Biosynthesis of a steroid metabolite by an engineered Rhodococcus erythropolis strain expressing a mutant cytochrome P450 BM3 enzyme

Venkataraman, Harini; Te Poele, Evelien M; Rosłoniec, Kamila Z; Vermeulen, Nico; Commandeur, Jan N M; van der Geize, Robert; Dijkhuizen, Lubbert

Published in:
Applied Microbiology and Biotechnology

DOI:
10.1007/s00253-014-6281-7

Publication date:
2015

Appl Microbiol Biotechnol (2015) 99:4713–4721
DOI 10.1007/s00253-014-6281-7

Biosynthesis of a steroid metabolite by an engineered *Rhodococcus erythropolis* strain expressing a mutant cytochrome P450 BM3 enzyme

Harini Venkataraman · Evelien M. te Poele · Kamila Z. Rosłoniec · Nico Vermeulen · Jan N. M. Commandeur · Robert van der Geize · Lubbert Dijkhuizen

Received: 14 August 2014 / Revised: 19 November 2014 / Accepted: 29 November 2014 / Published online: 16 December 2014

Abstract In the present study, the use of *Rhodococcus erythropolis* mutant strain RG9 expressing the cytochrome P450 BM3 mutant M02 enzyme has been evaluated for whole-cell biotransformation of a 17-ketosteroid, norandrostenedione, as a model substrate. Purified P450 BM3 mutant M02 enzyme hydroxylated the steroid with >95 % regioselectivity to form 16-β-OH norandrostenedione, as confirmed by NMR analysis. Whole cells of *R. erythropolis* RG9 expressing P450 BM3 M02 enzyme also converted norandrostenedione into the 16-β-hydroxylated product, resulting in the formation of about 0.35 g/L. Whole cells of strain RG9 itself did not convert norandrostenedione, indicating that metabolite formation is P450 BM3 M02 enzyme mediated. This study shows that *R. erythropolis* is a novel and interesting host for the heterologous expression of highly selective and active P450 BM3 M02 enzyme variants able to perform steroid bioconversions.

Keywords Cytochrome P450 BM3 M02 enzyme · *Rhodococcus erythropolis* · Steroids · Whole-cell bioconversion · Norandrostenedione

Introduction

The application of biocatalysts in synthetic chemistry has become indispensable for expanding the organic toolbox for chemists (Clouthier and Pelletier 2012). Enzymes catalyze oxidation reactions with high selectivity, which is difficult to achieve by conventional chemical methods (Hollmann et al. 2011; Ran et al. 2008; Schmid et al. 2001). Cytochrome P450 mono-oxygenases constitute an important group of oxidizing enzymes that are capable of inserting an oxygen atom in a highly regio- and or stereoselective manner (Bernhardt 2006; Julsing et al. 2008). Although human P450s are widely known for their biotransformation of xenobiotic compounds, their use at preparative scale is limited because of their low stability and membrane bound nature (Chefson and Auclair 2006). Over the past decades, several bacterial P450 mono-oxygenases have gained importance for biotransformation reactions (Urlacher et al. 2004). P450 BM3 is a widely studied bacterial P450 enzyme from *Bacillus megaterium*, which has one of the highest P450 activities ever reported. It is a soluble protein with the catalytic domain fused in a single polypeptide with the reductase domain, thereby making the electron transfer very efficient (Munro et al. 2002; Whitehouse et al. 2012). Protein engineering by rational, semi-rational, or directed evolution approaches have been successfully used in tailoring the P450 BM3 enzymes for different regio- and stereoselectivities towards a variety of substrates ranging from linear alkanes, alkenes, fine chemicals, and drug-like compounds (Whitehouse et al.

Harini Venkataraman and Evelien M. te Poele contributed equally.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-6281-7) contains supplementary material, which is available to authorized users.

H. Venkataraman · N. Vermeulen · J. N. M. Commandeur
AIMMS-Division of Molecular Toxicology, Department of Chemistry and Pharmaceutical Sciences, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

E. M. te Poele · K. Z. Rosłoniec · R. van der Geize · L. Dijkhuizen (✉)
Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
e-mail: l.dijkhuizen@rug.nl

2012). P450 BM3 mutants capable of selective hydroxylation of steroids have also been reported (Kille et al. 2011; Rea et al. 2012; Venkataraman et al. 2012a). Engineered P450 BM3 mutants are therefore promising candidates for biocatalysis.

Biotransformation reactions using either isolated P450 enzymes or whole cells have been widely described (Urlacher and Girhard 2011). For industrial scale reactions, the use of whole-cell catalysis systems is preferred because of the availability of the P450 co-factor under native conditions (Ishige et al. 2005). *Escherichia coli* is widely used as the recombinant host for expression and whole-cell biocatalysis using P450s (Fasan et al. 2011; Gudiminchi et al. 2012; Schewe et al. 2009; Siriphongphaew et al. 2012; Yim et al. 2010; Zehentgruber et al. 2010b). Various other microorganisms also have been explored as potentially suitable hosts for whole-cell biocatalysis (Bleif et al. 2012; Donova and Egorova 2012; Drăgan et al. 2005; Kolar et al. 2007; Petric et al. 2010; Zehentgruber et al. 2010a).

The genus *Rhodococcus* belongs to the nocardioform actinomycetes, with many strains acting as catabolic powerhouses, involved in numerous mineralization processes in natural environments (Martinkova et al. 2009; McLeod et al. 2006). *Rhodococcus* strains are well known for their robustness and solvent tolerance, possess a range of P450 enzymes, naturally metabolize a wide variety of hydrophobic substrates, including sterols/steroids, and possess active systems for their uptake into the cell (Mohn et al. 2008; van der Geize and Dijkhuizen 2004). These combined properties make rhodococci interesting candidates for whole-cell industrial biotransformation (P450) processes (Hughes et al. 1998; Plaggenborg et al. 2006). However, members of the *Rhodococcus* genus have not been targeted yet for the heterologous expression of bacterial P450 enzymes for steroid transformations. The use of *Rhodococcus* strains as biocatalyst for steroid synthesis also has been limited by the fact that they are able to completely degrade steroids (van der Geize and Dijkhuizen 2004).

Detailed targeted gene inactivation studies in *Rhodococcus erythropolis* strain SQ1 have led to the identification of 3-ketosteroid Δ1-dehydrogenase (KSTD) and 3-ketosteroid 9α-hydroxylase (KSH) genes involved in steroid ring degradation pathway (van der Geize et al. 2002a; van der Geize et al. 2001; van der Geize et al. 2002b; van der Geize et al. 2000). A triple gene deletion mutant of *R. erythropolis* SQ1, strain RG9, was unable to degrade 4-androstene-3,17-dione due to inactivation of the *kstD*, *kstD2*, and *kshA1* genes, which encode key enzymatic steps in steroid ring opening (van der Geize et al. 2002b). Mutant strain *R. erythropolis* RG9 therefore was selected by us to examine expression of steroid modifying P450 enzymes and to study its whole-cell biotransformation potential.

In the present study, hydroxylation of norandrostenedione (NAND) has been characterized in vitro using purified BM3 mutant M02 enzyme and in vivo using whole cells of strain RG9 expressing the BM3 M02 enzyme. P450 BM3 mutant M02 has been shown to be very active in the biotransformation of steroids and other drug-like compounds (Damsten et al. 2008; de Vlieger et al. 2010). The results show that the *R. erythropolis* mutant strain RG9 itself does not degrade or convert NAND but is efficient in whole-cell biotransformations of NAND when expressing P450 BM3 mutant M02 enzyme activity.

Materials and methods

Materials

All chemicals were of analytical grade and purchased from Sigma unless otherwise mentioned.

Bacterial strains and growth conditions

R. erythropolis (ATCC4277) strain SQ1 (Quan and Dabbs 1993) and mutant strain RG9 were cultivated at 29 °C and 180 rpm. Mutant strain RG9 (*kstD*, *kstD2*, *kshA1* mutant) was previously described (van der Geize et al. 2002a, b). Precultures of *Rhodococcus* were grown in LBP medium containing 1 % (wt/vol) Bacto peptone (Difco), 0.5 % (wt/vol) yeast extract (Difco), and 1 % (wt/vol) NaCl (Taguchi et al. 2004). Whole-cell bioconversions in *Rhodococcus* were performed in terrific broth (TB) medium supplemented with chloramphenicol (34 μg/mL).

Expression and purification of BM3 mutant M02 enzyme

The cytochrome P450 BM3 mutant M02 enzyme was produced using *E. coli* BL21 DE3 cells transformed with pET28-M02 vector and purified using nickel affinity chromatography as described previously (Damsten et al. 2008).

Determination of dissociation constant and coupling efficiency

The binding of NAND to P450 BM3 mutant M02 protein was analyzed by optical titrations using a Shimadzu UV-2501PC spectrophotometer (Shimadzu Duisburg, Germany) as described previously. The coupling efficiency was determined as the ratio of the total product formed to the amount of NADPH consumed expressed as percentage (Venkataraman et al. 2012b).

Product isolation and NMR analysis

Large-scale incubations were performed with 1 μM of purified BM3 mutant M02 enzyme, 1 mM of norandrostenedione in a volume of 25 mL in 100 mM potassium phosphate buffer (pH 7.4). The reaction was initiated with a NADPH regenerating system containing 0.2 mM NADPH, 1 mM glucose 6-phosphate, and 0.4 U/mL of glucose 6-phosphate dehydrogenase. The reaction was allowed to proceed for 3 h after which the products were extracted using 2×50 mL dichloromethane. The organic layers were collected in a round-bottom flask and evaporated using a rotary evaporator. The dried residue was dissolved in 1 mL methanol and applied by manual injection onto a semi-preparative high-performance liquid chromatography (HPLC) Luna 5 μm C18 (2) column (250 mm×10 mm i.d.) from Phenomenex. An isocratic flow rate of 2 mL/min of eluent containing 60 % methanol/40 % water was used to separate norandrostenedione and metabolites. Metabolites were detected using UV detection (254 nm) and collected manually. Collected fractions were first analyzed for purity by the HPLC method described below. The samples were evaporated to dryness under nitrogen stream and dissolved in 500 μL of methyl alcohol-*d4*. Structural identification of the metabolite was performed by ^{1}H- and ^{1}H-^{1}H-correlation spectroscopy (COSY) NMR experiments. NMR spectra were recorded at room temperature on a Bruker Avance 500 instrument (Fallanden, Switzerland), equipped with a cryoprobe operating at 500.13 MHz.

Samples were also analyzed by liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1200 Series Rapid resolution LC system connected to a time-of-flight (TOF) Agilent 6230 mass spectrometer equipped with electrospray ionization (ESI) source and operating in the positive mode. The MS ion source parameters were set with a capillary voltage at 3500 V. Nitrogen was used as drying gas (10 L/min) and nebulizing gas (pressure 50 psig) at a constant gas temperature of 350 °C. MS spectra were acquired using the Agilent TOF system. Data analysis was performed using the Agilent MassHunter Qualitative analysis software.

Cloning and heterologous expression of P450 BM3 mutant M02 enzyme in *R. erythropolis* strain RG9

The gene encoding P450 BM3 mutant M02 of plasmid pT1-P450BM3M02 (van Vugt-Lussenburg et al. 2007) was amplified with forward primer 5′-gcgcgccatggcaattaaagaaatgcctcagcc-3′ containing an *Nco*I restriction site and reverse primer 5′-ggccgctcgagcccagcccacacgtcttttgcgtatcgg-3′ containing an *Xho*I restriction site. The 3165-bp PCR product was digested with *Nco*I/*Xho*I and ligated into the *Nco*I/*Xho*I sites of pTip-QC1 (Nakashima and Tamura 2004). The resulting construct, pTip-QC1_P450BM3 M02, was transformed to *R. erythropolis* RG9 by electroporation (van der Geize et al. 2000).

In vivo biotransformation of NAND

In vivo biotransformation experiments with *R. erythropolis* strain RG9 were performed (in triplicate) in 250-mL shaking flasks in 25 mL TB supplemented with chloramphenicol (34 μg/mL) at 29 °C and 180 rpm. Expression of P450 BM3 mutant M02 in strain RG9 was induced in exponentially growing cultures (OD_{600} of 12–15) by the addition of thiostrepton (1 μg/mL) (Nakashima and Tamura 2004). NAND (1 g/L) was added immediately after induction, and bioconversion was followed for 48 h. Samples (2 mL) were taken at 0, 15, 22, 28, 39, and 48 h after induction. NAND bioconversion experiments with *R. erythropolis* wild type were performed (in triplicate) in 250-mL shaking flasks in 25 mL TB at 29 °C and 180 rpm. NAND (1 g/L) was added in exponentially growing cultures (OD_{600} of 12–15). From the wild-type cultures, 2-mL samples were taken at 0, 5, 9, and 22 h. For HPLC analysis, samples were diluted ten times with 80 % methanol/20 % water and filtered (0.2 μm). HPLC analysis was performed on a Luna 5 μm C18 column (250 mm×4.6 mm i.d.) from Phenomenex using a mobile phase (0.5 mL/min) consisting of 60 % methanol/40 % water. Steroid amounts were calculated based on a calibration curve of NAND. Representative samples were analyzed by the LC-MS method described above.

Results

In vitro biotransformation of NAND by P450 BM3 M02 using purified P450 BM3 M02

Incubations were performed with NAND and purified BM3 mutant M02 enzyme followed by identification of the products formed. The biotransformation of NAND by purified P450 BM3 mutant M02 resulted in 60 % conversion of initial substrate concentration (1 mM) with a total turnover number of 600. LC-MS analysis showed formation of one major metabolite with *m*/*z* value of 289, indicating that the product is mono-hydroxylated. The structure of the metabolite was elucidated by ^{1}H NMR and COSY analysis (Fig. 1). The signal at 3.97 ppm with a triplet multiplicity pattern was assigned to H-16. The characteristic fingerprint pattern at 3.97 ppm indicated that a group containing oxygen is geminal to H-16 (Kirk et al. 1990). Moreover, both the

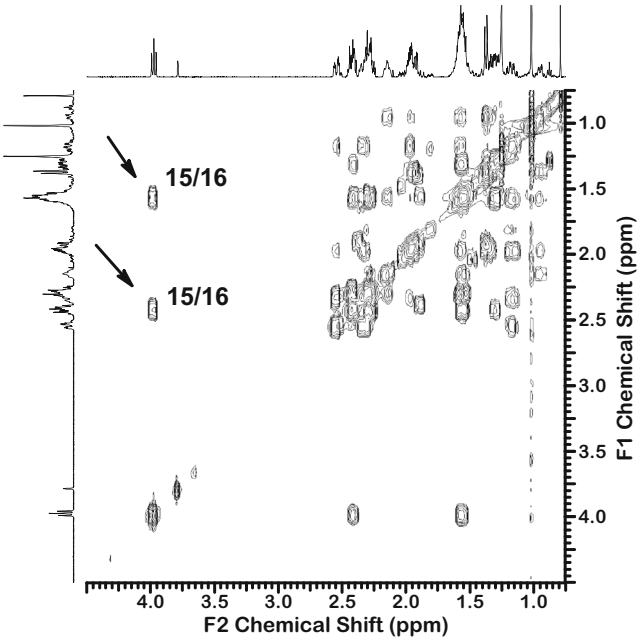

Fig. 1 COSY spectrum of the major metabolite formed during in vitro biotransformation of NAND by P450 BM3 mutant M02 enzyme. Both H-15 protons are connected to a single H-16. Moreover, the chemical shift of 3.97 ppm indicates that a group containing oxygen is geminal to H-16

H-15 protons were coupled to a single H-16 (Fig. 1). The stereoselectivity was assigned based on prior studies in which P450 BM3 mutant M02 was shown to hydroxylate steroids exclusively on the beta-face of the steroid (de Vlieger et al. 2010). Therefore, the major hydroxylated product of BM3 mutant M02 with NAND was assigned as 16-β-OH NAND (Fig. 2).

The binding of NAND to the P450 BM3 mutant M02 protein was studied by UV-VIS spectroscopy. Binding of the substrate resulted in a typical type I protein spectrum with a peak at 393 nm and trough at 420 nm. This is indicative for displacement of a water molecule by substrate with a decrease in the low spin character of the haem iron and increase in the high spin content (Fig. 3). The dissociation constant K_d was measured to be 4.2 μM (Fig. S1), indicating that NAND has high affinity for binding to P450 BM3 mutant M02 protein. The coupling efficiency for the oxidation of NAND to its product was found to be about 33 %

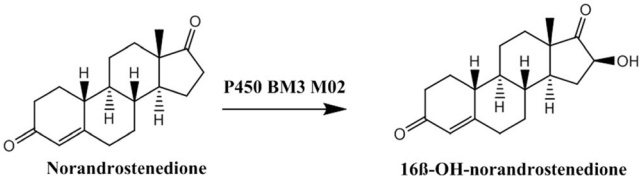

Fig. 2 In vitro biotransformation product of NAND into 16-β-OH NAND by P450 BM3 mutant M02

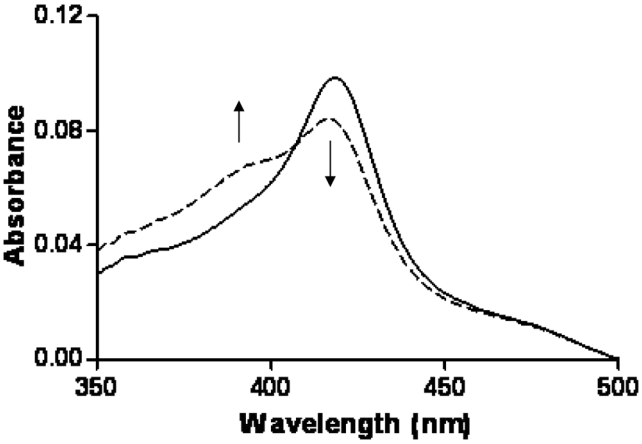

Fig. 3 Substrate-induced spin state shift of P450 BM3 mutant M02 protein. The *solid line* represents the resting state of the enzyme (ligand free). The *dashed line* represents the absolute absorbance spectrum of BM3 mutant M02 in the presence of NAND (100 μM). *Arrows* show the decrease in low spin (420 nm) and an increase in high spin (393 nm) after addition of substrate

(Table 1). This reflects the fact that the coupling efficiency of wild-type P450 BM3 for natural substrates (arachidonic acid) is high (100 %), but significantly lower for non-natural substrates (e.g., steroids) (Li et al. 2001).

In vivo biotransformation of NAND in *R. erythropolis* wild-type and RG9 cells

The wild-type *R. erythropolis* strain rapidly degraded NAND (Fig. 4). After 5 h, more than 80 % of the initial concentration of NAND was degraded. LC-MS

Table 1 In vitro characterization of the biotransformation of NAND by cytochrome P450 BM3 mutant M02

Enzyme	NADPH oxidation[a]	Product formation[b]	Coupling efficiency	Dissociation constant (K_d)[c]
BM3 M02	355.4±21.2	120.4±8.1	33.8±2.8	4.2±0.8

[a] Rates of NADPH oxidation were measured over 60 s at 340 nm as nanomole NADPH consumed/minute/nanomole enzyme. The reaction contained purified P450 BM3 mutant M02 (200 nM), NADPH (200 μM), and NAND (200 μM) in methanol (2 %) and potassium phosphate buffer (100 mM, pH 7.4)

[b] Rates of product formation were measured over 60 s as nanomole of total product formed/minute/nanomole enzyme under the same conditions as above. Coupling efficiency is reported in percentage

[c] Dissociation constants (μM) were determined by UV-VIS spectroscopy from two independent experiments using 1 μM enzyme in 100 mM potassium phosphate buffer, pH 7.4, titrated with a solution of NAND dissolved in methanol. Type I binding spectrum with a peak at 390 nm and trough at 420–422 nm was observed

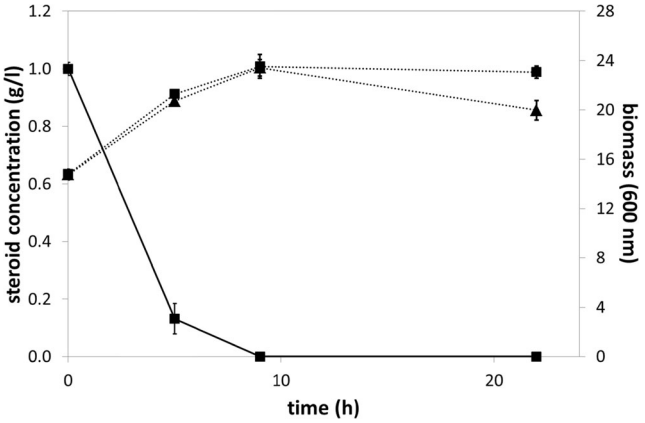

Fig. 4 Bioconversion of NAND by wild-type *R. erythropolis*. Experiments were performed (in triplicate) in 250-mL shaking flasks in 25 mL TB at 29 °C and 180 rpm. Legend: *solid line*, NAND conversion; *dotted lines*, biomass measured as optical densities of the cultures with NAND (*squares*) and without NAND (*triangles*) at 600 nm

analysis of a representative sample showed that NAND was converted to a mono-hydroxylated product (*m*/*z* 289) and a dehydrogenated product (*m*/*z* 271) (Fig. 5). Wild-type *R. erythropolis* is known to possess 3-ketosteroid 9α-hydroxylase and 3-ketosteroid dehydrogenase activity (van der Geize and Dijkhuizen 2004), and therefore, the degradation products are most likely 9α-OH NAND and 1,4-estradiene-3,17-dione (NANDD). After 12 h, almost all the 9α-OH NAND had disappeared again, indicating that catabolic enzymes present in the wild-type strain further degrade this initial product.

In view of the high product specificity of BM3 mutant M02 towards hydroxylation of NAND observed in vitro, the applicability of *R. erythropolis* RG9 as a suitable host for P450 BM3 mutant M02-mediated steroid biotransformation in vivo was further evaluated. Control cultures of mutant strain RG9 cells harboring the null vector pTip-QC1 incubated with NAND were unable to degrade the steroid (Fig. 6a). No significant decrease in the NAND peak was seen over an incubation period of 48 h. Next, we heterologously expressed P450 BM3 mutant M02 in *R. erythropolis* strain RG9 to study selective 16β-hydroxylation of NAND by whole-cell biotransformation.

Heterologous expression of P450 BM3 mutant M02 at a level of approximately 0.2 μM active P450 was achieved with thiostrepton-induced recombinant *R. erythropolis* RG9 cell cultures carrying pTip-QC1 P450 BM3 mutant M02. P450 BM3 mutant M02 protein production did not improve when expression was performed at different growth temperatures, i.e., 18 or 25 °C, or when using additives (δ-aminolevulinic acid (heme precursor), thiamine, iron (III) chloride, and trace elements) known to stimulate P450 protein expression in *E. coli* cells (data not shown).

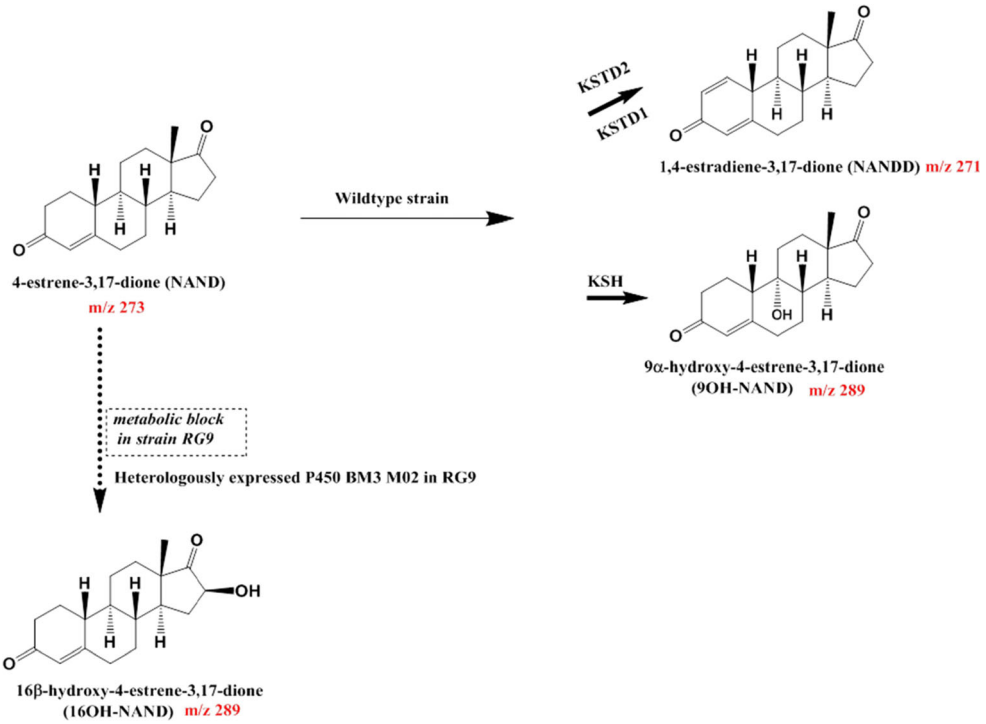

Fig. 5 Overview of degradation of NAND by the *Rhodococcus erythropolis* wild-type strain and conversion of NAND to the desired product by mutant strain RG9 expressing P450 BM3 mutant M02

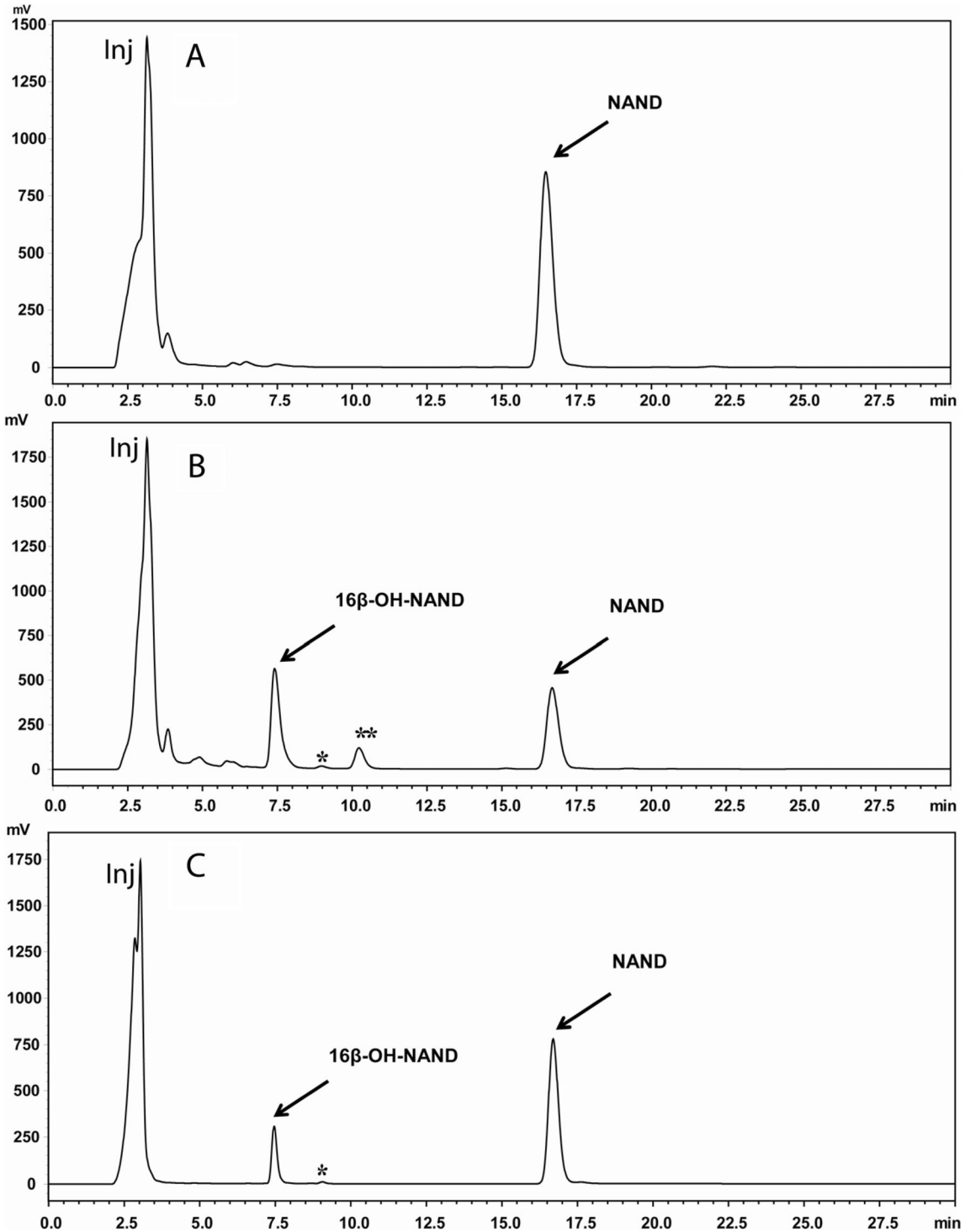

Fig. 6 Representative HPLC chromatograms of NAND bioconversions. **a** *Rhodococcus erythropolis* strain RG9 cells without P450 BM3 mutant M02 after incubation for 22 h (see Fig. 7). **b** Strain RG9 cells with heterologously expressed P450 BM3 mutant M02 after incubation for 22 h (see Fig. 7). **c** In vitro profile of purified BM3 mutant M02 enzyme after incubation for 3 h. Substrate NAND and its major metabolite 16-β-hydroxy norandrostenedione (16-β-OH NAND) have retention times of 17 and 7.5 min, respectively. *Single asterisk*, P450-BM3 mutant M02 enzyme-mediated metabolite peak; *double asterisks*, metabolite peak found in whole-cell reactions

Biotransformation was studied at a NAND concentration of 1 g/L. *R. erythropolis* strain RG9 cells carrying vector pTip-QC1 P450 BM3 mutant M02 converted NAND into the same mono-hydroxylated product

(16-β-OH NAND) (Fig. 6b) as observed in in vitro incubations with purified P450 BM3 mutant M02 protein (Fig. 6c). Given that strain RG9 cells containing the null vector pTip-QCI did not show product formation (Fig. 6a), 16-β-OH NAND product formation from NAND is clearly P450 BM3 mutant M02 enzyme dependent.

Whole-cell bioconversions started with 1 g/L NAND and yielded about 0.35 g/L of 16-β-OH NAND within 20 h. With about 1.2 mM product formed, the total turnover number for 16-β hydroxylation achieved by whole-cell bioconversion was tenfold higher (6000) compared to that observed under in vitro conditions, thus indicating the efficiency of the whole-cell reaction. Further conversion was not observed after 22 h (Fig. 7) and maybe due to the fact that the enzyme had become inactivated.

Discussion

Bioconversion of NAND with the heterologously expressed P450 BM3 mutant M02 enzyme in *R. erythropolis* mutant strain RG9, blocked in NAND degradation, clearly was successful. The whole-cell bioconversion yielded 16-β-OH NAND, the same monohydroxylated product as the in vitro biotransformation of NAND using purified P450 BM3 M02 enzyme. Whole-cell bioconversions with 1 g/L NAND yielded about 0.35 g/L of 16-β-OH NAND; formation of 16-β-OH NAND thus was not stoichiometric with substrate disappearance. This may be due to the degradation of the product by residual ketosteroid dehydrogenase and/or hydroxylase activity. The structure of the steroid scaffold may also play a significant role in inducing synthesis of other catabolic enzymes in *R. erythropolis*. Strain RG9 is unable to degrade androstenedione, but completely degraded its P450 BM3-dependent product, 16-β-OH-androstenedione (Rosłoniec 2010). This 16-β-OH-AD degradation was possibly due to other catabolic enzymes that are still present in this host, e.g., 3-ketosteroid Δ1-dehydrogenase KSTD3 and 3-ketosteroid 9α-hydroxylase KshA2 (Knol et al. 2008; van der Geize et al. 2008). Apparently, 16-β-OH-AD is able to induce such additional catabolic enzymes in strain RG9; most likely also 16β-OH NAND acts as an inducer but to a lesser extent. Alternatively, quantification of product was based on the parent steroid calibration curve which may have resulted in inaccurate estimation of the product concentration.

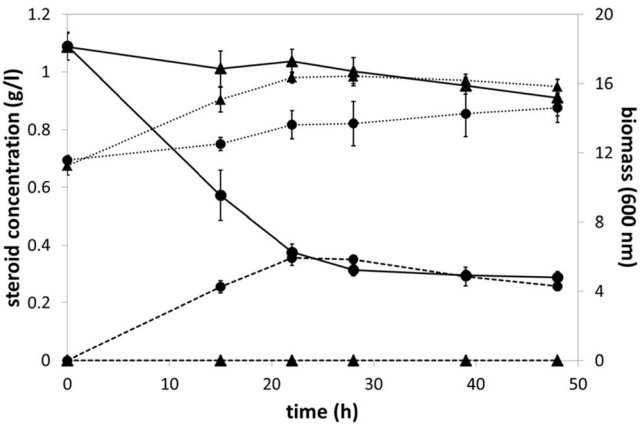

Fig. 7 Bioconversion of NAND by *R. erythropolis* RG9 expressing BM3 mutant M02 protein (*circles*) and *R. erythropolis* RG9 containing the null vector pTip-QC1 (*triangles*). Experiments were performed (in triplicate) in 250-mL shaking flasks in 25 mL TB supplemented with chloramphenicol (34 μg/mL) at 29 °C and 180 rpm. Legend: *solid lines*, NAND conversion; *dashed lines*, steroid product formation; *dotted lines*, biomass measured as optical densities of the cultures at 600 nm

Achieving a higher expression level of P450 enzymes in *Rhodococcus* mutant strains completely blocked in product degradation may improve the total yield of steroid products. The P450 BM3 mutant M02 enzyme expressed in *R. erythropolis* mutant strain RG9 achieved excellent steroid turnover numbers already. *E. coli* is commonly used as a host for biocatalysis as it lacks endogenous P450 enzymes and therefore provides a clean system. However, substrate uptake is also an important factor for the selection of a host organism in whole-cell biocatalysis. In *E. coli*, the uptake of hydrophobic substrates, like steroids, is relatively inefficient (Urlacher and Girhard 2011). *Rhodococcus* strains on the other hand are highly efficient in steroid substrate uptake due to a (more) accessible cell wall and the presence of active transporters (Mohn et al. 2008). Relatively high concentrations of hydrophobic substrates therefore can be used in whole-cell catalysis with *Rhodococcus* strains as host organisms.

In conclusion, the potential use of *R. erythropolis* strain RG9 expressing the P450 BM3 mutant M02 enzyme has been evaluated for whole-cell biocatalysis using a 17-ketosteroid as model compound: norandrostenedione. To our knowledge, the current study is the first report about whole-cell biotransformation of a heterologously expressed P450 BM3 enzyme in *Rhodococcus*. The combination of this P450 BM3 mutant enzyme with a genetically engineered *Rhodococcus* strain blocked at the level of steroid ring degradation provides a promising option for biocatalysis to further expand the range of possible steroid products formed.

Acknowledgments This work was financially supported by the Netherlands Organization for Scientific Research (NWO) within the IBOS (Integration of Biosynthesis and Organic Synthesis) program.

References

Bernhardt R (2006) Cytochromes P450 as versatile biocatalysts. J Biotechnol 124:128–145

Bleif S, Hannemann F, Zapp J, Hartmann D, Jauch J, Bernhardt R (2012) A new *Bacillus megaterium* whole-cell catalyst for the hydroxylation of the pentacyclic triterpene 11-keto-beta-boswellic acid (KBA) based on a recombinant cytochrome P450 system. Appl Microbiol Biotechnol 93:1135–1146

Chefson A, Auclair K (2006) Progress towards the easier use of P450 enzymes. Mol Biosyst 2:462–469

Clouthier CM, Pelletier JN (2012) Expanding the organic toolbox: a guide to integrating biocatalysis in synthesis. Chem Soc Rev 41: 1585–1605

Damsten MC, van Vugt-Lussenburg BM, Zeldenthuis T, de Vlieger JS, Commandeur JN, Vermeulen NP (2008) Application of drug metabolising mutants of cytochrome P450 BM3 (CYP102A1) as biocatalysts for the generation of reactive metabolites. Chem Biol Interact 171:96–107

de Vlieger JS, Kolkman AJ, Ampt KA, Commandeur JN, Vermeulen NP, Kool J, Wijmenga SS, Niessen WM, Irth H, Honing M (2010) Determination and identification of estrogenic compounds generated with biosynthetic enzymes using hyphenated screening assays, high resolution mass spectrometry and off-line NMR. J Chromatogr B Analyt Technol Biomed Life Sci 878:667–674

Donova MV, Egorova OV (2012) Microbial steroid transformations: current state and prospects. Appl Microbiol Biotechnol 94: 1423–1447

Drăgan CA, Zearo S, Hannemann F, Bernhardt R, Bureik M (2005) Efficient conversion of 11-deoxycortisol to cortisol (hydrocortisone) by recombinant fission yeast *Schizosaccharomyces pombe*. FEMS Yeast Res 5:621–625

Fasan R, Crook NC, Peters MW, Meinhold P, Buelter T, Landwehr M, Cirino PC, Arnold FH (2011) Improved product-per-glucose yields in P450-dependent propane biotransformations using engineered *Escherichia coli*. Biotechnol Bioeng 108: 500–510

Gudiminchi RK, Randall C, Opperman DJ, Olaofe OA, Harrison ST, Albertyn J, Smit MS (2012) Whole-cell hydroxylation of n-octane by *Escherichia coli* strains expressing the CYP153A6 operon. Appl Microbiol Biotechnol 96:1507–1516

Hollmann F, Arends IWCE, Buehler K, Schallmey A, Buhler B (2011) Enzyme-mediated oxidations for the chemist. Green Chem 13:226–265

Hughes J, Armitage YC, Symes KC (1998) Application of whole cell rhodococcal biocatalysts in acrylic polymer manufacture. Antonie Van Leeuwenhoek 74:107–118

Ishige T, Honda K, Shimizu S (2005) Whole organism biocatalysis. Curr Opin Chem Biol 9:174–180

Julsing MK, Cornelissen S, Buhler B, Schmid A (2008) Heme-iron oxygenases: powerful industrial biocatalysts? Curr Opin Chem Biol 12:177–186

Kille S, Zilly FE, Acevedo JP, Reetz MT (2011) Regio- and stereoselectivity of P450-catalysed hydroxylation of steroids controlled by laboratory evolution. Nat Chem 3:738–743

Kirk DN, Toms HC, Douglas C, White KA, Smith KE, Latif S, Hubbard RWP (1990) A survey of the high-field H-1-NMR spectra of the steroid-hormones, their hydroxylated derivatives, and related-compounds. J Chem Soc Perk T 2:1567–1594

Knol J, Bodewits K, Hessels GI, Dijkhuizen L, van der Geize R (2008) 3-Keto-5alpha-steroid Delta (1)-dehydrogenase from *Rhodococcus erythropolis* SQ1 and its orthologue in *Mycobacterium tuberculosis* H37Rv are highly specific enzymes that function in cholesterol catabolism. Biochem J 410:339–346

Kolar NW, Swart AC, Mason JI, Swart P (2007) Functional expression and characterisation of human cytochrome P45017alpha in *Pichia pastoris*. J Biotechnol 129:635–644

Li QS, Ogawa J, Schmid RD, Shimizu S (2001) Engineering cytochrome P450 BM-3 for oxidation of polycyclic aromatic hydrocarbons. Appl Environ Microbiol 67:5735–5739

Martinkova L, Uhnakova B, Patek M, Nesvera J, Kren V (2009) Biodegradation potential of the genus *Rhodococcus*. Environ Int 35:162–177

McLeod MP, Warren RL, Hsiao WW, Araki N, Myhre M, Fernandes C, Miyazawa D, Wong W, Lillquist AL, Wang D, Dosanjh M, Hara H, Petrescu A, Morin RD, Yang G, Stott JM, Schein JE, Shin H, Smailus D, Siddiqui AS, Marra MA, Jones SJ, Holt R, Brinkman FS, Miyauchi K, Fukuda M, Davies JE, Mohn WW, Eltis LD (2006) The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. Proc Natl Acad Sci U S A 103:15582–15587

Mohn WW, van der Geize R, Stewart GR, Okamoto S, Liu J, Dijkhuizen L, Eltis LD (2008) The actinobacterial *mce4* locus encodes a steroid transporter. J Biol Chem 283:35368–35374

Munro AW, Leys DG, McLean KJ, Marshall KR, Ost TW, Daff S, Miles CS, Chapman SK, Lysek DA, Moser CC, Page CC, Dutton PL (2002) P450 BM3: the very model of a modern flavocytochrome. Tr Biochem Sci 27:250–257

Nakashima N, Tamura T (2004) Isolation and characterization of a rolling-circle-type plasmid from *Rhodococcus erythropolis* and application of the plasmid to multiple-recombinant-protein expression. Appl Environ Microbiol 70:5557–5568

Petric S, Hakki T, Bernhardt R, Zigon D, Cresnar B (2010) Discovery of a steroid 11alpha-hydroxylase from *Rhizopus oryzae* and its biotechnological application. J Biotechnol 150:428–437

Plaggenborg R, Overhage J, Loos A, Archer JA, Lessard P, Sinskey AJ, Steinbuchel A, Priefert H (2006) Potential of *Rhodococcus* strains for biotechnological vanillin production from ferulic acid and eugenol. Appl Microbiol Biotechnol 72:745–755

Quan S, Dabbs ER (1993) Nocardioform arsenic resistance plasmid characterization and improved *Rhodococcus* cloning vectors. Plasmid 29:74–79

Ran N, Zhao L, Chen Z, Tao J (2008) Recent applications of biocatalysis in developing green chemistry for chemical synthesis at the industrial scale. Green Chem 10:361–372

Rea V, Kolkman AJ, Vottero E, Stronks EJ, Ampt KA, Honing M, Vermeulen NP, Wijmenga SS, Commandeur JN (2012) Active site substitution A82W improves the regioselectivity of steroid hydroxylation by cytochrome P450 BM3 mutants as rationalized by spin relaxation nuclear magnetic resonance studies. Biochemistry 51: 750–760

Rosłoniec KZ (2010) Steroid transformation by *Rhodococcus* strains and bacterial cytochrome P450 enzymes. PhD Dissertation, University of Groningen

Schewe H, Holtmann D, Schrader J (2009) P450(BM-3)-catalyzed whole-cell biotransformation of alpha-pinene with recombinant *Escherichia coli* in an aqueous-organic two-phase system. Appl Microbiol Biotechnol 83:849–857

Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B (2001) Industrial biocatalysis today and tomorrow. Nature 409: 258–268

Siriphongphaew A, Pisnupong P, Wongkongkatep J, Inprakhon P, Vangnai AS, Honda K, Ohtake H, Kato J, Ogawa J, Shimizu S, Urlacher VB, Schmid RD, Pongtharangkul T (2012) Development of a whole-cell biocatalyst co-expressing P450 monooxygenase and glucose dehydrogenase for synthesis of epoxyhexane. Appl Microbiol Biotechnol 95:357–367

Taguchi K, Motoyama M, Kudo T (2004) Multiplicity of 2,3-dihydroxybiphenyl dioxygenase genes in the Gram-positive polychlorinated biphenyl degrading bacterium *Rhodococcus rhodochrous* K37. Biosci Biotechnol Biochem 68:787–795

Urlacher VB, Girhard M (2011) Cytochrome P450 monooxygenases: an update on perspectives for synthetic application. Tr Biotechnol 30: 26–36

Urlacher VB, Lutz-Wahl S, Schmid RD (2004) Microbial P450 enzymes in biotechnology. Appl Microbiol Biotechnol 64:317–325

van der Geize R, Dijkhuizen L (2004) Harnessing the catabolic diversity of rhodococci for environmental and biotechnological applications. Curr Opin Microbiol 7:255–2261

van der Geize R, Hessels GI, van Gerwen R, Vrijbloed JW, van Der Meijden P, Dijkhuizen L (2000) Targeted disruption of the *kstD* gene encoding a 3-ketosteroid delta (1)-dehydrogenase isoenzyme of *Rhodococcus erythropolis* strain SQ1. Appl Environ Microbiol 66: 2029–2036

van der Geize R, Hessels GI, van Gerwen R, van der Meijden P, Dijkhuizen L (2001) Unmarked gene deletion mutagenesis of *kstD*, encoding 3-ketosteroid Delta1-dehydrogenase, in *Rhodococcus erythropolis* SQ1 using *sacB* as counter-selectable marker. FEMS Microbiol Lett 205:197–202

van der Geize R, Hessels GI, Dijkhuizen L (2002a) Molecular and functional characterization of the *kstD2* gene of *Rhodococcus erythropolis* SQ1 encoding a second 3-ketosteroid Delta (1)-dehydrogenase isoenzyme. Microbiol 148:3285–3292

van der Geize R, Hessels GI, van Gerwen R, van der Meijden P, Dijkhuizen L (2002b) Molecular and functional characterization of *kshA* and *kshB*, encoding two components of 3-ketosteroid 9alpha-hydroxylase, a class IA monooxygenase, in *Rhodococcus erythropolis* strain SQ1. Mol Microbiol 45:1007–1018

van der Geize R, Hessels GI, Nienhuis-Kuiper M, Dijkhuizen L (2008) Characterization of a second *Rhodococcus erythropolis* SQ1 3-ketosteroid 9alpha-hydroxylase activity comprising a terminal oxygenase homologue, KshA2, active with oxygenase-reductase component KshB. Appl Environ Microbiol 74:7197–7203

van Vugt-Lussenburg BM, Stjernschantz E, Lastdrager J, Oostenbrink C, Vermeulen NP, Commandeur JN (2007) Identification of critical residues in novel drug metabolizing mutants of cytochrome P450 BM3 using random mutagenesis. J Med Chem 50:455–461

Venkataraman H, Beer SB, Bergen LA, Essen N, Geerke DP, Vermeulen NP, Commandeur JN (2012a) A single active site mutation inverts stereoselectivity of 16-hydroxylation of testosterone catalyzed by engineered cytochrome P450 BM3. Chembiochem 13:520–523

Venkataraman H, de Beer SBA, Geerke DP, Vermeulen NPE, Commandeur JNM (2012b) Regio- and stereoselective hydroxylation of optically active α-ionone enantiomers by engineered cytochrome P450 BM3 mutants. Adv Synth Catal 354:2172–2184

Whitehouse CJ, Bell SG, Wong LL (2012) P450(BM3) (CYP102A1): connecting the dots. Chem Soc Rev 41:1218–1260

Yim SK, Kim DH, Jung HC, Pan JG, Kang HS, Ahn T, Yun CH (2010) Surface display of heme- and diflavin-containing cytochrome P450 BM3 in *Escherichia coli*: a whole cell biocatalyst for oxidation. J Microbiol Biotechnol 20:712–717

Zehentgruber D, Dragan CA, Bureik M, Lutz S (2010a) Challenges of steroid biotransformation with human cytochrome P450 monooxygenase CYP21 using resting cells of recombinant *Schizosaccharomyces pombe*. J Biotechnol 146:179–185

Zehentgruber D, Hannemann F, Bleif S, Bernhardt R, Lutz S (2010b) Towards preparative scale steroid hydroxylation with cytochrome P450 monooxygenase CYP106A2. Chembiochem 11:713–721