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Production of chiral hydroxy long chain fatty acids by whole cell biocatalysis of pentadecanoic acid with an *E. coli* recombinant containing cytochrome $P450_{BM-3}$ monooxygenase

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Abstract

A multi-step product recovery process for 12-, 13- and 14-hydroxypentadecanoic acids was developed. Biotransformation of pentadecanoic acid by whole cells of recombinant *E. coli* K27 (pCYP102, pGEc47), synthesizing cytochrome $P450_{BM-3}$ monooxygenase [EC 1.14.14.1], led to the production of 12-, 13- and 14-hydroxypentadecanoic acids. To prevent further oxidation of these products, the conversion was performed under oxygen-limited conditions. After filtration and hexane extraction of culture supernatants, the hydroxy fatty acids were methylated. The purification process allowed us to separate the mixture of 12-, 13- and 14-hydroxypentadecanoic acid methyl esters into their single regioisomers by reversed-phase HPLC, followed by precipitation of the compounds as white crystals at 4°C and −20°C. The purified hydroxymethyl esters were characterized by GC–MS analysis.

Chiral HPLC analysis of the ω-1 product, 14-hydroxypentadecanoic acid methyl ester, indicated that pentadecanoic acid is oxidized by cytochrome $P450_{BM-3}$ to optically pure (*S*)-(+)-14-hydroxypentadecanoic acid at an e.e. of over 95%. This result confirms recent observations by Capdevila[1] that oxidation by cytochrome $P450_{BM-3}$ monooxygenase is highly stereoselective.

1. Introduction

Saturated long chain fatty acids are regioselectively oxidized at the ω-1, ω-2 and ω-3 position of the alkyl chain by a unique cytochrome P450-dependent fatty acid monooxygenase from *Bacillus megaterium* ATCC 14581, cytochrome $P450_{BM-3}$.[2] The enzyme is a soluble, one component monooxygenase that carries FAD, FMN and a heme domain in a single polypeptide with a molecular weight of 119000 daltons. In the presence of NADPH and O_2, it can catalyze the oxygenation of long chain

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alkanes without the aid of any other protein.[3,4] The enzyme has a turnover number of 4600 nmol of pentadecanoic acid oxygenated per nmol of $P450_{BM-3}$ min^{-1}, the highest activity reported for a P450-dependent monooxygenase thus far.[5]

A growing demand for single stereoisomer chiral pharmaceuticals or fine chemicals has led to an increase in the production of chiral synthons by microbial biocatalysts. These optically active synthons have served as starting materials for the synthesis of a wide variety of natural products and biologically active compounds.[6–8] The asymmetric center that exists in ω-1, ω-2 and ω-3 hydroxy fatty acids raises the question of their stereochemistry and enantiomeric composition. Investigations on the oxidation of unsaturated long chain fatty acids by cytochrome $P450_{BM-3}$ monooxygenase have shown that these reactions occur with high enantioselectivity: oxidation of eicosapentenoic acid ($C_{20:5}$) and arachidonic acid ($C_{20:4}$) yielded 17-(*S*),18-(*R*)-epoxyeicosatetraenoic acid (94% e.e.) for the former and a mixture of 18-(*R*)-hydroxyarachidonic acid (92% e.e.) and 14-(*S*),15-(*R*)-epoxyeicosatrienoic acid at 98% e.e. for the latter substrate.[1]

However, there has been hitherto no determination of the extent to which cytochrome $P450_{BM-3}$ catalyzed hydroxylation of long chain saturated fatty acids is enantioselective, nor is it clear that such reactions can be carried out with whole cells containing cytochrome $P450_{BM-3}$ monooxygenase as well as with the isolated enzyme. The production of chiral 12-, 13- and 14-hydroxypentadecanoic acids on a preparative scale by biotransformations with whole cells could lead to various applications of these compounds as fine chemicals, pharmaceutical synthons or precursors in polymer chemistry. Hydroxycarboxylic acids can serve as precursors for polymers or cyclic lactones, which are used in fragrances and antibiotics. Since this class of compounds and their isomers of different chain lengths from C_{12}–C_{18} are not commercially available, such applications have never been explored. Although it is possible to synthesize hydroxy fatty acids by chemical means, the procedures are complicated and extensive purification is needed,[9] and chemical synthesis does not yet provide a feasible route to the production of these ω-1, ω-2 and ω-3 hydroxy long chain fatty acid metabolites. Thus far, only two synthetic reactions are known for the formation of subterminal oxidized pentadecanoic acid derivatives: starting either with 14-hydroxypentadeca-10,12-diynoic acid or (*R*)-(−)-14-(2,4,10-trioxatricyclo-(3.3.1.$1^{3,7}$)dec-3-yl)-2-tetradecanol as reactants, 14-hydroxypentadecanoic acid and (*R*)-(−)-14-hydroxypentadecanoic acid, respectively, have been prepared.[10,11] These are rather complex synthetic routes with low yields and expensive starting compounds. Moreover, the formation of the (*S*)-(+)-enantiomer has not yet been described.

In this work we describe a biocatalytic process to 12-, 13- and 14-hydroxypentadecanoic acids based on the oxidation of pentadecanoic acid by a recombinant strain *E. coli* K27 (pCYP102, pGEc47). The host strain is unable to oxidize either substrates or products, because it lacks acyl-CoA synthetase, the first enzyme in the β-oxidation pathway,[12] and there are therefore no undesirable chain modifications associated with the β-oxidation pathway. The recombinant expresses the cyp102 gene, encoding for cytochrome $P450_{BM-3}$ monooxygenase. It also expresses genes on pGEc47, which facilitates efficient substrate uptake.[13] The use of a whole cell system containing the monooxygenase ensured maintenance or continued synthesis of the biocatalyst and regeneration of the cofactor NADPH during the bioconversion. Oxidation of pentadecanoic acid catalyzed by whole cells of *E. coli* K27 (pCYP102, pGEc47) was performed under oxygen-limited conditions to produce 12-, 13- and 14-hydroxypentadecanoic acids. This is important, since we and others have shown that excess oxygen leads to multiple oxidation of substrate and products, yielding ketones and hydroxyketones as side-products.[14,15]

Below, we report on the development of a method for the biosynthesis and purification of single regioisomers of 12-, 13- and 14-hydroxypentadecanoic acids. We show further, that oxidation with cytochrome

$P450_{BM-3}$ monooxygenase is stereospecific and yields optically active (*S*)-(+)-14-hydroxypentadecanoic acid in an enantiomeric excess of better than 95%.

2. Experimental section

2.1. Chemicals

Pentadecanoic acid was obtained from Sigma (Buchs, Switzerland). Solvents for product purification and HPLC, GC, GC–MS, ^{1}H NMR and ^{13}C NMR measurements were purchased from Biosolve (Zurich, Switzerland). All other chemicals were from Fluka AG (Buchs, Switzerland).

2.2. Chemical racemization of (R)-(−)-14-hydroxypentadecanoic acid methyl ester to rac-14-hydroxypentadecanoic acid methyl ester

(*R*)-(−)-14-Hydroxypentadecanoic acid methyl ester was kindly provided by Dr. Gerlach from the Department of Organic Chemistry of the University Bayreuth, Germany.

To 20 mg of (*R*)-(−)-14-hydroxypentadecanoic acid methyl ester, 9.7 mg $Na_2Cr_2O_7$, 1 ml CH_3CO_2H and 0.3 ml benzene were added and the solution was refluxed at 60°C until a dark-brown colour was observed. Subsequently, the solution was heated to 70°C and turned green after 30 min. 14-Oxopentadecanoic acid methyl ester was distilled and saturated with NaCl; the extraction with ether was repeated twice and the extract was dried over Na_2SO_4. The filtrate was used for the reduction of the ketone to the 14-hydroxypentadecanoic acid methyl ester. A quantity (1.5 ml) of EtOH and $NaBH_4$ (approx. 10 g) were mixed for 3 h at room temperature. After ether extraction and concentration under vacuum, analysis by TLC, GC–MS and ^{1}H NMR and ^{13}C NMR spectroscopy confirmed the formation of 14-hydroxypentadecanoic acid methyl ester.[16] HPLC analysis utilizing a chiral column confirmed that this product was racemic.

2.3. Bioconversion of pentadecanoic acid

Biotransformation of pentadecanoic acid by *E. coli* K27 (pCYP102, pGEc47), a recombinant which synthesizes cytochrome $P450_{BM-3}$ monooxygenase, was performed in a 3 l bioreactor using 2 l M9 minimal medium as described elsewhere.[14] The conversion process was performed under a constant limited oxygen supply of 5% DOT. The biotransformation was stopped after 6 h, the broth was harvested and used for product recovery. A 1 ml sample of the 6 h broth was used for substrate and product analysis by GC–MS.

2.4. Methylation of fatty acid metabolites

Prior to GC, GC–MS and HPLC analysis, bioconversion samples were acidified with HCl (pH 2), methylated with diazomethane and extracted with hexane. The resulting methyl esters in hexane were dried over sodium sulfate.

2.5. Reversed-phase HPLC

Elution of 12-, 13- and 14-hydroxypentadecanoic acid methyl esters and their separation in single fractions was achieved by reversed-phase HPLC (Hewlett Packard HP1050 Ti) over a CC 250/4 Nucleosil 100-5 C_{18} column (Machery & Nagel AG, Switzerland), applying an acetonitrile/water (40/60 v/v) gradient over 30 min to 100% acetonitrile at a flow rate of 1 ml min^{-1}. The three methyl ester products were detected at 235 nm. Fractions of each hydroxy methyl ester isomer were collected from multiple HPLC runs, concentrated by evaporation of the solvent (rotary-evaporator) and used for further analysis.

2.6. Gas chromatography

A quantity (1 ml) of the hexane solution, containing the hydroxymethyl esters, was analyzed on a capillary gas chromatograph (Fisons instruments, HRGC MEGA2 series) using a 25 m CP-Sil 5CB column (Chrompack, Middelburg, The Netherlands). The temperature program used was: 80°C for 2 min, a temperature gradient of 8°C/min to 240°C and isothermic at 240°C for 10 min. Nonanoic acid (2.5 mM) was added to the assay mixture before acidification from a 100 mM stock solution in DMSO to produce the corresponding methyl ester, which served as an internal standard. Amounts of products were determined by comparison with the internal standard and corrected for differences in FID detector response.[17]

2.7. GC–MS analysis

The presence of products and the purity of the single hydroxy isomers eluted from reversed-phase HPLC were determined by GC–MS. Methyl esters of cytochrome $P450_{BM-3}$ oxidation products were identified by GC–MS using ionization by electron impact (EI) on a Fisons gas chromatograph GC800 coupled to a Fisons MD800 mass selective detector. The EI-mass spectra were recorded at 70 eV and 250°C (H_2 carrier gas, flow 1 ml min^{-1}). For GC–MS analysis, the cytochrome $P450_{BM-3}$ oxidation products were methylated as described above and the GC column and separation conditions were identical. Authentic standards for all products were not available and fragment distribution was therefore used to determine the absolute configuration of the ω-1, ω-2 and ω-3 hydroxy compounds produced by the bioconversion reaction.[18]

2.8. HPLC analysis by a chiral stationary phase

High pressure liquid chromatography utilizing a chiral stationary phase (CSP) was used for the enantiospecific determination of 14-hydroxypentadecanoic acid methyl ester at 235 nm. A Chiralcel OD column (Daicel Europe, Düsseldorf, Germany) eluted with a mobile phase of hexane/2-propanol (99/1 v/v) at a flow rate of 1 ml min^{-1} was used.

2.9. ^1H and ^{13}C NMR

Proton (^1H) and carbon (^{13}C) nuclear magnetic resonance spectra (NMR) were recorded on a Varian Gemini-200 at 200 and 50 MHz respectively, in deuterated chloroform with tetramethylsilane (TMS) as the internal reference. Chemical shifts are reported in δ-values (TMS, δ=0 ppm); the coupling-constants J are in hertz.

3. Results

3.1. Product recovery

The products formed from pentadecanoic acid oxidation by *E. coli* cells K27 (pCYP102, pGEc47), 12-, 13- and 14-hydroxypentadecanoic acids,[14] were isolated from the fermentation supernatant and purified (Fig. 1). Since 12-, 13- and 14-hydroxypentadecanoic acids are not commercially available, GC and GC–MS were used routinely as tools during all steps of the recovery process to follow quality and quantity of the single hydroxy isomers (Figs 2 and 3). After separation of the cells from the fermentation liquid by centrifugation (5 kg, 4°C, 30 min) and filtration to remove medium impurities (Whatman 3 mm, Ø 15 cm), HCl was added to the fermentation supernatant, followed by hexane in a 1:1 ratio for extraction of the protonated hydroxy fatty acids into the organic phase. This step was repeated three times. The extract was dried over Na_2SO_4, filtered and concentrated under vacuum by solvent evaporation (rotary-evaporator, 60°C). This solution contained a mixture of 12-, 13- and 14-hydroxypentadecanoic acids. The addition of diazomethane led to the formation of 12-, 13- and 14-hydroxypentadecanoic acid methyl esters, as confirmed by GC–MS (Fig. 2A), which were separated by reversed-phase HPLC, as described below (Fig. 2B–D). Single fractions containing either 12-, 13- or 14-hydroxypentadecanoic acids were collected and used for solvent evaporation under vacuum. Hexane was added to the concentrated products and such solutions were used for GC and GC–MS analysis. The products precipitated as white crystals in the remaining solvent at temperatures between 4°C and −20°C.

3.2. Reversed-phase HPLC

The methyl esters of the three monohydroxylated isomeric products obtained after hexane extraction and methylation by diazomethane were eluted by reversed-phase (RP) HPLC from a C_{18} column with 60–70% acetonitrile in water. They appeared at a retention time (t_R) of 10 min (14-hydroxypentadecanoic acid methyl ester), at t_R of 11 min (13-hydroxypentadecanoic acid methyl ester) and at t_R of 11.5 min (12-hydroxypentadecanoic acid methyl ester), respectively (Fig. 2).

The isolated products were characterized by GC–MS (Fig. 3). The total ion current (TIC) chromatogram indicated that both 14- and 12-hydroxypentadecanoic acids were pure. The fraction collected for 13-hydroxypentadecanoic acid methyl ester contained 5–10% of the 12-hydroxy isomer (Fig. 2C).

RP-HPLC was repeated 10 times and fractions containing the individual products were pooled. The three hydroxy products were used for analysis by GC–MS and products could be identified as 12-, 13- and 14-hydroxypentadecanoic acid methyl esters. As shown in Fig. 3, the most relevant mass peaks differed by multiples of 14 units (m/z 200, 214, 228, which correspond to $[M-C_4H_7OH]$, $[M-C_3H_5OH]$ and $[M-C_2H_3OH]$ and 229, 243, 257, which correspond to $[M-C_3H_7]$, $[M-C_2H_5]$ and $[M-CH_3]$ respectively). The cleavage pattern confirmed the successful isolation of 12-, 13- and 14-hydroxypentadecanoic acid methyl esters, respectively, as described elsewhere.[14]

3.3. Enantiomeric distribution

Product isomers were concentrated by evaporation of acetonitrile in a rotary evaporator and were resuspended in hexane. The fraction containing 14-hydroxypentadecanoic acid methyl ester (0.3 mg) was analyzed by HPLC using a chiral stationary phase. Only one standard, (*R*)-(−)-14-hydroxypentadecanoic acid methyl ester, was available for the determination of the enantiomeric distribution of the ω-1-hydroxy product from the bioconversion of pentadecanoic acid. A quantity (20 mg) of this standard

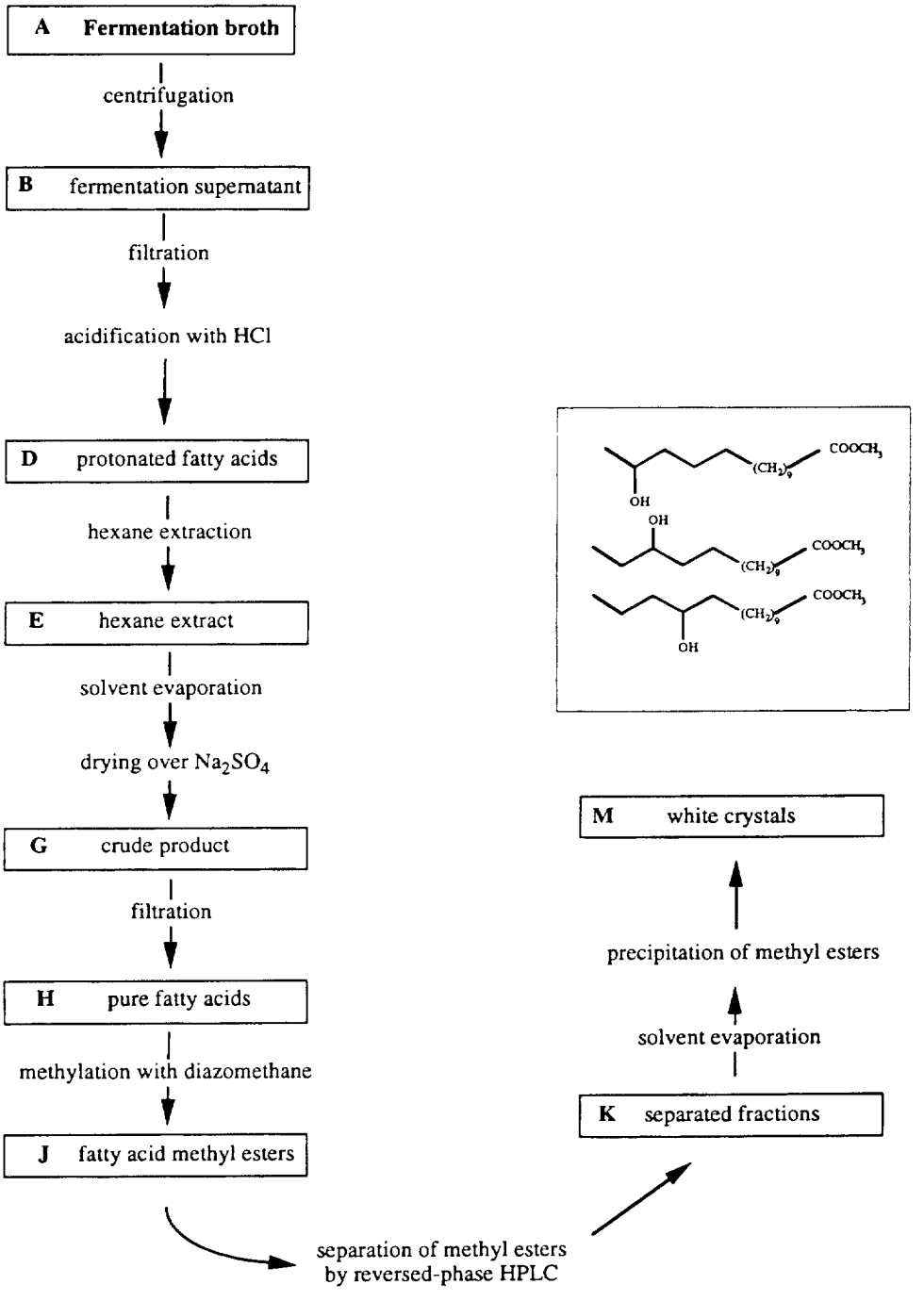


Fig. 1. Product recovery of 12-, 13- and 14-hydroxypentadecanoic acids as methylesters after bioconversion of $C_{15:0}$ by cytochrome $P450_{BM-3}$. The fermentation broth from a 2 l oxygen-limited bioconversion of pentadecanoic acid by *E. coli* K27 (pCYP102, pGEc47), containing 12-, 13- and 14-hydroxypentadecanoic acids, was processed as indicated. The isolated hydroxy fatty acid products precipitated as white crystals between 4°C and −20°C. The purity of the fractions was determined by GC–MS analysis

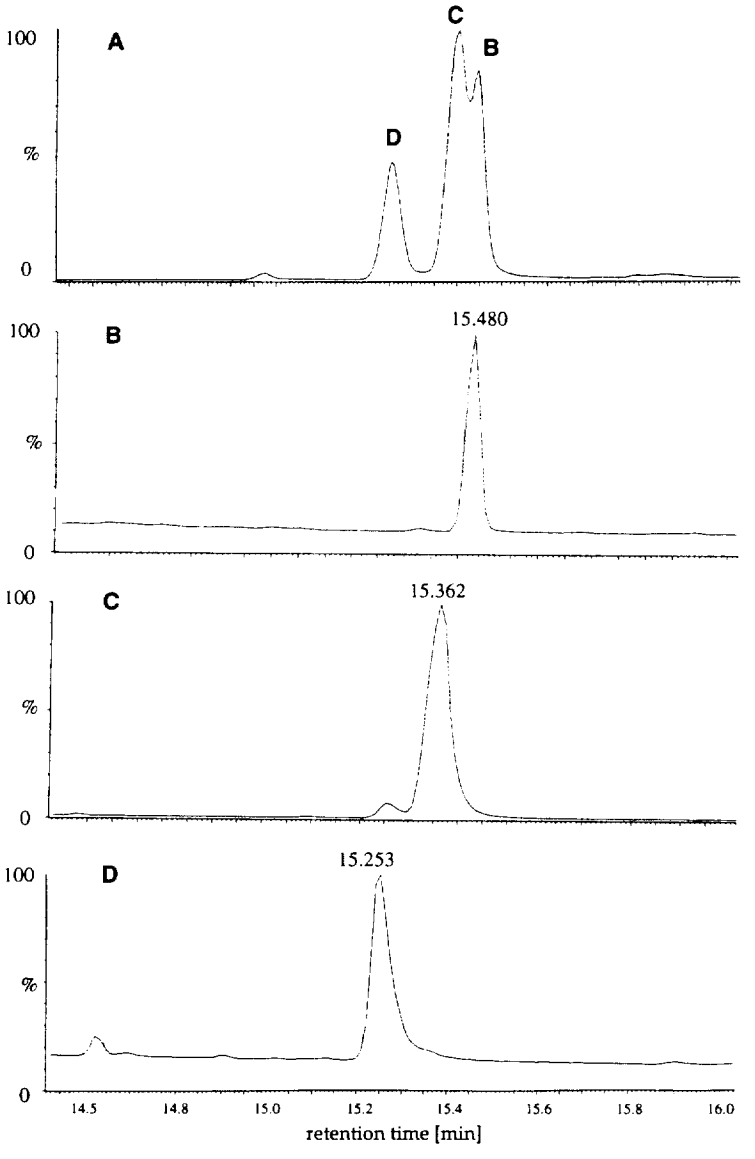


Fig. 2. Total ion current (TIC) chromatogram of the oxidation products isolated by RP-HPLC. Panel A: mixture of 12-, 13- and 14-hydroxypentadecanoic acid methyl esters (Fig. 1, fraction J). Panel B, 14-hydroxy-; panel C, 13-hydroxy- and panel D, 12-hydroxypentadecanoic acid methyl esters collected as single fractions from RP-HPLC (Fig. 1, fraction K). The retention time of the isolated product is shown above the peaks

was racemized to its (*S*)-(+)- and (*R*)-(−)- enantiomers by oxidation with $Na_2Cr_2O_7$ and reduction of 14-oxopentadecanoic acid methyl ester by $NaBH_4$ (Fig. 4) to serve as a racemic reference compound. The chemically synthesized product *rac*-14-hydroxypentadecanoic acid methyl ester was analyzed by TLC, GC–MS, ^1H NMR and ^{13}C NMR spectroscopy. By TLC, the product was detected at the same R_f as the starting material (data not shown). The ketone intermediate, which appeared as a separate spot on TLC during the racemization, was completely converted. GC–MS analysis confirmed identical mass spectra of (*R*)-(−)-14-hydroxypentadecanoic acid methyl ester and *rac*-14-hydroxypentadecanoic acid methyl ester (Fig. 5).

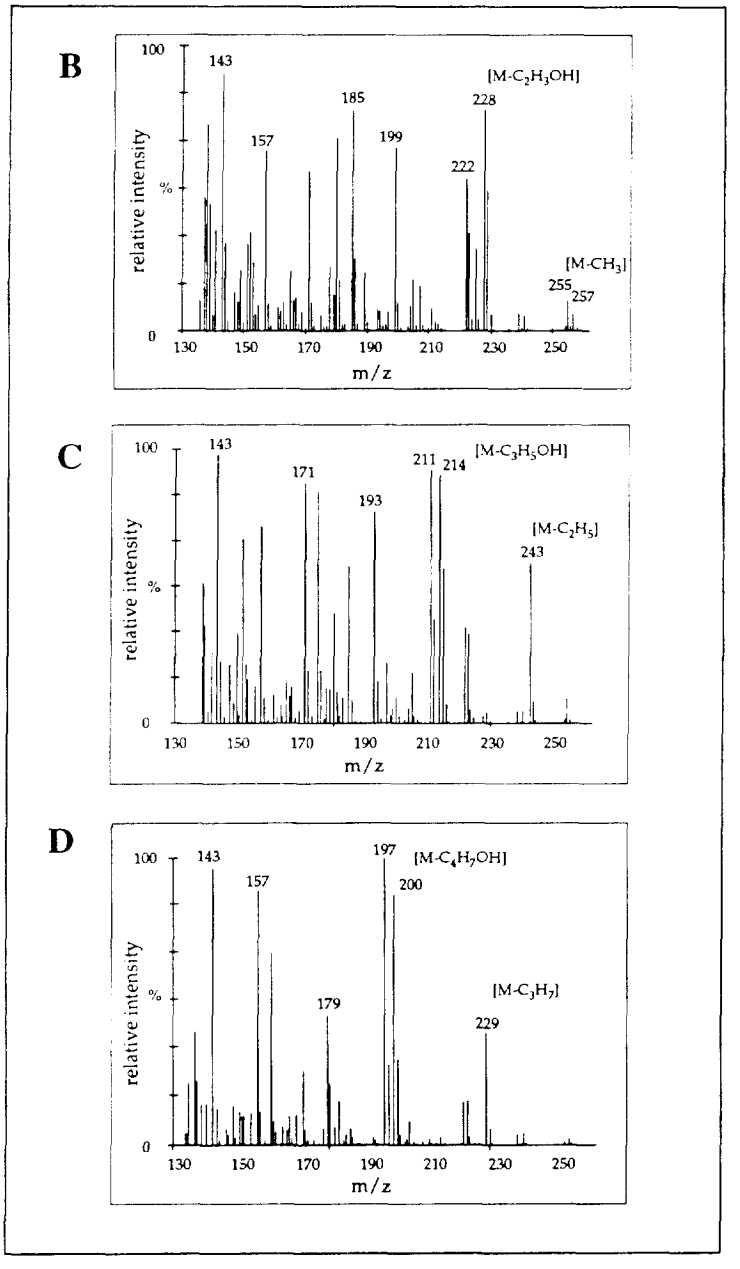


Fig. 3. Mass spectra of hydroxypentadecanoic acid methyl esters. Products B–D, separated by RP-HPLC (Fig. 2), were identified as 14-, 13- and 12-hydroxypentadecanoic acid methyl esters, respectively

^{1}H NMR and ^{13}C NMR spectra of the two samples were identical: ^{1}H NMR ($CDCl_3$, 200 MHz): 1.18 ppm (*d*, *J*=6.2, CH_3COH); 1.22–1.51 ppm (*m*, $10CH_2$); 1.52–1.68 ppm (*m*, $CH_2CH_2COOCH_3$); 2.30 ppm (*t*, *J*=7.5, CH_2COOCH_3); 3.67 ppm (*s*, OCH_3); 3.70–3.86 ppm (*m*, *CH*OH). ^{13}C NMR ($CDCl_3$, 50 MHz): 20.97; 22.47; 23.27; 26.66; 26.76; 26.95; 27.11; 31.64; 36.91; 48.98; 65.74.

HPLC analysis using a Chiralcel OD column resolved the (*R*)-(−)-14-hydroxypentadecanoic acid methyl ester **1** (standard) at a t_R of 7.2 min; the racemic mixture **3** was resolved to its (*R*)-(−)- and (*S*)-(+)-enantiomers at 7.2 and 7.7 min t_R, respectively (Fig. 6). 14-Hydroxypentadecanoic acid methyl ester,

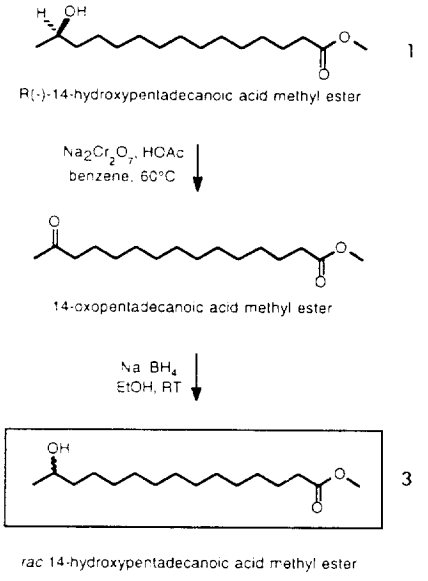


Fig. 4. Chemical racemization of (*R*)-(−)-14-hydroxypentadecanoic acid methyl ester. (*R*)-(−)-14-Hydroxypentadecanoic acid methyl ester was chemically converted to *rac*-14-hydroxypentadecanoic acid methyl ester as described in the experimental section

formed in the biotransformation (Fig. 2B and Fig. 3B), was detected at a t_R of 7.7 min, indicating it must be the (*S*)-(+)-enantiomer (e.e.>95%) as described in the experimental section. This demonstrates that the oxidation of pentadecanoic acid at the ω-1 position by whole cells of *E. coli* K27 (pCYP102, pGEc47) containing active cytochrome $P450_{BM-3}$ monooxygenase yields pure (*S*)-(+)-14-hydroxypentadecanoic acid **2** (Fig. 7).

4. Discussion

We have described a whole cell biocatalysis and product recovery process to obtain 12-, 13- and 14-hydroxypentadecanoic acids. Biotransformation of pentadecanoic acid with *E. coli* K27 (pCYP102, pGEc47) under low oxygen conditions improved the regioselectivity of the enzymatic reaction and only three monohydroxylated products, 12-, 13- and 14-hydroxypentadecanoic acids, were formed.

After the first recovery step, centrifugation, the remaining (unoxidized) pentadecanoic acid formed a layer on top of the cell pellett, while the hydroxylated products remained dissolved in the liquid. The separation of the three products by reversed-phase HPLC was the most cumbersome part of the recovery process. Due to their similar composition, the three hydroxy fatty acid methyl esters eluted at similar retention times.

The ultimate system for the production of ω-1, ω-2 and ω-3 hydroxy long chain fatty acids would be an engineered biocatalyst with a more restricted regiospecificity. This would facilitate the product recovery process. Different mutants of cytochrome $P450_{BM-3}$ monooxygenase might enable the production of either the ω-1, ω-2 and ω-3 hydroxy long chain fatty acid product. It has been shown that site-directed mutagenesis of the enzyme can transform cytochrome $P450_{BM-3}$ monooxygenase into an effective fatty acid ω-hydroxylase.[19]

The enzyme oxidizes $C_{15:0}$ to the ω-1 hydroxy fatty acid as the enantiomerically pure (*S*)-(+)-14-hydroxypentadecanoic acid, providing a possible route for the production of this highly enantiomeric

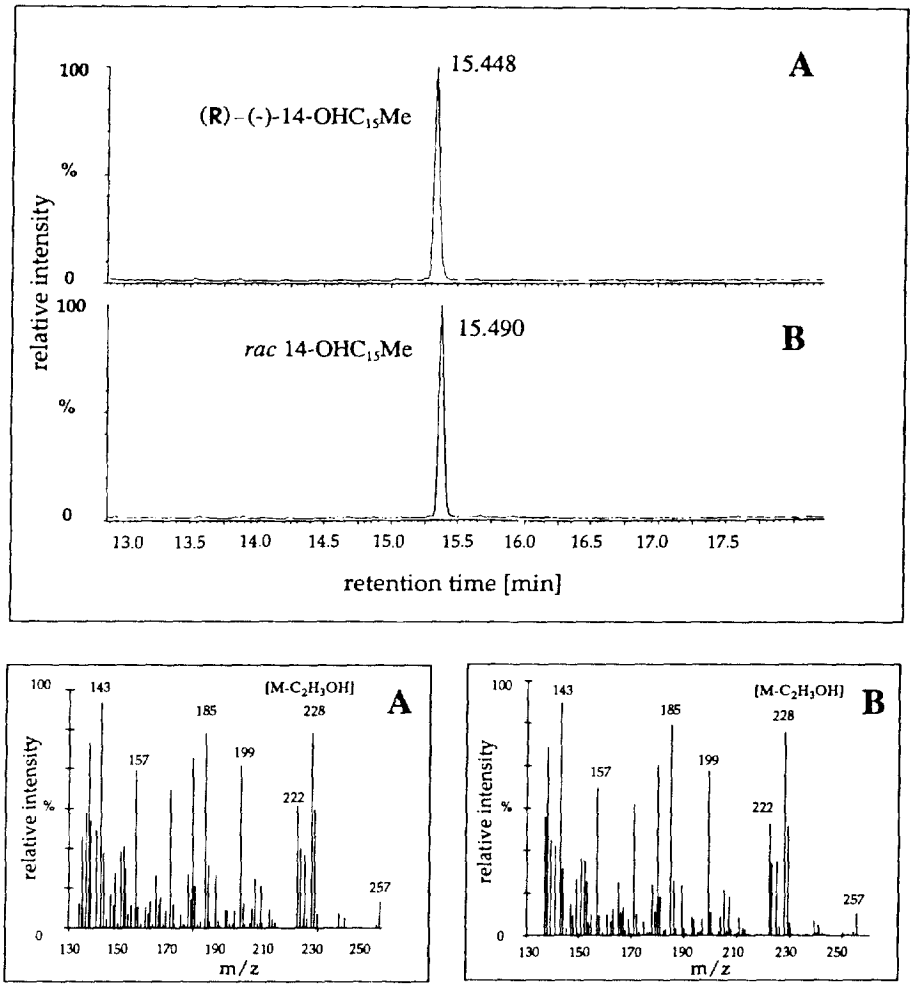


Fig. 5. Comparison of biocatalytically and chemically produced 14-hydroxypentadecanoic acid methyl esters. GC–MS spectra of (*R*)-(−)-14-hydroxypentadecanoic acid methyl ester produced biocatalytically (A) and *rac*-14-hydroxypentadecanoic acid methyl ester produced by chemical synthesis (B)

compound. An alternative route by chemical synthesis has not yet been described. The enantiomeric distribution of the 12- and 13-hydroxypentadecanoic acid products remains to be determined.

The overall biocatalysis and product recovery to obtain 12-, 13- and 14-hydroxypentadecanoic acids may be generally applicable to the preparation of ω-1, ω-2 and ω-3 hydroxy fatty acids (C_{12}–C_{18}) since the cytochrome $P450_{BM-3}$ biocatalyst is able to monooxidize this entire group of saturated long chain fatty acid substrates.

The present study adds another example of a highly stereoselective long chain fatty acid oxidation catalyzed by cytochrome $P450_{BM-3}$ monooxygenase, and is the first report of a stereoselective oxidation of a saturated fatty acid by this enzyme. The stereoselective oxidation of pentadecanoic acid as a representative long chain fatty acid substrate of cytochrome $P450_{BM-3}$ monooxygenase extends the usefulness of the enzyme as a biocatalyst in organic synthesis towards the production of homochiral compounds.

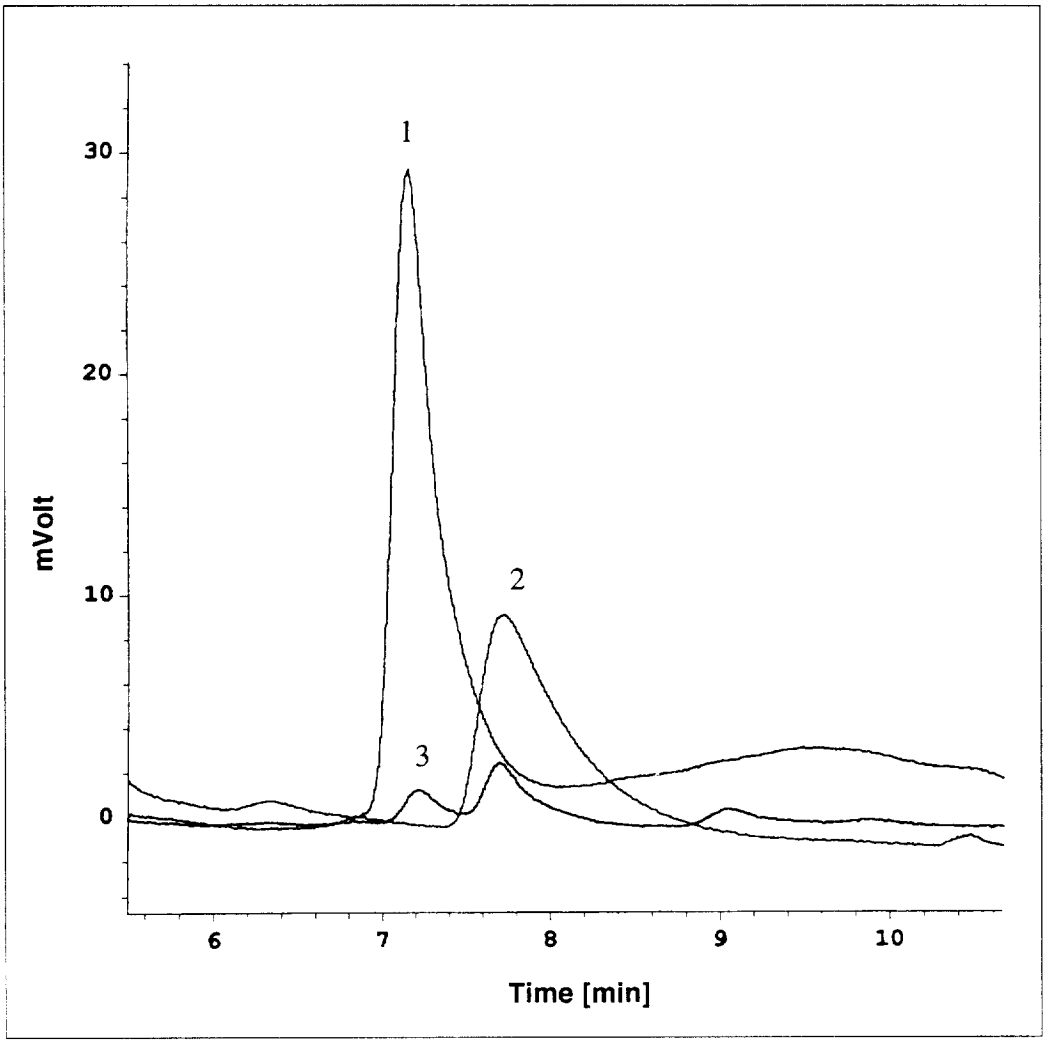
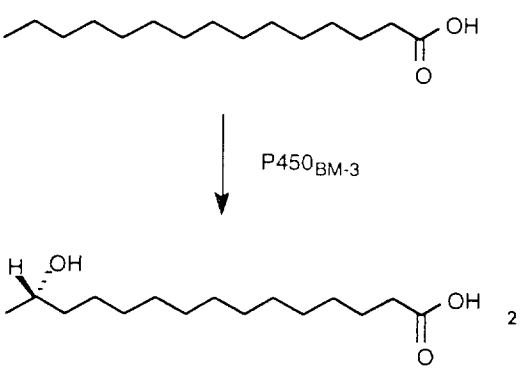


Fig. 6. Identification of 14-hydroxypentadecanoic acid methyl ester as the (*S*)-(+)-enantiomer by HPLC utilizing a chiral stationary phase. Elution of the standard (*R*)-(−)-14-hydroxypentadecanoic acid methyl ester (**1**), the racemic mixture *rac*-14-hydroxypentadecanoic acid methyl ester (**3**) and the cytochrome $P450_{BM-3}$ oxidation product (**2**) on a Chiralcel OD column. The product coeluted with (*S*)-(+)-12-hydroxypentadecanoic acid methyl ester present in the racemic mixture (**3**)

Acknowledgements

A gift of synthetic (*R*)-(−)-14-hydroxypentadecanoic acid methyl ester by Dr. Gerlach, University of Bayreuth, Germany, is greatly appreciated. This work was supported in part by the Swiss National Fonds.



S(+)-14-hydroxypentadecanoic acid

Fig. 7. Oxidation of pentadecanoic acid to the ω-1 hydroxy product by whole cell biocatalysis using cytochrome $P450_{BM-3}$ monooxygenase. Optically pure (*S*)-(+)-14-hydroxypentadecanoic acid is formed from pentadecanoic acid oxidation by a recombinant *E. coli* K27 (pCYP102, pGEc47), which synthesizes cytochrome $P450_{BM-3}$ monooxygenase and is capable of efficiently taking up long chain fatty acids

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