoA	6.9	0.1	0.9	0.0	0.2	0.0	0.0	0.0	0.3	2.1	13.7	75.8
Hexanoyl-CoA	26.1	0.0	1.4	0.3	1.0	1.9	5.1	3.1	6.9	17.3	19.7	17.2
Pentanoyl-CoA	72.9	0.0	0.4	0.0	0.1	0.1	0.2	2.4	8.5	15.4		
Propionyl-CoA	62.1	3.8	2.3	10.5	0.0	15.3	2.7					
4-Hydroxynonanoate	8.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4	13.3	76.8
4-Hydroxynonenoate	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	10.6	87.7
2-Hydroxyheptanoate	0	0	0	0	0	0	0	0	0	1.8	13.9	84.2

Table 2. Mass isotopomer distribution of 4-hydroxynonanoate (C9) and 4-hydroxypentanoate (C5) and metabolites in livers perfused with 100% ^2H_2O buffer.

Note that the two substrates were unlabeled when added to the labeled buffer. All data are corrected for natural enrichment. Also the mass isotopomer distributions of the 4-OH-acyl-CoAs and 4-phospho-acyl-CoAs have been corrected for the incorporation of ^2H in the CoA moiety. The total labeling represent the sum of the mol fractions of labeled isotopomers (M1, M2,…).

		M	M1	M2	M3	M4	M5	M6	M7	M8	M9	Sum labeling	Total corrected labeling
4-OH-acid	C5	72.5	11.4	10.4	5.5	0.0	0.0	0.1	0.0	0.0	0.0	27.5	
	C9	74.5	7.5	8.1	6.8	0.6	2.4	0.0	0.0	0.0	0.0	25.5	
Free CoA	C5	95.6	1.3	1.1	0.7	1.2	0.1	0.1	0.0	0.0	0.0	4.4	
	C9	87.5	4.7	2.6	1.7	2.9	0.3	0.2	0.0	0.0	0.0	12.5	
4-OH-acyl-CoA	C5	38.6	23.4	10.5	10.5	10.4	6.6	0.0	0.0	0.0	0.0	61.4	57.0
	C9	57.7	18.7	9.5	9.3	4.1	0.5	0.0	0.3	0.0	0.0	42.3	29.8
4-P-acyl-CoA	C5	13.1	30.1	13.4	15.1	15.6	8.8	2.8	0.8	0.4	0.0	86.9	82.5
	C9	13.1	14.4	15.0	18.0	17.1	10.0	4.8	2.4	1.4	1.4	84.5	72.0

Figure 1

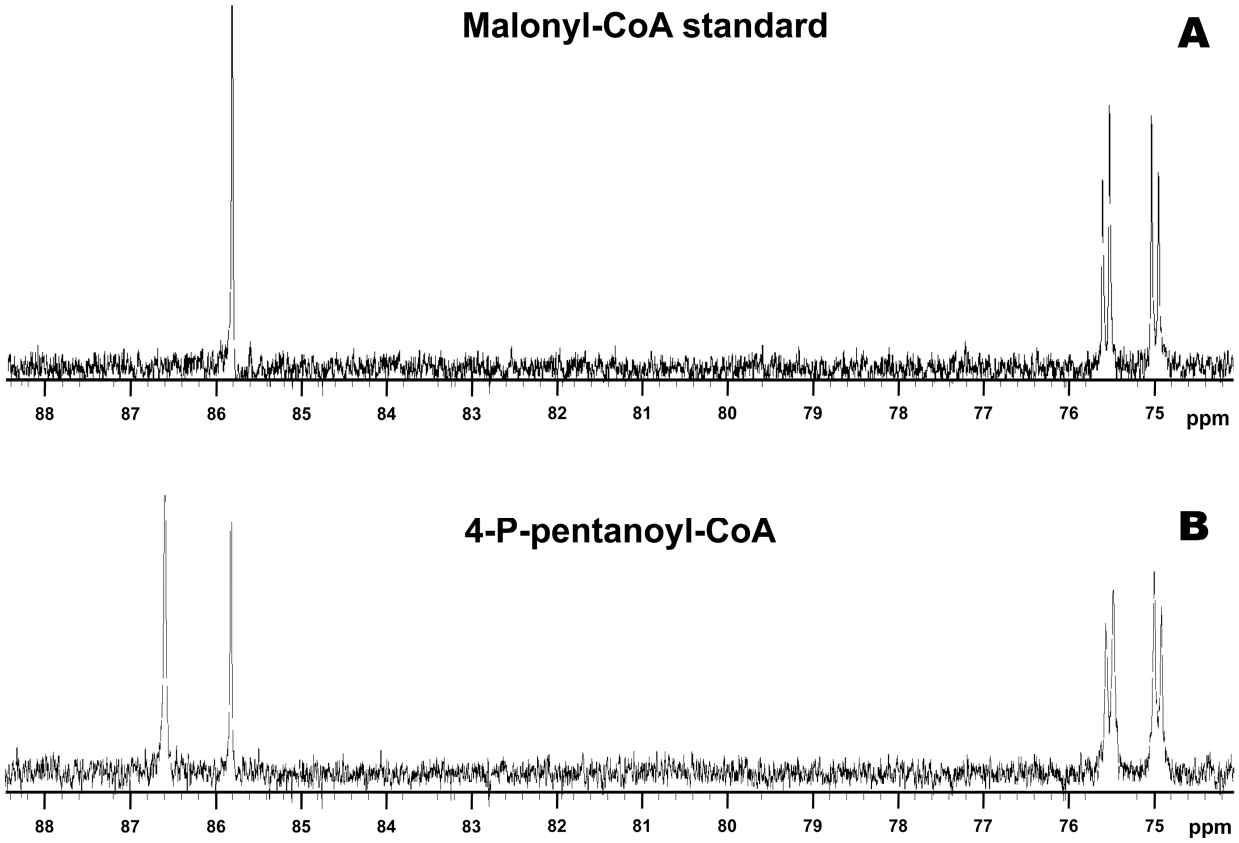


Figure 2

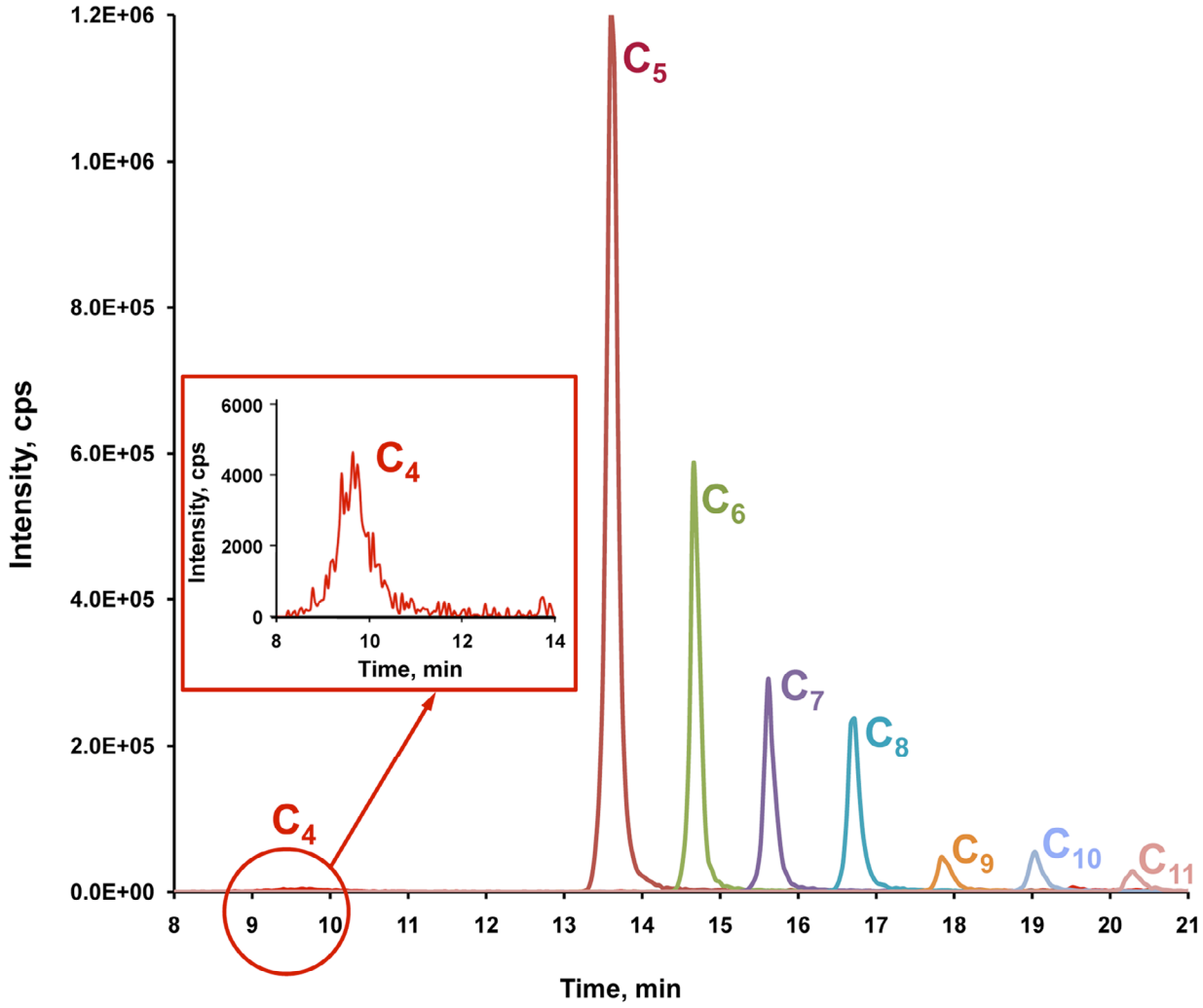
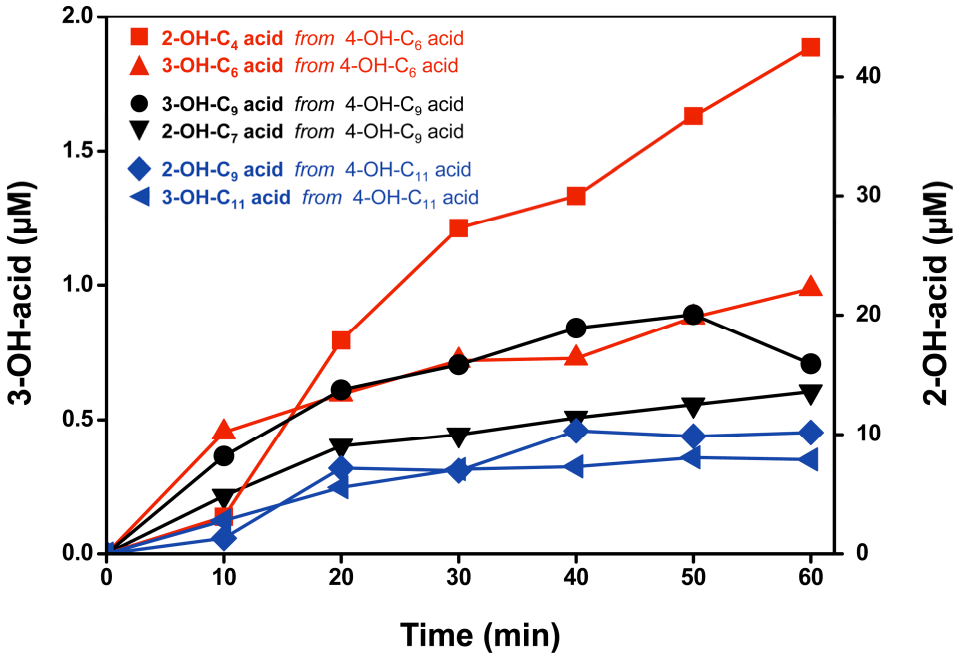


Figure 3



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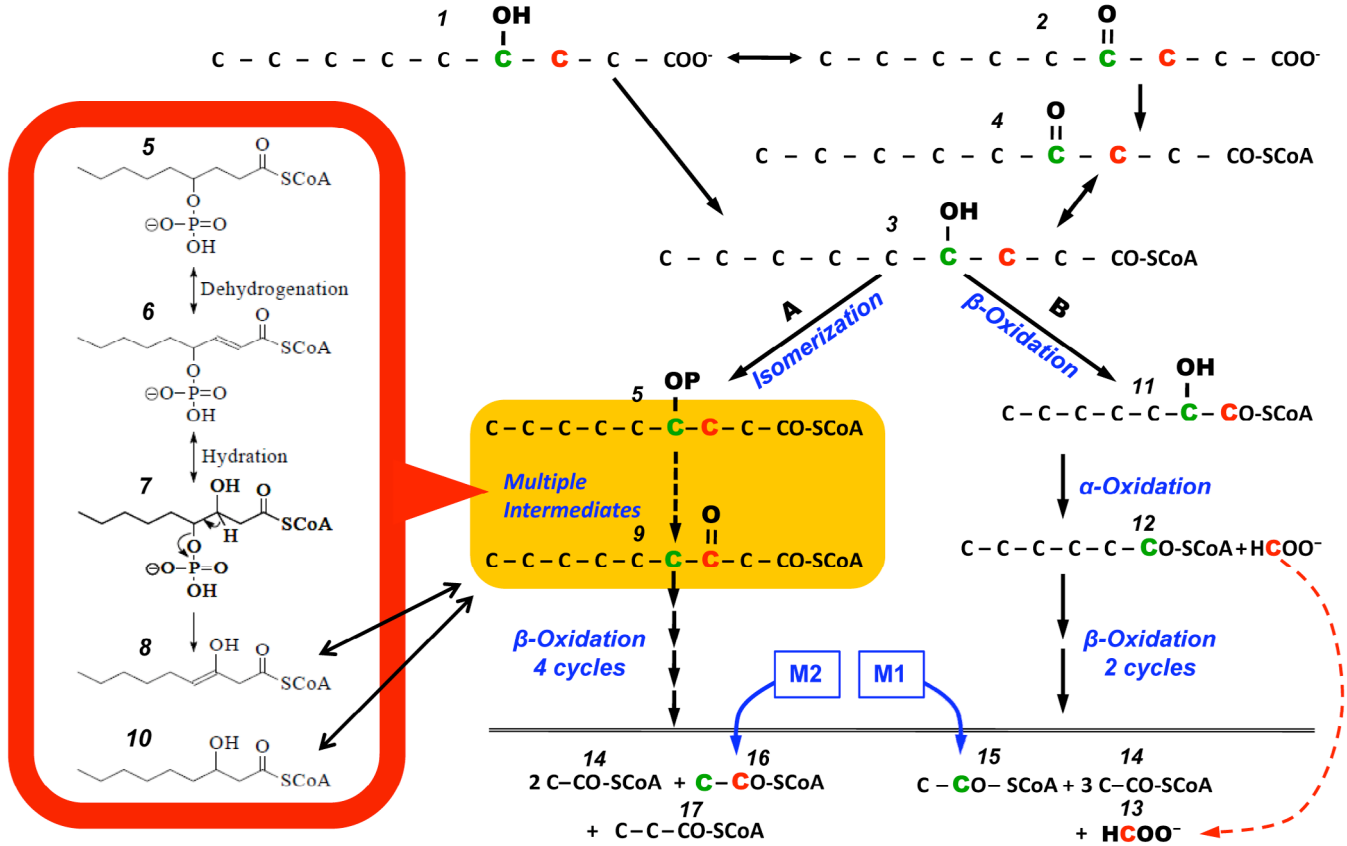


Figure 5

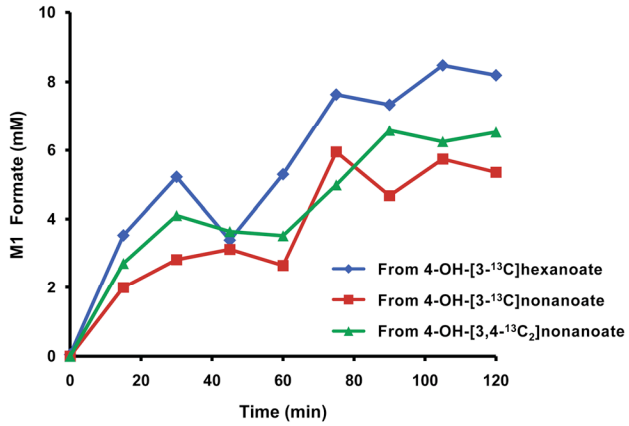


Figure 6

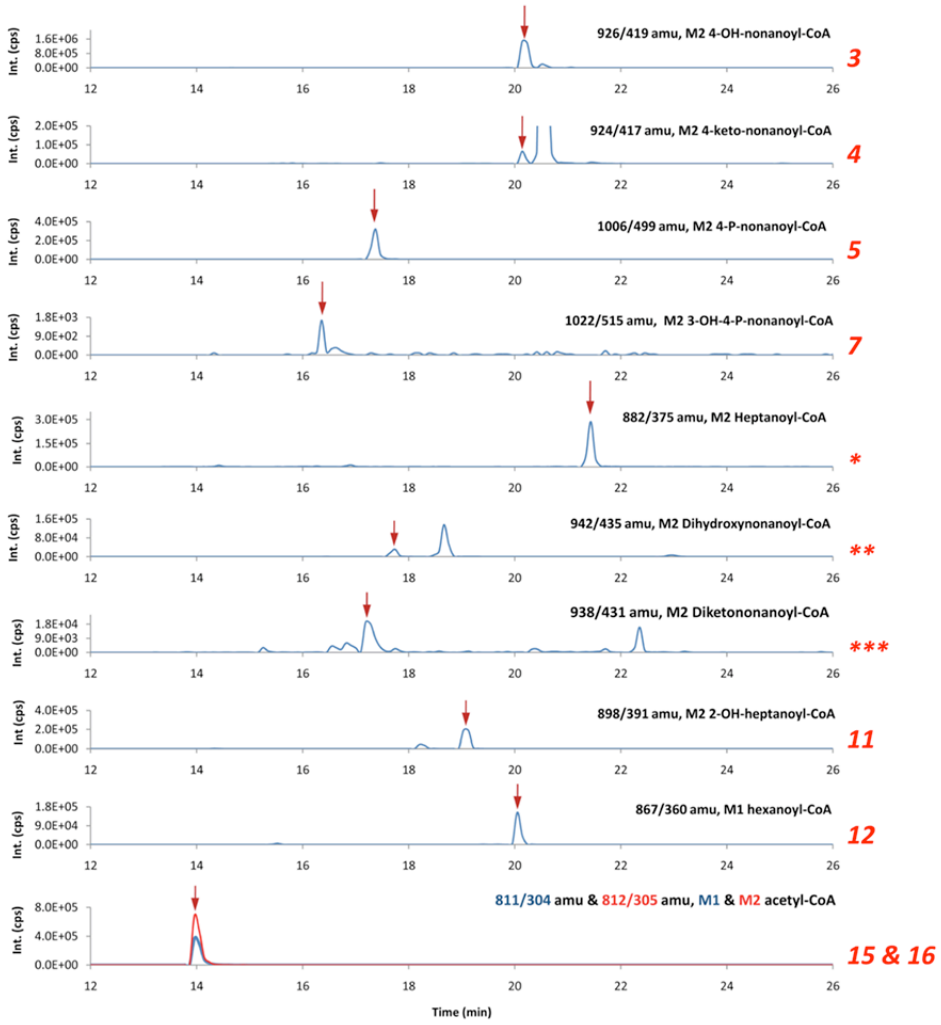


Figure 7

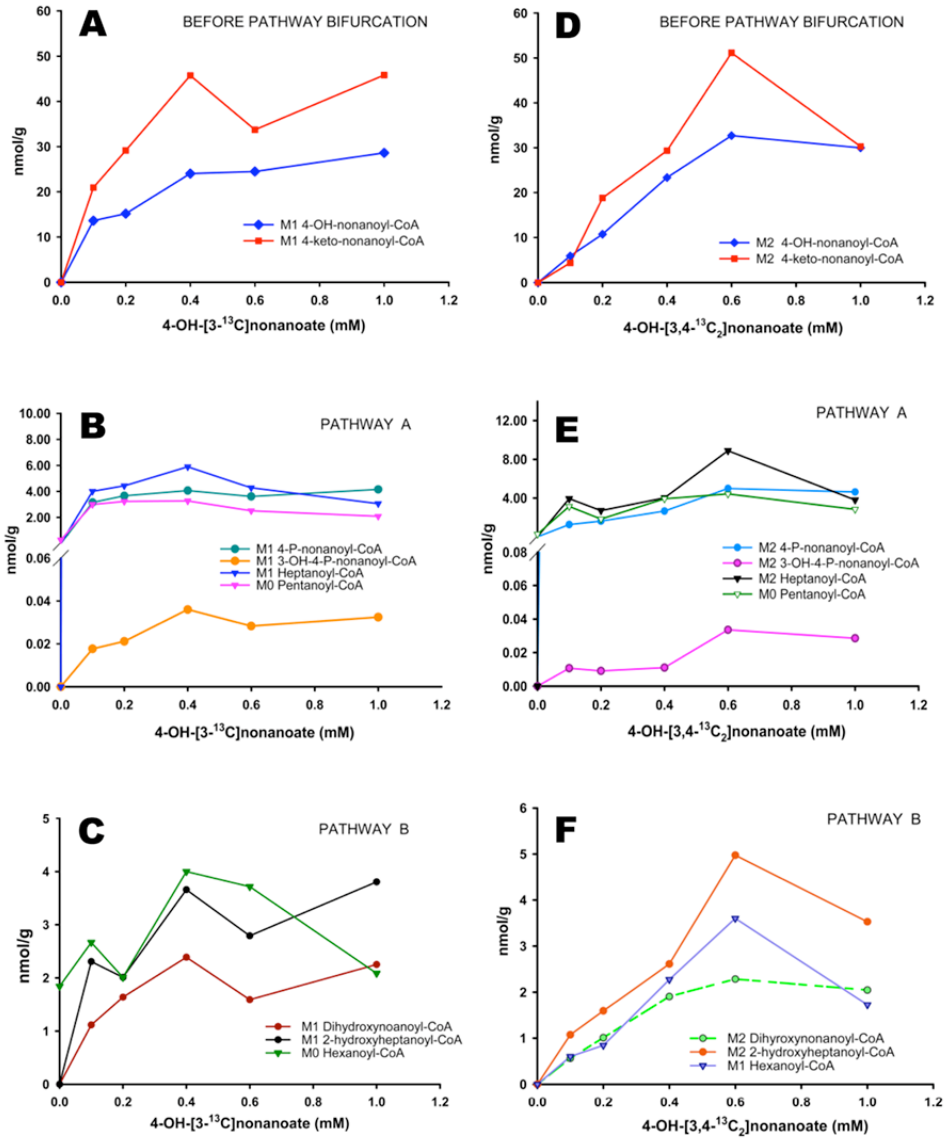


Figure 8

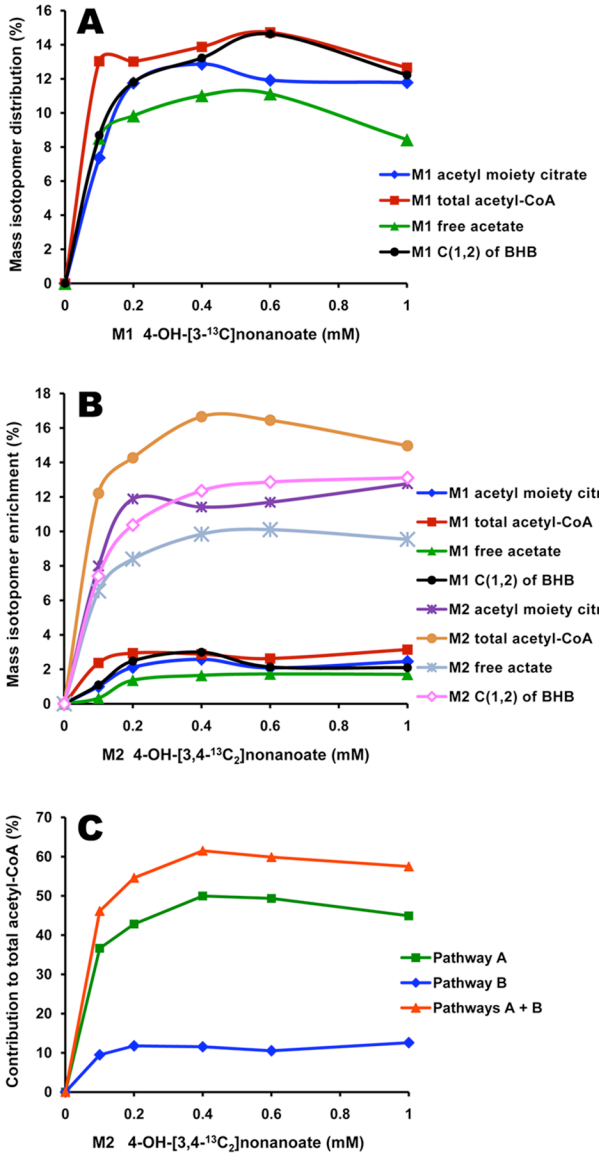


Figure 9

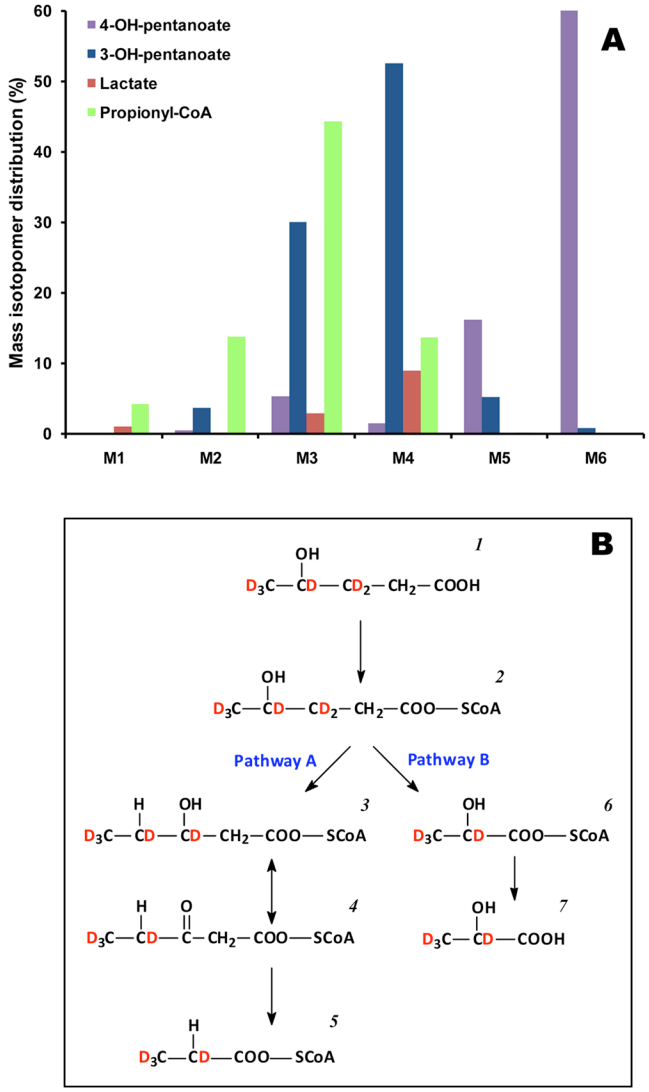
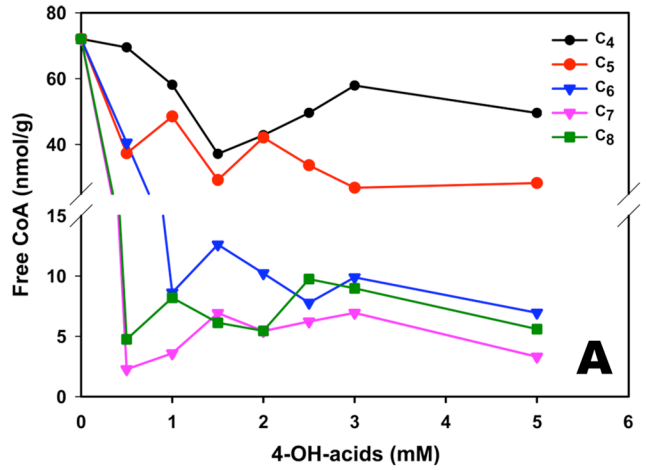
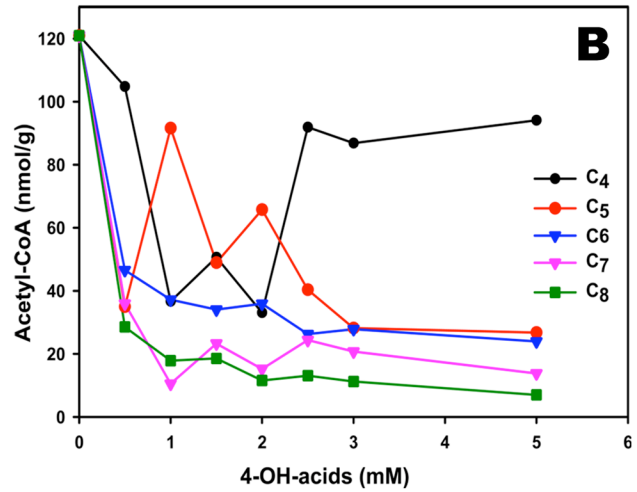


Figure 10





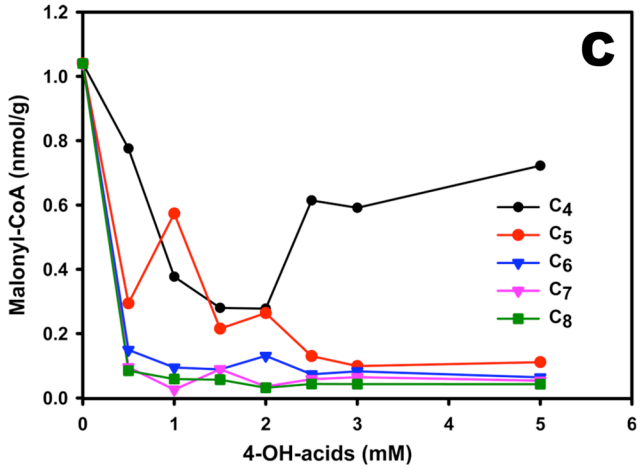


Figure 11