Oxidation of *threo*-9,10-Dihydroxystearic Acid Mediated by *Micrococcus luteus* as a Key Step in the Conversion of Oleic Acid into Pelargonic and Azelaic Acids

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A sequential one-pot chemoenzymatic procedure for the conversion of diol **1**, easily obtained from oleic acid, into pelargonic and azelaic acids is herein described. The oxidation of diol **1** into a mixture of the corresponding regioisomeric hydroxyketones was promoted by an alcohol dehydrogenase from *Micrococcus luteus*, selected after an extensive screening of commercial enzyme kits and strain collections. The stereo-chemistry of the oxidation process of chiral diol **1** was also investigated. The hydroxyketones obtained by the biocatalyzed oxidation were submitted to further oxidative cleavage by a mild treatment with aqueous NaClO in a biphasic mixture, affording pelargonic and azelaic acids in high yield (76 and 71 %, respectively) with no need of column chromatography.

Introduction

The limited availability of non-renewable petroleum-based sources and the increasing drive to reduce waste and energy consumption have prompted the scientific community to move towards the optimization of greener manufacturing processes. The use of agro-waste/bio-based renewable feedstocks, the development of catalytic systems, the reduction of solvents and additional reagents (such as protecting groups), the search for energy and cost-effective synthetic procedures, avoiding toxic and harmful chemicals, are key features of ongoing research. A definite warning comes from the European Union to increase environmental sustainability, help with the transition to climate neutrality, recover unavoidable waste and promote the market of secondary raw materials and by-products.[1]

Over the last few years, our research group has become interested in investigating the use of *soapstock* as a renewable source of fatty acids, to be submitted to further chemo-enzymatic manipulation for the synthesis of fine chemicals.
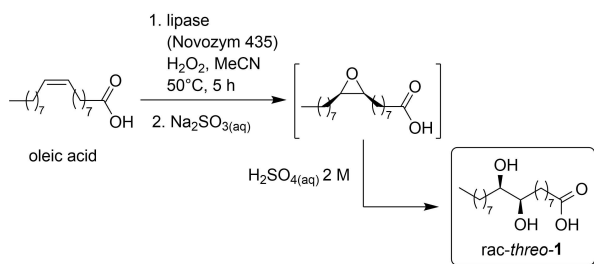
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Soapstock is the by-product obtained from the neutralization step of the refining process to which vegetable seed oil is submitted after extraction.[2] It consists of an alkaline aqueous emulsion of lipids, the composition of which depends on the botanical origin of the seeds and the conditions of refining and storage. It usually contains water (45–50 %, percentages vary according to the operating conditions), fatty acid sodium salts (determined as free acids, 10 %), triacylglycerols and diacylgly-cerols (12 %), residual phospholipids (9 %), pigments and other minor components (24 %).[3] During the refining process, soap-stock is collected by centrifugation and then submitted to a so-called *splitting* step: it is treated with sulfuric or hydrochloric acid, to recover free fatty acids with concomitant partial hydrolysis of residual oil, affording *oleins*, a mixture of free fatty acids in triglycerides.[4]

Samples of soapstock and oleins were provided to us by an Italian vegetable oil refining plant (Oleificio Zucchi, Cremona), obtained from the treatment of high-oleic sunflower oil and showing a high percentage of oleic acid (60–80 %). We started investigating a green and safe procedure for recovering this oleic acid and submitting it to oxidative cleavage, in order to produce azelaic and pelargonic acids. The aim of the research was to establish an alternative route to the ozonolysis of commercial oleic acid[5] and obtain value from waste, *i.e.*, azelaic acid for pharmaceutical and cosmetic industry and pelargonic acid for flavor and agrochemical products from soapstock, using chemo-enzymatic processes. We performed the preliminary investigations on commercial oleic acid as a model compound, to study each step of the most suitable oxidative procedure using a less complex starting material.[6] We identified as key intermediate of the synthetic sequence *threo*-9,10-dihydroxys-tearic acid (**1**), prepared by lipase-mediated epoxidation of oleic acid, in the presence of H$_2$O$_2$ 35 % aqueous solution, followed by oxirane hydrolysis catalyzed by 2 M sulfuric acid (Scheme 1). Diol **1** crystallized directly out of the reaction mixture and no further purification was needed. The following steps consisted

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Scheme 1. Chemo-enzymatic synthesis of rac-*threo*-diol **1**.

in the oxidation of diol **1** to dioxo derivative **2**, carried out by reaction with $Fe(NO_3)_3 \cdot 9H_2O$/TEMPO/NaCl in toluene solution at 100°C, and the final oxidative cleavage to pelargonic acid (**3**) and azelaic acid (**4**), promoted by H_2O_2 35% aq. solution at 30°C (Scheme 2a). We considered to further improve the procedure by investigating biocatalyzed alternatives to Fe(III)/TEMPO oxidation.

Unfortunately, the employment of commercial alcohol dehydrogenase kits (supplied by Evoxx technologies GmbH and Johnson Matthey) gave no successful hits. As an alternative approach, a screening of selected whole-cell microorganisms belonging to the Polish Collection of Microorganisms (PCM) Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wroclaw, and the German Collection of Microorganisms and Cell Cultures (DSMZ) was performed. The bacterium *Micrococcus luteus* PCM525 was considered for further studies, having shown good capability to oxidize rac-*threo*-**1** to the mixture of the corresponding hydroxyketones **5a** and **5b** (Scheme 2b).

In order to overcome some hurdles met in the development of the fermentation experiments, we decided to overproduce recombinantly in *Escherichia coli* the alcohol dehydrogenase (ADH) responsible for the oxidation, employing the genomic sequence already described in the literature for a secondary ADH from *M. luteus* NCTC2665.[7] Complete conversion of rac-*threo*-**1** to regioisomers **5** was achieved after process optimization. This mixture was submitted to one-pot *in situ* oxidative

cleavage promoted by 10% aqueous sodium hypochlorite solution to final products **3** and **4**. In this work, we describe the development of the synthetic strategy shown in Scheme 2b, along with the results obtained by studying the stereochemical course of the oxidation of rac-*threo*-**1** with recombinant ADH from *M. luteus* NCTC2665.

Results and Discussion

Screening of selected whole cells microorganisms

Racemic *threo*-diol **1**, prepared according to the procedure described in reference,[6] was employed to investigate the oxidative capability of 20 bacteria belonging to the genus of *Bacillus*, *Comamonas*, *Dietzia*, *Gordonia*, *Micrococcus*, *Pseudomonas*, *Rhodococcus*, *Streptomyces* from PCM and DSMZ microorganism collections. Most of the strains tested were unable to transform substrate **1**. A few of them (*Dietzia* sp. DSM44016, *Rhodococcus erythropolis* DSM2150, *R. erythropolis* DSM44534, *R. rhodnii* DSM2157, and *Gordonia* sp. DSM44456) afforded complex mixtures of transformation products.

Nevertheless, in the screening biotransformations with *Streptomyces griseus* DSM40395 and *Micrococcus luteus* PCM525, the formation of a mixture of the two regioisomeric hydroxyketones **5a** and **5b** was observed with a conversion of 30 and 40%, respectively, as shown by GC/MS analysis. *M. luteus* PCM525 was selected for further studies, also on the basis of our recently published results, showing the high ADH activity of the microorganism in the transformation of 10-hydroxystearic acid into 10-oxostearic acid.[8] When the oxidation of diol **1** with the growing cells of *M. luteus* PCM525 was performed on preparative scale (50 g/L of substrate **1** dissolved in EtOH) in TSB medium, derivatives **5** were obtained in maximum 30% conversion (estimated by ^{1}H NMR analysis as the molar ratio between compounds **5** and starting diol **1**) after three days. Based on the literature, the optimum pH for oxidation reactions catalyzed by commercial alcohol dehydrogenases is in the range 8.5–9.0, therefore the reaction with resting cells of *M. luteus* PCM525 was further investigated in this pH range.
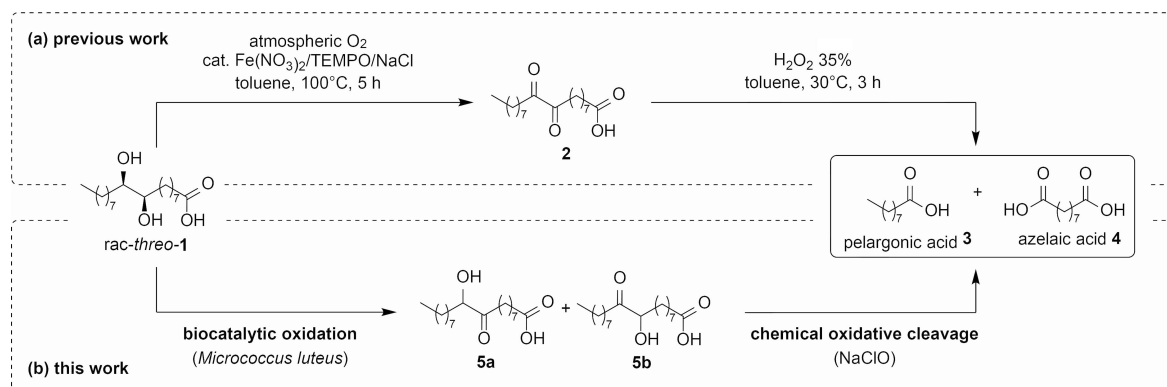
Scheme 2. Alternative routes to the conversion of **1** into pelargonic and azelaic acids: (a) previously described chemical procedure; (b) chemo-enzymatic sequence described in this work.

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Substrate **1**, dissolved in EtOH (50 mg/mL), was added to the resting cells of *M. luteus* in aqueous buffer (Tris-HCl, pH 9.0) and the reactions were monitored for three days. The two hydroxyketone derivatives **5** were obtained as the only oxidation products with a significantly higher conversion (65%, calculated by ^{1}H NMR analysis from the molar ratio between compounds **5** and starting diol **1**).

Despite this satisfactory result, the procedure showed some critical drawbacks. Complete conversion of compound **1** to the oxidation products **5a, b** could not be achieved, even exploring different reaction conditions, such as increased cell loadings (from 0.05 g to 0.6 g cells for 25 mg substrate) and different pH values (8.0, 8.5, 9.0). Furthermore, the presence of *erythro*-**1** in the final mixture was observed in most cases, likely originating from the concurrent reduction of hydroxyketones **5** by the same microorganism. A sample of diol *erythro*-**1**, to be used as a reference standard, was prepared starting from elaidic acid (see Supporting Information), according to the same procedure shown in Scheme 1 to prepare *threo*-**1** from oleic acid. Finally, it was apparent that the final conversion strongly depended on the stability of the pH value during the reaction course. In some tests, a very low conversion was correlated to a strong decrease of the pH value (from 9.0 to 6.0–5.5) of the aqueous medium, probably due to cell stress. The complete unpredictability of this phenomenon raised some concerns about the effective reproducibility of the process.

Overproduction of recombinant ADH from *M. luteus* in *E. coli* BL21(DE3)

In an attempt to overcome the critical issues that emerged with the use of *M. luteus* cells we envisaged to clone and overexpress the gene coding for the ADH responsible for the oxidative activity. A literature search revealed that a secondary ADH from *M. luteus* NCTC2665 had been overproduced in functional form in *E. coli* cells and employed by Bornscheuer and Park to oxidize 10-hydroxystearic acid to 10-oxostearic acid.[7] According to the information available from the PCM website,[9] *M. luteus* PCM525 and NCTC2665 are the same strain. The coding sequence of the ADH from *M. luteus* NCTC2665 was cloned in a pET-24(+) vector which was used to transform *E. coli* BL21(DE3), in order to overproduce the recombinant ADH.

The reactions were set up under the same experimental conditions described above for *M. luteus* PCM525, using *E. coli* resting cells expressing the *M. luteus* ADH. The reaction led to the exclusive formation of hydroxyketones **5**, and the conversion was increased to 75% (calculated by ^{1}H NMR analysis from the molar ratio between compounds **5** and starting diol **1**). Rewardingly, neither the formation of the other diastereoisomer of the starting diol **1**, nor any problem related to the acidification of the reaction medium were observed. Thus, the reliability of the oxidation procedure towards the production of hydroxyketones **5a, b** was considerably improved.

Use of recombinant *M. luteus* ADH in the oxidation of *threo*-1 (cell-free extract and purified enzyme)

A further development consisted in the investigation of both cell-free extract of *E. coli* BL21(DE3) expressing recombinant *M. luteus* ADH and the corresponding purified enzyme (obtained through standard affinity chromatography) as biocatalysts for the oxidation. A commercial NADH oxidase[10] (NOX) was added to the system, for the regeneration of the NAD$^+$ cofactor at the expense of O$_2$ from air. Due to the low solubility of starting substrate in aqueous media, we explored the use of different co-solvents, considering both common organic solvents, such as DMF, acetonitrile and ethanol, and two unconventional solvents, N-methylmorpholine N-oxide (N-MMO), and Cyrene™. The results of the oxidation reactions are reported in Table 1.

Complete conversion was obtained with both cell-free extract and purified enzyme, using N-MMO as a co-solvent (for the screening reaction a commercially available 50% solution of N-MMO in water was employed). N-MMO is a particularly polar compound, currently used as a solvent for the dissolution of cellulose in Lyocell process.[11] The polar nature of N-MMO allowed us to achieve a good solubility of the starting substrate *threo*-**1** in the reaction buffer, leading to complete oxidation to the mixture of compounds **5** using both the cell-free extract and the purified enzyme. NMR analysis of the crude reaction mixture confirmed that no *erythro*-**1** was formed during this biocatalyzed reaction.

In order to avoid unnecessary purification steps and make the procedure more appealing to scale-up and industrial application, all further optimization experiments were performed using the cell-free extract only. Under optimized conditions, it was possible to achieve quantitative oxidation of *threo*-**1** (50 mg) to regioisomeric hydroxyketones **5a, b** (30 h, N-MMO as a co-solvent) in 81% isolated yield after reaction work-up and purification by column chromatography.

Table 1. Effect of the co-solvent on the oxidation of *threo*-**1** with cell-free extract of *E. coli* expressing *M. luteus* ADH and purified enzyme.[a]

Co-solvent	Conversion to hydroxyketones **5a, b** [%][b]	
	Cell-free extract	Purified enzyme
DMF	72	94
MeCN	60	90
EtOH	58	99
N-MMO	99	99
Cyrene™	80	91

[a] Experimental conditions: 5 μmol substrate, 50 μL cosolvent, 0.5 μmol NAD$^+$, 0.5 mL cell-free extract or 250 μg purified enzyme, 300 μg NOX, 100 mM Tris-HCl, pH 9.0, total volume 1.5 mL, 30 °C, 24 h, [b] Molar percentage of hydroxyketones **5** in the crude mixture as determined by ^{1}H NMR analysis. The other component is always unreacted starting *threo*-**1**. Two replicates were performed for each biotransformation: no significant differences (<5%) were observed for conversion.

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Stereochemical course of the oxidation of *threo*-1 by *M. luteus* ADH

Diol *threo*-1 is a chiral compound with two stereogenic centers, and it was obtained in racemic form with excellent diastereoisomeric excess (de > 99% by ^{1}H NMR analysis, see Supporting Information), according to the procedure in Scheme 1. A few experiments were carried out to establish whether the course of the *M. luteus* catalyzed oxidation was influenced by the absolute configuration of the two stereocenters.

The optical rotation value of the methyl ester of (9*R*,10*R*)-1 was available in the literature,[12] and we developed an HPLC method to separate the four stereoisomers of the ester derivative **6** (Scheme 3) on a chiral stationary phase,[13] in order to calculate the enantiomeric and diastereoisomeric excess (ee and de) of diol samples.

The samples of diol *threo*-1 to be submitted to derivatization and chiral HPLC analysis were produced according to the following procedures:

(a) recovery (by column chromatography) of diol *threo*-1, left unreacted by the oxidation with *E. coli* resting cells producing recombinant *M. luteus* ADH, as described above (Scheme 4a);

(b) reduction of diketone **2** by the corresponding cell-free extract, using glucose as co-substrate and glucose dehydrogenase (GDH) as a NADH regeneration system (Scheme 4b).

The oxidation of (9*S*,10*S*)-1 is faster than that of the (9*R*,10*R*) enantiomer and when the reaction does not reach complete conversion, the unreacted diol is enriched in the (9*R*,10*R*) enantiomer (ee = 53%). If the reaction is carried out in reductive conditions starting from diketone **2**, complete enantioselectivity and high diastereoselectivity are observed, obtaining (9*S*,10*S*)-1 (ee = 99%, de = 93%).
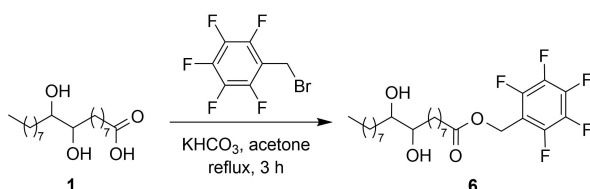
Scheme 3. Derivatization of diol **1** for HPLC analysis.
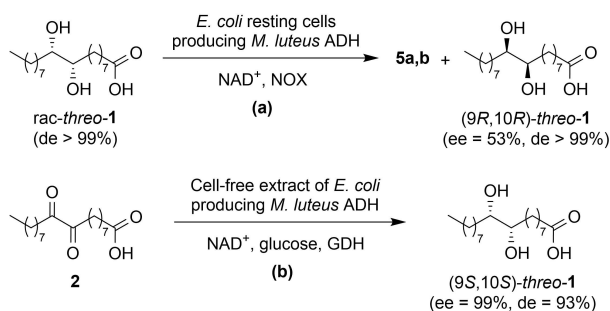
Scheme 4. Investigation of the stereochemical course of *M. luteus* ADH mediated reactions.

The high stereoselectivity observed in the reduction of diketone **2**, in spite of the structural similarity of the two aliphatic fragments linked to the carbonyl groups, paves the way for interesting synthetic applications of ADH from *M. luteus* in the synthesis of enantiomerically enriched vicinal *threo*-diols. These structural motifs are widespread in natural products, pheromones, and active pharmaceutical ingredients, and the availability of novel synthetic methods, especially for the aliphatic series, is greatly appreciated.[14]

Oxidative cleavage of hydroxyketones 5 a, b by NaClO 10% aq. solution

During our investigation on green methods for the oxidative cleavage of the oxygenated derivatives of unsaturated fatty acids, we considered the capability of bleach (NaClO 10% aq. solution) to perform this kind of reaction as described in 2007 by Khurana *et al.*[15] The authors employed 10–20 equiv. of NaClO (1.082 M aq. solution) in acetonitrile at room temperature to convert 1,2-diols, α-hydroxyketones and 1,2-diketones into the corresponding mixture of carboxylic acids. In our preliminary experiments, starting from a 25 mg/mL solution of diol **1**, the use of 10% NaClO aq. solution afforded only 55% oxidation (conversion by GC/MS) to pelargonic and azelaic acids over 3 days, and hydroxyketones **5 a**, **b** were observed among reaction products. Prompted by this result, we investigated the oxidation of the mixture of compounds **5 a**, **b** by aq. NaClO 10%. Complete conversion into pelargonic and azelaic acids was obtained only in a biphasic medium, using EtOAc as an organic co-solvent and Triton X-100 as a surfactant, after 18 h at 45 °C. The reaction work-up and the isolation of the final products were extremely simple and straightforward. Pelargonic acid could be isolated directly from the organic phase, which was immediately separated from the aqueous phase of the reaction when it was still warm. Azelaic acid, characterized by a higher water solubility, was recovered in high purity from the aqueous phase after treatment with aq. Na$_2$SO$_3$ and acidification. No column chromatography was required for the purification of compounds **3** and **4**.

The NaClO-promoted oxidative cleavage was performed directly on the reaction mixture obtained from *M. luteus* (cell-free extract) oxidation without isolation of derivatives **5**. A sequential one-pot conversion of *threo*-1 (50 mg) into acids **3** (isolation yield 76%, 85% purity by GC/MS analysis) and **4** (isolation yield 71%, 99% purity GC/MS analysis) was thus accomplished by a first step of oxidation with recombinant *M. luteus* ADH (cell-free extract, 30 °C, 30 h) and a further oxidative treatment after addition of NaClO 10%, EtOAc and Triton X-100 (45 °C, 18 h).

NaClO aq. solution is particularly advantageous for promoting the last oxidative step: it is inexpensive, easily available, and safe procedures for handling and storage are already common practice. Further studies on NaClO oxidation of diol *threo*-1 are in progress.

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Conclusion

A sequential one-pot oxidative procedure for the conversion of diol **1** into pelargonic and azelaic acids has been developed. Based on our previous work,[6] diol *threo*-**1** can be obtained in a one-pot process from oleic acid by lipase-mediated epoxidation in the presence of H_2O_2 and subsequent acid-catalyzed oxirane opening in 70–75% yields. An alternative path for further manipulating compound *threo*-**1** and achieving final cleavage to acids **3** and **4** is here presented. Diol *threo*-**1** can be effectively oxidized to hydroxyketones **5a,b** by the cell-free extract of *E. coli* BL21 (DE3) producing recombinant ADH from *M. luteus* NCTC2665. NOX is employed to recycle NAD^+ at the expense of atmospheric O_2, affording H_2O as an end-product.[16] Complete conversion can be obtained in 30 h, starting from 50 mg of diol in buffer solution with 1.6% v/v N-MMO as a cosolvent. The isolation of the corresponding oxidation products **5a,b** can be avoided, and final cleavage can be promoted by addition of NaClO 10% aq. solution directly to the reaction medium, together with EtOAc (15% v/v) and Triton X-100. Pelargonic and azelaic acids can be obtained as separated pure compounds in 76% and 71% isolated yields, respectively.

The overall procedure offers a number of advantages. H_2O_2 aq. solution, molecular O_2 and NaClO aq. solution are the stoichiometric oxidants employed in this sequence. Two steps are catalyzed by easily available biocatalysts: commercial Novozym 435 and recombinant ADH from *M. luteus*. The isolation and recovery of the reaction products throughout the sequence are carried out avoiding time-consuming and solvent-demanding column chromatography: (i) diol *threo*-**1** crystallizes from the reaction medium after epoxidation of oleic acid and oxirane hydrolysis; (ii) intermediates **5a,b** are submitted directly to the following step without isolation; (iii) the use of a biphasic medium for NaClO treatment enables the selective recovery of acids **3** and **4** from organic and aqueous phase, respectively.

The stereochemical course of ADH from *M. luteus* reduction and oxidation has been carefully investigated, highlighting the synthetic potential of the ADH in the reduction process of prochiral ketones showing similar aliphatic side chains. Additional work is in progress to use as a starting material a mixture of fatty acids, enriched in oleic acid, obtained by hydrolysis of soapstock from high-oleic sunflower oil refining process.

Experimental Section

General information

Chemicals and solvents were purchased from Merck (Merck Life Science S.r.l., Milan, Italy) and used without further purification. Trimethylsilyldiazomethane 10% solution in hexane (TCI Europe N.V.) was purchased from Zentek S.r.l. (Milan, Italy). TLC analyses were performed on Macherey-Nagel pre-coated TLC sheets Polygram® SIL G/UV$_{254}$ purchased from Chimikart S.r.l. (Naples,

Italy). All the chromatographic separations were carried out on a PuriFlash XS-420+ (Interchim) using Purezza-Daily Standard Flash cartridges (Sepachrom, Italy). ^1H and ^{13}C NMR spectra were recorded on a 400 MHz spectrometer (Bruker AV 400) in CDCl$_3$ solution at r.t. The chemical shift scale was based on internal tetramethylsilane. GC/MS analyses were performed using an HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies Italia Spa, Cernusco sul Naviglio, Italy). The following temperature program was employed: 50 °C/10 °C min^{-1}/250 °C (5 min)/ 50 °C min^{-1}/300 °C (10 min). The samples for GC/MS were treated with MeOH and trimethylsilyl diazomethane 10% in hexane, to derivatize carboxylic acids by transformation into the respective methyl esters. HPLC analyses were performed on a CHIRALART amylose-SA column (4.6 mm × 250 mm, 5 μm, YMC Europe), using the following conditions: hexane:*i*-PrOH 97:3, λ = 210 nm, 0.4 mL min^{-1}: *threo*-(9*R*,10*R*)-**6** t$_R$ = 86.5 min, *erythro*-**6** (1st enantiomer) t$_R$ = 95.3 min, *threo*-(9*S*,10*S*)-**6** t$_R$ = 99.4 min, *erythro*-**6** (2nd enantiomer) t$_R$ = 106.5 min.

Sources of microorganisms and enzymes

Bacillus benzoevorans DSM5391, *B. subtilis* DSM1088, *Comamonas testosteroni* DSM50244, *Dietzia* sp. DSM44016, *Gordonia* sp. DSM44456, *G. bronchialis* PCM2167, *Micrococcus* sp. DSM30771, *M. luteus* PCM525, *Pseudomonas fluorescens* PCM717, *Rhodococcus* sp. DSM364, *R. aetherivorans* DSM44541, *R. coprophilus* PCM2174, *R. erythropolis* PCM2150, *R. erythropolis* DSM44534, *R. rhodnii* PCM2157, *R. rhodochrous* PCM909, *R. ruber* PCM2166, *R. ruber* DSM7512, *Streptomyces griseus* PCM2331, *S. griseus* DSM40395 were obtained from the Polish Collection of Microorganisms (PCM) Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw and the German Collection of Microorganisms and Cell Cultures (DSMZ) in Braunschweig. Secondary ADH from *M. luteus* NCTC2665[17] with a His$_6$-tag, was overproduced in *Escherichia coli* BL21(DE3) strains. The expression construct pET-24(+)-ADH was purchased from Twist Bioscence (South San Francisco, California). NADH oxidase was purchased from Prozomix (Northumberland, UK). GDH (from *Bacillus megaterium* DSM509), fused with a His$_6$-tag, was overproduced in *Escherichia coli* BL21(DE3) strains. The preparation of expression constructs (pKTS-GDH) has been described previously.[18]

Screening oxidation with whole cells of bacteria

The bacterial strains were transferred from the agar slants to a sterilized 100 mL Erlenmeyer flask containing 25 mL of medium. Cultures of microorganisms were incubated aerobically on a rotary shaker (150 rpm) at 23–35 °C, depending on the strain. The following growth media for bacteria were used: TSB (tryptic soy broth) and PCM (g/L H_2O) consisting of 20 g glucose, 10 g peptone, 2 g casein hydrolysate, 2 g yeast extract, and 6 g NaCl. After 2–3 days of microorganism cultivation at 23–35 °C, 10 mg of substrate dissolved in 0.2 mL of EtOH was added to the shaken cultures. To check the progress of the biotransformation, samples (7 mL) of the reaction mixtures were taken at determined intervals (1, 2, 3 days). To the aqueous phase, 2 ml of saturated NaCl was added and the resulting mixture was extracted with ethyl acetate (3 mL). Then, the samples were centrifuged (7000 *g*, 10 min) and the organic layer was dried (Na$_2$SO$_4$), filtered, derivatized with MeOH and trimethylsilyl diazomethane 10% in hexane and analyzed by GC/MS.

Preparation of the resting cells of *M. luteus* PCM525

A 100 mL Erlenmeyer flask containing sterile TSB medium (30 mL) was inoculated with *M. luteus* PCM525 and incubated in an orbital shaker (150 rpm, 28 °C) for 1 day. The content of the pre-culture flask was aseptically poured into a final volume of 300 mL in a 1 L flask and the culture was grown for the next 2 days in the same conditions. The cells were centrifuged (7000 *g*, 20 min, 4 °C) and liquid media were discarded, then the cells were washed twice with Tris-HCl buffer (100 mM, pH 8.5–9.0). A final mass of 3.2/3.5 g of wet cells was obtained.

Oxidation of *threo*-9,10-dihydroxystearic acid (1) with *M. luteus* PCM525 resting cells

Portions of cells (0.6 g), prepared as described in the previous paragraph, were resuspended in Tris-HCl buffer (30 mL, 100 mM, pH 9.0) and a solution of substrate **1** (50 mg, 0.16 mmol) in EtOH (1 mL) was added. The reactions were conducted in an orbital shaker (147 rpm) at 28 °C and monitored by TLC. After 3 days an extraction with EtOAc (15 mL) was performed. The organic phase was dried (Na$_2$SO$_4$), concentrated under reduced pressure and submitted to ^{1}H NMR analysis.

Preparation of the resting cells of *E. coli* BL21(DE3) producing ADH from *M. luteus* NCTC2665

A 100 mL Erlenmeyer flask containing sterile LB medium (30 mL) supplemented with kanamycin (30 μg mL^{-1}) was inoculated with a single colony from a fresh plate of *E. coli* BL21(DE3) harboring pET-24(+)-ADH and grown overnight at 37 °C and 220 rpm. This starting culture was used to inoculate 1 L of LB medium, which was incubated at 37 °C and 220 rpm until OD$_{600}$ reached 0.5–0.6, then enzyme expression was induced by the addition of IPTG (0.125 mM final concentration). After 5 h, the cells were harvested by centrifugation (7000 *g*, 20 min, 4 °C), and the liquid media were discarded. The cells were washed twice with Tris-HCl buffer (100 mM, pH 8.5/9). A final mass of 3.8/4 g of wet cells was obtained.

General procedure for the oxidation of *threo*-9,10-dihydroxystearic acid (1) with resting cells of *E. coli* BL21(DE3) producing ADH from *M. luteus* NCTC2665 (preparative scale)

Portions of cells (0.6 g), prepared as described in the previous paragraph, were resuspended in Tris-HCl buffer (30 mL, 100 mM, pH 9.0) and a solution of substrate **1** (50 mg, 0.16 mmol) in EtOH (1 mL) was added. Reactions were conducted in an orbital shaker (147 rpm) at 30 °C and monitored by TLC. After 3 days an extraction with EtOAc (15 mL) was performed. The organic phase was dried (Na$_2$SO$_4$), concentrated under reduced pressure and submitted to ^{1}H NMR analysis.

The crude mixture, obtained according to this procedure on 100 mg of *threo*-**1** and containing 50% (molar percentage from ^{1}H NMR analysis) of unreacted starting diol, was purified by column chromatography, eluting with hexane–EtOAc mixtures with an increasing amount of the more polar solvent, to recover unreacted (9*R*,10*R*)-*threo*-9,10-dihydroxystearic acid ((9*R*,10*R*)-**1**) (42.5 mg, 42%, ee = 53% and de > 99% by HPLC analysis on a chiral stationary phase of the corresponding ester derivative **6**). Spectroscopic data of *threo*-**1**: ^{1}H NMR (400 MHz, CD$_3$OD, ppm)[19] δ 3.45–3.35 (2H, m, 2*CH*OH), 2.29 (2H, t, *J* = 7.4 Hz, *CH$_2$*COOH), 1.70–1.15 (26H, m, 13 *CH$_2$*), 0.97–0.82 (3H, m, *CH$_3$*). ^{13}C NMR (100.6 MHz,

DMSO-*d$_6$*, ppm):[20] δ 174.4, 73.14, 73.13, 33.7, 32.4, 31.3, 29.3, 29.2, 29.1, 28.8, 28.7, 28.6, 25.64, 25.61, 24.5, 22.1, 13.9. GC/MS (EI) after esterification by treatment with MeOH and trimethylsilyl diazomethane 10% in hexane, and acetylation of OH groups with Ac$_2$O and pyridine, t$_r$ = 23.96 min: m/z (%) = 383 (M$^+$−31, 5), 229 (50), 187 (100), 155 (76).

Preparation of cell-free extract of *E. coli* BL21(DE3) expressing ADH from *M. luteus* NCTC2665 and purification of the recombinant enzyme

Cells collected as described in the previous paragraph were resuspended in 30 mL of lysis buffer (20 mM potassium phosphate buffer pH 7.0, 300 mM NaCl, 10 mM imidazole), disrupted by sonication (Omni Ruptor 250 ultrasonic homogenizer, five sonication cycles, 15 s each, 50% duty) and centrifuged (20000 *g*, 20 min, 4 °C) to obtain the cell-free crude lysate.

Further purification of his-tagged ADH from *M. luteus* NCTC2665 was performed by affinity chromatography on IMAC stationary phase (Ni-Sepharose Fast Flow, GE Healthcare) with a mobile phase composed of 20 mM potassium phosphate buffer pH 7.0, 300 mM NaCl and a 10–300 mM imidazole gradient. Protein elution was monitored at 280 nm, the fractions were collected according to the chromatogram, stored frozen at −80 °C after adding 10% v/v of glycerol.

General procedure for the oxidation of *threo*-9,10-dihydroxystearic acid (1) with cell-free extract or purified ADH from *M. luteus* NCTC2665 (analytical scale)

A solution of the substrate (5 μmol) in the selected cosolvent (50 μL) was added to a Tris-HCl buffer solution (100 mM, pH 9.0) containing NAD$^+$ (0.5 μmol), NOX (300 μg) and the required cell-free extract (500 μL) or purified ADH (150 μg) with a final reaction volume of 1500 μL. The mixture was incubated for 24 h in an orbital shaker (150 rpm, 30 °C). The solution was extracted with EtOAc (2 × 250 μL), centrifuged after each extraction (15000 *g*, 1.5 min), and the combined organic solutions were dried over anhydrous Na$_2$SO$_4$. Two replicates were performed for each biotransformation: no significant differences (< 5%) were observed for conversion values.

General procedure for the oxidation of *threo*-9,10-dihydroxystearic acid (1) with cell-free extract of *E. coli* BL21(DE3) producing ADH from *M. luteus* NCTC2665 (preparative scale)

A solution of diol *threo*-**1** (50 mg, 0.16 mmol) in N-MMO (800 μL) was added to a Tris-HCl buffer solution (100 mM, pH 9.0) containing NAD$^+$ (10 mg), and NOX (5 mg) and cell-free extract (15 mL) to reach a total volume of 50 mL. The reaction was monitored by TLC until complete conversion (generally 30 h). The mixture was extracted with EtOAc (3 × 3 mL), dried (Na$_2$SO$_4$), concentrated under reduced pressure, to obtain a mixture of 10-hydroxy-9-oxostearic acid (**5a**) and 9-hydroxy-10-oxostearic acid (**5b**) (40.2 mg, 81%): ^{1}H NMR (400 MHz, CDCl$_3$, ppm, the signals of the two regioisomers are overlapped) δ = 4.20–4.12 (1H, m, *CH*OH), 2.54–2.28 (4H, m, *CH$_2$*CO and *CH$_2$*COOH), 1.90–1.15 (24H, m, 12 *CH$_2$*), 0.88 (3H, d with *J* = 6.6 Hz, *CH$_3$*). ^{13}C NMR (100.6 MHz, CDCl$_3$, ppm): δ = 212.7, 212.6, 179.54, 179.49, 76.6, 76.5, 38.0, 37.9, 34.2, 34.1, 33.9, 33.8, 32.0, 31.9, 29.60, 29.56, 29.44, 29.38, 29.36, 29.3, 29.23, 29.19, 29.15, 29.11, 29.08, 29.0, 25.0, 24.9, 24.79, 24.75, 23.8, 23.7, 22.78, 22.77, 14.22, 14.21. GC/MS (EI) after esterification by treatment with MeOH and trimethylsilyl diazomethane 10% in hexane, and acetylation of the OH group with

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Ac$_2$O and pyridine, t$_r$ = 22.76 min: m/z (%) = 328 (M$^+$−42, 5), 297 (13), 185 (100).

Reduction of diketone 2 with cell-free extract of *E. coli* BL21 (DE3) expressing ADH from *M. luteus* NCTC2665

A solution of diketone **2** (100 mg, 0.32 mmol) in *i*-PrOH (1 mL) was added to a Tris-HCl buffer solution (100 mM, pH 9.0) containing glucose (4 eq), GDH (5 mg), NADH (10 mg) and cell-free extract (8 mL) to reach a total volume of 18 mL. The reaction was monitored by TLC until complete conversion (24 h). The mixture was then extracted with EtOAc (3×3 mL), dried (Na$_2$SO$_4$), concentrated under reduced pressure, to obtain a crude mixture that was purified by column chromatography, eluting with hexane and increasing amount of EtOAc to recover a pure sample of (9*S*,10*S*)-*threo*-9,10-dihydroxystearic acid ((9*S*,10*S*)-**1**) (88 mg, 87%, ee = 99% and de = 93% by HPLC analysis on a chiral stationary phase of the corresponding ester derivative **6**. ^{1}H and ^{13}C NMR spectra were in agreement with those described for (9*R*,10*R*)-**1**. The diol was converted into the corresponding methyl ester by treatment with CH$_2$N$_2$ in Et$_2$O solution for the determination of optical rotatory power: $[\alpha]_D = -21.5$ (*c* 1.5, MeOH) [lit. ref [12] for (9*R*,10*R*)-**1** as methyl ester $[\alpha]_D = 22.5$ (*c* 1.2, MeOH)); ^{1}H NMR (400 MHz, CDCl$_3$, ppm) δ = 3.66 (3H, s, COO*CH$_3$*), 3.45–3.35 (2H, m, 2*CH*OH), 2.29 (2H, t with *J* = 7.5 Hz, *CH$_2$*COOMe), 1.71–1.15 (26H, m, 13 CH$_2$), 0.97–0.80 (3H, m, *CH$_3$*). ^{13}C NMR (100.6 MHz, CDCl$_3$, ppm): δ = 174.3, 74.7, 74.6, 51.5, 34.2, 33.9, 33.8, 32.0, 29.8, 29.7, 29.6, 29.4, 29.3, 29.2, 25.8, 25.7, 25.1, 22.8, 14.2.

Derivatization of diol 1 for HPLC analysis: synthesis of the corresponding pentafluorobenzyl ester 6[13]

To a solution of diol *threo*-**1** (100 mg, 0.32 mmol) in acetone (52 mL), pentafluorobenzyl bromide (125 mg, 0.48 mmol) and KHCO$_3$ (35 mg, 0.35 mmol) were added. The mixture was refluxed for 3 h, then diluted with Et$_2$O (260 mL) and EtOAc (10 mL) and washed with water. The organic phase was separated, dried (Na$_2$SO$_4$), and concentrated under reduced pressure. ^{1}H NMR (400 MHz, CDCl$_3$, ppm) δ = 5.19 (2H, s, *CH$_2$*O—CO), 3.45–3.35 (2H, m, 2*CH*OH), 2.32 (2H, t with *J* = 7.4 Hz, *CH$_2$*COO), 1.80–1.2 (26H, m, 13 CH$_2$), 0.91–0.85 (3H, m, *CH$_3$*). ^{13}C NMR (100.6 MHz, CDCl$_3$, ppm): δ = 173.2, 145.8 (dm, *J*$^1_{C-F}$ = 255 Hz), 141.9 (dm, *J*$^1_{C-F}$ = 256 Hz), 137.6 (dm, *J*$^1_{C-F}$ = 253 Hz), 109.8 (m), 74.7, 74.6, 34.0, 33.8, 33.7, 32.0, 29.8, 29.7, 29.6, 29.4, 29.2, 29.1, 25.8, 25.7, 24.9, 22.8, 14.2. GC/MS (EI) t$_r$ = 28.7 min: m/z (%) = 353 (M$^+$−143, 15), 181 (100), 155 (85), 109 (20).

General procedure for the oxidative cleavage of hydroxyketones 5 a, b with NaClO solution

A mixture of NaClO 10% (7.5 mL), EtOAc (10 mL) and Triton X-100 (50 μg) was added to the reaction mixture containing hydroxyketones **5 a, b**, after complete enzymatic oxidation with cell-free extract of *E. coli* BL21(DE3) producing ADH from *M. luteus* NCTC2665 (according to already described general procedure) of 50 mg of diol **1**. The mixture was shaken at 150 rpm at 45 °C in an orbital shaker. Oxidative cleavage was monitored by TLC and complete formation of pelargonic and azelaic acid generally occurred in 18 h. The still warm organic phase was separated, cooled to r.t. washed first with aq. Na$_2$SO$_3$, then with water, dried (Na$_2$SO$_4$), and concentrated under reduced pressure to afford pelargonic acid (19.0 mg, 76%). The aqueous phase was treated with aq. Na$_2$SO$_3$, acidified with HCl 10% and extracted with EtOAc. The reunited organic phases were dried

(Na$_2$SO$_4$), and concentrated under reduced pressure, to obtain azelaic acid (21.1 mg, 71%).

Data of pelargonic acid (**3**): ^{1}H NMR (CDCl$_3$, 400 MHz, ppm):[21] δ = 9.81 (1H, s, COOH), 2.37 (2H, t with *J* = 7.5 Hz, *CH$_2$*COOH), 1.75–1.2 (12H, m, 6CH$_2$), 0.80–0.95 (3H, m, CH$_3$). ^{13}C NMR (CDCl$_3$, 100.6 MHz, ppm):[21] δ = 180.3, 34.2, 31.9, 29.3, 29.23, 29.21, 24.8, 22.8, 14.2. GC/MS (EI) as a methyl ester, obtained by treatment with MeOH and trimethylsilyl diazomethane 10% in hexane, t$_r$ = 9.33 min: m/z (%) = 172 (M$^+$, 0.5), 141 (15), 129 (18), 87 (45), 74 (100).

Data of azelaic acid (**4**): ^{1}H NMR (CD$_3$OD, 400 MHz, ppm):[22] δ = 2.28 (4H, t with *J* = 7.4 Hz, 2CH$_2$COOH), 1.75–1.55 (4H, m, 2CH$_2$), 1.4–1.2 (6H, m, 3CH$_2$). ^{13}C NMR (CD$_3$OD, 100.6 MHz, ppm):[22] δ = 177.5, 34.9, 29.97, 29.93, 26.0. GC/MS (EI) as a methyl ester, obtained by treatment with MeOH and trimethylsilyl diazomethane 10% in hexane, t$_r$ = 13.9 min: m/z (%) = 185 (M$^+$, −31, 55), 152 (100), 143 (47), 111 (63).

Conflict of Interest

The authors declare no conflict of interest.

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