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Improvement of a P450-Based Recombinant *Escherichia coli* Whole-Cell System for the Production of Oxygenated Sesquiterpene Derivatives

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ABSTRACT: Sesquiterpenes are common constituents of essential oil in plants. Their oxygenated derivatives often possess desirable flavor, fragrance, and pharmaceutical properties. Recently, the CYP264B1-based recombinant *Escherichia coli* whole-cell system has been constructed for the oxidation of sesquiterpenes. However, limiting factors of this system related to the high volatility of substrates and the suitability of the P450 redox partner need to be addressed. In this work, the improvement of the system was implemented with (+)-α-longipinene as a model substrate. By using 2-hydroxypropyl-β-cyclodextrin and an alternative ferredoxin reductase, the conversion of (+)-α-longipinene was improved 77.1%. Applying the optimized conditions, the yields of the main products were 54.2, 34.2, and 47.2 mg L^{-1}, corresponding to efficiencies of 82.1, 51.8, and 71.5% for the conversion of (+)-α-longipinene, (−)-isolongifolene, and α-humulene, respectively, at a 200 mL scale. These products were characterized as 12-hydroxy-α-longipinene, isolongifolene-9-one, and 5-hydroxy-α-humulene, respectively, by nuclear magnetic resonance spectroscopy.

KEYWORDS: *cytochrome P450, CYP264B1, adrenodoxin, adrenodoxin reductase, ferredoxin reductase, sesquiterpenes, whole-cell conversion, cyclodextrin*

INTRODUCTION

Sesquiterpenes are terpenes consisting of three isoprene units that are present as common constituents of essential oil in plants. Their oxygenated derivatives play significant roles in the fragrance and perfume industry, or they can be used as a starting material for synthesizing higher value compounds.[1−3] In addition, they may exhibit biological activities such as antioxidant, antibacterial, anti-inflammatory, and anticarcinogenic effects.[4,5] This makes these compounds a potentially rich reservoir for the discovery of new drugs, flavors, and fragrances.

To synthesize a desired oxygenated sesquiterpene, regio- and stereoselective oxidations are required. These reactions are difficult to conduct by organic synthesis, especially if performed on inactive carbon atoms.[6,7] Biooxidation systems, however, are able to perform such reactions under mild conditions, which are environmentally friendly and more advantageous in terms of cost and time efficiency. Of these, cytochrome P450 monooxygenases (P450s) emerged as highly interesting oxidative biocatalysts. Biosynthesis of the sesquiterpene antibiotic albaflavenone from epi-isozizaene can be achieved through two sequential allylic oxidations by CYP170A1.[8] Regioselective oxidation of (+)-valencene at allylic position C2 by CYP109B1 gives nootkatone, a highly sought-after fragrance in the food and flavor industry.[9] Another example is the oxidation from amorpha-4,11-diene to artemisinic acid mediated by CYP71AV1, the key step of the biosynthesis of the antimalarial drug artemisinin.[10] With their ability to introduce oxygen into allylic positions, or even into nonactivated C−H bonds, P450s thus have great potential for synthesizing high-value compounds from natural products.[11,12]

To date, several P450 enzymes have been reported to be capable of oxidizing diverse sesquiterpene substrates, such as premnaspirodiene oxygenase from the plant *Hyoscyamus muticus*,[13] CYP71AV8 from the plant *Cichorium intybus* L.,[14] and CYP260A1 and CYP264B1 from the myxobacterium *Sorangium cellulosum* So ce56. Among them, CYP264B1 is a promising enzyme for the biotransformation of sesquiterpenes due to its ability to perform regioselective hydroxylation on various structural sesquiterpene classes.[15,16] Because P450s from bacteria are often not membrane associated and normally exhibit higher stabilities and activities, they are more desirable for practical application than those from plants.[17] Thus, CYP264B1 is an attractive candidate for the oxidation of sesquiterpene compounds.

For the usage of P450s, one of the most important drawbacks is the cofactor dependence of this enzyme group. P450s do not function independently like other enzymes, but utilize electrons from an external donor, mostly from NAD(P)H, to activate molecular oxygen for substrate conversion. Moreover, these redox equivalents are transferred via an electron-transfer chain, which consists of one or more proteins, also referred to as redox partners.[18] To overcome this challenge, the current trend

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in P450 research is the creation of whole-cell biocatalysts using recombinant microorganisms carrying genes coding for P450s and the respective redox partners. The advantages of this solution are that (i) the cofactors are supplied by host cells themselves, (ii) the enzymes are more stable within intact cells, and (iii) the proteins in whole cells do not require laborious purification processes.[12,18]

In the whole-cell conversion of sesquiterpenes, a factor that decreases the efficiency of the system is the volatility of these substrates. Sesquiterpenes are lipophilic and volatile compounds; they are thus scarcely soluble in water and easily escape from the culture. A strategy to address this problem is the use of cyclodextrins (CDs) to retain the volatile substrate in the medium. CDs are known as useful tools in enhancing the bioconversion of hydrophobic compounds due to their biocompatibility with microbes.[19−23] These oligosaccharides are composed of (1→4)-linked α-D-glucosyl units that form a shape of truncated cones.[24,25] The external surface of CDs is sufficiently hydrophilic to render them soluble in water. In contrast, their interior is considerably less hydrophilic than the aqueous environment, and thus it is able to hold appropriately sized nonpolar moieties of other hydrophobic molecules within the cavity, without the formation or disturbance of covalent bonds.[26] The binding between the host CD and the guest molecule is rather in a dynamic equilibrium than being fixed or permanent.[27] In addition, when contacting cells, CDs were supposed to extract and form inclusion complexes with lipids and other hydrophobic moieties from the cell membrane, making it more permeable, which facilitates uptake of substrate into the *Escherichia coli* cells.[28] Therefore, CDs are able to improve the solubility of substances and fixation of volatile compounds.

Another important issue in the application of P450-based whole-cell systems is the selection of an appropriate electron-transfer system. As the cytochrome P450 enzyme family is widespread in nature, their electron-transfer chains are variable.[29] Especially in the case of bacterial P450s, the corresponding redox chain is difficult to identify. Because of the random distribution of the natural redox partners of a P450 in the genome of an organism, the search for these proteins is challenging. However, there are heterologous redox partners available, which can interact with a broad range of P450s, such as spinach ferredoxin−ferredoxin reductase, putidaredoxin−putidaredoxin reductase from *Pseudomonas putida* (Pdx-PdR), *E. coli* flavodoxin−flavodoxin reductase (Fld-Fpr), and mammalian mitochondrial adrenodoxin−adrenodoxin reductase (Adx-AdR). In addition, $Adx_{4\text{-}108}$ (the truncated form of Adx) was shown to interact with many P450s and to be reduced by several reductases.[30] Recently, a versatile electron-transfer system containing $Adx_{4\text{-}108}$ and Fpr for P450-based *E. coli* whole-cell biotransformation has been constructed.[31] This system was successfully applied for whole-cell systems using CYP264A1 and CYP106A2,[31] CYP260A1,[32] and CYP260B1 and CYP267B1.[33] By applying this system, the whole-cell system containing CYP264B1-$Adx_{4\text{-}108}$-Fpr was also constructed for the conversion of sesquiterpenes.[16] The reason for the use of Fpr instead of AdR—the natural redox partner of Adx—in these studies was that AdR is a membrane-associated protein, which is challenging to express in *E. coli*. Although the higher expression level of Fpr was suspected to be the reason for the higher efficiency of the P450-based whole-cell system,[31] this might not be the case for all other P450 systems, because the biotransformation depends not only on the content of the reductase but also on the other components of the electron-transfer chain as well as the molar ratio between them.[34,35] The optimal molar ratio of CYP264B1/Adx/AdR for the in vitro conversion of its substrate was found to be 1:20:1.[15] The ratio of Adx/AdR expressed in *E. coli* by Salamanca-Pinzón and Guengerich is 920 nmol/500 nmol.[36] Schiffer et al. were able to enhance product level in their whole-cell system by increasing the Adx amount, confirming that the AdR level was not the limiting factor for the efficiency of the whole-cell conversion when using the *E. coli* C43(DE3) strain.[35] Moreover, the *E. coli* C43(DE3) strain used in this study has previously been reported as advantageous for the synthesis of membrane proteins.[37,38] Many membrane-associated proteins were overexpressed in this strain in high yields.[39] Furthermore, the slowest and rate-limiting step in the P450 electron transport chain is the interaction between the ferredoxin and the P450.[30] Therefore, when expressed in *E. coli* C43(DE3), the expression level of AdR should be sufficient and ready for substrate conversion by resting cells in comparison to the expression level of Adx and CYP264B1.

For all of these reasons, the two CYP264B1-based whole cell biocatalysts containing either AdR or Fpr were compared to find the better system for the transformation of sesquiterpenes. In addition, the limiting factors related to the substrate's volatility were investigated. As a model reaction, the conversion of (+)-α-longipinene was chosen because CYP264B1 converts this sesquiterpene substrate in a highly specific way. By using 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and alternative redox partner systems, the efficiency of the system was improved. Applying the optimized conditions to the transformation of other sesquiterpenes, (−)-isolongifolene and α-humulene, we were able to demonstrate that our new whole-cell system is an efficient biocatalyst for the production of oxygenated sesquiterpene derivatives.

MATERIALS AND METHODS

Materials. All sesquiterpene substrates (GC grade) including (+)-α-longipinene, (−)-isolongifolene, and α-humulene were purchased from Sigma-Aldrich. The expression vector pET17b and the expression host *E. coli* C43(DE3) were obtained from Invitrogen and Novagen, respectively. 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) and other chemicals were obtained from Sigma-Aldrich.

Protein Expression and Purification. The expression and purification of the CYP264B1 were performed as described previously.[15,16] The bovine adrenodoxin reductase (AdR) and the truncated bovine adrenodoxin ($Adx_{4\text{-}108}$) used for the in vitro conversion were expressed and purified according to procedures published by Sagara et al.[40] and Uhlmann et al.,[41] respectively.

Vector Construction. The vector pETC4FA was constructed as described previously.[16] The vector pETC4AA was constructed as follows: A DNA fragment containing adrenodoxin reductase (AdR) and truncated adrenodoxin ($Adx_{4\text{-}108}$) encoding genes with a Shine−Dalgarno site upstream of each gene was cut from the vector pETMR5[31] by *Hin*dIII and *Eco*RI. This fragment was then inserted into the vector pETC4 containing the gene coding for CYP264B1, using the same restriction sites, resulting in the tricistronic vector pETC4AA containing CYP264B1, AdR, and Adx encoding genes.

In Vitro Conversion. The in vitro conversions of substrates were carried out in a final volume of 500 μL in 20 mM potassium phosphate buffer, pH 7.4, with 20% glycerol (KppG buffer). The reaction mixture contained 2 μM CYP264B1, 200 μM substrate, 40 μM $Adx_{4\text{-}108}$, 2 μM AdR, and a NADPH regenerating system consisting of 5 mM glucose-6-phosphate, 1 U of glucose-6-phosphate dehydrogenase, and 1 mM $MgCl_2$. Reactions were initiated by the addition of 0.1 mM NADPH. After incubation at 30 °C for 30 min in a thermomixer (Eppendorf), the reactions were stopped by adding 500 μL of ethyl acetate. The

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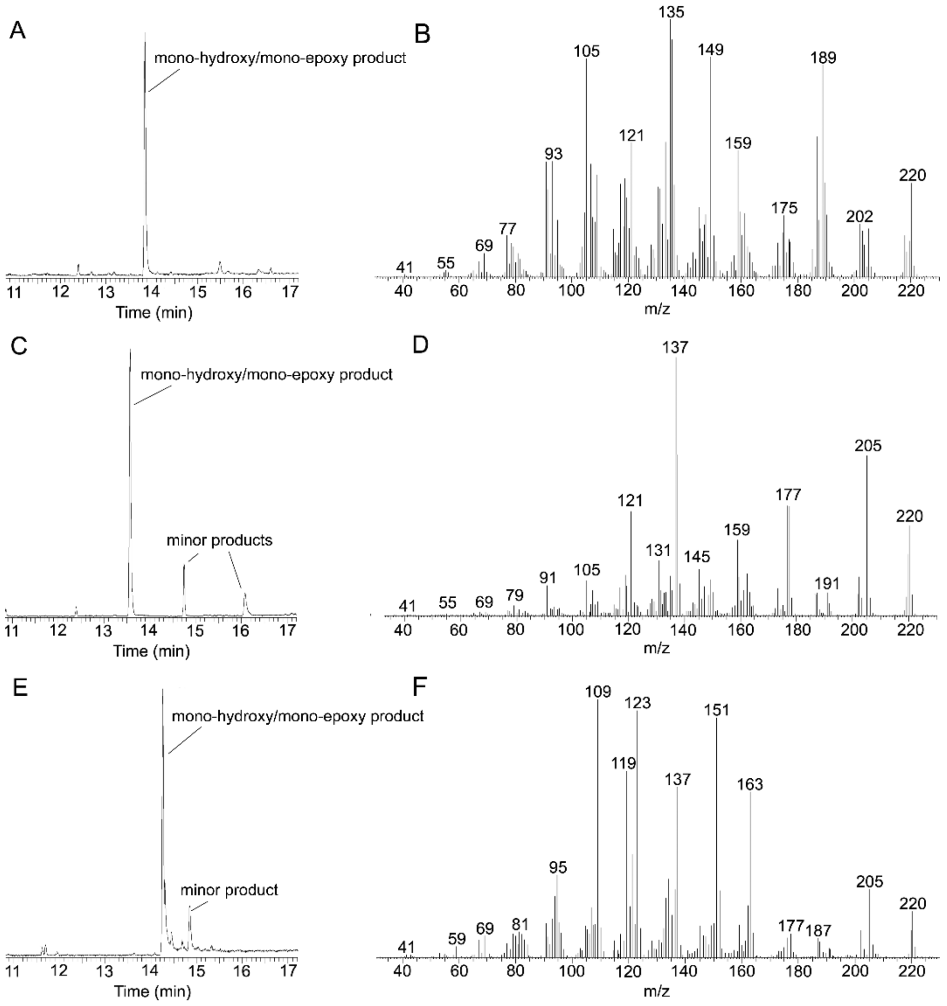

Figure 1. Total ion chromatograms and mass spectra of the major products obtained by GC-MS analysis from the in vitro conversions of (+)-α-longipinene (A, B), (−)-isolongifolene (C, D), and α-humulene (E, F).

samples were extracted twice and the extracts dried in a vacuum-dryer. The residuals were dissolved in 50 μL of ethyl acetate for gas chromatography−mass spectrometry (GC-MS) analysis.

In Vivo Conversion. *Protein Expression.* *E. coli* C43(DE3) was transformed with the plasmid pETC4AA. Transformants were grown in 10 mL of Terrific broth (TB) medium (Becton Dickinson) containing ampicillin (100 mg mL^{-1}) at 37 °C with shaking at 120 rpm overnight. The overnight culture (3 mL) was diluted in 300 mL of TB medium containing 100 mg mL^{-1} ampicillin in a 2 L baffled flask and grown at 37 °C under constant shaking until the OD_{600} reached 0.8−1.0. The protein expression was then induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. δ-Aminolevulinic acid (δ-Ala) was also added to a final concentration of 0.5 mM at this time to support the heme synthesis. The culture was further incubated at 28 °C under constant shaking at 200 rpm.

Incorporation of Sesquiterpene Substrate with HP-β-CD. The sesquiterpenes were dissolved in 100% ethanol to a final concentration of 1 M (stock solution). To incorporate sesquiterpenes into HP-β-CD, the sesquiterpene stock solution was added dropwise to the HP-β-CD solution (10% in sterile distilled water) to a final sesquiterpene concentration of 2 mM. The (+)-α-longipinene−HP-β-CD solution was further stirred constantly for 3 h.

Standard in Vivo Conversion. After 24 h of protein expression, the cells were harvested and washed with Kpp buffer (50 mM potassium phosphate, pH 7.4) by centrifugation at 4000*g* for 10 min at 4 °C. The cells were then resuspended in KppG buffer (50 mM potassium phosphate, pH 7.4, containing 2% glycerol, 1 mM IPTG, 0.5 mM δ-Ala, 100 μg mL^{-1} ampicillin), whereupon the cell wet weight was adjusted to 25 g L^{-1}. The sesquiterpenes as substrates in HP-β-CD solution were added to a final concentration of 200 μM for the sesquiterpenes (approximately 44 mg L^{-1}) and 1% for HP-β-CD. The conversion was carried out in baffled flasks at 30 °C and a shaking speed of 200 rpm. After 24 h, the reactions were stopped by cooling them to 4 °C for 1 h. An equal volume of ethyl acetate was added, and the products were extracted twice. The organic phases were pooled, the solvent was evaporated in a rotary vacuum system, and the crude extract was kept at −20 °C until purification.

Evaluation of the Influence of HP-β-CD on Product Yield. The transformations with and without HP-β-CD were carried out in both aluminum foil wrapped baffled flasks and screw-cap sealed baffled flasks. The conditions for each experiment were the same as the standard in vivo conversion with total reaction volume of 10 mL in a 100 mL flask. After 24 h, samples of 500 μL were taken for GC-MS analysis.

Purification of Products. The extracts of sesquiterpene conversions were dissolved in ethyl acetate and loaded on a silica gel column (35−70 mesh particle size, 1.5 cm × 60 cm). A mobile phase containing ethyl acetate and hexane (1:9) was used. The fractions were analyzed by thin layer chromatography (as described below) using the extract from the in vitro conversions of each substrate as the positive control and the same solvent mixture as the mobile phase. The fractions containing the product were combined and dried in a rotary vacuum system.

Thin Layer Chromatography (TLC) Analysis. Samples were spotted onto TLC aluminum plates (silica gel 60, Fluka). The plates

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were developed in a solvent tank containing a mixture of *n*-hexane/ethyl acetate (1:9). After developing, the TLC plates were dried and stained with a vanillin solution of 1% (w/v) in ethanol/sulfuric acid (95:5) and subsequently heated for 2 min at 110 °C to visualize the patterns.

GC-MS Analysis. The GC-MS analysis was carried out using a Focus GC/DSQ II GC/MS (Thermo Scientific) equipped with an HP-5 capillary column (25 m × 0.32 mm i.d., 0.52 μm film; Agilent Technologies) under the following conditions: column flow, 10 mL min^{-1}; injection volume, 1 μL; transfer line, 280 °C; electron energy, 70.5 eV. The starting oven temperature was 50 °C for 1 min, and then the temperature was increased by 10 °C min^{-1} to 310 °C and held for 5 min (operated in splitless mode). The mass spectra were recorded in a *m*/*z* range of 20−350.

For the quantification of sesquiterpene products, a standard curve using eudesmol was built with a set of concentrations of 15, 30, 60, 120, and 240 μM. The chromatographic peak areas of each sample were plotted against their respective concentrations, and a calibration curve was generated by linear regression analysis. The eudesmol internal standard (50 μM) was added to the samples before extraction with 1 volume of ethyl acetate for GC-MS analysis. The sesquiterpene product peak areas were normalized corresponding to the internal standard eudesmol. The sesquiterpene concentration in the sample was calculated by applying the linear equation of the calibration curve to each adjusted product peak area.

NMR Characterization of the Products. The NMR spectra of the products were recorded in $CDCl_3$ with a Bruker DRX 500 NMR (Billerica, MA, USA). All chemical shifts are relative to $CHCl_3$ or $CDCl_3$ (δ 77.00 for ^{13}C NMR, δ 7.24 for ^{1}H NMR) using the standard δ notation in parts per million.

12-Hydroxy-α-longipinene: ^{1}H NMR ($CDCl_3$; 500 MHz) δ 2.13 (1H, m, H-1); 1.56−1.65 (4H, m, H-4b, H-5b, and H-3); 1.35 (2H, m, H-4a and H-5a); 1.48 (1H, m, H-7); 2.24 (1H, d, J = 6.5 Hz); 5.46 (1H, brs, H-10); 2.30 (2H, brs, H-11); 3.99 (2H, brs, H-12); 0.82 (3H, s, H-13); 0.91 (3H, s, H-14); 0.82 (3H, s, H-15). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 40.1 (C-1); 39.8 (C-2); 40.9 (C-3); 21.7 (C-4); 39.1 (C-5); 32.8 (C-6); 59.1 (C-7); 42.7 (C-8); 151.0 (C-9); 119.1 (C-10); 34.1 (C-11); 65.9 (C-12); 23.9 (C-13); 27.9 (C-14); 27.8 (C15).

Isolongifolene-9-one. ^{1}H NMR ($CDCl_3$; 500 MHz) δ 1.91−1.98 (2H, m, H-3 and H-5a); 1.58 (1H, m, H-4a); 1.73−1.77 (1H, m, H-4b); 1.39 (1H, m, H-5b); 2.06 (1H, d, J = 15 Hz, H-8a); 2.38 (1H, d, J = 15 Hz, H-8b); 5.69 (1H, s, H-10); 1.41 (2H, m, H-11); 1.06 (3H, s, H-12); 1.12 (3H, s, H-13); 1.03 (3H, s, H-14); 0.97 (3H, s, H-15). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 184.0 (C-1); 44.1 (C-2); 46.4 (C-3); 24.3 (C-4); 27.8 (C-5); 58.7 (C-6); 34.5 (C-7); 49.9 (C-8); 200.3 (C-9); 116.9 (C-10); 36.7 (C-11); 27.1 (C-12); 24.6 (C-13); 25.8 (C-14); 25.4 (C-15).

5-Hydroxy-α-humulene. The NMR data for this compound are presented in Schifrin et al.[16]

Statistical Methods. Data are represented as the mean values ± SD of three independent experiments. Statistical analysis was performed using Student's t test, and the values were taken as significantly different when $p \leq 0.05$.

RESULTS AND DISCUSSION

In Vitro Conversion of Sesquiterpenes by the CYP264B1 System. The in vitro assays were carried out to provide the product pattern standards for the in vivo conversion. In this study, the three sesquiterpenes (+)-α-longipinene, (−)-isolongifolene, and α-humulene were used. The mitochondrial redox partners from bovine adrenals (AdR and Adx_{4-108}) were used because they have been proven to be efficient in supporting the CYP264B1 activity.[15] The in vitro CYP264B1-dependent conversions of (+)-α-longipinene, (−)-isolongifolene, and α-humulene were carried out as described (see Materials and Methods) and analyzed by GC-MS. The results showed that in the reaction mixtures of all three substrates new peaks occurred, which did not appear in the control experiments. (+)-α-Longipinene was converted into only one product at a retention time of 13.85 min (Figure 1A). The major ions with their intensities (in parentheses) in the mass spectrum of this product were as follows: 41 (1), 55 (3), 69 (10), 77 (16), 93 (46), 105 (85), 121 (53), 135 (100), 149 (84), 159 (49), 175 (25), 189 (82), 202 (201), and 220 (38) (Figure 1B). (−)-Isolongifolene was converted into a major product (73%) at a retention time of 13.57 min and two minor products at retention times of 14.75 and 16.07 min, respectively (Figure 1C). The major ions with their intensities in the mass spectrum of the major product of this sesquiterpene were as follows: 55 (1), 69 (2), 79 (5), 91 (12), 105 (13), 121 (41), 131 (22), 137 (100), 145 (18), 149 (56), 159 (30), 177 (43), 191 (10), 205 (64), and 220 (35) (Figure 1D). α-Humulene was converted into a major product (90%) at a retention time of 14.26 min and a minor product at a retention time of 14.84 min (Figure 1E). The major ions with their intensities in the mass spectrum of the major product of α-humulene were as follows: 41 (1), 59 (5), 69 (10), 81 (11), 95 (32), 109 (100), 119 (72), 123 (96), 137 (66), 151 (92), 163 (64), 177 (10), 187 (8), 205 (26), and 220 (18) (Figure 1F). According to the mass fragmentation spectra (MS) and molecular weights (M_w = 220), all major products from the conversions of (+)-α-longipinene, (−)-isolongifolene, and α-humulene were monohydroxy/monoepoxy compounds (Figure 1B,D,F).

Among 10 sesquiterpenes that were identified here and in previous studies as substrates of CYP264B1,[15,16] only (+)-α-longipinene was converted specifically into one product. Therefore, this compound was chosen for investigating the whole-cell system under different conditions.

Improvement of the CYP264B1-Based Whole-Cell System for the Conversion of Sesquiterpenes. *Effect of Cyclodextrin on Substrate Conversion.* In our previous study, a whole-cell system containing CYP264B1-Adx_{4-108}-Fpr was constructed for the conversion of sesquiterpenes.[16] However, this system did not always function and converted some of the substrates with low efficiencies. The main reason for this drawback was probably due to the loss of the volatile substrates from the culture medium. Our strategy to overcome this shortcoming was the application of HP-β-CD, one the most often used CDs, in biotransformation.[42,43] CDs are well-known as enhancers of the permeation of poorly soluble compounds through biological membranes as well as for their ability to fix volatile compounds.[28,44] Using HP-β-CD-incorporated (+)-α-longipinene, whole-cell conversions were performed as described under Materials and Methods. Resting cells were used to avoid *E. coli* metabolites such as indole, which are accumulated during bacterial growth and can inhibit product formation.[31] We observed that (+)-α-longipinene was converted into only one product, which was identical to that obtained in the in vitro conversion. In the control culture of *E. coli* containing the pET17b vector, this product was not present (data not shown).

To evaluate the influence of HP-β-CD on product yield, whole-cell conversions with and without HP-β-CD were performed in two types of flasks. The aluminum foil wrapped baffled flasks are normally used for the transformation of nonvolatile compounds, whereas the screw-cap sealed baffled flasks are used for volatile compound conversion to prevent their evaporation.[16] For the experiments carried out in the aluminum foil wrapped baffled flasks, the conversions with HP-β-CD showed about 57.1% product formation (~25.1 mg L^{-1})

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(AluF-CD; Figure 2), whereas in the controls (without HP-β-CD) only 5.2% was reached (AluF; Figure 2). This result

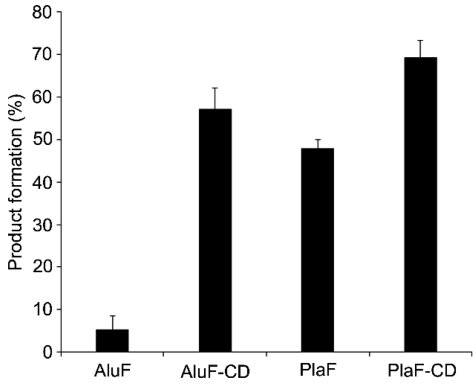

Figure 2. Influence of HP-β-CD on the biotransformation of (+)-α-longipinene. AluF, conversions without HP-β-CD carried out in aluminum foil wrapped baffled flasks; AluF-CD, conversions with HP-β-CD carried out in aluminum foil wrapped baffled flasks; PlaF, conversions without HP-β-CD carried out in screw-cap sealed baffled flasks; PlaF-CD, conversions with HP-β-CD carried out in screw-cap sealed baffled flasks. All values are represented as a mean from three independent experiments including standard deviations.

clearly shows the advantage of HP-β-CD. By using HP-β-CD, the efficiency of (+)-α-longipinene conversion was improved >10 times. For the experiments carried out in the screw-cap sealed baffled flask (performed as in the previous study[16]), the conversion with HP-β-CD reached about 69.2% product (~30.4 mg L^{-1}) (PlaF-CD; Figure 2), whereas the control (without HP-β-CD) showed a lower yield, about 47.8% (~21 mg L^{-1}) (PlaF; Figure 2). This result indicated that HP-β-CD helped to improve the conversion efficiency by approximately 44.8%.

Biotransformation of (+)-α-Longipinene by the Two CYP264B1 Systems with Different Reductases. Our previous study has shown that two autologous redox chains from the myxobacterium *S. cellulosum* So ce56 can transfer electrons to CYP264B1. However, the heterologous redox chain containing bovine AdR and bovine $Adx_{4\text{-}108}$ was found to support the activity of CYP264B1 in vitro much more efficiently.[15] In addition, recent studies in our group have observed that the ratio of P450 and the different redox partners affects not only the catalytic activity but also the product pattern in vitro and in vivo.[45] Therefore, the vector pETC4AA (containing genes coding for CYP264B1, $Adx_{4\text{-}108}$, and AdR) was constructed. The efficiency of the substrate conversion was compared to that of a system containing the reductase from *E. coli* (Fpr), which is soluble and not membrane-associated as AdR, using plasmid pETC4FA (containing genes coding for CYP264B1, Adx, and Fpr).

pETC4AA and pETC4FA were transformed into *E. coli* C43(DE3) and cultivated in TB medium for protein expression. Whole-cell conversions using resting cells were performed in KppG buffer in screw-cap sealed baffled flasks with 200 μM substrate incorporated into 1% HP-β-CD as described under Materials and Methods. After 0, 2, 4, 8, and 24 h, samples were taken for GC-MS analysis. The results showed that the product yield of the system pETC4AA was always slightly higher than the product yield of the system pETC4FA during the investigated time course. With regard to the end point after 24 h, the yield of the system with AdR was approximately 37.2 mg L^{-1}, about 24.8% higher than the one with Fpr (~29.8 mg L^{-1}) (Figure 3).

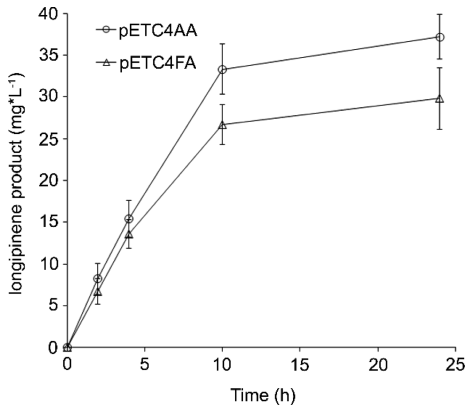

Figure 3. Time dependency of (+)-α-longipinene product formation by the two CYP264B1-based whole-cell systems with different reductases, AdR (plasmid pETC4AA containing the genetic information for the expression of CYP264B1, adrenodoxin reductase, and adrenodoxin) and Fpr (plasmid pETC4FA containing the genetic information for the expression of CYP264B1, *E. coli* reductase, and adrenodoxin). Whole-cell conversions were performed by using resting cells in KppG buffer (20 mM potassium phosphate, pH 7.4, with 20% glycerol). Samples were taken at time points of 0, 2, 4, 10, and 24 h. The product yield was determined by GC-MS using eudesmol as internal standard. All values are represented as a mean from three independent experiments including standard deviations.

Taken together, in comparison to the previous system, which was performed in the screw-cap sealed baffled flasks,[16] the yield of the (+)-α-longipinene conversion was increased from about 21 to 37.2 mg L^{-1} (equivalent to 77.1% conversion improved) by using AdR instead of Fpr and by using HP-β-CD to incorporate the hydrophobic substrate. Therefore, the system containing CYP264B1-$Adx_{4\text{-}108}$-AdR and 1% HP-β-CD was used for further studies.

Whole-Cell Conversion and Product Identification. The whole-cell system containing CYP264B1-$Adx_{4\text{-}108}$-AdR was used to scale up the conversion of (+)-α-longipinene to a reaction volume of 200 mL. At this scale, the influence of the substrate concentration on the product yield of (+)-α-longipinene conversion was investigated. The optimized conditions were then also applied for the conversion of (−)-isolongifolene and α-humulene.

Whole-cell conversions with different concentrations of (+)-α-longipinene within a range from 50 to 500 μM were performed. After 24 h, aliquots of 1 mL of culture were extracted with ethyl acetate for GC-MS analysis. The highest conversion was about 94.7%, obtained at substrate concentrations up to 200 μM. At the substrate concentration of 300 μM, the overall amount of product reached a maximal value of about 54.2 mg L^{-1} day^{-1} (equivalent to 82.1% conversion). When using substrate concentrations >300 μM, the conversion efficiencies and product concentrations decreased (Figure 4). With the product yield taken into account, a substrate concentration of 300 μM was chosen for further experiments.

To evaluate the efficiency of the whole-cell system with other sesquiterpenes, the optimized conditions for (+)-α-longipinene were applied for the conversion of (−)-isolongifolene and α-humulene. The biotransformations were performed at the scale of 200 mL with substrate concentrations of 300 μM. After 24 h,

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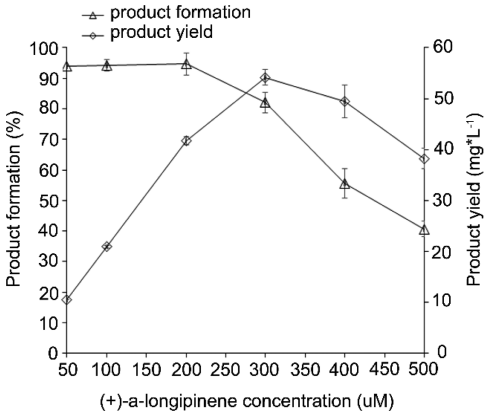

Figure 4. Dependency of the product formation on substrate concentrations for the in vivo conversion of (+)-α-longipinene by CYP264B1 using $Adx_{4\text{-}108}$ and AdR as redox partners. All values are represented as a mean from three independent experiments including standard deviations.

aliquots of 1 mL of culture were extracted with ethyl acetate for GC-MS analysis. (−)-Isolongifolene was converted into a major product and a minor product at retention times of 13.57 and 14.75 min, respectively. These product peaks were identical to those obtained in the in vitro conversion (Figure 1C). The yield of the (−)-isolongifolene conversion accounting for the major product was about 34.2 mg L^{-1}, corresponding to the conversion of 51.8%. α-Humulene was converted into one product at a retention time of 14.26 min. This product peak was also identical to that in the in vitro conversion (Figure 1E). The minor in vitro product at a retention time of 14.84 min did not appear in the in vivo conversion. The yield of the major α-humulene product obtained by the whole-cell system was about 47.2 mg L^{-1}, corresponding to a conversion of 71.5% (Table 1).

Table 1. Production Yield of Major Products and Corresponding Bioconversion of Three Sesquiterpene Substrates[a]

substrate	major product yield per liter per day (mg)	bioconversion (%)
(+)-α-longipinene	54.2 ± 2.1	82.1
(−)-isolongifolene	34.2 ± 3.4	51.8
α-humulene	47.2 ± 3.6	71.5

[a]All values are represented as a mean from three independent experiments including standard deviation.

To produce a sufficient amount of product for NMR identification, biotransformations of (+)-α-longipinene, (−)-isolongifolene, and α-humulene were carried out in 1 L of KppG buffer. After 24 h, the cultures were extracted with ethyl acetate. The organic extract was evaporated in a rotary vacuum system and then separated on a silica gel column with *n*-hexane and ethyl acetate (9:1) as mobile phase. All fractions were checked by TLC. The pure fractions containing product were confirmed by GC-MS and evaporated to dryness under vacuum at 40 °C. The products were then characterized by ^{1}H and ^{13}C NMR.

According to NMR data, the product of (+)-α-longipinene conversion were idenfified as 12-hydroxy-α-longipinene. The data were fully consistent with the previous NMR analysis of 12-hydroxy-α-longipinene.[46] The major product of α-humulene conversion by this system was identified as 5-hydroxy-α-humulene. The detailed NMR data for this compound were described in our previous study.[16]

For the (−)-isolongifolene conversion, the product was identified by NMR as isolongifolene-9-one. This compound was not the expected monohydroxy/monoepoxy product of isolongifolene (see In Vitro Conversion and In Vivo Conversion). We recognized that the GC-MS data (with the molecular ion at *m*/*z* 220) of the product in the pure fraction measured right after the purification process were identical to the product obtained in vitro (Figure 1C,D) but not identical to the GC-MS data (with the molecular ion at *m*/*z* 218) of the product in the same fraction measured after the NMR measurement (data not shown). Indeed, there was no evidence for the presence of isolongifolene-9-one in the collected fractions obtained after the purification process. This result suggests that the monohydroxy/monoepoxy derivative of isolongifolene oxidized to isolongifolene-9-one after the concentration process and during the storage (for 3 weeks, at 4 °C) for NMR measurement. The isolongifolene-9-one was, therefore, designated the follow-up product of the isolongifolene oxidation (Table 2).

To date, transformations of (+)-α-longipinene, (−)-isolongifolene, and α-humulene have received remarkable attention from researchers because they are major constituents of plant essential oils, which have interesting biological properties. (+)-α-Longipinene is one of the major components of essential oils from many herbs and plants, such as *Cedrus atlantica* and *Monticalia andicola* with antioxidant and antibacterial activities.[47,48] Biotransformation of (+)-α-longipinene by *Aspergillus niger* was performed to investigate its derivatives.[49] So far, 12-hydroxy-α-longipinene has been found in the heartwood of *Juniperus chinensis* Linn. var. *tsukusiensis* Masam.[46] (−)-Isolongifolene is present in the essential oil of the cone of pine and Japanese cedar.[50] This compound is a sesquiterpene with an extremely rich woody and floral odor. Because of their fragrance properties, oxygenated derivatives of (−)-isolongifolene are used in perfumes and cosmetics.[51] Conversion of (−)-isolongifolene to obtain isolongifolen-9-one was performed through a chemical route. The product occupies a vintage place in modern perfume industry.[52] Biotransformation of (−)-isolongifolene by *Glomerella cingulate* was also investigated. The metabolites were shown to have a suppressive effect against the chemical mutagen-induced SOS response.[53] α-Humulene has been found as a major constituent in the essential oils of many aromatic plants such as *Cordia verbenacea* and *Humulus lupulus*.[54,55] Biotransformation of this compound by fungi has been described to produce derivatives for further studies.[56] Moreover, α-humulene was reported to have the ability to inhibit cancer cell growth,[57] and it can be used as a precusor for the synthesis of zerumbone, a compound possessing anti-inflammatory and anti-HIV effects as well as antiproliferative activities in a wide variety of cancer cell lines.[58]

Taken together, by the use of HP-β-CD and alternative redox partners, the whole-cell biocatalyst for the production of oxygenated sesquiterpene derivatives based on CYP264B1 was further optimized. This system was successfully applied for the efficient conversion of (+)-α-longipinene, (−)-isolongifolene, and α-humulene. The corresponding products were analyzed by GC-MS and NMR, and their structures were determined as 12-hydroxy-α-longipinene, isolongifolene-9-one, and 5-hydroxy-α-humulene. With the ability to selectively convert various structural sesquiterpene classes, this system can be

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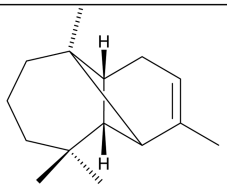
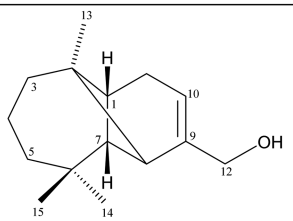
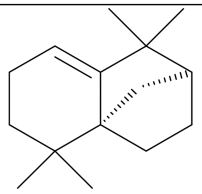
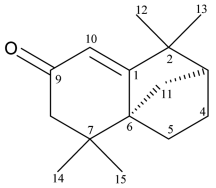
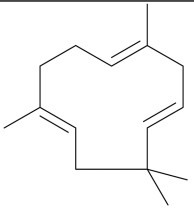
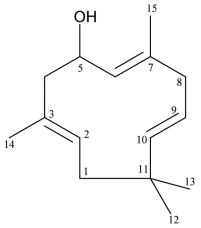
Table 2. Conversion of (+)-α-Longipinene, (−)-Isolongifolene, and α-Humulene Catalyzed by CYP264B1

Substrates	Product
 (+)-α-longipinene	 12-hydroxy-α-longipinene
 (-)-isolongifolene	 isolongifolene-9-one (follow-up of the major product, 73%)
 α-humulene	 5-hydroxy-α-humulene (major product, 90%)

considered a promising candidate for the biosynthesis of novel sesquiterpene derivatives. Thus, the potential of sesquiterpene compounds for their application in the pharmaceutical, flavor, and fragrance industries can be exploited in the future.

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Notes

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