

# Multistep Enzymatic Synthesis of Long-Chain $\alpha,\omega$ -Dicarboxylic and $\omega$ -Hydroxycarboxylic Acids from Renewable Fatty Acids and Plant Oils\*\*

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Global climate change and oil depletion have stimulated the development of routes to produce renewable biofuels and chemicals in an environmentally friendly way. Tremendous scientific and technological developments in biocatalysis have enabled sustainable processes for the production of biofuels, chemicals, and polymers from carbohydrate-based biomass.<sup>[1]</sup> However, when using the current technology it is sometimes difficult to obtain high productivity and product yield; this is particularly true for the production of energy-rich molecules from plant-derived carbohydrates. For instance, oxygenated long-chain carboxyl synthons (e.g.,  $\alpha,\omega$ -dicarboxylic acids,  $\omega$ -hydroxycarboxylic acids, alcohols with chain length  $\geq C_8$ ), are difficult to produce in high yields from plant carbohydrates through biocatalysis. This difficulty is due to problems in the tight regulation of carbon chain length, especially for carboxylic acids having chains with an odd number of carbon atoms, in the deregulation of cellular regulatory systems, in the efficient regeneration of costly nicotinic amide cofactors, and in the regiospecific oxygenation of the fatty-acid moiety involved in the biosynthesis.<sup>[2]</sup>

Long-chain  $\alpha,\omega$ -dicarboxylic acids and  $\omega$ -hydroxycarboxylic acids are used in the production of a variety of chemical products and intermediates, such as, nylons and other polyamides, polyesters, resins, hot-melt adhesives, powder coatings, corrosion inhibitors, lubricants, plasticizers, greases, and perfumes.<sup>[3]</sup> For instance, several 10 000 tons of sebacic acid (1,10-decanedioic acid), which is produced from ricino-

leic acid, are used annually for the preparation of nylon-6,10. The majority of dicarboxylic acids are produced from petrochemical feedstocks or fatty acids by chemical routes under harsh reaction conditions that require high temperature and pressure, strong acids (e.g.,  $H_2SO_4$ ,  $HNO_3$ ), and/or toxic oxidants (e.g., ozone), which cause serious problems and harm our environment.<sup>[3a-c]</sup> However, in the past few years milder methods based on, for example, ruthenium catalysis in combination with peracetic acid to replace ozonolysis, or the metathesis reaction of unsaturated fatty acids to yield linear diacids, have been developed.<sup>[4]</sup> Biotransformation routes that use whole cell microorganisms for the preparation of dicarboxylic acids and their precursors  $\omega$ -hydroxycarboxylic acids have been also reported.<sup>[5]</sup> Sebacic acid and azelaic acid can be produced by fermentation of *Candida tropicalis*.<sup>[3c]</sup> However, in this case, petrochemical feedstocks, such as decane and nonane, are used as starting materials. Dicarboxylic acids and  $\omega$ -hydroxyfatty acids can be produced from vegetable oil fatty acids by *C. tropicalis*.<sup>[6]</sup> However, the diversity of the products is significantly constrained by the limitations in the availability of the reactants used and the range of intermediates accepted by the metabolic pathways in *C. tropicalis*.

Herein, a biocatalytic process was designed and evaluated for the production of long-chain  $\alpha,\omega$ -dicarboxylic acids (e.g.,  $C_{10}$ ) and  $\omega$ -hydroxycarboxylic acids (e.g.,  $C_9$ ,  $C_{11}$ ,  $C_{13}$ ) from renewable fatty acids of vegetable and animal origin (e.g., oleic acid, ricinoleic acid), which are currently the most important renewable feedstock of the chemical industry.<sup>[4]</sup> Hydration of internal carbon atoms of fatty acids by a hydratase, oxidation of the hydroxy group to the ketone by an alcohol dehydrogenase (ADH), Baeyer–Villiger monooxygenase (BVMO) catalyzed oxidation to the ester, and hydrolysis of this ester, yielded  $\alpha,\omega$ -dicarboxylic acids or  $\omega$ -hydroxy fatty acids.

The first example was the cleavage of ricinoleic acid (**1**) into *n*-heptanoic acid (**4**) and  $\omega$ -hydroxyundec-9-enoic acid (**5**; Scheme 1). The recombinant *Escherichia coli* BL21(DE3) expressing the ADH of *Micrococcus luteus* NCTC2665 and the BVMO of *Pseudomonas putida* KT2440, which were reported to catalyze oxidation of long-chain secondary alcohols<sup>[7]</sup> and long-chain keto hydrocarbons (e.g., 4-decanone),<sup>[8]</sup> respectively, catalyzed the transformation of ricinoleic acid (**1**) into **3** via 12-ketooleic acid (**2**; Figure 1 and Supporting Information, Figure S1B). The ester (**3**) was produced in a final concentration of 0.76 mM from 1.0 mM ricinoleic acid. Addition of the cell extract of *E. coli* BL21,

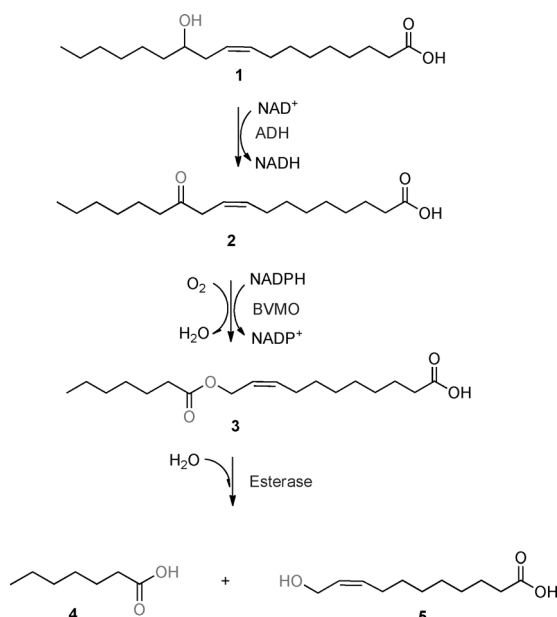
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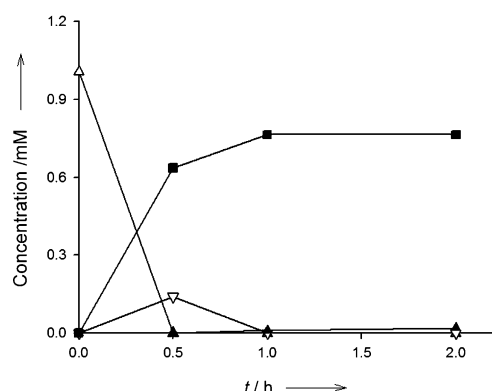
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**Scheme 1.** Designed biotransformation pathway. Ricinoleic acid (1) is converted into *n*-heptanoic acid (4) and ω-hydroxyundec-9-enoic acid (5) by multistep enzyme-catalyzed reactions.

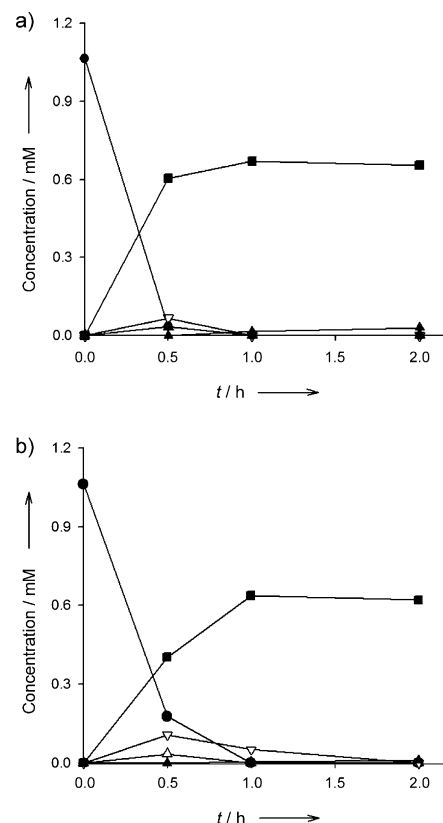


**Figure 1.** Time course of the biotransformation of ricinoleic acid (1) by the recombinant *E. coli* BL21(DE3), expressing the ADH from *M. luteus* and the BVMO from *P. putida* KT2440. The experiments were carried out in triplicate and the standard deviation was less than 10%. The symbols indicate concentration of ricinoleic acid (1;  $\Delta$ ), 10-ketooleic acid (2;  $\nabla$ ), 3 ( $\blacksquare$ ), and *n*-heptanoic acid (4) and ω-hydroxyundec-9-enoic acid (5;  $\blacktriangle$ ).

expressing the esterase gene of *P. fluorescens* SIK WI, which has been reported to hydrolyze long-chain esters (e.g., *n*-octylacetate),<sup>[9]</sup> resulted in the formation of *n*-heptanoic acid (4) and ω-hydroxyundec-9-enoic acid (5) from 3 (Supporting Information, Figure S1C). According to GC–MS analysis, the final products (4 and 5) were generated from ricinoleic acid in a greater than 70% yield. Compound 5 was isolated from the reaction broth in a yield of over 70% with a purity of approximately 90% and identified by NMR analysis (Supporting Information, Figures S1D and S6B).

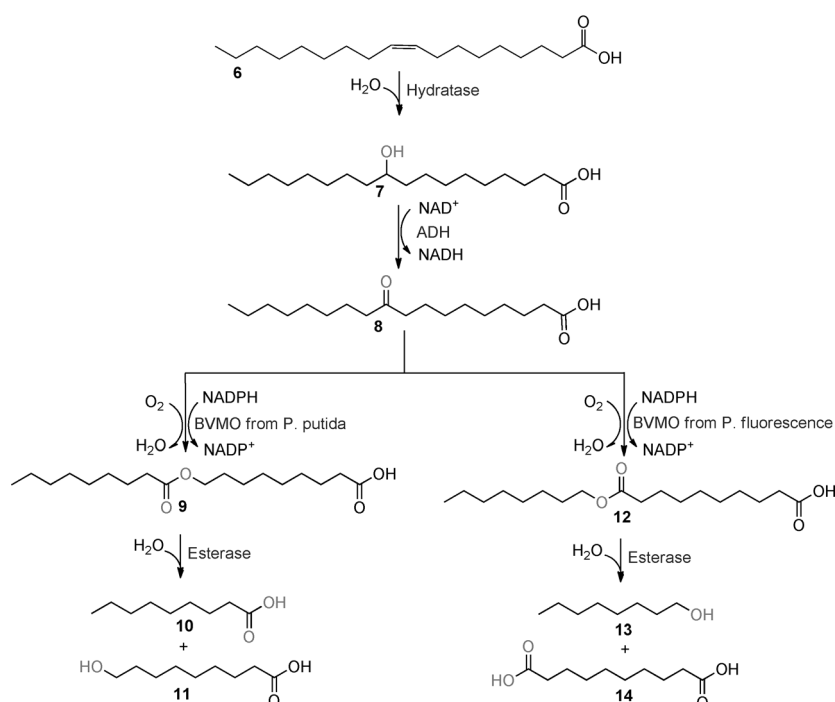
Most fatty acids that are available from plant oils occur as nonhydroxylated forms.<sup>[4]</sup> Therefore, an enzyme to catalyze the formation of hydroxylated fatty acids was introduced into

the cleavage pathway (Scheme 2). In addition, some BVMOs were reported to catalyze abnormal-ester formation, which is driven by migration of the less-substituted carbon center during catalysis.<sup>[10]</sup> As the formation of abnormal esters may generate different products, another source of BVMO was also adopted in the synthetic pathway. To evaluate the performance of the extended pathway, recombinant *E. coli* cells that expressed the gene encoding oleate hydratase (OhyA) from *Stenotrophomonas maltophilia*<sup>[11]</sup> in addition to the ADH of *M. luteus* and the BVMO of *P. putida* KT2440, were constructed and used for the biotransformation of oleic acid (Figure 2a). Addition of oleic acid (6) to 1.0 mM in the reaction medium allowed the recombinant biocatalyst to



**Figure 2.** Time course of the biotransformation of oleic acid by the recombinant *E. coli* BL21(DE3), expressing the oleate hydratase from *S. maltophilia*, the ADH from *M. luteus* and the BVMO from *P. putida* KT2440 (a) or the BVMO from *P. fluorescens* (b). The experiments were carried out in triplicate and the standard deviation was less than 10%. The symbols indicate concentration of oleic acid (6;  $\bullet$ ), 10-hydroxystearic acid (7;  $\Delta$ ), 10-ketostearic acid (8;  $\nabla$ ), 9 or 12 ( $\blacksquare$ ), and a) *n*-nonanoic acid (10) and ω-hydroxynonanoic acid (11;  $\blacktriangle$ ) or b) *n*-octanol (13) and 1,10-decanedioic acid (14;  $\blacktriangle$ ).

produce ester 9 via 10-hydroxystearic acid (7) and 10-ketostearic acid (8; Supporting Information, Figure S2C). The ester (9) was produced to a concentration of 0.67 mM and was completely converted into *n*-nonanoic acid (10) and ω-hydroxynonanoic acid (11) by the esterase from *P. fluorescens* (Supporting Information, Figure S2D). Remarkably, the replacement of the gene encoding BVMO of *P. putida*



**Scheme 2.** Extended biotransformation pathway. Oleic acid (**6**) is converted into either *n*-nonanoic acid (**10**) and  $\omega$ -hydroxynonanoic acid (**11**) or *n*-octanol (**13**) and 1,10-decanedioic acid (**14**) by multistep enzyme-catalyzed reactions.

$\omega$ -hydroxytridec-11-enoic acid were produced from lesquerolic acid (Supporting Information, Figure S4). Linoleic acid was converted in a similar fashion into oleic acid (Supporting Information, Figure S5). This result indicated that versatile carboxyl synthons can be produced from renewable fatty acids through multistep biocatalysis involving fatty acid hydroxylases and BVMOs.

Fatty acids are produced from plant oils. To further extend the applicability of the biocatalytic cascade reaction, we finally developed a biotransformation process that directly uses a plant oil (i.e., olive oil) as starting material. Olive oil was hydrolyzed with lipase and the resulting hydrolyzate was subjected to the oxidative cleavage reactions. Lipase treatment of 5 g L<sup>-1</sup> olive oil resulted in the production of 11.3 mM oleic acid and 1.2 mM palmitic acid. The subsequent biotransformation was conducted by the recombinant *E. coli* BL21 expressing hydratase, ADH, and BVMO from *P. putida* KT2440. The ester **9** was produced to 6.9 mM in the medium from oleic acid (**6**; Figure 3). Hydrolysis of ester **9**

KT2440 with that of *P. fluorescens* DSM 50106<sup>[12]</sup> enabled the recombinant cells to transform 10-ketostearic acid (**8**) into **12**, which differs from **9** in the position of the ester oxygen atoms in the fatty acid skeleton, as confirmed by MS analysis (Supporting Information, Figure S2E). This change in the reaction pathway resulted in the production of *n*-octanol (**13**) and 1,10-decanedioic acid (i.e., sebacic acid; **14**) instead of *n*-nonanoic acid (**10**) and  $\omega$ -hydroxynonanoic acid (**11**; Figure 2b and Supporting Information, Figure S2F). This result indicates that the diversity of the products of the synthetic pathway could be broadened by employing fatty acid hydratases and different types of BVMOs.

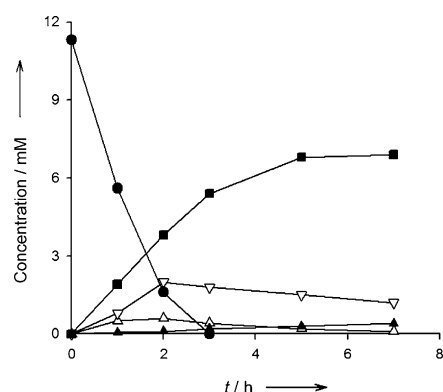
In addition to ricinoleic acid and oleic acid, 5-hydroxydecanoic acid, lesquerolic acid ((11*Z*, 14*R*)-14-hydroxyicos-11-enoic acid), and linoleic acid were subjected to the oxidative cleavage by the enzyme cascade (Table 1). 5-Hydroxydecanoic acid was degraded into either 1,5-dicarboxylic acid and *n*-heptanol or  $\gamma$ -hydroxybutyric acid and *n*-hexanoic acid depending on the BVMO used (Supporting Information, Figure S3). *n*-Heptanoic acid and

**Table 1:** Products accessible through the multistep biocatalysis.

Starting material	Origin of BVMO	Final products	Yield [%] <sup>[a]</sup>
5-hydroxydecanoic acid	<i>P. fluorescens</i>		> 60
	<i>P. putida</i>		> 60
lesquerolic acid	<i>P. fluorescens</i>		> 50
	<i>P. putida</i>		> 50
ricinoleic acid	<i>P. fluorescens</i>		> 70
	<i>P. putida</i>		> 70
oleic acid <sup>[b]</sup>	<i>P. fluorescens</i>		> 60
	<i>P. putida</i>		> 60
linoleic acid <sup>[b]</sup>	<i>P. fluorescens</i>		> 50
	<i>P. putida</i>		> 50

[a] The product yield was calculated based on the substrate depletion and the product concentration determined by GC–MS 2 h after biotransformation. The substrate was added to 1.0 mM in 50 mM Tris-HCl buffer (pH 8.0), containing 3.6 g dry cells L<sup>-1</sup> of recombinant *E. coli* biocatalysts.<sup>[15]</sup> [b] With coexpression of the oleate hydratase from *S. maltophilia*.

was mediated by cell extract of *E. coli* BL21 expressing the esterase gene from *P. fluorescens*, because the esterase reaction products (i.e., nonanoic acid and  $\omega$ -hydroxynonanoic acid) were toxic to viable *E. coli* cells. At the end,  $\omega$ -



**Figure 3.** Biotransformation of olive oil with lipase and the recombinant *E. coli* BL21, expressing oleate hydratase from *S. maltophilia*, the ADH from *M. luteus*, and BVMO from *P. putida* KT2440. Olive oil was hydrolyzed with lipase in 50 mM Tris-HCl buffer (pH 7.5; data not shown). The recombinant cells grown with Riesenberg medium were harvested and added directly to the olive oil hydrolyzate (see the Supporting Information for details). The experiments were carried out in triplicate and the standard deviation was less than 10%. The symbols indicate concentration of oleic acid (6; ●), 10-hydroxystearic acid (7; △), 10-ketostearic acid (8; ▽), 9 (■), and *n*-nonanoic acid (10) and  $\omega$ -hydroxynonanoic acid (11; ▲).

hydroxynonanoic acid was produced at a total concentration of 6.7 mM, which equals a molar yield from oleic acid of approximately 60%.

In summary, we developed a synthetic biocatalytic cascade reaction, which can be used to produce high value long-chain  $\alpha,\omega$ -dicarboxylic and  $\omega$ -hydroxycarboxylic acids from cost-effective renewable fatty acids and/or plant oils. The biocatalytic cascade reactions allowed the production of 1,10-decanedioic acid as well as  $\omega$ -hydroxycarboxylic acids with chain lengths of C<sub>9</sub>, C<sub>11</sub>, and C<sub>13</sub>; these carboxylic acids can be converted into  $\alpha,\omega$ -dicarboxylic acids by simple oxidation of the hydroxy group. In addition, carboxylic acids with chain lengths of C<sub>7</sub> and C<sub>9</sub>, and primary alcohols with chain length of C<sub>8</sub> were produced from ricinoleic acid, lesquerolic acid, oleic acid, and linoleic acid (Table 1). The diversity of the carboxylic products can be extended by a number of ways. One of them could be to engineer oleate hydratases to accept other unsaturated fatty acids, such as petroselinic acid ((*Z*)-octadec-6-enoate) and erucic acid ((*Z*)-docos-13-enoic acid), which are commercially available.<sup>[4]</sup> Another way is to employ new sources of fatty acid hydroxylases. For instance, cytochrome P450 monooxygenases<sup>[13]</sup> and lipoxygenases<sup>[14]</sup> were reported to catalyze regiospecific hydroxylation of internal carbon atoms in the fatty acid skeleton. Therefore, we expect that the applicability of our synthetic method for multistep

biocatalysis can be further broadened by adopting new fatty acid hydroxylases.

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