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Completing the series of BVMOs involved in camphor metabolism of *Pseudomonas putida* NCIMB 10007 by identification of the two missing genes, their functional expression in *E. coli*, and biochemical characterization

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Abstract The camphor-degrading Baeyer–Villiger monooxygenases (BVMOs) from *Pseudomonas putida* NCIMB 10007 have been of interest for over 40 years. So far the FMN- and NADH-dependent type II BVMO 3,6-diketocamphane 1,6-monooxygenase (3,6-DKCMO) and the FAD- and NADPH-dependent type I BVMO 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase (OTEMO) have not been entirely studied, since it was not possible to produce those enzymes in satisfactory amounts and purity. In this study, we were able to clone and recombinantly express both enzymes and subsequently use them as biocatalysts for various mono- and bicyclic ketones. Full conversion could be reached with both enzymes towards (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one and with 3,6-DKCMO towards (−)-camphor. Further OTEMO gave full conversion with norcamphor. OTEMO was found to have a pH optimum of 9 and a temperature optimum of 20 °C and converted (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one with a k_{cat}/K_M value of 49.3 mM^{-1} s^{-1}.

Keywords Baeyer–Villiger monooxygenase · Camphor · *Pseudomonas putida* · Bicyclic ketones

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Introduction

Camphor (**1**) is a bicyclic monoterpene that naturally occurs as two enantiomers. While the (+)-enantiomer is located, e.g., in the wood of *Cinnamocum camphora*, (−)-**1** can be found in plant essential oils. The microbial degradation of **1** is one of the best-studied examples for the occurrence of Baeyer–Villiger monooxygenases in natural catabolic processes. While initial attempts to characterize the intermediates in the microbial decomposition of **1** by *Pseudomonas putida* NCIMB 10007 were already made in 1959 (Bradshaw et al. 1959), the complete elucidation of the enzymatic processes involved took a further 30 years. In 1960, ring opening via oxygen insertion by chemical Baeyer–Villiger reactions with **1** and other bicyclic ketones was proven (Meinwald and Frauenglass 1960). This was followed by the discovery of an enzymatic system for lactonization in camphor-grown *Pseudomonads*. Moreover mechanistic similarities to the chemical Baeyer–Villiger reaction and other enzymatic reactions like the lactonization of the D-ring of testosterone were observed (Conrad et al. 1961; Meinwald and Frauenglass 1960). The first BVMO identified in camphor metabolism was the 2,5-diketocamphane 1,2-monooxygenase (2,5-DKCMO, Fig. 1, Enzyme a), which is a complex of two identical oxygenating subunits combined with a reductase. After initial hydroxylation of (+)-**1** by a P450 monooxygenase and oxidation by a dehydrogenase, the 2,5-diketocamphane (**3**) is degraded by 2,5-DKCMO. The corresponding enzyme for (−)-**1** decomposition, namely 3,6-diketocamphane 1,6-monooxygenase (3,6-DKCMO, Fig. 1, Enzyme b), was first reported in 1965 (Conrad et al. 1965). A mixture of both enzymes was formerly referred to as MO1. The

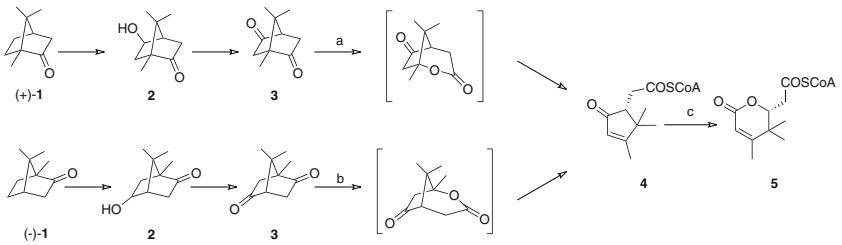

Fig. 1 Camphor degradation in *Pseudomonas putida* NCIMB 10007: In the first step, camphor (**1**) is hydroxylated by the $P450_{Cam}$-monooxygenase (Unger et al. 1986) followed by an oxidation by the 5-exo-alcohol dehydrogenase (Koga et al. 1989) yielding the corresponding diketocamphane (**3**). (+)-**1** is degraded by the 2,5-diketocamphane 1,2-monooxygenase (**a**), while (−)-**1** requires the 3,6-diketocamphane 1,6-monooxygenase (**b**). Both resulting lactones are unstable and lead to spontaneous formation of the 2-oxo-Δ^3-4,5,5-trimethylcylopentenylacetic acid, which as a coenzyme A derivative (**4**) is the substrate of OTEMO (**c**) (Ougham et al. 1983)

dependency of these enzymes on the cofactors FMN and NADH was postulated. The ability to degrade **1** turned out to be inducible by a plasmid with a size of 230 kDa (CAM plasmid; Rheinwald et al. 1973).

During further metabolism of camphor, the necessity of a second BVMO for cleavage of the second ring of the bicycle was found. The insertion of an oxygen into 2-oxo-Δ^3-4,5,5-trimethylcylopentenylacetyl-CoA (**4**) forming a substituted pimelic acid lactone (**5**) was already postulated in 1971 (Hartline and Gunsalus 1971). Nevertheless, it was not discussed if this reaction is catalyzed by the DKCMOs or another enzyme and it took 10 more years to purify that BVMO and characterize it as an FAD- and NADPH-dependent enzyme composed of 2 identical subunits with an overall size of 106 kDa (Ougham et al. 1983). Formerly referred to as MO2, nowadays this enzyme is mostly named after its substrate as 2-oxo-Δ^3-4,5,5-trimethylcylopentenylacetyl-CoA monooxygenase (OTEMO, Fig. 1, Enzyme c).

The differences in cofactor dependency lead to the classification of the three involved BVMOs into different subtypes (Leisch et al. 2011). While OTEMO belongs to the well-characterized type I, the 2,5- and 3,6-DKCMO are counted as type II enzymes. Type II BVMOs are of special interest for industrial applications because of the lower costs of NADH compared to NADPH, but hold more challenges regarding the structural feature that the subunits oxygenase and FMN reductase are distinct polypeptide chains forming only a loose complex. This might be an explanation for the availability of only a few examples of type II enzymes. Despite the two DKCMOs from *P. putida*, only two FMN- and NADH-dependent luciferases from *Photobacterium phosphoreum* NCIMB 844 and *Vibrio fischeri* ATCC 7744 are reported to perform Baeyer–Villiger oxidation of 2-tridecanone and several monocyclic and bicyclic [3.2.0] ketones (Villa 1997). A BVMO from *Rhodococcus erythropolis* involved in the metabolism of limonene was furthermore described to be a type II enzyme (Van der Werf et al. 1999). The conversions were measured in whole cell preparations of the wild type strain, where the required reductase was available.

During the 1990s, conversions of different substrates by the three BVMOs involved in metabolism of **1** were investigated. The conversion of bicyclo[3.2.0]ketones and norbonanone by *P. putida* NCIMB 10007 whole cells was reported to be highly stereo-controlled and products complementary to those formed by *Acinetobacter calcoaceticus* whole cells were observed (Grogan et al. 1993a, b). At this time, difficulties connected to investigations using whole cells, namely over-metabolism and side reactions due to other enzymes in the cell, occurred and therefore the necessity of studying isolated enzymes was recognized. The NADPH-dependent activity of partially purified preparations (MO2) towards bicyclic ketones was analyzed in the same year and turned out to be less regiospecific. Cyclopentanones bearing alkyl side-chains were biotransformed in a regio- and stereoselective manner by both, MO1 and MO2 (Grogan et al. 1993a, b), but enantiocomplementary behavior was observed when they were subjected to 3-substituted cyclobutanones (Gagnon et al. 1995a, b). Based on that, the enantioselective oxidation of a variety of cycloalkanones by MO2 and the application of these reactions in the total synthesis of (*R*)-(+)-lipoic acid could be reported (Adger et al. 1997; Adger et al. 1995). This substance is used in the therapy of liver diseases. After the separation of the 2,5- and 3,6-BVMOs by ion exchange chromatography and subsequent exposure to bicyclic ketones, it became apparent that the 2,5-DKCMO was highly stereoselective for bicyclo[3.2.0]ketones, while the 3,6-DKCMO converted norcamphor-derived compounds most selectively (Gagnon et al. 1994). Opposite selectivity for 2,5- and 3,6-DKCMO towards hydroxylated bicyclic ketones, which are precursors for the industrial relevant compound azadirachtin, was furthermore observed (Gagnon et al. 1995a, b).

Although these previous investigations led to considerable knowledge of many properties of the three BVMOs involved in natural camphor degradation, the first heterologous

overexpression of 2,5-DKCMO in *Escherichia coli* was reported by us recently (Kadow et al. 2011). This recombinant overexpression overcomes whole-cell derived problems and enables more comprehensive studies and application of these enzymes. To complete the series of the camphor-degrading BVMOs, the oxygenating subunit of 3,6-DKCMO and the OTEMO were expressed recombinantly, characterized and compared to each other within this study.

Material and methods

Enzymes, chemicals, and media

Pfu$^+$ polymerase was obtained from Roboclon (Berlin, Germany), dNTPs from Roth (Karlsruhe, Germany), and restriction enzymes from New England Biolabs (Beverly, MA, USA). For SDS-PAGE analysis, the prestained PAGE ruler from Fermentas (St. Leon-Rot, Germany) was used. All other chemicals were purchased from Fluka (Buchs, Switzerland), Sigma-Aldrich (Munich, Germany), or Acros Organics (Geel, Belgium). Genomic DNA was isolated with the DNeasy Kit from Qiagen. For DNA purification from PCR, the MinElute PCR purification Kit by Qiagen (Hilden, Germany) was used. Furthermore the Miniprep Kit from Qiagen was used for plasmid purification. For subcloning DNA fragments, the TOPO TA Cloning Kit from Invitrogen (Darmstadt, Germany) was employed. HisTrap 5 ml FF columns and Sephadex G25 were obtained from GE Healthcare (Uppsala, Sweden). The plasmid pET-28b(+) was from Novagen (Darmstadt, Germany). The BCA kit was purchased from Interchim (Montluçon, France). The Chaperone Plasmid Set was obtained from TaKaRa (Saint-Germain-en-Laye, France).

Amplification and cloning

Identification of the oxygenating subunit of the 3,6-DKCMO gene was accomplished by using a degenerate oligonucleotide primer (N-term3,6-DKCMO_4: 5′-GCCCARACCTTYGAYTGGGGBATYAAG-3′) that was derived from the N-terminal amino acid sequence of the 3,6-DKCMO (Willetts 1997) by a gene walking PCR with genomic DNA. After initial denaturation for 4 min at 95 °C, the cycling program was followed for 30 cycles: 30 s, 95 °C denaturation; 30 s, 55 °C primer annealing; 3 min, 72 °C elongation; unspecific annealing of the primer: 30 s, 95 °C denaturation; 30 s, 40 °C primer annealing; 3 min, 72 °C elongation; and complementary strand synthesis for 30 cycles: 30 s, 95 °C denaturation; 30 s, 60 °C primer annealing; 3 min, 72 °C elongation. The final elongation step was performed over 15 min at 72 °C. Obtained DNA fragments were subcloned with the TOPO TA cloning kit, transformed into DH5α cells and colonies were checked in a colony PCR with M13 primers (M13-FP: 5′-TGTAAAACGACGGCCAGT-3′, M13-RP: 5′-CAGGAAACAGCTATGACC-3′). After initial denaturation for 10 min at 95 °C, the cycling program was followed for 25 cycles: 30 s, 95 °C denaturation, 30 s, 56 °C primer annealing, 3 min, 72 °C elongation. The final elongation step was performed over 10 min at 72 °C. Plasmids of colonies, which contained DNA fragments were then isolated and sequenced. The 5′-sequence of the 3,6-DKCMO gene was identified by using an oligonucleotide reverse primer (2.GW_3,6-DKCMO_1: 5′-TATAGGACGCTTGCGGATAGGCCC-3′), that had been derived from the newly obtained sequence of the 3,6-DKCMO gene in a gene walking PCR. The gene walking PCR was carried out as described above. Again all obtained DNA fragments were subcloned and processed as described above.

Amplification of the whole 3,6-DKCMO gene was performed with genomic DNA containing the CAM-plasmid with oligonucleotides supplemented with restriction sites for *Nde*I at the N-terminus and *Hind*III at the C-terminus (*Nde*I_3,6-DKCMO_fw: 5′-GGAATTCCATATGGCAATGGAAACTGGTTTGATCTTC-3′; 3,6-DKCMO_*Hind*III_rv: 5′-CCCAAGCTTTCAACGCTTAGGCAGGAGAATCTTT-3′). After initial denaturation for 5 min at 95 °C, the cycling program was followed for 5 cycles: 45 s, 95 °C denaturation; 30 s, 60 °C primer annealing; 70 s, 72 °C elongation and then for 25 cycles: 45 s, 95 °C denaturation; 30 s, 57 °C primer annealing; and 70 s, 72 °C elongation. The final elongation step was performed over 10 min at 72 °C. The resulting 1156 bp fragment was digested with *Nde*I and *Hind*III and ligated into pET-28b digested with the same enzymes. The resulting plasmid with a N-terminal His-tag fusion was called pET-28_3,6-DKCMO.

Identification of the OTEMO gene was accomplished by performing a gene walking PCR starting from the known sequence of the 2,5-DKCMO using genomic DNA (walk III_1rv: 5′-TCCACATACACCGACGTTGC-3′). After initial denaturation for 4 min at 95 °C, the cycling program was followed for 30 cycles: 30 s, 95 °C denaturation; 30 s, 55 °C primer annealing; and 3 min, 72 °C elongation; unspecific annealing of the primer: 30 s, 95 °C denaturation; 30 s, 40 °C primer annealing; 3 min, 72 °C elongation, and complementary strand synthesis for 30 cycles: 30 s, 95 °C denaturation; 30 s, 60 °C primer annealing; and 3 min, 72 °C elongation. The final elongation step was performed over 15 min at 72 °C. The generated DNA fragments were purified from agarose gel and then sequenced (walk III_5 rv: 5′-GTGACCTCCTGAATCGGTGC-3′).

Amplification of the whole OTEMO gene was performed with genomic DNA containing the CAM-plasmid with oligonucleotides supplemented with restriction sites for *Nde*I at the N-terminus and *Hind*III at the C-terminus (*Nde*I_OTEMO_fw: 5′-GGAATTCCATATGAGCAATAGAG

CAAAAAGTCCGGCA-3′; OTEMO_*Hind*III_rv: 5′-CCCAAGCTTTTAGGCGAGTTCAAATCCGTTGTAGT TATTAT-3′). After initial denaturation for 5 min at 95 °C, the cycling program was followed for 5 cycles: 45 s, 95 °C denaturation; 30 s, 60 °C primer annealing; and 70 s, 72 °C elongation and then for 25 cycles: 45 s, 95 °C denaturation; 30 s, 57 °C primer annealing; and 70 s, 72 °C elongation. The final elongation step was performed over 10 min at 72 °C. The resulting 1,657 bp fragment was digested with *Nde*I and *Hind*III and ligated into pET-28b digested with the same enzymes. The resulting plasmid with a N-terminal His-tag fusion was called pET-28_OTEMO.

Bacterial strains and culture conditions

P. putida NCIMB 10007 (equivalent to ATCC 17453) was purchased from the German National Resource Center for Biological Material (DSMZ). For cultivation of *P. putida*, basal salt medium without antibiotics as described previously was used (Gagnon et al. 1994). *E. coli* cells were cultivated in terrific broth (TB) medium (12 g tryptone, 24 g yeast, 4 g glycerol in 1 l buffer autoclaved separately). Overnight cultures were grown in Luria Bertani (LB) medium (10 g tryptone, 5 g yeast, 5 g NaCl in 1 l dest H_2O). LB and TB media were supplemented with 50 μg/ml kanamycin. In the case of coexpression of the chaperones GroES-GroEL, which correspond to plasmid pGro7 in the TaKaRa Chaperone Plasmid Set, 50 μg/ml chloramphenicol was added.

Transformation of *E. coli* strain BL21-DE3 (Novagen, genotype: [95 F–*ompT hsdSB* (rB-mB-) *gal dcmrne131* (DE3)]) with pET-28_3,6-DKCMO and pET-28_OTEMO was carried out by the heat shock method described by Chung et al. (1989). Expression of recombinant OTEMO in *E. coli* BL21 was performed by cultivation at 37 °C to an OD_{600} of 0.5, than addition of IPTG to a final concentration of 0.1 mM and shifting the culture to 20 °C and 200 rpm for another 16 h of cultivation. For soluble expression of recombinant 3,6-DKCMO, co-expression of chaperones GroES and GroEL was necessary. The culture was incubated at 37 °C to an OD_{600} of 0.5, then the chaperones were induced by addition of 2 mg/ml L-arabinose. After incubation for another 30 min, the recombinant protein expression was induced with 0.1 mM IPTG and the culture was transferred to 20 °C and 200 rpm.

To obtain larger amounts of cells, the two enzymes of interest were expressed in a 4-l batch cultivation in a New Brunswick Scientific (Edison, NJ, USA) fermenter. The temperature was set to 20 °C and an oxygen saturation of 50% and a pH of 7.5 were aimed at. As already described for shake flask cultivations, TB medium and antibiotics in concentrations of 50 μg/ml of kanamycin and 50 μg/ml of chloramphenicol (in the case of chaperone co-expression) were used. At an $OD_{600\ nm}$ 1–2, the cells were induced with 0.1 mM IPTG and 2 mg/ml L-arabinose in the case of chaperone co-expression. The cells were harvested 16 h after induction.

Gene expression analysis

Gene expression analysis was performed with crude cell extract. Samples standardized to cell amount were taken during cultivation. Cells were harvested by centrifugation and resuspended in sodium phosphate buffer (100 mM, pH 7.5) containing 300 mM sodium chloride. Cell disruption was performed by FastPrep (40 s, 4 m/s; MP Biomedicals, Solon, OH, USA). For SDS-PAGE analysis, the supernatant was substituted with Laemmli buffer (Laemmli 1970). SDS-PAGE was carried out on 10% resolving gels for OTEMO and on 12% resolving gels for 3,6-DKCMO. Proteins were stained with a Coomassie R250/G250 solution.

Enzyme purification

Cells were harvested by centrifugation and resuspended in sodium phosphate buffer (50 mM, pH 7.5). Cell disruption was performed by a single passage through a French pressure cell. Recombinant enzymes were purified by affinity chromatography via N-terminal His-tag on an automated Äkta purifier system. After centrifugation of disrupted cells for 45 min at (10,000×*g*), the supernatant with recombinant enzyme was added to the column. A 5 ml HisTrap FF crude column with bound Ni^{2+} was equilibrated with sodium phosphate buffer (100 mM, pH 7.5) supplemented with 300 mM NaCl and 30 mM imidazole. After passing through of the crude extract, the column was washed with three column volumes of sodium phosphate buffer (100 mM, pH 7.5) supplemented with 300 mM NaCl and 30 mM imidazole followed by two column volumes of sodium phosphate buffer (100 mM, pH 7.5) supplemented with 300 mM NaCl and 45 mM imidazole to remove unspecific bound proteins. Elution was performed by adding three column volumes of 300 mM imidazole in sodium phosphate buffer (100 mM, pH 7.5) supplemented with 300 mM NaCl. Fractions of washing and elution steps were collected to analyze purity by SDS-PAGE. In order to remove imidazole and NaCl from the eluate, the pooled elution fractions were loaded to a 60 ml size exclusion column (Sephadex G25 matrix), which was equilibrated with sodium phosphate buffer (50 mM, pH 7.5) before. Protein fractions were recognized via online absorption measurement at 280 nm and collected. Determination of protein content of purified and desalted protein as well as crude extract was carried out with the BCA kit and a standard curve of BSA in the same buffer in a range of 2–0.005 mg/ml was used. Samples were measured in triplicates in three different dilutions.

Activity measurements

Determination of pH or temperature optimum, temperature stability and kinetics for OTEMO was performed by spectrophotometric measurement of NADPH consumption in 1 ml cuvettes. Ten to fifty microliters of purified enzyme solution (4–5 mg/ml) was mixed with 0.3 mM of NADPH and 1 mM substrate solution in DMF and the volume was adjusted to 1 ml with Tris buffer (100 mM, pH 9.0). Decrease in absorption was measured at 340 nm for 60–180 s except for acetophenone, which was measured at 370 nm. For determination of pH optimum, 100 mM Tris buffers with varying pH values were used. Temperature optimum was determined by incubating reaction mixtures for 3–5 min at specific temperatures, subsequent addition of enzyme and NADPH and measurement as described above. In order to determine temperature stability, the enzyme was incubated at different temperatures for 0.5, 1, 2, 4, and 20 h and was then used in the above described activity assay. Kinetic parameters K_M and k_{cat} were ascertained by determining specific activities for different substrate concentrations from 10^{-3} to 60 mM.

Biocatalytic reactions and GC analysis

For biocatalysis, His-tag purified enzyme and crude extracts of *E. coli* BL21 pET28_3,6-DKCMO and pET28_OTEMO cultivations were used. Reactions were carried out in sodium phosphate buffer (50 mM, pH 7.5) in the case of 3,6-DKCMO or in Tris buffer (100 mM, pH 9.0) in the case of OTEMO. A substrate concentration of 2 mM was used; the cofactor FMN was used at a final concentration of 0.3 mM. NADH/NADPH was used in equimolar amounts to the substrate. Purified enzyme was employed in concentrations of 1–2 mg/ml, crude extracts in concentrations of 10–15 mg/ml. Incubation was performed in 24-well MTP at 800 rpm and 25 °C. Sample volume was 500 µl. Extraction of substrates and products was performed by vortexing of samples with 300 µl of ethyl acetate and 200 µl of hexane subsequently. Separation of aqueous and organic phase was achieved by centrifugation. Samples were dried over anhydrous sodium sulfate and analyzed by GC on a GC 2010 (Shimadzu Europa GmbH, Duisburg, Germany) with a BPX5 column (5% phenyl-/95% methylpolysilphenylene siloxane, SGE GmbH, Darmstadt, Germany).

Injection temperature was set to 220 °C. Column temperature for (+)-**1**, (−)-**1**, and **12**–**14** was 60 °C for 5 min followed by a gradient of 10 °C/min to 180 °C maintained for 3 min. Column temperature for **7** and **8** was 60 °C and for **6** it was 50 °C. For **9** and **20**, 80 °C for 10 min followed by a gradient of 10 °C/min to 180 °C was used and maintained for 5 min. **15** was measured at 50 °C for 15 min followed by a gradient of 10 °C/min to 180 °C maintained for 5 min. **16** and **18** were measured at 60 °C for 15 min followed by a gradient of 10 °C/min to 180 °C maintained for 5 min. **17** and **19** were measured at 70 °C for 15 min followed by a gradient of 10 °C per min to 180 °C maintained for 5 min. **10** was detected isothermal at 90 °C. Column temperature for **11** was 100 °C.

For quantification standard curves of substrates and/or products with concentrations from 0.5 to 10 mM were measured. Specific activity is given in units per milligram (U/mg) protein. One unit is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of substrate per minute.

GenBank accession number

The nucleotide sequence of the gene encoding 3,6-diketocamphane 1,6-monooxygenase was deposited to the NCBI with the accession number JQ034404 and the gene encoding 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase with the accession number JQ034405. The protein sequence of 3,6-DKCMO has been published before by the group of Littlechild (pdb code: 2WGK, www.pdb.org; McGhie et al. 1996).

Results

Identification and cloning of the 3,6-DKCMO and OTEMO genes

The 3,6-Diketocamphane 1,6-monooxygenase (3,6-DKCMO) and the 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase (OTEMO) from *P. putida* NCIMB 10007 are encoded on the CAM operon on the transmissible 230 kb CAM plasmid (Rheinwald et al. 1973). First genomic DNA, which also contained the CAM plasmid, was isolated from the *P. putida* strain NCIMB 10007 cultivated with camphor as sole carbon source.

The N-terminal amino acid sequences of several BVMOs including the one from 3,6-DKCMO had been published by A. Willets (1997). Employing the codon usage of *P. putida*, several degenerate primers for the N-terminal region were designed on the basis of this sequence information, and then used in gene walking PCRs with genomic DNA as the template (Pilhofer et al. 2007). With this method, a PCR fragment of about 2.5 kb was obtained, which contained the whole 3,6-DKCMO gene except for 150 nucleotides from the 5′-end. In order to identify the missing part of the gene, specific reverse primers were designed from the determined sequence, which were then used in a further gene walking PCR. The complete gene sequence was verified by translation of the sequence and

Fig. 2 Phylogenetic tree illustrating the integration of OTEMO and the two DKCMOs in the BVMO family. The tree was constructed using Clustal W2 (Larkin et al. 2007) and edited with Dendroscope v. 3.0.9 (Huson et al. 2007). The *scale bar* represents 0.1 substitutions per site. For those enzymes, which have been studied experimentally, the literature reference is given in *parenthesis*. The remaining enzymes are from genome sequence data and not yet characterized biochemically

comparison of the resulting amino acid sequence with the already published amino acid sequence of the 3,6-DKCMO (pdb code: 2WGK, www.pdb.org).

After the identification of the gene encoding 3,6-DKCMO, OTEMO was the last missing non-cloned BVMO involved in camphor degradation. During the cloning of 2,5-DKCMO, the orientation of genes located on the CAM plasmid was assembled using all parts available in NCBI (Kadow et al. 2011). Gene walking downstream putidaredoxin led to putative 3-ketoacid-CoA-transferase subunits A and B, but no further BVMOs could be found. Therefore we supposed the encoding part for OTEMO could be located upstream of the gene encoding the 2,5-DKCMO. Thus, a walking PCR in the upstream direction was performed and a 1,641-bp long ORF was found, which had similarity to several BVMOs and luciferases when compared to entries in the NCBI database. Also the N-terminal amino acid sequence of OTEMO had been published by A. Willets (1997) as well and translation of the newly found gene sequence resulted in a protein sequence with the postulated N terminus. Therefore we concluded that this gene encodes OTEMO. The primary protein structure of OTEMO exhibits the characteristic FAD- and NADPH-binding domains, which were described previously (Fraaije et al. 2002). The first GXGXXG-motif is G_{16}AGVTG_{21} and the second is G_{192}TGATG_{197}. These motifs incorporate the BVMO fingerprint sequence FXFXXXHXXXW-(P/D), which is resembled by F_{160}KGESFHSSRWP$_{171}$ in OTEMO's primary structure.

The genes were amplified by gradient touchdown PCR using gene specific primers with restriction sites for the enzymes *Nde*I and *Hind*III, and the resulting PCR products were afterwards cloned into the expression vector pET-28b. The obtained construct has an N-terminal His-tag fused to the gene to allow a functional expression of the 3,6-DKCMO and OTEMO in *E. coli* and easy protein purification by affinity chromatography. The N-terminal tag was preferred over the C-terminal tag, because earlier studies on BVMOs suggested decreased expression levels when a C-terminal tag was used (Rehdorf et al. 2009).

The genetic relationship between the newly identified OTEMO and several other BVMOs is shown in Fig. 2. In this phylogenetic tree, it is also illustrated that the DKCMOs are not closely related to the type I BVMOs.

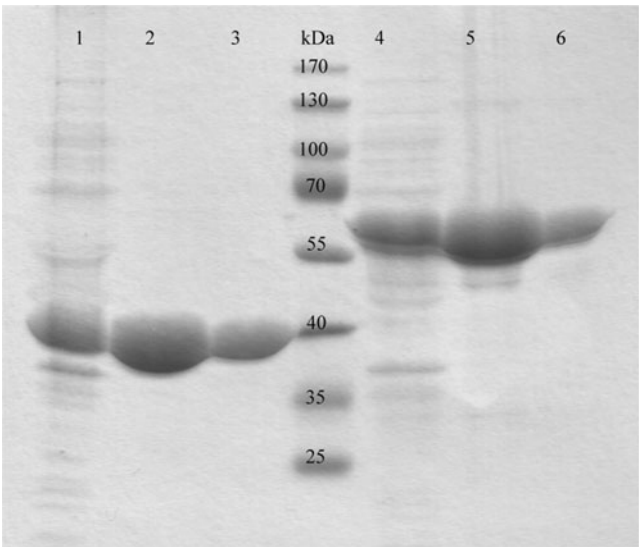

Fig. 3 SDS-PAGE of purified 3,6-DKCMO and OTEMO. *1* Crude extract 3,6-DKCMO. *2* 3,6-DKCMO after His-tag purification. *3* 3,6-DKCMO after final desalting step. *4* Crude extract OTEMO. *5* OTEMO after His-Tag purification. *6* OTEMO after final desalting step

Table 1 Purification data for OTEMO as determined spectrophotometrically for **13** and for 3,6-DKCMO as determined from biocatalysis reactions with (−)-**1**

Enzyme	Step	Volume [ml]	Volumetric activity [U/ml]	Activity [U]	Specific activity [mU/mg]	Yield [%]	Factor
OTEMO	Crude extract	22	15.6	343.2	1,000	100	1
	Purified	9	24	216	1,900	62.9	1.8
	Desalted	14.5	9.1	132	2,200	38.4	2.2
3,6-DKCMO	Crude extract	22	0.0017	0.037	0.09	100	1
	Purified and desalted	10	0.0016	0.016	0.46	42.6	4.96

Expression and purification of recombinant 3,6-DKCMO and OTEMO

The utilization of *E. coli* BL21(DE3) as expression host yielded primarily soluble OTEMO protein after 16 h cultivation at 20 °C in TB medium. The determined theoretical protein size of the His-tagged OTEMO of 63.5 kDa was confirmed in SDS-PAGE analysis where a protein band of about 60 kDa was detectable (Fig. 3). 3,6-DKCMO expressed under the same conditions was mainly present as insoluble inclusion bodies. Therefore co-expression of different chaperones was investigated to improve soluble expression of 3,6-DKCMO. The chaperones GroES and GroEL were found to be the most effective ones. SDS-PAGE analysis of crude cell extract confirmed an increased level of soluble protein and led to a clear band at approximately 40 kDa (Fig. 3), which corresponds to the theoretical estimated molecular weight of 44.5 kDa of the His-tagged protein.

After successful recombinant expression, a nickel-based affinity chromatography of the His-tagged proteins and the subsequent removal of imidazole by size exclusion chromatography on a G25 column was performed and yielded pure protein (Fig. 3, lane 3). OTEMO seems to bind FAD since fractions that contained the pure protein were yellow. Purification of recombinant OTEMO with a purification factor of 2.2 (Table 1) was achieved, which shows that OTEMO is expressed at a high level and the crude extract already contains mostly OTEMO protein. In the case of 3,6-DKCMO, a purification factor of 4.9 was determined (Table 1). The fractions containing purified protein were colorless, which confirmed previous studies, in which FMN was reported to be not covalently bound to the enzyme (Taylor and Trudgill 1986). In contrast to OTEMO, the 3,6-DKCMO needs an additional flavin reductase, which is a distinct polypeptide, for full activity. The crude cell extract of *E. coli* is assumed to provide such a reductase. For this reason, biocatalysis experiments were performed with crude cell extract although the 3,6-DKCMO could be successfully purified.

Characterization and substrate specificity of OTEMO

A variety of ketones representing different classes of BVMO substrates were used for spectrophotometric measurements and biocatalysis (Fig. 4). For example substrates **6**–**8** represent monocyclic ketones, **9**–**10** aromatic ketones. **11** served as an example for aliphatic and **12**–**14** for bicyclic ketones. **15**–**20** resemble α,β-unsaturated substituted monocyclic ketones.

Since OTEMO is a type I BVMO, its activity could be determined by measuring the consumption of NADPH spectrophotometrically utilizing the crude cell extract and purified enzyme. For the determination of pH or temperature optimum and temperature stability, we used substrate **13**, as it turned out to be the favored substrate of OTEMO.

OTEMO is active over a broad range of basic pH values (Fig. 5a). Indeed, the pH optimum was determined to be 9

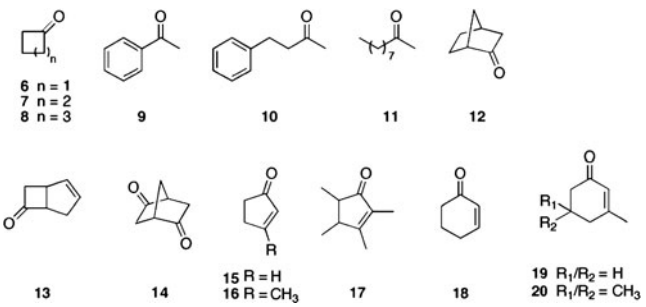

Fig. 4 Substrates used for biocatalysis. Cyclobutanone (**6**), cyclopentanone (**7**), and cyclohexanone (**8**) represent the monocyclic ketones. Acetophenone (**9**) and 4-phenyl-2-butanone (**10**) resemble aromatic ketones. 2-Decanone (**11**) served as an example for aliphatic ketones. Norcamphor (**12**), (±)-cis-bicyclo[3.2.0]hept-2-en-6-one (**13**), and (*R*, *R*)-bicyclo[2.2.1]heptane-2,5-dion (**14**) are bicyclic ketones. 2-Cyclopenten-1-one (**15**), 3-methyl-2-cyclopenten-1-one (**16**), 2,3,4,5-tetramethyl-2-cyclopenten-1-on (**17**), 2-cyclohexen-1-one (**18**), 3-methyl-2-cyclohexen-1-one (**19**), and 3,5,5-trimethyl-2-cyclohexen-1-one (**20**) resemble α, β-unsaturated substituted monocyclic ketones

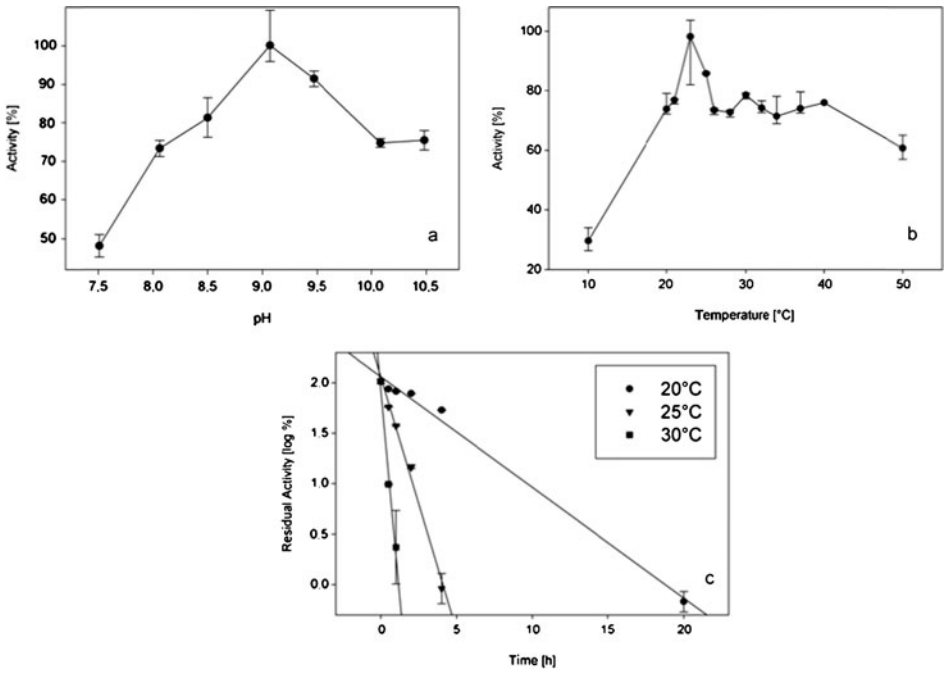

Fig. 5 Spectrophotometric characterization of OTEMO using substrate **13**: **a** pH optimum, **b** temperature optimum, **c** temperature stability

for purified OTEMO, which coincides with the results previously published (Ougham et al. 1983). Thus, all the following experiments were conducted with 100 mM Tris buffer at pH 9.

The temperature optimum of OTEMO is 23 °C, but the enzyme is also reasonably active at all temperatures between 20 °C and 40 °C (Fig. 5b). However, since the temperature stability of this enzyme seems to decrease rapidly at temperatures above 20 °C, all biocatalysis overnight reactions with OTEMO were carried out at 20 °C. The half life of the enzyme was determined to be 196 min at 20 °C, 43 min at 25 °C, and 9 min at 30 °C. When incubated at 25 °C for 4 h, OTEMO was completely inactive and incubated at 30 °C the enzyme lost all its activity after only 1 h (Fig. 5c). Storage of purified OTEMO at 4 °C for 24 h leads to loss of about half of its activity.

The substrates favored by OTEMO were firstly determined by the spectrophotometric activity assay and kinetic data were collected using purified enzyme. The k_{cat}/K_M value for OTEMO towards cyclobutanone (**6**) was 14.7 mM^{-1} s^{-1} and towards (±)-*cis*-bicyclo[3.2.0]hept-2-en-6-one (**13**) 49.3 mM^{-1} s^{-1} (Table 2).

In order to determine the substrate specificity of OTEMO, the substrates shown in Fig. 4 were used in biocatalytic reactions with purified enzyme and OTEMO converted all of them (Tables 3 and 4). However, the conversion rates for bicyclic ketones such as **12**–**14** were the highest. The best conversion towards a monocyclic substrate was observed with **6** and the aromatic compounds **9** and **10** were also converted at reasonable rates. The aliphatic ketone **11** was only poorly converted.

Additionally upscaling of biocatalysis experiments to a 35 ml reaction volume substituted with 0.07 mmol of substrates was performed. Using OTEMO crude extract, complete conversion of substrates **12** and **13** could be obtained.

Characterization and substrate specificity of 3,6-DKCMO

Spectrophotometric activity assays using crude cell extracts of 3,6-DKCMO could not be performed, because the decrease in absorption of NADH was not significantly different from controls. Hence, only biocatalysis reactions could be utilized to investigate the 3,6-DKCMO. The same substrates, which were used to characterize OTEMO, were also used in biocatalysis reactions with 3,6-DKCMO crude extract. Under the chosen conditions, mainly bicyclic ketones were converted by 3,6-DKCMO and almost complete conversion was found for (−)-**1** and **13** (Table 3). The substrates (+)-**1**, **9** and **12** were also converted with high yields. In contrast, conversion of monocyclic ketones and the aliphatic substrate **11** took place at much lower rates.

Upscaling of biocatalysis experiments to a 35 ml reaction volume substituted with 0.07 mmol of substrates with crude

Table 2 Kinetic data for OTEMO for **6** and **13** as determined spectrophotometrically

Substrate	V_{max} [mmol mg^{-1} min^{-1}]	K_M [mM]	k_{cat}/K_M [mM^{-1} s^{-1}]
6	1.77 10^{-3}	0.127	14.7
13	1.29 10^{-3}	0.028	49.3

Table 3 Conversion of several ketones by 3,6-DKCMO crude extract and purified OTEMO as determined by GC analysis

Substrate	(+)-**1**[a]	(−)-**1**[a]	**6**[b]	**7**[b]	**8**[b]	**9**[a]	**10**[a]	**11**[b]	**12**[a]	**13**[a]	**14**[a]
3,6-DKCMO [%]	88	91	13	24	3	80	48	11	77	99	26
OTEMO [%]	44	22	36	19	19	67	54	7	96	100	87

[a] Based on substrate peak areas

[b] Based on product peak areas

extract of 3,6-DKCMO gave 92% conversion with substrate **13** and 85% with (−)-**1**.

Biocatalytic reactions with unsaturated cyclopentanone and cyclohexanone derivatives

A few α,β-unsaturated monocyclic ketones that are similar to the proposed natural OTEMO substrate were subjected to biocatalytic reactions with purified OTEMO and crude extract of *E. coli* cells expressing 3,6-DKCMO and 2,5-DKCMO (Table 4).

Generally, the unsubstituted ketones were converted better than the substituted ones. All three enzymes exhibit similar preferences and converted substrates **15** and **18** best, but OTEMO led to higher conversion for these substrates than the DKCMOs, e.g., 74% for substrate **18** in comparison to about 58% reached by 2,5-DKCMO.

Discussion

For over 50 years, three BVMOs were known to be involved in the camphor degradation pathway, but detailed investigations of these enzymes were not performed using recombinant proteins so far. Within this work, the oxygenating subunit of 3,6-diketocamphane 1,6-monooxygenase and OTEMO were successfully cloned and overexpressed recombinantly in *E. coli* as heterologous expression host. The recombinant expression of 2,5-DKCMO was recently performed in our group as well (Kadow et al. 2011). Hence, all monooxygenases involved in the camphor degradation pathway are now easily available at stable quality and protein engineering studies are possible for the first time. The purification using the N-terminal His-tag via nickel based affinity chromatography turned out to be efficient and fast for all enzymes investigated.

Table 4 Conversion of several ketones by crude cell extract of 2,5- and 3,6-DKCMO and pure OTEMO as determined by GC analysis

Substrate	**15**	**16**	**17**	**18**	**19**	**20**
2,5-DKCMO [%]	38	11	44	58	19	27
3,6-DKCMO [%]	48	10	43	50	20	21
OTEMO [%]	62	13	34	74	13	22

As it was previously shown for the 2,5-DKCMO, recombinant expression and purification via His-Tag leads to much higher protein yields from less cultivation volume than protein expression in the *P. putida* wild-type strain. In the case of the 3,6-DKCMO enzyme we obtained 56 mg of pure protein from 600 ml culture, in contrast to former studies, where a yield of 16 mg purified 3,6-DKCMO from 10 l of wild-type cultivation could be reached (Jones et al. 1993). When purifying the OTEMO protein, we obtained 63 mg from 300 ml culture volume, which is a much better yield than published by Ougham et al. who isolated 13.8 mg pure OTEMO protein from 30 l of wild-type cultivation (Ougham et al. 1983).

Previous studies on the purified enzymes determined a molecular size of 76 kDa for the 3,6-DKCMO protein and 78 kDa for the 2,5-DKCMO protein (Jones et al. 1993). Under denaturing conditions, two identical subunits with a molecular weight of 40.3 kDa each were identified for the 3,6-DKCMO. The 2,5-DKCMO subunits were supposed to be 3–4 kDa larger. However, we estimated the mass from the amino acid sequence of one subunit of 3,6-DKCMO to be 42.3 kDa and fused to the His-tag 44.5 kDa. SDS-PAGE analysis of *E. coli* crude extract and pure protein resulted in protein bands corresponding to approximately 40 kDa, which coincides to those molecular weights. Furthermore, similar to McGhie, Littlechild, and colleagues (1996), we determined a molecular size of 40.7 kDa for the 2,5-DKCMO subunit, which means that the 2,5-DKCMO is smaller than the 3,6-DKCMO and not otherwise.

OTEMO was previously found to consist of two identical subunits, with a size of 56 kDa each (Ougham et al. 1983). The molecular size for the OTEMO dimer of 106 kDa has been determined in the same work by a different method. We calculated the size of 61.4 kDa for one OTEMO subunit and 63.5 kDa for the His-tagged protein, which was confirmed in SDS-PAGE analysis, where a clear band at about 60 kDa could be detected.

As already discussed for the 2,5-DKCMO (Kadow et al. 2011), the 3,6-DKCMO does not seem to bind its cofactor FMN tightly, since fractions containing 3,6-DKCMO collected by affinity chromatography turned out to be colorless. However, fractions containing purified OTEMO protein were yellow, which leads to the assumption that FAD is bound

covalently in this enzyme. In fact, addition of FAD to purified enzyme did not lead to higher activities in spectrophotometric activity assays, which were used to characterize OTEMO. Characterization of 3,6-DKCMO was not possible, using the spectrophotometric activity assay, because there are different competing factors in the crude cell extract, like soluble NADH oxidases and free flavins (Jones et al. 1993) that also utilize NADH. Theoretically the competing enzymes could be removed by purification of the enzyme. However, type II BVMOs need a flavin-reducing subunit for full activity and we assume that there is a reductase in the *E. coli* crude extract, which can complement the 3,6-DKCMO, but is lost during purification. For this reason, spectrophotometric activity assays were not possible with purified enzyme either, unless a suitable NADH dehydrogenase would be available.

In biocatalytic reactions, we were able to show that the 3,6-DKCMO, like the 2,5-DKCMO, converts mainly bicyclic ketones. The favored substrates of the 3,6-DKCMO turned out to be **1**, **12**, and **13**. These substrate preferences are similar to those observed for 2,5-DKCMO (Kadow et al. 2011). OTEMO accepts bicyclic ketones as well, but also monocyclic ketones with a reasonable conversion. In fact the rates for several monocyclic ketones were at least three times higher as for 3,6-DKCMO converting the same substrates. However, the favored substrates in terms of conversion of OTEMO are bicyclic ketones such as **12** and **13**. This is surprising since the natural substrate of OTEMO was proposed to be a cyclopentenone derivative and earlier studies always claimed that the OTEMO is specific towards monocyclic ketones. Our assumption that α,β-unsaturated monocyclic ketones, similar to the natural substrate, would be better accepted than saturated ones could be confirmed, but nevertheless no higher conversions than with bicyclic substrates could be obtained. From literature, it was known that the 2-oxo-Δ^3-4,5,5-trimethylcylopentenylacetic acid occurs as a CoA-derivative in the natural reaction (Ougham et al. 1983), which might explain why the unsaturated monocycles are not converted as well as the bicyclic compounds. Nevertheless, we conclude that by identifying OTEMO, a new biocatalyst showing unusual substrate preferences towards α,β-unsaturated monocyclic ketones and bicyclic ketones is available now.

Since the OTEMO and the 3,6-DKCMO belong to two different classes of BVMOs, it is not surprising that the homology between those enzymes is only 3%. The enzyme most homologous to the OTEMO (78%) based on the protein sequence is the CHMO from *Novosphingobium aromaticivorans*, followed by CHMOs from other organisms. Although the sequence of OTEMO has not been available in the database so far, the enzyme was included in a phylogenetic tree in a recently published review (Leisch et al. 2011). The enzymes appearing in close relationship of OTEMO as shown in this reference are not the ones we found in BLAST search to be closely related to the OTEMO sequence determined by us.

Even though a homology of only 46% could be determined for the 3,6-DKCMO and the 2,5-DKCMO based on the protein sequence, these enzymes seem to have similar general properties, which is actually not surprising since they are involved in the degradation of the two isomers generated through degradation of (+)- or (−)-camphor. However, in earlier studies it was claimed that 3,6-DKCMO is specific for (−)-**1**, whereas the 2,5-DKCMO was supposed to be specific for (+)-**1** (Jones et al. 1993). We were already able to show that the 2,5-DKCMO also converts (−)-**1**, even though not as good as (+)-**1**. Surprisingly the conversion of (+)- and (−)-**1** for the 3,6-DKCMO is not that different, even if the 3,6-DKCMO converts (−)-**1** slightly better. However, definite conclusions regarding substrate specificity cannot be drawn as long as the missing reducing subunit is not available for coupling to the oxygenating subunit. It might be that substrate binding is affected when the two subunits form a complex.

In conclusion, we were able to show that 3,6-DKCMO and OTEMO can easily be expressed in *E. coli* and therefore together with the 2,5-DKCMO the series of camphor metabolizing BVMOs from *P. putida* is completed. Although these results facilitate the work with these biocatalysts, several limitations still make their application in the future challenging. In the case of OTEMO, problems resulting from its low temperature stability might be solved in the future by protein engineering approaches as it was shown for many other enzymes. Regarding the two DKCMOs, it will be obligatory to identify a suitable FMN reductase to generate an efficient system for application in industrial processes.

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