DOI: 10.1002/adsc.201600216

Advanced Synthesis & Catalysis

Combined Biocatalytic and Chemical Transformations of Oleic Acid to ω-Hydroxynonanoic Acid and α,ω-Nonanedioic Acid

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Received: February 27, 2016; Revised: July 26, 2016; Published online: ■■ ■■, 0000

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201600216.

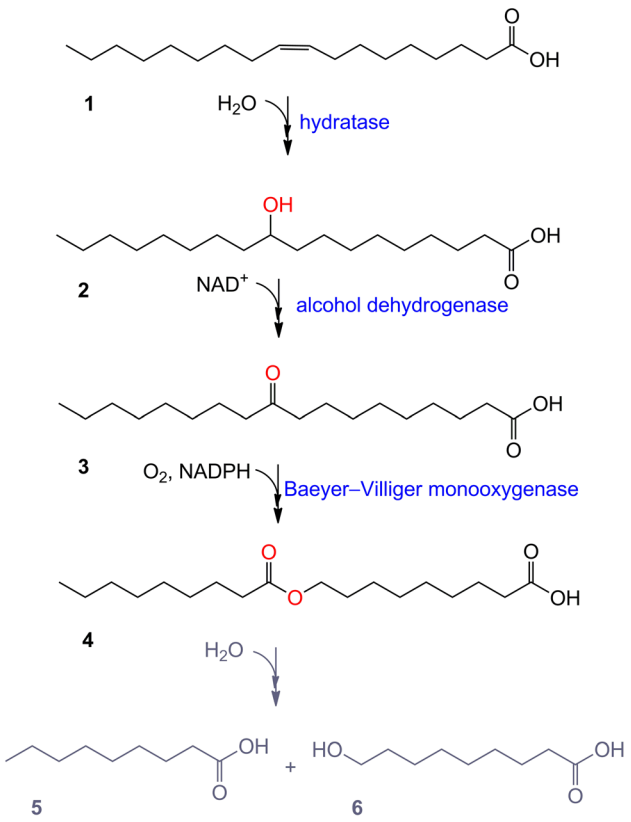
Abstract: A practical chemoenzymatic method for the synthesis of 9-hydroxynonanoic acid and 1,9-nonanedioic acid (i.e., azelaic acid) from oleic acid [(9*Z*)-octadec-9-enoic acid] was investigated. Biotransformation of oleic acid into 9-(nonanoyloxy)nonanoic acid *via* 10-hydroxyoctadecanoic acid and 10-keto-octadecanoic acid was driven by a C-9 double bond hydratase from *Stenotrophomonas maltophilia*, an alcohol dehydrogenase from *Micrococcus luteus*, and a Baeyer–Villiger monooxygenase (BVMO) from *Pseudomonas putida* KT2440, which was expressed in recombinant *Escherichia coli*. After production of the ester (i.e., the BVMO reaction product), the compound was chemically hydrolyzed to *n*-nonanoic acid and 9-hydroxynonanoic acid because *n*-nonanoic acid is toxic to *E. coli*. The ester was also converted into 9-hydroxynonanoic acid and the *n*-nonanoic acid methyl ester, which can be oxygenated into the 9-hydroxynonanoic acid methyl ester by the AlkBGT from *P. putida* GPo1. Finally, 9-hydroxynonanoic acid was chemically oxidized to azelaic acid with a high yield under fairly mild reaction conditions. For example, whole-cell biotransformation at a high cell density (i.e., 10 g dry cells/L) allowed the final ester product concentration and volumetric productivity to reach 25 mM and 2.8 mM h^{-1}, respectively. The overall molar yield of azelaic acid from oleic acid was 58%, based on the biotransformation and chemical transformation conversion yields of 84% and 68%, respectively.

Keywords: carboxylic acids; enzyme catalysis; fatty acids; oxidation

Introduction

Azelaic acid (1,9-nonanedioic acid) is widely used as a building block and/or intermediate for the production of polyamides and polyesters, pharmaceuticals, plasticizers, lubricants, or hydraulic fluids.[1] It is also utilized for food packaging (e.g., paper, film, and foil laminates) and in the electronics, textiles, and automotive industries. 9-Hydroxynonanoic acid can be used for the production of nonanolactone, which is a 10-membered ring lactone, and is an important intermediate and a useful monomer for producing aliphatic polyesters or polylactones *via* ring-opening polymerization.[2] *n*-Nonanoic acid (i.e., pelargonic acid) is also used in the preparation of flavors, plasticizers, and lacquers.

n-Nonanoic acid, 9-hydroxynonanoic acid, and azelaic acid are manufactured *via* the ozonolysis of C_9 and C_{10} unsaturated fatty acids (e.g., oleic acid) followed by the reduction of the intermediate ozonides.[1a,3] A biological synthesis of azelaic acid has also been reported.[1a] Azelaic acid is naturally produced in small amounts from oleic acid by *Malassezia furfur* on human skin. It is biotechnologically produced from nonane *via* fermentation with *Candida tropicalis*.[3] However, *C. tropicalis* is known as a pathogen and nonane should be obtained from non-renewable biomass and petrochemical feedstocks. Recently, the biosynthesis of 9-hydroxynonanoic acid and azelaic acid



Scheme 1. Designed biotransformation pathway. Oleic acid (**1**) is enzymatically converted into ester **4** (see the Results section for details), which can be hydrolyzed into *n*-nonanoic acid (**5**) and 9-hydroxynonanoic acid (**6**). Adopted from our previous study.[4]

from oleic acid was reported.[4] Oleic acid was first hydrated to 10-hydroxystearic acid by an oleate hydratase[4a,5] and then further oxidized to 9-(nonanoyloxy)-nonanoic acid (**4**) *via* 10-ketostearic acid (**3**) (Scheme 1).

The ester (**4**) was hydrolyzed by an esterase to yield *n*-nonanoic acid and 9-hydroxynonanoic acid. 9-Hydroxynonanoic acid was then further oxidized to azelaic acid by the alcohol dehydrogenase (AlkJ) of *Pseudomonas putida* GPo1.[4b,6] The multistep biotransformation was carried out by recombinant *Escherichia coli* expressing the catalytic enzymes in whole-cell assay conditions after cell cultivation, harvest, and resuspension into a buffer solution. The final product concentration was a few mM in the reaction medium.

The goal of this study was to develop a chemo-enzymatic process that allows the efficient production of high concentrations of *n*-nonanoic acid, 9-hydroxynonanoic acid, and azelaic acid from renewable biomass (i.e., oleic acid). To realize this goal, the ester (**4**) was chemically hydrolyzed into *n*-nonanoic acid (**5**) and 9-hydroxynonanoic acid (**6**). The ester (**4**) was also converted into 9-hydroxynonanoic acid and *n*-nonanoic acid methyl ester (**8**), which can be oxygenated into the 9-hydroxynonanoic acid methyl ester (**9**) by the AlkBGT from *P. putida* GPo1.[7] Finally, 9-hydroxynonanoic acid was chemically oxidized to azelaic acid with a high yield under fairly mild reaction conditions.

Results and Discussion

Production of the Ester 4 from 10-Hydroxystearic Acid

The preparation of 10-hydroxystearic acid (i.e., 10-hydroxyoctadecanoic acid) with a high concentration by biotransformation of oleic acid with an oleate hydratase from *Stenotrophomonas maltophilia* has been reported previously.[5a] Therefore, the bioconversion of 10-hydroxystearic acid into the ester (**4**) (Scheme 1) was first investigated by using the recombinant *E. coli* BL21(DE3):pACYC-ADH, pJOE-BVMO expressing the ADH from *M. luteus* and the BVMO from *P. putida* KT2440, which has been previously reported (Figure 1).[8]

The addition of 5 mM 10-hydroxystearic acid into the recombinant *E. coli* culture at the stationary growth phase led to the initiation of the biotransformation. The ester **4** was produced with a final concentration of 4.1 mM in the medium, 12 h after the initiation of the biotransformation (Figure 1A). The concentration of the reaction intermediate [i.e., 10-ketostearic acid (**3**)] in the reaction medium remained below 0.1 mM until t = 8 h. After this period, it began to accumulate in the medium, indicating that the BVMO reaction rate was gradually decreasing.

The next step was to increase the final product concentration and volumetric productivity. This was accomplished by enhancing the substrate concentrations and biocatalyst concentrations in the reaction medium. The biotransformation was carried out after fed-batch cultivation in a 1-L scale bioreactor, as reported previously.[8] When the recombinant *E. coli* BL21(DE3):pACYC-ADH, pJOE-BVMO had grown to 10 g cell dry weight (CDW)/L, 30 mM 10-hydroxystearic acid were added into the culture broth (Figure 1B). The reaction dynamics were similar to those of the whole-cell biotransformation in the flask; 10-hydroxystearic acid was stoichiometrically converted into the ester **4** *via* 10-ketostearic acid (**3**). However, the final product concentration and volumetric productivity were increased up to 21 mM and 1.4 mM h^{-1}, respectively, which were 5- and 4-fold higher than in the experiment shown in Figure 1A.

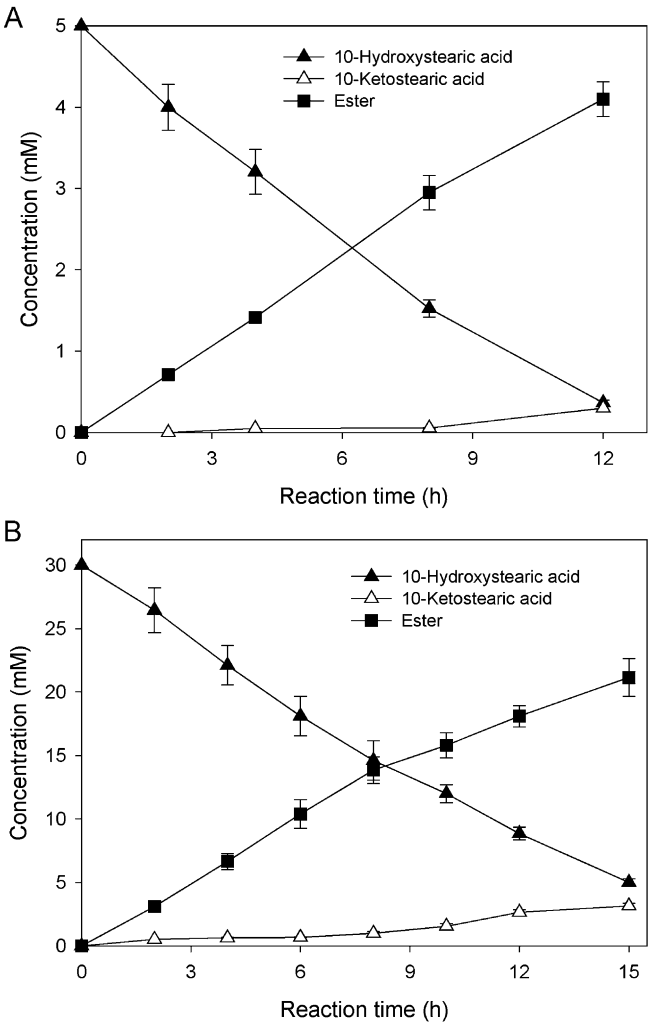


Figure 1. Time course of the biotransformation of 10-hydroxystearic acid (**2**) by the recombinant *E. coli* BL21(DE3):pACYC-ADH, pJOE-BVMO, expressing the ADH from *M. luteus* and the BVMO from *P. putida* KT2440. The biotransformation was initiated at the stationary growth phase of the batch culture (cell density: 3 g CDW/L) (A) or at the cell density of 10 g CDW/L (B) after fed-batch cultivation in a Riesenberg medium. 5 mM (A) or 30 mM (B) of the reaction substrate was added into the culture broth, to which 0.5 g L^{-1} of Tween 80 was then added. The symbols indicate the concentrations of 10-hydroxystearic acid (**2**) (▼), 10-ketooleic acid (**2**) (▽), and **3** (■). The error bars indicate standard deviations.

Biosynthesis of the Ester 4 from Oleic Acid

The whole-cell biotransformation rate in the experiment shown in Figure 1B was 2.3 U/g CDW, which is *ca.* 4-fold lower than the whole-cell biotransformation rate of ricinoleic acid under similar conditions.[8] One of the factors influencing the biotransformation rate might include substrate transport to the catalytic enzymes inside cells because the starting material (i.e., 10-hydroxystearic acid) is barely soluble and exists as a solid in water. The solubility of the compound in solvents is also quite low. As a consequence, it can be very difficult to distribute into an aqueous reaction medium. Therefore, we investigated the bioconversion of oleic acid (**1**) into the ester (**4**). The first approach was to use the recombinant *E. coli* BL21(DE3):pET28a-OhyA, pACYC-ADH-BVMO, expressing the hydratase from *S. maltophilia*, the ADH from *M. luteus*, and the BVMO from *P. putida* KT2440. The recombinant biocatalysts were able to catalyze the conversion of oleic acid into the ester (**4**) (Table 1 and Supporting Information, Figure S2A). However, the rate of ester formation was very low, probably because of low enzyme activity of the BVMO and ADH in the recombinant cells. Therefore, the target gene expression system was changed to pACYC-OhyA-ADH, pJOE-BVMO to reduce the expression of the OhyA while enhancing the expression of the BVMO and ADH. The newly constructed recombinant *E. coli* BL21(DE3):pACYC-OhyA-ADH, pJOE-BVMO showed a rather higher BVMO reaction rate compared to the *E. coli* BL21(DE3):pET28a-OhyA, pACYC-ADH-BVMO (Table 1 and Supporting Information, Figure S2B). To further improve the rate of ester formation by increasing the expression levels of both ADH and hydratase, *E. coli* BL21(DE3):pCOLA-ADH-OhyA, pJOE-BVMO was constructed; the pCOLA vector has a *ca.* 3-fold higher copy number (i.e., 20–40) than pACYC.[9] The enhanced expression of ADH and OhyA in the recombinant cells allowed the increase of the biotransformation rate (Table 1 and Supporting Information, Figure S2C). Another approach was to use a two-cell system, as previously reported for the production of ε-caprolactone from cyclohexanol via cyclohexanone[10] and of perillyl acetate from glucose *via* limonene.[11] *E. coli* BL21(DE3):pET28a-OhyA and *E. coli* BL21(DE3):-pACYC-ADH, pJOE-BVMO were independently grown to enhance the expression levels of the cascade enzymes. Following this, *E. coli* BL21(DE3):pET28a-OhyA was added into culture of *E. coli* BL21(DE3):-pACYC-ADH, pJOE-BVMO at a ratio of 1 to 3. Notably, the rate of ester formation from oleic acid was significantly increased up to 4.6 U/g CDW, which was *ca.* 7 times greater than that of *E. coli* BL21(DE3):-pET28a-OhyA, pACYC-ADH-BVMO (Table 1 and Supporting Information, Figure S2D). These results indicate that the rate of biotransformation of oleic acid to the ester (**4**) was dependent upon the expression level of the cascade enzymes in the *E. coli* cells.

Next, the oleic acid biotransformation was conducted at a high cell density, based on the experiment shown in Figure 1B (Figure 2). Mixing of the *E. coli* BL21(DE3):pET28a-OhyA and *E. coli* BL21(DE3):-pACYC-ADH, pJOE-BVMO strains in a ratio of 1 to 5 in the reaction medium allowed the whole-cell ester formation rate to increase up to 2.8 mM h^{-1} and

Table 1. Catalytic performance of recombinant *E. coli*-based biocatalysts[a]

Strains/biocatalysts	Initial ester (**4**) production rate [U/g CDW][b]	Final ester (**4**) concentration [mM][c]
E. coli BL21(DE3):pET28a-OhyA, pACYC-ADH-BVMO	0.6	0.8
E. coli BL21(DE3):pACYC-OhyA-ADH, pJOE-BVMO	1.1	1.5
E. coli BL21(DE3):pCOLA-ADH-OhyA, pJOE-BVMO	1.7	2.8
E. coli BL21(DE3):pET28a-OhyA and *E. coli* BL21(DE3):pACYC-ADH, pJOE-BVMO	4.6	5.2

[a] The cell cultivation and reaction conditions were the same as the experiment shown in Figure 1A, except for the substrate used. The cell density was 3 g CDW/L. Oleic acid was added instead of 10-hydroxystearic acid into the culture broth at the stationary growth phase.

[b] The initial ester (**4**) production rates were calculated based on the product concentration, which was measured at $t = 1.0$ or 2.0 h by GC/MS (see the reaction profile in the Supporting Information, Figure S2).

[c] The final ester (**4**) concentrations were measured at $t = 11$ or 9 h by GC/MS (see the reaction profile in the Supporting Information, Figure S2).

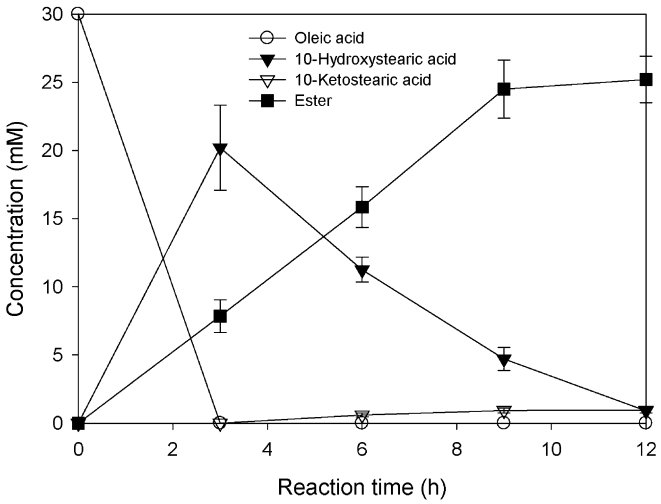


Figure 2. Time course of the biotransformation of oleic acid by the recombinant *E. coli* BL21(DE3):pET-OhyA expressing the oleate hydratase of *S. maltophilia* and *E. coli* BL21(DE3):pACYC-ADH, pJOE-BVMO. The biotransformation was initiated by adding 30 mM oleic acid, 0.5 g L^{-1} Tween 80, and 1 g CDW/L of *E. coli* BL21(DE3):pET-OhyA into the *E. coli* BL21(DE3):pACYC-ADH, pJOE-BVMO culture broth (cell density: 10 g CDW/L). Symbols indicate the concentrations of oleic acid (**1**) (○), 10-hydroxystearic acid (**2**) (▼), 10-ketostearic acid (**3**) (▽), and **4** (■). The error bars indicate standard deviations.

4.6 U/g CDW. The specific ester formation rate was identical to that achieved in the flask experiment shown in the Supporting Information, Figure S2D. However, this value is two-fold greater than that obtained in the experiment shown in Figure 1B (Table 2). *In situ* generation of the 10-hydroxystearic acid appeared to contribute to the substantial increase of the specific product formation rate.

In summary, overexpression of the cascade enzymes in whole-cell biocatalysts, *in situ* generation 10-hydroxystearic acid *via* a two-strain approach, and the application of higher cell densities allowed a high volumetric productivity and high product concentration in the culture medium.

Regarding the regeneration of the nicotinamide cofactors (Scheme 1) during whole-cell biotransformation, we assume that a small fraction of the fatty acid substrates were used as a carbon and energy source for *E. coli*. Cofactor regeneration by the intracellular transhydrogenases (e.g., PntAB) is another possibility. The NADH cofactor, which is generated from the second reaction by the alcohol dehydrogenase, could be converted into NADPH by the PntAB, which is oxidized during the third reaction by the BVMO. The serial reactions by the alcohol dehydrogenase, PntAB, and BVMO might allow the coupling of cofactor regeneration and maintenance of redox balance in cells.

Table 2. Biocatalytic performance of recombinant *E. coli*-based biocatalyst[a]

	Reaction at fed-batch cultivation I	Reaction at fed-batch cultivation II
Substrate concentration [mM]	30	30
Biocatalyst concentration [g CDW/L]	10	10
Biotransformation time [h]	15	9
Final product concentration [mM]	21	25
Spedific product formation rate [U/g CDW]	2.3	4.6
Volumetric productivity [mM h^{-1}]	1.4	2.8

[a] Reaction in fed-batch cultivation I and II was shown in Figure 1B and Figure 2, respectively.

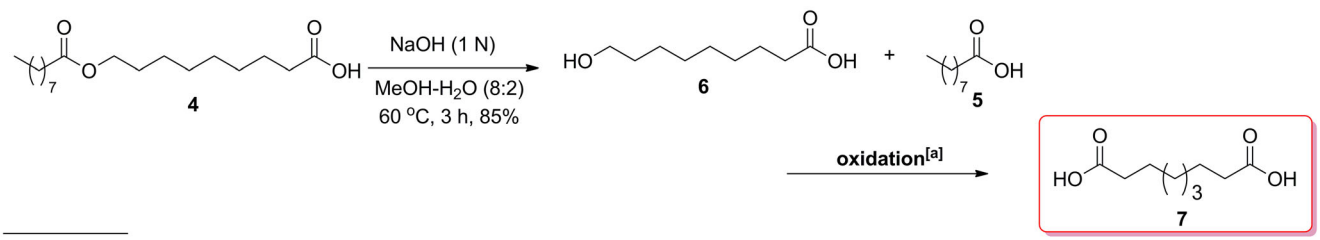
Optimization of the transhydrogenase activity would lead to further increases of the whole-cell biotransformation rates.

Chemical Conversion of the Ester Intermediate to Azelaic Acid

One of the major factors limiting the fatty acid biotransformation productivity of whole-cell biocatalysts may include the toxic effects of fatty acids on the host cells (e.g., *E. coli*).[8,12] For instance, the specific growth rate of *E. coli* was reduced by approximately 50% in the presence of 5 mM *n*-nonanoic acid (Supporting Information, Figure S1). Therefore, we investigated the chemical hydrolysis of the ester into *n*-nonanoic acid and 9-hydroxynonanoic acid.

The ester intermediate (**4**) was purified by extraction and column chromatography to approximately 85%, based on the measured concentration of the ester after biotransformation. Then, the ester intermediate (**4**) was successfully converted to azelaic acid (**7**) by successive two-step reactions, including the hydrolysis of the ester functionality and the oxidation of the alcohol functionality (Scheme 2). At first, the ester functional group was efficiently hydrolyzed with sodium hydroxide in a co-solvent of methanol/water (4/1) at 60 °C to afford 9-hydroxynonanoic acid (**6**) in approximately 85% yield. Finally, the oxidation of the terminal hydroxyl group of compound **6** to carboxylic acid was examined.

Initially, periodic acid in the presence of a catalytic amount of chromium trioxide was used, as in a method previously developed by us.[8] 9-Hydroxynonanoic acid (**6**) was treated with periodic acid (H_5IO_6) (2.5 equiv.) in the presence of a catalytic amount of chromium trioxide (CrO_3) (0.01 equiv.) in wet acetonitrile (0.75% water) at 0 °C to give azelaic acid (**7**) in approximately 80% yield after recrystallization from ethyl acetate-hexane (Method A). The periodic acid-mediated oxidation conditions are likely useful because the actual conversion efficiency might be more than 95% before recrystallization. However, these oxidation conditions are relatively expensive and the chromium-based oxidant is generally considered eco-unfriendly. Thus, other oxidation conditions were investigated with the aim to develop a green catalytic process that is feasible for large-scale applications. Many oxidation conditions were tried by employing different oxidants. The two best oxidation conditions were successfully developed, and further modified and optimized by many screening reactions from the reported conditions.[13] At first, hydroxynonanoic acid (**6**) was easily oxidized to azelaic acid (**7**) with 94% yield with $NaClO_2$ (1.2 equiv.), 2,2,6,6-tetramethyl-piperidin-1-yl oxyl (TEMPO) (4 mol%), and NaOCl (2 mol%) in aqueous acetonitrile at room temperature for 8 h (Method B).[13a] In addition, hydroxynonanoic acid (**6**) was also oxidized to azelaic acid (**7**) with 93% yield by using trichloroisocyanuric acid (TCCA) (1.5 equiv.), TEMPO (2 mol%), NaBr (10 mol%), and 10% aqueous $NaHCO_3$ in acetone at room temperature for 4 h (Method C).[13b] The TEMPO-catalyzed oxidation procedure allowed excellent conversion yields under mild reaction conditions and easy isolation of the target products. Additionally, the procedure required only cheap reagents and a catalytic amount of TEMPO under no buffer conditions, indicating a rather low production cost. Method B with $NaClO_2$, TEMPO, and NaOCl is preferable because it might be more advantageous in terms of economic cost and the product (azelaic acid), which was very pure after simple extractive work-up without any purification techniques such as column chromatography and recrystallization. The overall yields were approximately 80% over two chemical steps and approximately 68% based on both purification of the ester intermediate after biotransformation and the two-step chemical syntheses, respectively. All the intermediates were fully confirmed by their NMR spectra (see the Experimental Section and the Supporting Information for details).



[a] **Oxidation conditions**

Method A: H_5IO_6 (2.5 equiv.), CrO_3 (0.01 equiv.), wet CH_3CN, 0 °C, 75 min, 79% (after recrystallization).
Method B: $NaClO_2$ (1.2 equiv.), TEMPO (4 mol%), NaOCl (2 mol%), aqueous CH_3CN, r.t., 8 h, 94%.
Method C: TCCA (1.5 equiv.), TEMPO (2 mol%), NaBr (10 mol%), 10% aqueous $NaHCO_3$, acetone, r.t., 4 h, 93%.

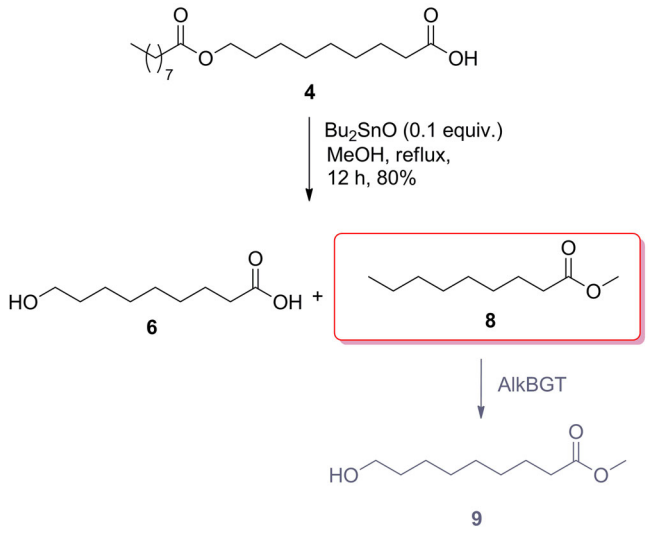
Scheme 2. Chemical conversion of the ester intermediate (**4**) to azelaic acid (**7**).

Similarly to the preparation of undecanedioic acid through biotransformation and chemical reactions,[8] azelaic acid (**7**) was prepared *via* direct coupling of hydrolysis and oxidation, followed by simple recrystallization from the crude ester intermediate after biotransformation. The overall yield of azelaic acid (**7**) was approximately 58% by recrystallization, which is expected to be improved in a large-scale preparation.

Production of Methyl Nonanoate and 9-Hydroxynonanoic Acid from the Ester (4)

Transesterification of the ester intermediate (**4**) with methanol into 9-hydroxynonanoic acid (**6**) and *n*-nonanoic acid methyl ester (**8**) was investigated because the methyl ester (**8**) can be oxygenated into the 9-hydroxynonanoic acid methyl ester (**9**) with a high yield by the AlkBGT from *P. putida* GPo1 (Scheme 3).[7,14] The methyl ester (**8**) can also be converted into the 9-aminononanoic acid methyl ester by serial combinations of the AlkBGT from *P. putida* GPo1 and ω-transaminase from *Chromobacterium violaceum*.[15]

The treatment of the ester (**4**) with sodium methoxide in methanol did not work well, generating only the hydrolysis products (**5**, **6**). By thorough screening of several reaction conditions, dibutyltin oxide-catalyzed methanolysis was found to be very successful. When the ester (**4**) was treated with a catalytic amount of dibutyltin oxide in methanol at reflux conditions for 12 h, the desired products, methyl nonanoate (**8**) and 9-hydroxynonanoic acid (**6**) were produced with rather high yields (80%). This indicates that the industrially relevant chemicals may be produced *via* combined whole-cell biotransformation of oleic acid and methanolysis of the ester (**4**).



Scheme 3. Chemical conversion of the ester intermediate (**4**) to methyl nonanoate (**8**) and 9-hydroxynonanoic acid (**6**).

Conclusions

A practical chemo-enzymatic synthesis of C_9 carboxylic acids, including 9-hydroxynonanoic acid (**6**) and azelaic acid (**7**), from oleic acid was examined. The chemo-enzymatic process allowed the production of the target compounds with rather high concentrations by circumventing the toxic effects of medium-chain fatty acids on the host cells; after biotransformation of oleic acid into 9-(nonanoyloxy)nonanoic acid (**4**) by the novel whole-cell biocatalytic process developed in this study (Figure 2), the ester was chemically hydrolyzed into 9-hydroxynonanoic acid and *n*-nonanoic acid with a high yield. The ester (**4**) was also converted into 9-hydroxynonanoic acid and the *n*-nonanoic acid methyl ester (**8**), which can be oxygenated into the 9-hydroxynonanoic acid methyl ester (**9**) by the AlkBGT from *P. putida* GPo1. Finally, 9-hydroxynonanoic acid was chemically oxidized to azelaic acid (**7**) with a high yield under mild reaction conditions. This study demonstrates that industrially relevant C_9 chemicals can be prepared from oleic acid with great efficiency, in terms of carbon economy and environmental friendliness compared to the ozonolysis-based chemical processes. This study will contribute to development of sustainable processes for the production of medium-chain fatty acids from renewable long-chain fatty acids and plant oils.

Experimental Section

Microbial Strains and Culture Media

Recombinant *E. coli* BL21(DE3):pACYC-ADH, pJOE-BVMO and recombinant *E. coli* BL21(DE3):pET-OhyA were grown in a LB medium (5 $g\,L^{-1}$ yeast extract, 10 $g\,L^{-1}$ tryptone, and 10 $g\,L^{-1}$ NaCl) supplemented with the appropriate antibiotics (i.e., chloramphenicol and ampicillin) for seed cultivation. The Riesenberg medium,[16] supplemented with 10 $g\,L^{-1}$ glucose and the appropriate antibiotics, was used for the main cultivation and biotransformation. The Riesenberg medium consisted of 4 $g\,L^{-1}$ $(NH_4)_2HPO_4$, 13.5 $g\,L^{-1}$ KH_2PO_4, 1.7 $g\,L^{-1}$ citric acid, 1.4 $g\,L^{-1}$ $MgSO_4$, and 10 $mL\,L^{-1}$ trace metal solution [10 $g\,L^{-1}$ $FeSO_4$, 2.25 $g\,L^{-1}$ $ZnSO_4$, 1.0 $g\,L^{-1}$ $CuSO_4$, 0.5 $g\,L^{-1}$ $MnSO_4$, 0.23 $g\,L^{-1}$ $Na_2B_4O_7$, 2.0 $g\,L^{-1}$ $CaCl_2$, and 0.1 $g\,L^{-1}$ $(NH_4)_6Mo_7O_{24}$]. Recombinant gene expression was induced by adding 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and/or 2 $g\,L^{-1}$ rhamnose to the culture broth.

Preparation of the Recombinant Plasmid

The pCOLA-ADH-OhyA was constructed by transferring the alcohol dehydrogenase gene from *M. luteus* from pACYC-ADH[4b] and the hydratase gene from *S. maltophilia* from pET28a-OhyA[17] into the pCOLA-Duet vector (Supporting Information, Table S1). The ADH gene was amplified by a polymerase chain reaction (PCR) with P1 and P2

as the primers (see the primer sequences in the Supporting Information, Table S2). The hydratase gene was amplified with P3 and P4 primers. The vector and PCR product were digested using appropriate restriction enzymes. The digested vector and PCR product were mixed at a ratio of 1 to 3 and ligated using T4 DNA ligase (ELPIS-Biotech, Daejeon, Republic of Korea).

Biotransformation in a Flask and Bioreactor

The biotransformation was carried out as previously reported.[12a] In brief, the biotransformation was initiated at the stationary growth phase, usually 8 h after the induction of gene expression with 0.1 mM IPTG and/or 2 g L^{-1} rhamnose. After changing the pH of the culture broth to 8.0 and increasing the temperature to 35 °C, 5–30 mM oleic acid or 10-hydroxystearic acid and 0.5 g L^{-1} Tween80 were added into the culture broth containing a cell concentration of 3 to 10 g dry cells/L. Cultivation and biotransformation was performed in a 250-mL flask (working volume: 20 mL) in a shaking incubator (200 rpm). The bioreactor experiment was conducted in a 1-L scale reactor (Biotron, Bucheon, Korea). The agitation speed and aeration rate were set at 400–1000 rpm and 1 vvm, respectively, to avoid any oxygen limitation during cultivation and biotransformation.

Product Analysis by Gas Chromatography/Mass Spectrometry (GC/MS)

Concentrations of the remaining fatty acids and accumulating carboxylic acids in the medium [e.g., oleic acid (**1**), 10-hydroxystearic acid (**2**), 10-ketostearic acid (**3**), and the ester (**4**)] were determined as described previously.[4a] The reaction medium was mixed with an equal volume of ethyl acetate containing 0.1 or 0.5 g L^{-1} methyl palmitate as an internal standard. The organic phase was harvested after vigorous vortexing and then subjected to derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (TMS). The TMS derivatives were analyzed using a Thermo Ultra Trace GC system connected to an ion trap mass detector (Thermo ITQ1100 GC-ion Trap MS, Thermo Scientific, Indianapolis, IN, USA). The derivatives were separated on a non-polar capillary column (30 m length, 0.25 μm film thickness, HP-5MS, Agilent Technologies, Palo Alto, CA, USA). A linear temperature gradient was programmed as 90 °C, 5 °C min^{-1} to 280 °C. The injection port temperature was 230 °C. Mass spectra were obtained by electron impact ionization at 70 eV. Scan spectra were obtained within the range of 100–600 *m*/*z*. Selected ion monitoring was used for the detection and fragmentation analysis of the reaction products.

Step-by-Step Chemical Conversion of the Ester Intermediate (4) to Azelaic Acid (7)

9-(Nonanoyloxy)nonanoic acid (4): The purification of the crude ester, which was obtained by extraction, was done by column chromatography (silica gel) with eluting by 20% ethyl acetate-hexane. The isolation/recovery yield of the ester (**4**) was approximately 85%, based on the measured concentration of the ester by GC/MS analysis after the biotransformation reaction. In addition to the following NMR analysis, the structure of the ester (**4**) was also thoroughly characterized by GC/MS analysis after the silylation of the ester, as reported previously by us.[4a,5a] Low melting white solid; mp 38–40 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 4.05$ (t, $J = 6.0$ Hz, 2H), 2.40–2.26 (m, 4H), 1.63–1.59 (m, 6H), 1.32–1.26 (m, 18H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 179.17$, 174.09, 64.34, 34.41, 33.85, 31.85, 31.81, 29.23, 29.14, 29.11, 29.05, 29.03, 28.95, 28.61, 25.86, 25.02, 22.65, 14.10.

9-Hydroxynonanoic acid (6): The ester intermediate (**4**) (260 mg, 0.83 mmol) was treated with NaOH (1 N) in MeOH-H_2O (4/1, 10 mL) at 60 °C for 3 h. The reaction mixture was cooled down and its pH was adjusted to 2 by slow addition of aqueous HCl (6 N) under ice-cooled conditions. The reaction mixture was evaporated to reduce the volume to approximately 2 mL. Water (5 mL) was added, and the residual solvent was saturated with NaCl and extracted with ethyl acetate (3 × 15 mL). The combined organic extract was washed with brine (10 mL) and water (10 mL), and dried over anhydrous Na_2SO_4, filtered, and evaporated to obtain a yellow semi-solid material. The crude product was purified by silica gel column chromatography using 50% ethyl acetate-hexane as an eluent to afford 9-hydroxynonanoic acid (**6**) as a low melting white solid; yield: 122 mg (85%); mp 50–52 °C (lit.[18] mp 49.5–51.0 °C). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.64$ (t, $J = 6.0$ Hz, 2H), 2.35 (t, $J = 7.5$ Hz, 2H), 1.66–1.52 (m, 4H), 1.38–1.32 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 178.85$, 63.03, 33.83, 32.68, 29.14 (2C), 28.93, 25.61, 24.63.

Azelaic acid (7), Method A:[8] A stock solution of H_5IO_6/CrO_3 was prepared by dissolving periodic acid (H_5IO_6) (11.4 g, 50 mmol) and CrO_3 (23 mg, 0.23 mmol) in wet acetonitrile (0.75% water) to a volume of 114 mL (complete dissolution typically required 1–2 h). The H_5IO_6/CrO_3 stock solution (3.25 mL, 2.5 equiv. of H_5IO_6 and 0.01 equiv. of CrO_3) was then added to a solution of compound **6** (100 mg, 0.57 mmol) in wet acetonitrile (4 mL, 0.75% water) while maintaining the reaction temperature at 0–5 °C for 30 min. The mixture was aged at 0–5 °C for 45 min. The reaction was quenched by adding an aqueous solution of Na_2HPO_4 (0.2 g in 2 mL H_2O). Ethyl acetate (10 mL) was added and stirred. The organic layer was separated. The aqueous part was saturated with NaCl and extracted further with 10% MeOH-ethyl acetate (4 × 10 mL). The combined organic layer was washed with brine (10 mL), 5% $NaHSO_3$ (10 mL), and brine (10 mL). The organic layer was dried over anhydrous Na_2SO_4 and then concentrated to give the crude dicarboxylic acid, which was further purified by recrystallization from ethyl acetate-hexane to yield pure azelaic acid (**7**) as a white solid; yield: 85 mg (79%).

Azelaic acid (7), Method B:[13a] A 50-mL two-necked, round-bottomed flask, equipped with a condenser was charged with compound **6** (0.13 g, 0.74 mmol), TEMPO (2,2,6,6-tetramethyl-piperidin-1-yl oxyl) (4.6 mg, 0.029 mmol, 4 mol%), and 6 mL of acetonitrile. A solution of sodium chlorite was prepared by dissolving 80% $NaClO_2$ (81 mg, 0.89 mmol) in 1.0 mL of water, and a solution of dilute sodium hypochlorite (NaOCl, 0.5 mL) was prepared by diluting household bleach (5.25% NaOCl, 0.53 mL, *ca.* 2.0 mol% with 9.5 mL of water). Approximately 20% of the $NaClO_2$ solution was added from a syringe, followed by 20% of the diluted bleach solution. The remaining portions of both reagents were then added simultaneously over 30 min. The resulting mixture was stirred at room tempera-

ture for 8 h. Water (7.5 mL) was added and the pH was adjusted to 8.0 by the addition of *ca.* 1.2 mL of 2.0 N NaOH. The reaction mixture was then poured into ice-cold sodium sulfite solution (0.34 g in 4.4 mL of water), maintained below 20 °C with an ice/water bath. After stirring for 15 min at room temperature, 8 mL of methyl *tert*-butyl ether (MTBE) was added and the resulting mixture was stirred for 15 min. The organic layer was separated and discarded. More MTBE (8 mL) was added and the rapidly stirred mixture was acidified with 2.0 N HCl to pH 3–4. The organic layer was separated and the aqueous layer was extracted with 2×8 mL portions of MTBE. The combined organic phases were washed with two 6 mL portions of water and 6 mL of brine, and then concentrated to give almost pure azelaic acid (**7**) as a white solid without further recrystallization; yield: 131 mg (94%).

Azelaic acid (7), Method C:[13b] A 10% aqueous solution of $NaHCO_3$ (2.25 mL) was added to a solution of compound **6** (0.13 g, 0.74 mmol) in acetone (8 mL) at 0 °C, followed by solid NaBr (7.6 mg, 10 mol) and TEMPO (2,2,6,6-tetramethyl-piperidin-1-yl oxyl) (2.3 mg, 0.015 mmol, 2 mol%). Trichloroisocyanuric acid (TCCA) (0.258 g, 1.11 mmol) was then slowly added within 20 min at 0 °C. The resulting mixture was warmed to room temperature and stirred for the required time (4 h) until the completion of the reaction, and then 2-propanol (0.4 mL) was added. The mixture was filtered on celite, concentrated under vacuum, and treated with 2.7 mL of a saturated solution of Na_2CO_3. The aqueous phase was washed with two portions of 8 mL of MTBE, treated with 1 N HCl, and extracted twice with 10 mL of 10% MeOH-ethyl acetate. The organic layers were dried (Na_2SO_4), and the solvent was evaporated to yield almost pure azelaic acid (**7**) as a white solid without further recrystallization; yield: 130 mg (93%).

Azelaic acid (7): Mp 109–110 °C (lit.[19] mp 105–107 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.17 (t, J = 7.5 Hz, 4H), 1.47 (m, 4H), 1.24 (m, 6H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 174.95 (2C), 34.07 (2C), 28.90, 28.86 (2C), 24.89 (2C).

Direct Chemical Conversion of the Ester Intermediate (4) to Azelaic Acid (7)

The final product, azelaic acid (**7**), was directly synthesized without column chromatography through a two-step reaction of the crude ester. The crude ester (**4**) (approximately 430 mg, calculated amount of the ester: 290 mg), which was obtained by extraction using organic solvents after biotransformation, was subjected to hydrolysis [1 N NaOH in MeOH-H_2O (4/1) at 60 °C for 3 h], followed by direct oxidation [$NaClO_2$ (1.2 equiv.), TEMPO (4 mol%) and NaOCl (2 mol%) in acetonitrile at room temperature for 8 h). Azelaic acid (**7**) was isolated by recrystallization in the final step; yield: 100 mg (58% overall yield over two steps based on the measured concentration of the crude ester).

Methanolysis of the Ester Intermediate (4) to Methyl Nonanoate (8)

The ester intermediate (**4**) (150 mg, 0.75 mmol) was dissolved in methanol (5 mL). After the addition of dibutyltin oxide (20 mg, 0.075 mmol), the mixture was heated under reflux for 12 h. After the completion of the reaction, the mixture was poured into a saturated sodium bicarbonate solution (5.0 mL) and extracted three times with ethyl acetate. The combined organic layers, which contained the dibutyltin oxide as a fine white precipitate, were filtered through celite, dried over sodium sulfate, and filtered. After removal of the solvent under vacuum, methyl nonanoate (**8**) was purified by vacuum distillation as a colorless liquid; yield: 110 mg (80%); bp 72 °C (10 mmHg) (lit.[20] bp 122 °C (45 mmHg)); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.62 (s, 3H), 2.26 (t, J = 7.5 Hz, 2H), 1.60–1.56 (m, 2H), 1.29–1.20 (m, 10H), 0.84 (t, J = 6.0 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.84, 51.03, 33.83, 31.68, 29.10, 29.02, 29.00, 24.79, 22.49, 13.82.

Acknowledgements

This study was supported by the R&D Program of MOTIE/KEIT, Republic of Korea [10044604 and 10044647]. J.-H. Seo was partially supported by Ewha Womans University (RP-Grant 2015).

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10 Combined Biocatalytic and Chemical Transformations of Oleic Acid to ω-Hydroxynonanoic Acid and α,ω-Nonanedioic Acid

Adv. Synth. Catal. **2016**, *358*, 1 – 10

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