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PAPER

Regioselective ω-hydroxylation of medium-chain *n*-alkanes and primary alcohols by CYP153 enzymes from *Mycobacterium marinum* and *Polaromonas* sp. strain JS666†‡

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The oxofunctionalization of saturated hydrocarbons is an important goal in basic and applied chemistry. Biocatalysts like cytochrome P450 enzymes can introduce oxygen into a wide variety of molecules in a very selective manner, which can be used for the synthesis of fine and bulk chemicals. Cytochrome P450 enzymes from the CYP153A subfamily have been described as alkane hydroxylases with high terminal regioselectivity. Here we report the product yields resulting from C_5–C_{12} alkane and alcohol oxidation catalyzed by CYP153A enzymes from *Mycobacterium marinum* (CYP153A16) and *Polaromonas* sp. (CYP153A *P.* sp.). For all reactions, byproduct formation is described in detail. Following cloning and expression in *Escherichia coli*, the activity of the purified monooxygenases was reconstituted with putidaredoxin (CamA) and putidaredoxin reductase (CamB). Although both enzyme systems yielded primary alcohols and α,ω-alkanediols, each one displayed a different oxidation pattern towards alkanes. For CYP153A *P.* sp. a predominant ω-hydroxylation activity was observed, while CYP153A16 possessed the ability to catalyze both ω-hydroxylation and α,ω-dihydroxylation reactions.

Introduction

ω-Hydroxylated hydrocarbons are of great interest in the chemical industry due to their use as versatile solvents, plasticizers, surfactants and diol precursors for polymer intermediates, among other applications. The selective terminal hydroxylation of alkanes remains a challenging task as it is still difficult to direct the introduction of hydroxyl groups on primary non-activated C–H bonds. There are basically three approaches to oxo-functionalize *n*-alkanes: heterogeneous catalysis, organometallic activation and biological/biomimetic catalysis.[1,2] Relevant inorganic and organometallic catalysts include platinum-chloride complexes,[3,4] shape-selective metalloporphyrins,[5] Lewis acid-assisted metal compounds,[6–9] immobilized mononuclear iron carboxylates[10] and molecular sieves based on microporous transition metal-coordinated aluminophosphates,[11] as well as the Shell Hydroformylation technology.[12–14] Nevertheless, the application of these methods generally results in the undesirable mixture of at least two regioisomeric alcohols and further oxidized products such as aldehydes, ketones and fatty acids.

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† We dedicate this work to Prof. Dr Franz Lingens on the occasion of his 85th birthday

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§ Both authors contributed equally to this work

The use of biocatalysts offers important advantages bioinorganic catalysts cannot yet provide: high C_1 selectivity and low byproduct formation under mild reaction conditions. For their biotechnological potential, several alkane ω-oxidizing microorganisms and their enzymes have been studied more intensively during the last two decades.[15–19] It is known that alkane ω-hydroxylases are complex oxygenase systems whose substrate ranges depend on the chain-length of the hydrocarbon. In general, short-chain alkanes (C_1–C_4) are hydroxylated by methane, propane and butane monooxygenases.[20–22] Medium-chain alkanes (C_5–C_{16}) are oxidized by integral-membrane non-heme diiron monooxygenases (AlkB)[23] or alternatively by heme iron-containing cytochrome P450 monooxygenases (P450s or CYPs).[24,25] Longer alkanes ($\geq C_{16}$) can be oxidized by yeast P450s,[26] integral-membrane monooxygenases (AlkM)[27] and other flavin-containing alkane monooxygenases (LadA)[28] and dioxygenases.[29]

P450s with the natural ability to ω-hydroxylate medium-sized alkanes belong to the CYP153 family.[30] CYP153s are bacterial class I P450s that operate as three-component systems, comprised by the P450 itself and two additional redox proteins, namely an iron–sulfur electron carrier (ferredoxin) and a FAD-containing reductase (ferredoxin reductase), which are necessary for the transfer of electrons from NAD(P)H to the P450 active site.[25,31] CYP153 enzymes are regarded as promising biocatalysts due to their functional expression in soluble form in contrast to the AlkB-related enzymes[30] as well as their high regioselectivity (>95%) for the ω-position compared to other P450 families, including engineered CYP102A1 (P450-BM3)[32,33] and CYP102A3.[34]

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CYP153A16 from *Mycobacterium marinum* M. and CYP153A from *Polaromonas* sp. strain JS666 (CYP153A *P.* sp.) share respectively 69% and 86% amino acid sequence similarity with CYP153A6 from *Mycobacterium* sp. HXN-1500, known to ω-oxidize C_6–C_{11} alkanes, alkenes, cycloalkenes and alicyclic compounds.[35,36] In this study, we report the product yields and regioselectivities of cloned and expressed CYP153A16 and CYP153A *P*. sp. monooxygenases whose activity was reconstituted with NADH-dependent putidaredoxin reductase (CamA) and putidaredoxin (CamB). *In vitro* biocatalytic reactions with purified proteins were carried out using C_5–C_{12} alkanes and C_6–C_{12} primary alcohols as substrates within a cofactor regenerating system (Fig. 1). The alkanes and primary alcohols were respectively oxidized to primary alcohols and α,ω-diols. Interesting to our view is the formation of α,ω-diols, as such ability has been directly demonstrated only with CYP153 from *Acinetobacter* sp. OC4.[37]

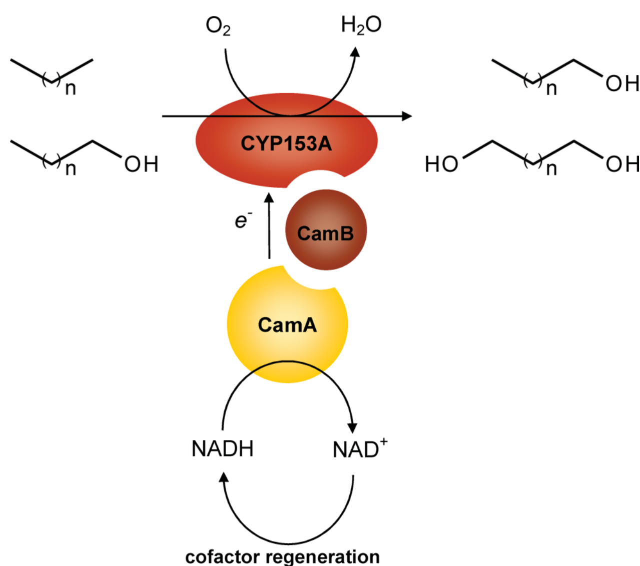

Fig. 1 Schematic representation of the terminal hydroxylation of *n*-alkanes and primary alcohols catalyzed by CYP153A enzymes using putidaredoxin reductase (CamA) and putidaredoxin (CamB) for electron (e^-) transfer and an additional NADH regenerating system.

Materials and methods

Chemicals, enzymes, vectors and strains

All chemicals, solvents and buffer components were obtained from Sigma-Aldrich (Schnelldorf, Germany). *Pfu* DNA polymerase, endonucleases, T4 DNA ligase and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Fermentas (St. Leon-Rot, Germany). NADH disodium salt was obtained from Codexis (Jülich, Germany). Glucose-6-phosphate dehydrogenase (1000 U) from *Leuconostoc mesenteroides* was obtained from Roche Diagnostics (Mannheim, Germany). Expression vector pKK223-3 was obtained from Amersham Biosciences (Glattbrugg, Switzerland). Plasmid pET28a(+) and *E. coli* strain BL21(*DE3*) originated from Novagen (Madison, Wisconsin, USA). *E. coli* strains DH5α and JM105 were purchased from Invitrogen (Darmstadt, Germany).

Cloning, expression and purification of CYP153A, CamA and CamB

CYP153A enzymes. The gene *cyp153A16* (MMAR_3154) derived from genomic DNA from *Mycobacterium marinum* M. ATCC BAA-535 was amplified with primers 5′-AATTGAATTCGCGTGCATCATCATCATCATCATAGCAATATTCGC -3′ (encoding an N-terminal His_6-tag sequence, underlined) and 5′-AGCTAAGCTTTCATGCGCCACCTTTCGGGG-3′ using a standard PCR protocol. The resulting fragment was cloned into the *tac*-promoter-based pKK223-3 expression system using *Eco*RI and *Hind*III restriction sites.

CYP153A (Bpro_5301) from *Polaromonas* sp. strain JS666 ATCC BAA-500 (CYP153A *P.* sp.) was introduced into the *Nde*I and *Eco*RI cloning sites of the T7 promoter-based pET28a(+) vector. The gene coding for CYP153A *P.* sp. was amplified by PCR using oligonucleotides 5′-GGTCATATGAGATCATTA ATGAGTGAAGCGATTGTGGTAAACAACC-3′ and 5′-ATTGAATTCAATGATGA TGATGATGATGAGCGTTGATGCGGACGGG-3′ (encoding a C-terminal His_6-tag sequence).

The ligated plasmids were used to transform competent *E. coli* DH5α cells. Successful cloning was verified by automated DNA-sequencing (GATC-Biotech, Konstanz, Germany).

For protein expression, each vector construct was introduced into *E. coli* BL21(*DE3*). The resulting transformants were grown at 37 °C, 180 rpm in 400 ml TB medium containing 100 μg ml^{-1} ampicillin or 30 μg ml^{-1} kanamycin. When the cultures reached an OD_{600} of 0.6–0.8, they were supplemented with 0.1 mM IPTG, 0.5 mM δ-aminolevulinic acid and 0.1 mM $FeSO_4$ for P450 expression. After 12–14 h of incubation at 30 °C, 140 rpm, cells were harvested by centrifugation (10 000 × *g*, 20 min, 4 °C) and resuspended in binding buffer (50 mM potassium phosphate buffer pH 7.5 containing 300 mM KCl, 20 mM imidazole and 0.1 mM PMSF). Cells were disrupted by sonication on ice (4 × 2 min, 2 min intervals) and the cell debris was removed by centrifugation (37 000 × *g*, 30 min, 4 °C) and the supernatants were recovered.

Protein purification was performed by immobilized metal ion affinity chromatography (IMAC) utilizing a Ni-NTA column (Qiagen, Germany) pre-equilibrated with binding buffer. The P450 enzymes were eluted with 200–300 mM imidazole and dialyzed against 50 mM potassium phosphate buffer pH 7.5 for 4 h. The purity of the enzymes was estimated by SDS-PAGE on 15% polyacrylamide gels. Purified protein solutions were concentrated by ultrafiltration and stored in aliquots at −20 °C

CamA and CamB. Putidaredoxin reductase (CamA) and putidaredoxin (CamB) from *Pseudomonas putida* ATCC 17453 were expressed as His-tagged proteins in *E. coli* BL21(*DE3*) using the plasmid constructs pET28a(+)-camA and pET28a(+)-camB, which have been described previously.[38] Protein expression and purification were carried out as detailed therein.

Determination of P450, CamA and CamB concentration

Concentrations of the P450 enzymes were determined by the carbon monoxide (CO) differential spectral assay, based on the formation of the characteristic Fe^{II}–CO complex at 448 nm. Enzymes were reduced by the addition of 10 mM dithionite from a freshly prepared 1 M stock solution, and the carbon

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monoxide complex was formed by slow bubbling with CO gas for approximately 30 s. The concentrations were calculated using the absorbance difference at A_{450} and A_{490} (Ultrospec 3100pro spectrophotometer, Amersham Biosciences) and an extinction coefficient of 91 M^{-1} cm^{-1}.[39,40]

The concentration of CamA was determined as the average of the concentration calculated at 378, 454 and 480 nm using extinction coefficients of 9.7, 10.0 and 8.5 mM^{-1} cm^{-1}, respectively.[41]

The concentration of CamB was determined as the average concentration calculated from the two wavelengths 415 and 455 nm using the extinction coefficients 11.1 and 10.4 mM^{-1} cm^{-1}, respectively.[41]

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Reconstitution of P450 activity

Amino acid sequence alignments of CamA and CamB against the putative natural redox partners of CYP153A16 and CYP153A *P.* sp. were performed. These included the proteins encoded by genes MMAR_3153 (ferredoxin reductase) and MMAR_3155 (ferredoxin) flanking *cyp153A16* in the genome of *Mycobacterium marinum* M., as well as the gene products of Bpro_5300 (ferredoxin reductase) and Bpro_5299 (ferredoxin) located downstream the CYP153A gene within the genome of *Polaromonas* sp. strain JS666.

In order to assess the electron transfer potential of the non-physiological redox proteins CamA and CamB to the CYP153A enzymes, a preliminary test was performed. The alkane hydroxylase activity of CYP153A16 was assayed in the absence or presence of CamA/CamB using *E. coli* cell-free extracts containing the P450 monooxygenase. Reaction mixtures (0.5 ml) contained 50 mM potassium phosphate buffer pH 7.5, a lysate volume equivalent to 1.5 μM P450 and a cofactor regenerating system comprising 12 U ml^{-1} glucose-6-phosphate dehydrogenase (G6PDH), 5 mM glucose-6-phosphate (G6P) and 1 mM $MgCl_2$. Samples with CamA/CamB contained 7.5 μM of each redox protein. Two hundred micromolar *n*-octane was added to the samples from a 20 mM stock solution in ethanol. Reactions were started by addition of 0.5 mM NADH, followed by incubation at 30 °C, 500 rpm for 4 h.

Prior to the *in vitro* bioconversions of alkanes and primary alcohols, substrate and P450 concentrations as well as the P450-to-redox partner ratios were adjusted using purified proteins and *n*-octane as model substrate. Octane and NADH concentrations were increased to 1 mM, while the P450 concentration was raised to 3 μM. At the same time, samples with P450-CamA-CamB ratios of 1 : 1 : 5, 1 : 2 : 10, 1 : 2 : 20 and 1 : 5 : 10 were analyzed.

In vitro biotransformations

The activity of each CYP153A enzyme was reconstituted with CamA and CamB and assayed *in vitro* using C_5–C_{12} *n*-alkanes and C_6–C_{12} primary alcohols as substrates. Biotransformations were performed using a final volume of 0.5 ml in 1.5 ml Eppendorf tubes. Reaction mixtures contained 50 mM potassium phosphate buffer pH 7.5, 3 μM CYP153A, 15 μM CamA, 30 μM CamB and the G6PDH/G6P system described above. One millimolar alkane or 0.2 mM 1-alcohol was added from a 100 mM or 20 mM stock solution in ethanol, respectively. NADH was added in the same concentration of that of the substrate and samples were incubated at 30 °C, 500 rpm for 4 h.

Sample treatment

The substrates and formed products were extracted twice with 0.5 ml ethyl acetate containing internal standard (0.05 mM 1-tetradecanol for the C_5–C_{10} substrates or 0.05 mM 1-hexadecanol for the C_{11}–C_{12} substrates). After vigorous mixing, the extraction fractions were pooled and centrifuged (15 000 × *g*, 2 min). The organic phase was collected and stored in a capped GC-vial without headspace.

Product analysis

Samples were analyzed by gas chromatography coupled to mass spectrometry on a GC-MS QP-2010 instrument (Shimadzu, Japan) equipped with a FS-Supreme-5-column (30 m × 0.25 mm × 0.25 μm, Chromatographie Service GmbH, Langerwehe, Germany) and with helium as carrier gas (flow rate, 0.69 ml min^{-1}; linear velocity 30 cm s^{-1}). Mass spectra were collected using electrospray ionization. The injector and detector temperatures were set at 250 °C. For the C_5–C_{10} compounds, the column oven was set at 40 °C for 5 min, raised to 160 °C at a rate of 10 °C min^{-1}, held isotherm for 1 min, and then raised to 310 °C at 45 °C min^{-1}. For the C_{11}–C_{12} compounds, the temperature was maintained at 90 °C for 5 min, raised to 200 °C at 10 °C min^{-1}, held isotherm for 1 min, and then raised to 310 °C at a rate of 30 °C min^{-1}.

Reaction products were identified by their characteristic mass fragmentation patterns.

For quantification of the products, a series of standard solutions consisting of 0.01–0.4 mM of the primary alcohols or 0.01–0.2 mM of the α,ω-diols in 50 mM potassium phosphate buffer were extracted with ethyl acetate containing internal standard and analyzed by GC-MS to obtain straight-line calibration plots.

Results

Expression and stability of the CYP153 enzymes

The CYP153 genes were cloned with a His-tag to facilitate protein purification by immobilized metal ion affinity chromatography. CYP153A16 and CYP153 *P.* sp. were expressed in soluble form in *E. coli* BL21 (DE3), yielding 1.25 and 32 mg active protein L^{-1} culture respectively after purification. An SDS gel for the purified P450 enzymes is shown in the supplementary material section (Fig. S1‡).

The reduced yield of CYP153A16 might be attributed to low expression levels caused by the use of GTG as start codon (originally present in the *cyp153A16* gene sequence). Our attempts to express the gene with the ATG initiation codon failed, probably due to toxicity upon overexpression and no further codon usage optimization was done. In terms of protein stability, it was noticed from CO-differential spectral assays that the CO-binding activity of purified CYP153A *P.* sp. remained unchanged at room temperature for at least 4 h, while CYP153A16 lost such ability after 5 min. The addition of 5–20% glycerol to prevent the conversion of the CO-bound P450 into the inactive P420 form did not contribute to stabilize the enzyme. Furthermore, the CO-binding capacity of CYP153A16 was tested in cell-free extracts

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using sodium dithionite, NADH or NADPH as reducing agents. A stable 450 nm peak could be obtained only in the presence of the pyridine nucleotides, suggesting that CYP153A16 was labile to sodium dithionite. Nevertheless, the conventional CO-differential spectral assay with sodium dithionite was used as a standard method to determine the concentration of both purified CYP153 enzymes.

Cam A and CamB as redox partners

Putidaredoxin reductase (CamA) and putidaredoxin (CamB) from *Pseudomonas putida* were selected as redox partners to reconstitute the activity of CYP153A16 and CYP153A *P.* sp. due to their amino acid sequence similarity (ranging from 63 to 68%) with the FAD-containing oxidoreductases and the [2Fe-2S] ferredoxins located in the vicinity of the CYP153A genes within the genomes of *Mycobacterium marinum* M. and *Polaromonas* sp. strain JS666 (supplementary material, Table S1‡). In addition, CamA and CamB are well-characterized redox proteins that have been expressed in *E. coli* in functional form.[42–44]

Prior to their use in hydroxylation reactions, the purity of the expressed and purified redox proteins was verified by SDS-PAGE (supplementary material, Fig. S1‡). Without CamA and CamB, CYP153A16 (as *E. coli* cell lysate) showed negligible P450 oxidation activity towards *n*-octane, thereby implying that the constitutive redox partners from *E. coli* could not interact with the CYP153 enzyme or they were not present in a stoichiometric ratio to support P450 activity. In contrast, when purified CamA and CamB were added to the reaction, the P450 was able to catalyze the conversion of *n*-octane to 1-octanol. This preliminary assay was merely qualitative, but it served to demonstrate that CamA and CamB were together able to transfer electrons from NADH to a CYP153 enzyme. Once this fact was established, the P450 concentration was increased by twofold and different P450–CamA–CamB ratios were tested. The maximum product concentration (140 µM 1-octanol) was obtained with a P450–CamA–CamB ratio of 1 : 5 : 10, while the activity dropped by 23–49% at ratios of 1 : 1 : 5, 1 : 2 : 10 and 1 : 2 : 20. The 1 : 5 : 10 ratio was subsequently used for bioconversions of *n*-alkanes and primary alcohols.

Table 1 Concentrations of terminally hydroxylated products (primary alcohols (C_n-1ol) and α,ω-diols) obtained from alkane oxidation catalyzed by CYP153A enzymes

	CYP153A16		CYP153A *P.* sp.	
Substrate	C_n-1ol [µM]	α,ω-diol [µM]	C_n-1ol [µM]	α,ω-diol [µM]
n-Pentane	34 ± 3[b]	—[a]	30 ± 1	—[a]
n-Hexane	24 ± 1[b]	—[a]	62 ± 5	—[a]
n-Heptane	81 ± 8	—[a]	103 ± 3	—[a]
n-Octane	120 ± 9	30 ± 3	165 ± 13	16 ± 1
n-Nonane	35 ± 2	113 ± 6	114 ± 3	16 ± 1
n-Decane	16 ± 1	65 ± 3	99 ± 4	—[a]
n-Undecane	<10	N.D. (59%)[c]	<10	—[a]
n-Dodecane	<10	<10	<10	—[a]

The initial substrate concentration was 1 mM. Reactions were set up for a final volume of 0.5 ml and run at 30 °C for 4 h.[a] Not detected; N.D. not determined. [b] CYP153A16 produced more 1-pentanol than 1-hexanol in a reproducible manner. [c] Percentage relative to total product estimated from the GC peak areas. The amount of 1,11-undecanediol was not be quantified as no standard was available.

Bioconversion of *n*-alkanes

The hydroxylation activities of CYP153A16 and CYP153A *P.* sp. towards C_5–C_{12} alkanes were investigated *in vitro* using CamA and CamB as electron transfer partners. The product distributions observed with each enzyme system are shown in Fig. 2. Amongst the formed products, primary alcohols (C_n-1ol), secondary alcohols (C_n-2ol), α,ω-diols and further oxidized products (aldehydes and fatty acids) could be identified. Table 1 shows the concentrations of the desired primary alcohol and α,ω-diol products obtained after 4 h incubation with each P450 system.

Similarly to previously reported CYP153s,[30,35,36] octane was the preferred alkane for both enzymes. Although their substrate ranges covered alkanes from pentane to dodecane, different activity patterns with each P450 were revealed. Compared to CYP153A16, CYP153A *P.* sp. exhibited higher terminal hydroxylase activity but lower to inexistent diterminal hydroxylase activity towards C_8–C_{12} alkanes. In other words, CYP153A *P.* sp. produced larger amounts of primary alcohols from alkanes than CYP153A16, but the latter yielded considerably higher concentrations of α,ω-diols than CYP153A *P.* sp. did. The α,ω-diols were the result of the subsequent hydroxylation of the primary

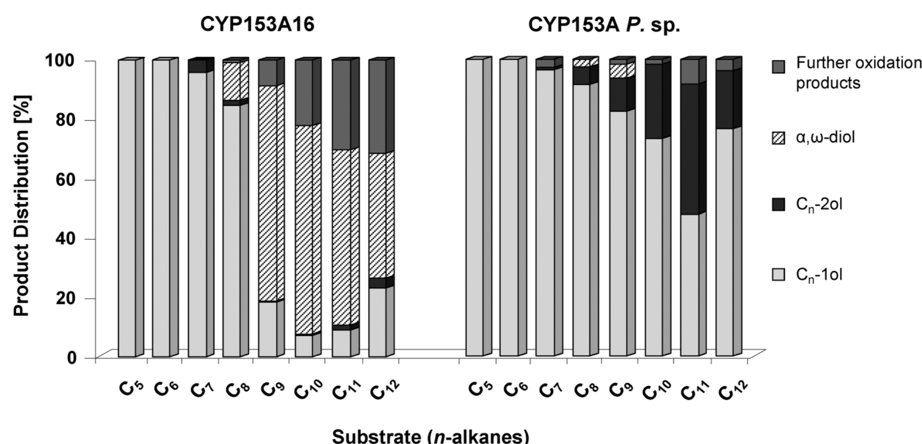

Fig. 2 Product distribution using *n*-alkanes as substrates. C_n-1ol, primary alcohol; C_n-2ol, secondary alcohol.

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Table 2 Concentrations of terminally hydroxylated products (α,ω-diols) obtained from primary alcohol oxidation catalyzed by CYP153A enzymes

Substrate	CYP153A16 α,ω-diol [μM]	CYP153A *P.* sp. α,ω-diol [μM]
1-Hexanol	—[a]	—[a]
1-Heptanol	—[a]	—[a]
1-Octanol	39 ± 3	18 ± 0.2
1-Nonanol	93 ± 4	16 ± 1
1-Decanol	80 ± 6	—[a]
1-Undecanol	N.D. (74%)[b]	—[a]
1-Dodecanol	43 ± 2	—[a]

The initial substrate concentration was 0.2 mM. Reactions were set up for a final volume of 0.5 ml and run at 30 °C for 4 h.[a] Not detected; N.D. not determined. [b] Percentage relative to total product

alcohol products. CYP153A *P.* sp. showed diterminal hydroxylase activity only towards octane and nonane, yet the product yields for 1,8-octanediol and 1,9-nonanediol were lower than those obtained with CYP153A16 by 2- and 7-fold, respectively. The accumulation of α,ω-diols suggests that CYP153A16 has a higher affinity towards C_8–C_{12} primary alcohols than CYP153 *P.* sp. This, however, should be confirmed by spin-state shift and substrate binding constant determinations.

In terms of product distribution (Fig. 2), both enzymes oxidized pentane and hexane to primary alcohols with absolute ω-regioselectivity, suggesting that the products 1-pentanol and 1-hexanol were not accepted as substrates. In addition, if we consider both ω-alcohols and α,ω-diols as the total terminally hydroxylated product, the ω-regioselectivities for C_7–C_9 compounds were 90–96% for CYP153A16 and 83–95% for CYP153A *P.* sp., with the maximum product yield for the mixture 1-octanol/1,8-octanediol.

Although CYP153A16 is more ω-regioselective than CYP153A *P.* sp., it tends to further oxidize the ω-hydroxylated products. For instance, with CYP153A16 not more than 5% of the total product was composed of secondary alcohols, while the concentration of aldehydes and fatty acids increased (representing a maximum of 32% of the total product) with the increase of the carbon chain length. Two other products from C_9–C_{12} substrates were observed (supplementary material, Table S2‡), though they could not be identified. Compared to CYP153A16, CYP153A *P.* sp. yielded more secondary alcohols (up to 37% of the total product in the case of longer chain alkanes), though smaller proportions of further oxidized products. Other byproducts different than aldehydes or fatty acids were not produced by CYP153A *P.* sp.

Bioconversion of primary alcohols

To further explore the substrate spectra of CYP153A16 and CYP153A *P.* sp., C_6–C_{12} primary alcohols were used as substrates. No oxidation activity towards 1-hexanol and 1-heptanol could be observed with both enzymes. Consistent with the results obtained with the alkane substrates, CYP153A16 was able to ω-hydroxylate primary alcohols ranging from C_8 to C_{12}, displaying higher activity for 1-nonanol. Likewise, CYP153A *P.* sp. could only oxidize 1-octanol and 1-nonanol to the corresponding α,ω diol products, but once more in significantly lower yields than those obtained with CYP153A16 (Table 2).

The product profiles observed with each primary alcohol substrate are shown in Fig. 3. With CYP153A16, 74–96% of

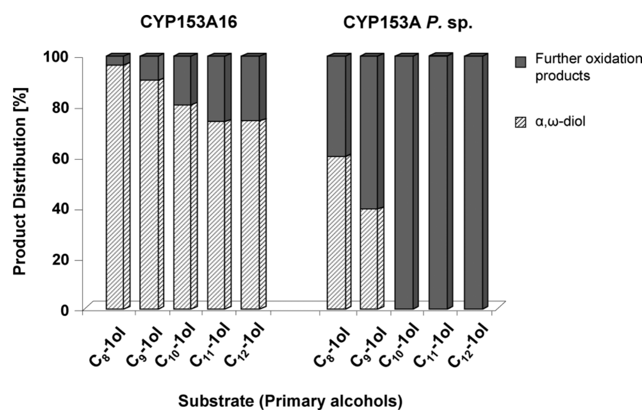

Fig. 3 Product distribution using primary alcohols (C_n-1ol) as substrates.

the total product obtained from the C_8–C_{12} primary alcohols was composed of α,ω-diols, while the rest comprised aldehydes, fatty acids and the byproducts mentioned in the previous section. Another result observed with CYP153A16 includes the formation of larger proportions of α,ω-diols from the C_{11} and C_{12} primary alcohols than from the corresponding alkanes. To be concrete, the concentrations of 1,11-undecanediol and 1,12-dodecanediol obtained from the primary alcohols were respectively 1.2- and 4-fold higher than those obtained from the corresponding alkane substrates. When using alkanes as substrates, the alkanes and the formed primary alcohols presumably compete for binding the active site of the enzyme, therefore, lower yields of α,ω-diols are obtained. On the other hand, substrate competition no longer occurs when using primary alcohols as start substrate material, which would result in higher α,ω-diol production. Concerning CYP153A *P.* sp., only 40–58% of the total product corresponded to the C_8 and C_9 α,ω-diols, while the remaining products consisted of aldehydes and fatty acids. Even though CYP153A *P.* sp. could bind C_{10}–C_{12} ω-alcohols, it showed low catalytic activity towards these substrates and they were exclusively converted to aldehydes.

Discussion

We have investigated the oxidation activities of ω-hydroxylases CYP153A16 and CYP153A *P.* sp. towards alkanes and primary alcohols in the presence of the non-physiological electron transfer proteins putidaredoxin reductase (CamA) and putidaredoxin (CamB).

The CYP153 enzyme from *Mycobacterium marinum* M. is herein referred as CYP153A16, based on the CYP nomenclature system.[45] Although there are no previous reports on CYP153A16, one CYP153 enzyme from *Mycobacterium marinum* M. (referred as CYP153A14) was formerly expressed in *Pseudomonas putida* and demonstrated to ω-oxidize C_6–C_{10} alkanes, among other compounds.[36] According to the Cytochrome P450 Homepage,[45] CYP153A14 and CYP153A16 seem to correspond to two different P450 sequences. However, assuming that only the *cyp153A16* gene can be found in the genome of *M. marinum* M., it is plausible that A14 and A16 refer to the same CYP153 enzyme.[46]

The CYP153 monooxygenase from *Polaromonas* sp. has not been assigned a CYP systematic name yet. Hence, we use the term CYP153A *P.* sp. in agreement with the Cytochrome P450 Engineering Database (CYPED),[47] where this enzyme is classified

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as a member of the CYP153 homologous family and accordingly named CYP153A *P.* sp. Even though CYP153A *P.* sp. has not been characterized yet, a 2008 publication on the genome of *Polaromonas* sp. strain JS666 suggests its physiological role in the pollutant-degrading microorganism.[48] Mattes and collaborators noticed the high amino acid sequence identity between CYP153A *P.* sp. (gene Bpro_5301) and CYP153A6 from *Mycobacterium* sp.[35] They additionally demonstrated that strain JS666 could grow on heptane or octane as sole carbon and energy source, though not on shorter aliphatic alkanes such as hexane, propane and ethane.[48] Consequently, CYP153A *P.* sp. is presumably involved in the *n*-alkane metabolic pathway within the strain.

While the studies outlined above are a significant precedent for this investigation, we consider that our results provide further insights into the capabilities of these CYP153A enzymes. As a matter of fact, we showed that CamA and CamB together can alternatively be used as electron transfer partners of CYP153A16 and CYP153 *P.* sp. Considering that P450 activity relies on the coupling efficiency of the electron transfer chain (*i.e.* reducing equivalents from the cofactor used for substrate oxidation), the product yields shown here are not to be solely attributed to the CYP153 enzymes, but to the complete CamA/CamB/CYP153 complexes. In our artificial systems, the substrate oxidation rate might be determined by the reduction rate of the P450 by CamB, since CamA and CamB are already known to interact reasonably well *in vitro*.[43,49] In this context, the fact that CamB is more similar to the ferredoxin from *Polaromonas* sp. than to that from *M. marinum* M. (supplementary material, Table S1‡) could explain the relatively higher hydroxylation activity of CYP153A *P.* sp. compared to CYP153A16. Nevertheless, even if CamA and CamB were shown to be able to transfer electrons to CYP153A16 and CYP153A *P.* sp., the oxidation activities of both P450s are most likely to be improved by using their physiological electron transfer proteins.

In addition, our *in vitro* biotransformations served to demonstrate that CYP153A16 and CYP153A *P.* sp., albeit their different substrate activities and ranges, are able to oxidize medium-chain length alkanes to primary alcohols and these further to α,ω-diols. This ability has been only reported for CYP153 from *Acinetobacter* sp. OC4 and CYP153A13a (P450balk), which were co-expressed with redox proteins in *E. coli* and demonstrated to *in vivo* oxidize octane to the corresponding primary alcohol and α,ω-diol in rather high yields.[37,50] Moreover, CYP153A13a has been recently fused to the reductase domain (RhFred) from *Rhodococcus* sp., expressed in *E. coli* and shown to hydroxylate C8–C12 alkanes and cyclohexane both *in vitro* and *in vivo*.[51] However, α,ω-diols were not observed as products. From our two P450 systems, CYP153A16 displays the highest diterminal hydroxylase activity. This finding is consistent with the fact that CYP153 from *Acinetobacter* sp. OC4 shares a higher similarity with CYP153A16 (80%) than with CYP153A *P.* sp. (70%) (supplementary material, Table S3‡). However, CYP153 from *Acinetobacter* sp. OC4 prefers 1-octanol, whereas the substrate preference of CYP153A16 seems to be shifted towards 1-nonanol.

The substrate range and regiospecificities of CYP153A *P.* sp. are similar to those of CYP153A6 from *Mycobacterium* sp. HXN-1500.[35,36] It is therefore not surprising to find a striking similarity (86%) between these two enzymes (supplementary material, Table S3‡). Curiously, the formation of α,ω-diols by CYP153A6 has not been reported in previous publications, while this activity, albeit low, was noticed in our CYP153A *P.* sp. system. If any α,ω-diols were produced in *in vitro* experiments with CYP153A6, they might had remained undetected due to their low concentration.

In summary, two oxidation patterns towards alkanes were observed with the CYP153 enzymes studied here: (1) predominantly ω-hydroxylase and (2) both ω-hydroxylase and α,ω-dihydroxylase. According to our protein similarity analysis, we would expect that enzymes with sequences similar to CYP153A6 display the first activity pattern, while those resembling CYP153A from *Acinetobacter* sp. OC4 behave according to the second one. For example, we recently carried out *in vitro* biotransformations with CYP153A (Maqu_0600) from *Marinobacter aquaeolei* and obtained primary alcohols and α,ω-diols from C_8–C_{12} alkanes (unpublished data). Once again, this result is correlated with the high protein similarity (83%) between the gene product of Maqu_0600 and CYP153A from *Acinetobacter* sp. OC4. Although we cannot establish the minimal sequence similarity needed to anticipate the oxidation pattern of a CYP153 enzyme, we consider that our results will be helpful to predict the activities of other homologous CYP153 enzymes.

A further contribution of our work is that byproduct formation with each CYP153A system is described in detail. A ω-regioselectivity of 83–100% could be observed only with C_5 to C_9 compounds, while the production of undesired aldehydes and fatty acids increased with the chain length of the substrates. Additionally, since our *in vitro* reactions were performed with purified proteins, it becomes evident that these byproducts originated from the oxidation activity of CYP153 enzymes and not from other oxidoreductases that could be present in cell-free extracts or whole cells. Besides, it is noteworthy to mention that these further oxidized products have not been detected in *in vivo* bioconversions before, though we ignore whether they were not formed at all or they were further metabolized after being produced. Concerning the formation of subterminally hydroxylated products, we suspect that the ω-regiospecificity of our systems might be increased in *in vivo* experiments, as observed with other CYP153 enzymes. For instance, Funhoff *et al.* reported that *in vitro* octane hydroxylation experiments with CYP153A6 yielded 5% 2-octanol, while the formation of secondary alcohols was not observed in *Pseudomonas putida* cultures.[35] Apparently, only terminal hydroxylation occurs in whole-cell biotransformations.[52,53]

An important issue to address in the research of CYP153s is the increase of their hydroxylation activities. Compared to other well-characterized P450 enzymes like P450-BM3 from *Bacillus megaterium* and CYP102A3 from *Bacillus subtilis*, the activity of CYP153s is rather low. Attempts to mutate the former enzymes aiming at the terminal hydroxylation of alkanes have resulted in low ω-regiospecificities (*e.g.* around 50% 1-octanol from *n*-octane),[33,34] indicating that engineered P450s from other families are not yet able to compete against CYP153 enzymes in terms of selectivity. Fortunately, the panorama seems favourable as *in vivo* directed evolution of CYP153A6 has demonstrated to be effective in shifting its substrate range towards short-chain alkanes with increased activity compared with the wild type enzyme and without affecting its terminal regioselectivity.[54] Combined rational design and random mutagenesis of these enzymes would lead to the optimization of desired properties, including higher activity, substrate specificity, expression level, and others. Taking

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into account that *in vivo* biotransformations might also result in the reduction of unwanted byproducts, CYP153s are overall promising biocatalysts which can be tailored for the production of chemicals of industrial importance.

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