Purification and characterization of a Baeyer–Villiger mono-oxygenase from *Rhodococcus erythropolis* DCL14 involved in three different monocyclic monoterpene degradation pathways

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A Baeyer–Villiger mono-oxygenase (BVMO), catalysing the NADPH- and oxygen-dependent oxidation of the monocyclic monoterpene ketones 1-hydroxy-2-oxolimonene, dihydrocarvone and menthone, was purified to homogeneity from *Rhodococcus erythropolis* DCL14. Monocyclic monoterpene ketone mono-oxygenase (MMKMO) is a monomeric enzyme of molecular mass 60 kDa. It contains 1 mol of FAD/monomer as the prosthetic group. The N-terminal amino acid sequence showed homology with many other NADPH-dependent and FAD-containing (Type 1) BVMOs. Maximal enzyme activity was measured at pH 9 and 35 °C. MMKMO has a broad substrate specificity, catalysing the lactonization of a large number of monocyclic monoterpene ketones and substituted cyclohexanones. The natural substrates 1-hydroxy-2-oxo-limonene, dihydrocarvone and menthone were converted stoichiometrically into 3-isopropenyl-6-oxoheptanoate (the spontaneous rearrangement product of the lactone formed by MMKMO), 4-isopropenyl-7-methyl-2-oxo-oxepanone and 7-isopropyl-4-methyl-2-oxo-oxepanone respectively. The MMKMO-catalysed conversion of iso-dihydrocarvone showed an opposite regioselectivity to that of dihydrocarvone; in this case, 6-isopropenyl-3-methyl-2-oxo-oxepanone was formed as the product. MMKMO converted all enantiomers of the natural substrates with almost equal efficiency. MMKMO is involved in the conversion of the monocyclic monoterpene ketone intermediates formed in the degradation pathways of all stereoisomers of three different monocyclic monoterpenes, i.e. limonene, (dihydro)carveol and menthol.

Key words: carvone, flavoprotein, limonene, menthol, regioselectivity.

INTRODUCTION

Terpenes are the largest family of natural products [1], and over 30000 different naturally occurring monoterpenes have been identified [2]. Volatile monoterpene emission from trees is estimated at 127×10^{14} g carbon/year [3]. Remarkably, little is known about the metabolism of monoterpenes; especially, information regarding the enzymes involved in the monoterpene degradation pathways is scarce [4–6].

Monoterpenes can be divided into three major subgroups: linear monoterpenes, monocyclic monoterpenes and bicyclic monoterpenes. The degradation of alicyclic monoterpenes is generally believed to involve a lactone-forming mono-oxygenase activity as the first step towards ring opening [4]. These Baeyer–Villiger mono-oxygenases (BVMOs) catalyse the insertion of one atom of oxygen next to an alicyclic keto group. The lactone thus formed is subsequently hydrolysed by a lactone hydrolase, or spontaneously rearranges to the corresponding oxo-acid when the oxygen atom is inserted between a hydroxy and a keto group. To date, only BVMOs involved in bicyclic monoterpene degradation pathways have been (partially) purified [7–9]. However, BVMO activity has also been detected in the degradation pathways of the monocyclic monoterpenes limonene [10], (dihydro)carveol [10a] and menthol [11].

Rhodococcus erythropolis DCL14, a Gram-positive bacterium, is able to grow on a large number of monocyclic monoterpenes, including limonene, carveol, dihydrocarveol and menthol, as the sole source of carbon and energy [10]. Cells grown on limonene and carveol contain BVMO activities catalysing the conversion of 1-hydroxy-2-oxo-limonene into unstable 7-hydroxy-4-isopropenyl-7-methyl-2-oxo-oxepanone [10], and the conversion of dihydrocarvone into 4-isopropenyl-7-methyl-2-oxo-oxepanone [10a] respectively (Scheme 1). In this report, the purification and characterization of the Baeyer–Villiger mono-oxygenase, monocyclic monoterpene ketone mono-oxygenase (MMKMO) from *R. erythropolis* DCL14 involved in the conversion of these monocyclic monoterpene ketones is described.

MATERIALS AND METHODS

Strain used and growth conditions

R. erythropolis DCL14 was previously isolated on (4*R*)-dihydrocarveol [10], and is maintained at the division of Industrial Microbiology (CIMW 0387B, Wageningen University, The Netherlands).

R. erythropolis DCL14 was subcultured once a month and grown at 30 °C on a yeast extract/glucose agar plate for 2 days, after which the plates were stored at room temperature. *R. erythropolis* DCL14 was grown in 5-l Erlenmeyer flasks containing 1 litre of mineral salts medium [12] with 0.01 % (v/v) monoterpene, and fitted with rubber stoppers. The flasks were incubated at 30 °C on a horizontal shaker oscillating at 1 Hz with an amplitude of 10 cm. After growth was observed, the concentration of the toxic substrates was increased with steps of 0.01 % (v/v), until a total of 0.1 % (v/v) carbon source had been added. With (−)-(1*S*,3*S*,4*R*)-menthol, a 4 M stock solution was prepared in acetone (acetone is not a growth substrate for *R. erythropolis* DCL14), and, as described above, this monoterpene

Abbreviations used; BVMO, Baeyer–Villiger mono-oxygenase; DTT, dithiothreitol, MMKMO, monocyclic monoterpene ketone mono-oxygenase.

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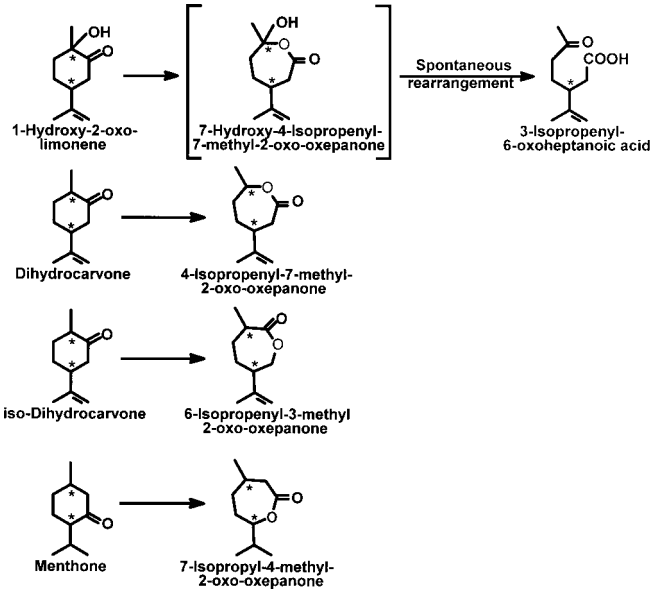

Scheme 1 Reactions catalysed by MMKMO from *R. erythropolis* DCL14

was added in steps of 0.01 % (v/v). With ethanol and succinate, cultures were grown in a 5-l Erlenmeyer flask containing 1 litre of mineral salts medium supplemented with 3 g of the pertinent carbon source per litre. Cells for enzyme purification were cultivated in a fed-batch fermenter on (4*R*)-limonene, as described previously [13]. Cells were collected by centrifugation (4 °C, 10 min at 16000 ***g***) and washed with 50 mM potassium phosphate buffer, pH 7.0. The pellet was resuspended in 7 ml of this buffer, and stored at −20 °C until used.

Purification of MMKMO

Cell extracts were prepared by sonication, as described previously [13]. All purification steps were performed at 4 °C and pH 7, unless stated otherwise. Buffers contained 10 % (v/v) glycerol, 5 μM FAD and 0.1 mM dithiothreitol (DTT). If necessary, the pooled fractions were concentrated by ultra-filtration with an Amicon ultra-filtration unit, using a membrane with a cut-off of 10000 under nitrogen at a pressure of 4 bar.

Hydrophobic interaction

The cell extract (16 ml) was applied on to an octyl-Sepharose column (2.5 cm × 35 cm) equilibrated with 300 mM NaCl in 20 mM potassium phosphate buffer (flow rate 0.75 ml/min; collected fraction volume was 7.5 ml). Fractions containing MMKMO activity were pooled.

Anion-exchange chromatography

The pooled fractions from the hydrophobic interaction step were diluted to an NaCl concentration of 100 mM, and applied on to a DEAE-Sepharose CL-6B column (2.5 cm × 31 cm) equilibrated with 100 mM NaCl in 20 mM potassium phosphate buffer. The column was washed with 100 ml of the same buffer (flow rate 0.75 ml/min; collected fraction volume was 7.5 ml) and subsequently the enzyme was eluted with a linear gradient of NaCl (0.1–1 M) in the same buffer (total volume of 1 litre). MMKMO activity was eluted at an NaCl concentration of 315 mM [10]. Fractions exhibiting MMKMO activity were pooled and concentrated.

Gel filtration

The concentrated MMKMO solution after anion-exchange chromatography was applied to a Sephacryl-S300 column (2.5 cm × 98 cm) equilibrated with 100 mM NaCl in 20 mM potassium phosphate buffer (flow rate 0.75 ml/min; collected fraction volume was 7.5 ml). Fractions containing MMKMO activity were pooled and concentrated.

Mono Q chromatography

Samples (0.5 ml) of the concentrated MMKMO solution after the gel-filtration step (approx. 0.15 mg of protein) were applied to a Mono Q column (1.2 cm × 15 cm) operated with a fast protein-liquid chromatography system (Pharmacia, Roosendaal, The Netherlands) at room temperature. The column was equilibrated with 25 mM Tris/HCl buffer (pH 8.0). The enzyme was eluted using a 0–1 M NaCl gradient in the same buffer (flow rate 1.0 ml/min, collected fraction volume was 1 ml). MMKMO eluted at an NaCl concentration of 450 mM. Fractions exhibiting MMKMO activity were pooled and concentrated.

Determination of molecular mass

The molecular mass of the denatured protein was determined by SDS/PAGE. An SDS/12.5 % polyacrylamide gel was prepared by the method of Laemmli [14]. Proteins were stained with Coomassie Brilliant Blue G. The Pharmacia low-molecular-mass calibration kit, containing phosphorylase b (M_r 94000), BSA (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000) and soya-bean trypsin inhibitor (M_r 20100), was used for the estimation of the molecular mass.

The molecular mass of the native protein was determined by gel-filtration on a Sephacryl S300 column as described above. BSA (M_r 67000), ovalbumin (M_r 43000), chymotrypsinogen A (M_r 25000) and limonene-1,2-epoxide hydrolase (M_r 16500) [13] were used as the reference proteins.

Assay of MMKMO activity

MMKMO activity was determined spectrophotometrically by monitoring the reduction of NADPH at 340 nm (ϵ_{340} 6.23 $mM^{-1} \cdot cm^{-1}$). Activity was measured at 30 °C in an incubation mixture (1 ml) containing cell extract, 50 mM glycine/NaOH buffer (pH 9.5), 0.2 mM NADPH and 1 mM (4*R*)-dihydrocarvone. The reaction was initiated by the addition of substrate. Specific activities were calculated from the linear part of the reaction, and were corrected for endogenous activity. For the determination of the pH-optimum of enzyme catalysis, the glycine/NaOH (pH 9.5) buffer in the standard activity assay was replaced with 50 mM of the other buffers. Steady-state kinetic parameters were determined from incubations (1 ml; 30.0 °C) containing 40 μg of MMKMO, 50 mM glycine/NaOH buffer (pH 9.5), 0.2 mM NADPH, and substrate at concentrations ranging between 0.05 and 1 mM. Kinetic parameters were calculated from Lineweaver–Burk plots, as described previously [15]. The substrate specificity of MMKMO was determined by replacing (4*R*)-dihydrocarvone in the standard activity assay with 1 mM of the other substrates.

Identification of the prosthetic group of MMKMO

The spectra of the native protein were recorded using a dialysed MMKMO solution. MMKMO was dialysed using a Sephadex

G-25 column (2.0 cm × 37 cm) equilibrated with 10 % (v/v) glycerol, 100 mM NaCl, 20 mM potassium phosphate buffer (pH 7.0), and 0.1 mM DTT (flow rate 0.75 ml/min; collected fraction volume was 3.75 ml). Active fractions were pooled and concentrated by ultra-filtration. The filtrate was used as the reference solution.

The prosthetic group of MMKMO was released by heat treatment (1 min at 100 °C). The precipitate was removed by centrifugation (15 min, 13000 ***g***, 4 °C). The supernatant contained the liberated prosthetic group, and was used either directly for spectral analysis, or was concentrated by freeze-drying for TLC analysis.

Product accumulation and identification studies

Reaction mixtures (1 ml) consisting of MMKMO, 50 mM glycine/NaOH buffer (pH 9.5), 1.5 mM NADPH and 1 mM monocyclic monoterpene ketone were prepared in 15-ml vials. The vials were fitted with Teflon Mininert valves (Supelco, Zwijndrecht, the Netherlands), preventing the evaporation of the monocyclic monoterpene ketones, and placed in a shaking water bath at 30 °C. After different time points, a vial was removed from the water bath and the reaction was terminated by the addition of 1 ml of ethyl acetate. For the detection of 3-isopropenyl-6-oxoheptanoate, 20 μl of a 1M H_2SO_4 solution was also added to the reaction mixture to accomplish the extraction of the fatty acid in the organic phase. The vials were vigorously shaken to achieve quantitative extraction of the terpenes. The ethyl acetate layer was pipetted into a microcentrifuge tube, and centrifuged (3 min, 13000 ***g***) to achieve separation of the two layers; subsequently, 1 μl of the ethyl acetate layer was analysed by GC.

For product-identification studies, the biotransformations were performed in an identical way, but on a ten-times-larger scale. At the end of the reaction, the liquid was extracted three times with half a volume of hexane. The organic layers were combined, dried over $MgSO_4$, and evaporated to dryness in a rotary evaporator under reduced pressure. The stereoisomers were assigned using a combination of chiral and non-chiral GC, and GC–MS. For the products of (4*R*)-dihydrocarvone, ^{1}H-NMR and ^{13}C-NMR spectral analyses were also performed. The data obtained were compared with those of authentic samples and/or literature data.

Analytical methods

Protein concentration was determined by the method of Bradford [16] with BSA as the standard. The N-terminal amino acid sequence of purified MMKMO was determined by Edman degradation at the Protein Sequencing Facility Leiden, Department of Medical Biochemistry, Sylvius Laboratory, Leiden, The Netherlands.

All monoterpene ketones and their corresponding lactones were analysed by chiral GC on a fused silica cyclodextrin α-DEX 120 capillary column (30 m × 0.25 mm internal diam., 0.25 μm film coating; Supelco). GC was performed on a Chrompack CP9000 GC equipped with a flame ionization detector using N_2 (1 ml·min^{-1}) as the carrier gas. The detector and injector temperatures were 250 and 200 °C respectively, and the split ratio was 1:50. Monocyclic monoterpenoids, lactones and fatty acids were analysed isocratically at an oven temperature of 120 °C, 140 °C and 170 °C respectively.

GC-MS analysis was performed on an HP5973 MSD GC equipped with afused silica capillary column (HP-5; 30 m × 0.25 mm internal diam., film thickness 0.25 μm). Other specifications were as follows: carrier gas and flow, He at 1.0 ml·min^{-1}; injector temperature, 220 °C; temperature program, 70–250 °C at 7 °C min^{-1}; on-line injection volume, 5 μl. Electron-impact mass spectra were obtained at 70 eV. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DPX 400 spectrometer at 400 Hz.

Absorption spectra were recorded at 25 °C on a Perkin–Elmer lambda 10 spectrophotometer; fluorescence spectra were recorded at 25 °C with a Perkin–Elmer LS50B Luminescence spectrometer. Measurements were performed in a quartz cuvette (10-mm path length) in a total volume of 2 ml. Fluorescence emission and excitation spectra were recorded between 300 and 700 nm (band width 5 nm), with the emission wavelength fixed at 445 nm, or the excitation wavelength fixed at 525 nm (band width 5 nm). All fluorescence and absorption spectra were corrected against the appropriate reference solution.

The flavin nucleotide was identified by TLC using Silicagel B12 precoated TLC plates (J. T. Baker, Deventer, The Netherlands) developed with 1-butanol/acetic acid/water (5:3:3, by vol.). Authentic FMN, FAD and riboflavin were used as standards. Flavins were detected by direct visual observation.

Chemicals

In the present paper, the sequence rule of Cahn–Ingold–Prelog is used to differentiate between the stereochemistries of the monoterpene stereoisomers rather than the older, still frequently used but somewhat confusing, (+)- and (−)-nomenclature. The carbon atom numbering is on the basis of the standard carbon atom numbering of limonene.

(+)-(4*R*)-Limonene was obtained from Acros (Den Bosch, The Netherlands) and (−)-(1*R*,4*S*)-menthone was from Aldrich (Zwijndrecht, The Netherlands). NADPH was purchased from Sigma (Zwijndrecht, The Netherlands), and FAD and FMN were from Boehringer Mannheim (Mannheim, Germany). (+)-(4*R*)-Dihydrocarvone [mixture of ≈ 77 % (+)-(1*R*,4*R*)- and ≈ 20 % (+)-(1*S*,4*R*)-iso-dihydrocarvone] and (+)-(1*S*,4*R*)-menthone were obtained from Fluka (Zwijndrecht, The Netherlands). (1*S*,4*R*)- and (1*R*,4*S*)-1-Hydroxy-2-oxolimonene were prepared as described previously [10]. DEAE-Sepharose CL6B, octyl-Sepharose, Sephacryl S300, Sephadex G-25 and Mono-Q were purchased from Pharmacia. Reference samples of optically pure (1*R*,4*R*)-dihydrocarvone and (1*S*,4*R*)-iso-dihydrocarvone were kindly provided by H. Swarts (Laboratory of Organic Chemistry, Wageningen University, The Netherlands). 3-Isopropenyl-6-oxoheptanoate was prepared as described previously [10]. A mixture of (4*R*)-4-isopropenyl-7-methyl-2-oxo-oxepanone and (6*R*)-6-isopropenyl-3-methyl-2-oxo-oxepanone was synthesized from (4*R*)-dihydrocarvone by oxidation with peracetate [17]. Peracetic acid (200 μl of a 32 % solution) was added to a solution of 100 μl (4*R*)-dihydrocarvone in 0.5 ml of acetic acid containing 0.03 g of anhydrous sodium acetate. The slurry was stirred vigorously for 2 h (longer reaction times resulted in less lactone and more lactone-epoxide) at room temperature. Subsequently, 10 ml of water was added to the reaction mixture, and the pH of the solution was adjusted to 7 with NaOH. The lactones thus formed were a mixture of 4-isopropenyl-7-methyl-2-oxo-oxepanone (79 %) and 6-isopropenyl-3-methyl-2-oxo-oxepanone (21 %) {4-isopropenyl-7-methyl-2-oxo-oxepanone, MS: *m*/*z* 168 [M^+] (3), 153 (3), 138 (16), 125 (17), 110 (71), 95 (39), 81 (27), 68 (100), 55 (24), 41 (43); 6-isopropenyl-3-methyl-2-oxo-oxepanone, MS: *m*/*z* 168 [M^+] (7), 153 (7), 139 (16), 125 (11), 108 (80), 93 (37), 81 (37), 67 (100), 55 (43), 41 (55)}. (4*S*,7*R*)- and (4*R*,7*S*)-7-Isopropyl-4-methyl-2-oxo-oxepanone were synthesized in the same manner from (1*S*,4*R*)- and (1*R*,4*S*)-menthone respectively, except that the reaction time was 36 h {7-isopropyl-4-methyl-2-

oxo-oxepanone, MS: m/z 170 [M^+] (1), 152 (1), 141 (1), 127 (83), 99 (100), 81 (88), 69 (64), 55 (49), 41 (35); similar to literature values [11,18]}. All other chemicals were of the highest purity commercially available.

RESULTS

Induction of BVMO activities in *R. erythropolis* DCL14

R. erythropolis DCL14 can grow on a large number of monocyclic monoterpenes [10]. During growth on these substrates, NADPH-dependent BVMO activities with monocyclic monoterpene ketones (i.e. 1-hydroxy-2-oxolimonene, dihydrocarvone and menthone) were induced (Table 1). The BVMO activities in cell extracts from different sources with different terpenoid substrates did not conform to a constant ratio, but did show the same trend. The ratio was probably not constant because of the presence of alcohol dehydrogenase-catalysed ketone reductase activity in these cell extracts, catalysing the conversion of the ketone substrates into their corresponding alcohols [19].

Enzymes present in cell extracts of cells grown on (4*R*)-limonene, (+)-(4*S*)-carveol or (1*R*,3*R*,4*S*)-menthol were separated by DEAE-Sepharose anion-exchange chromatography, and the BVMO activities with the different stereoisomers of 1-hydroxy-2-oxo-limonene, dihydrocarvone and menthone were determined in the different fractions. These experiments suggested that *R. erythropolis* DCL14 contains only one BVMO activity involved in the conversion of these monocyclic monoterpene ketones (results not shown). This BVMO was subsequently named MMKMO. Cells for further experiments were grown on (4*R*)-limonene.

Purification of MMKMO

During our initial efforts to purify the inducible MMKMO, the enzyme appeared to be rather unstable, especially at high ionic strength. Remarkably, when proteins were separated by gel filtration in the absence of added NaCl, a large loss in MMKMO activity was observed. When the pooled MMKMO fractions were subsequently applied on to a DEAE-Sepharose anion-exchange column, the lost activity was regained to a large extent. No significant loss of MMKMO activity was observed when the gel-filtration step was performed in the presence of 100 mM NaCl. NaCl concentrations higher than 1 M inactivated the enzyme irreversibly. A mixture of 10% (v/v) glycerol, 5 μM FAD and 0.1 mM DTT was used to stabilize MMKMO during purification.

The purification scheme for MMKMO is presented in Table 2. The enzyme was purified 20-fold with an overall yield of 25%. The ratio of the activity of MMKMO with (4*R*)-dihydrocarvone and (1*R*,4*S*)-1-hydroxy-2-oxolimonene remained constant during purification, suggesting further that only one BVMO is present in terpene-induced cells of *R. erythropolis* DCL14.

SDS/PAGE analysis of the purified enzyme revealed one distinct band, corresponding to a protein with a subunit mass of 60 kDa (results not shown). Gel filtration revealed one symmetric

Table 1 BVMO activities (nmol · min^{-1} · [mg protein]$^{-1}$) in cell extracts of *R. erythropolis* DCL14 grown on various carbon sources with different monoterpene ketones as the substrates for the enzyme

n.d., not determined.

	Enzyme substrate		
Growth substrate	(1*R*,4*S*)-1-Hydroxy-2-oxolimonene	(4*R*)-Dihydrocarvone	(1*R*,4*S*)-Menthone
(4*R*)-Limonene	191	140	109
(4*S*)-Limonene	326	253	181
(4*R*)-Carveol	296	236	174
(4*S*)-Carveol	298	209	152
(4*R*)-Dihydrocarveol	204	144	102
(1*R*,3*R*,4*S*)-Menthol	59	76	55
(1*R*,3*R*,4*S*)-Isopulegol	53	84	61
Cyclohexanol	57	38	n.d.
ϵ-Caprolactone	60	40	n.d.
Succinate	< 1	2	< 1
Ethanol	< 1	< 1	< 1

Table 2 Purification of MMKMO from (4*R*)-limonene grown cells of *R. erythropolis* DCL14

The specific activity (in nmol · min^{-1} · [mg protein]$^{-1}$ was measured with (4*R*)-dihydrocarvone as the substrate. Ratio (4*R*)-DCN/(1*R*,4*S*)-HK is defined as the ratio in specific activity of MMKMO with (4*R*)-dihydrocarvone and (1*R*,4*S*)-1-hydroxy-2-oxolimonene as the substrates.

Purification step	Total protein (mg)	Specific activity (nmol · min^{-1} · [mg protein]$^{-1}$	Purification (-fold)	Yield (%)	Ratio (4*R*)-DCN/(1*R*,4*S*)-HK
Cell-free extract	286	153	1	100	0.84
Hydrophobic interaction	166	190	1.24	72	0.88
Anion exchange	24	1190	7.81	63	0.87
Gel filtration	5.8	3170	20.8	42	0.90
Mono Q	3.8	2890	18.9	25	0.87

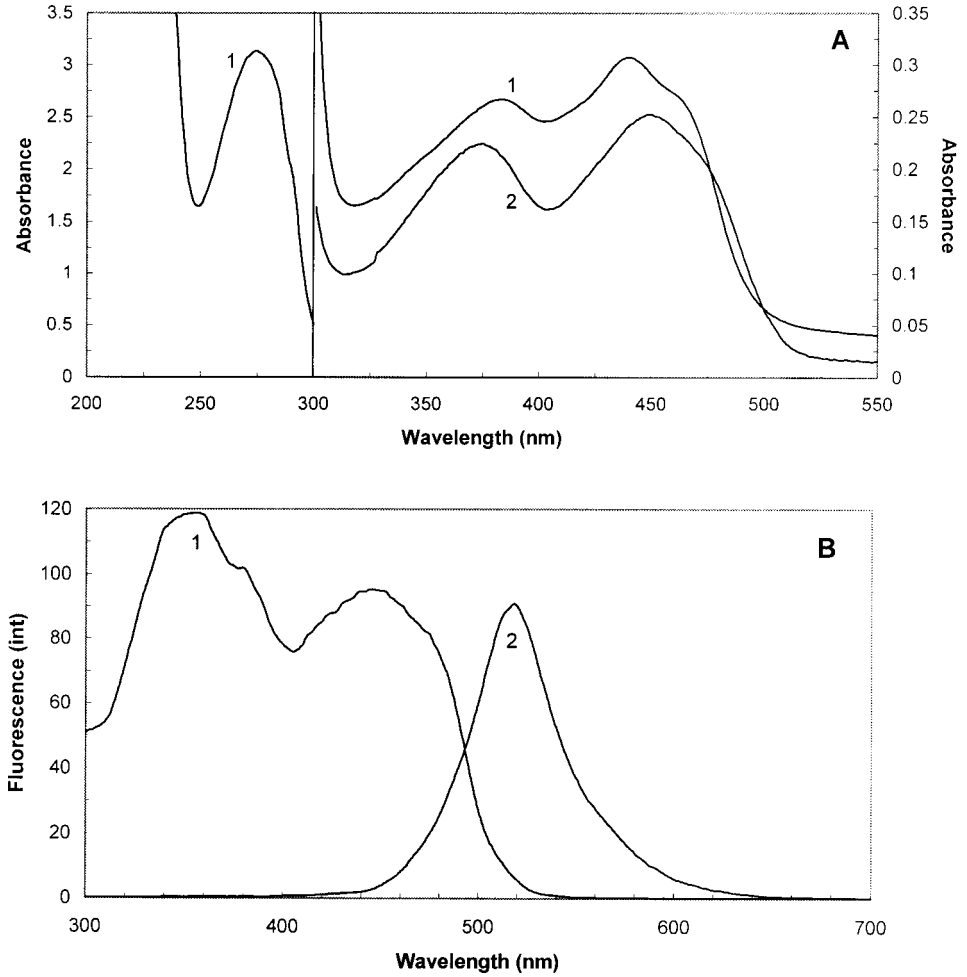

Figure 1 Spectral properties of the purified MMKMO from *R. erythropolis* DCL14

(**A**) Absorption spectra: curve 1 is the absorption spectrum of native MMKMO (1.83 mg · ml^{-1}), and curve 2 is that of the flavin liberated from the MMKMO (26 μM). (**B**) Fluorescence spectra of purified MMKMO (1.83 mg · ml^{-1}). Curve 1 is the excitation spectrum with emission at 525 nm, and curve 2 is the emission spectrum with excitation at 445 nm.

protein peak corresponding to an M_r of approx. 57000, indicating that the native enzyme is a monomer.

The last purification step (Mono Q anion-exchange chromatography) resulted in a purer enzyme preparation, but also resulted in a loss of specific activity (Table 2). This was the case for all of five independent purification procedures of MMKMO. This indicates that MMKMO either is partially unfolded or has partially lost the prosthetic group after Mono Q anion-exchange chromatography. Efforts to stabilize MMKMO during the Mono Q purification step failed. Since, after gel filtration, MMKMO was found to be $> 95\%$ pure (results not shown), the MMKMO characterization studies were performed with the enzyme preparation after gel filtration.

Spectral properties

The absorption spectrum of native MMKMO displayed a typical maximum at 275 nm, and two bands of relatively low intensity at approx. 368 and 437 nm (Figure 1A, trace **1**). The excitation spectrum showed maxima at 350 (with a shoulder near 380 nm) and 445 nm (Figure 1B, trace **1**), and the maximum of fluorescence emission was at 525 nm (Figure 1B, trace **2**). This indicates that MMKMO is a flavoprotein.

The cofactor of MMKMO was released by heat treatment (1 min, 100 °C). The released cofactor exhibited absorption maxima near 375 nm and 449 nm (Figure 1A, trace **2**). This spectrum was identical with that of FAD, but differed slightly from that of FMN (absorption maxima at 373 and 446 nm). Fluorescence spectral analysis revealed that, upon excitation at 525 nm, the released cofactor showed fluorescence emission at 380 and 445 nm, typical of flavins. The released cofactor had a fluorescence intensity similar to that of FAD, but a 10-fold lower intensity compared with that of FMN. TLC experiments established conclusively that MMKMO contained FAD as the prosthetic group.

Assuming a molar absorption coefficient (ϵ_{450}) of 11.3 mM^{-1} · cm^{-1}, the amount of FAD per MMKMO subunit was estimated. Different enzyme preparations yielded values ranging from 0.84–1.15 mol of FAD per subunit, indicating that each MMKMO monomer contains one FAD molecule as the cofactor.

N-terminal amino acid sequencing

The N-terminus of MMKMO was largely blocked (approx. 98 %). The N-terminal amino acid sequence was determined as Met-Gln-Thr-Ile-Pro-Ser-Thr-Thr(Gly?)Glu-Thr-Ala-Ala-Asp-

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MMKMO          XMQTIPSTTXETAADFDAVIXGAGFXGLYALHR
SMO_RR      MNGQHPRSVVTAPDATTGTTS-YDVVVVGAGIAGLYAIHRFRSQGLTVR
CHMO_AC                  MSQKMDFDAIVIGGGFGGLYAVKKLRDELELKV
CPMO_Psp     TTMTTMTTEQLGMNNSVNDKLDVLLIGAG
SMO_CR                    AEWAEEFDVLVVGAGAGG
CHMO_RC                  TAXTIHXVDAVVIGAGFGGIYAVHK
OTP_PP                   NRAKSPALDAVVIGAGVTGIYQAFLINQAGMAVL
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Figure 2 Alignment of the N-terminal amino acid sequence of MMKMO from *R. erythropolis* DCL14 and other NADPH-dependent and FAD-containing BVMOs

The Rossmann-fold motif (GxGxxG) and the conserved aspartate (D) in Type 1 BVMOs, five amino acids N-terminal to the phosphate-binding consensus sequence are highlighted in bold [21,22]. The BVMOs shown are MMKMO from *R. erythropolis* DCL14 (the present study), steroid mono-oxygenase (SMO-RR) from *R. rhodochrous* IFO 3338 [20], cyclohexanone mono-oxygenase (CHMO-AC) from *A. calcoaceticus* NCIMB 9871 [23], cyclopentanone mono-oxygenase (CPMO-Psp) from *Pseudomonas* sp. NCIMB 9872 [24], steroid mono-oxygenase (SMO-CR) from *Cylindrocarpon radiciola* ATTCC 11011 [25], cyclohexanone mono-oxygenase (CHMO-RC) from *R. caprophilus* WT1 [26], and 4,5,5-trimethyl-2-oxo-cyclopent-3-enylacetyl-CoA mono-oxygenase (OTP-PP) from *P. putida* NCIMB 10007 [21].

Phe-Asp-Ala-Val-Ile-Xaa-Gly-Ala-Gly-Phe-Xaa-Gly-Leu-Tyr-Ala-Leu-His-Arg. When this amino acid sequence was compared with that of other proteins present in the databases using the BLAST search programme, the highest homology (63% similarity) was found with steroid mono-oxygenase from *R. rhodochrous* IFO 3338 [20]. The N-terminus shows also substantial homology with many other previously determined N-termini of NADPH-dependent and FAD-containing (Type 1) BVMOs (Figure 2) [21], and it contains the GxGxxG (where 'x' denotes 'any residue') phosphate-binding consensus sequence involved in nucleotide binding [22], and an aspartate conserved in Type 1 BVMOs, five amino acids N-terminal of this consensus sequence [21].

Temperature- and pH-optima

MMKMO was active between pH 4.5 and 10.5. Depending on the buffer used, MMKMO was optimally active at pH 8.3 (Tris/HCl buffer; 3100 nmol·min^{-1}·mg^{-1}) or at pH 9.5 (glycine/NaOH buffer; 3150 nmol·min^{-1}·mg^{-1}). The fact that two different pH-optima were observed was, in part, an effect of the buffer used in the assay, but was also due to a different stability of MMKMO in these buffers: during the time of the activity assay (4 min), inactivation of MMKMO in Tris/HCl buffer was observed above pH 8.5, but in glycine/NaOH buffer, inactivation of MMKMO was only observed above pH 10.

The temperature optimum of MMKMO activity was near 36 °C. At pH 9.5 (glycine/NaOH buffer), the activity gradually increased from 600 nmol·min^{-1}·mg^{-1} at 4 °C to 3970 nmol·min^{-1}·mg^{-1} at 36 °C. At temperatures above 40 °C, enzyme inactivation was observed during the time of the activity assay (4 min), and at 50 °C activity was no longer detected.

Inhibitors and metal ions

A variety of enzyme inhibitors were tested for their ability to inhibit MMKMO activity. The thiol reagents iodoacetate and iodoacetamide (both at 1 mM) did not inhibit MMKMO activity, whereas *p*-chloromercuriobenzoate (0.1 mM) inhibited MMKMO only slightly (14%). Furthermore, the carbonyl reagent phenylhydrazine (1 mM) slightly inhibited MMKMO activity (by 10%), whereas MMKMO activity was not affected by either of the chelating agents, EDTA and α,α'-dipyridyl (both at 1 mM). DTT (1 mM) did not cause any inhibition, but SDS (1 mM) resulted in a drastic decrease (97%) in MMKMO activity. ATP was found to be a weak competitive inhibitor for MMKMO (K_i 9.5 mM).

The effect of several ions on MMKMO activity was also tested. None of the metals tested stimulated MMKMO activity; nor did any of the metal salts examined, i.e. $CaCl_2$, $CuCl_2$, $CoSO_4$, $MnCl_2$, $NiCl_2$ and $MgCl_2$ (all at 1 mM) inhibit enzymic activity. In contrast, $HgCl_2$ and $ZnCl_2$ (both at 1 mM) were found to inhibit MMKMO activity by 85% and 33% respectively.

Table 3 Substrate specificity of MMKMO from *R. erythropolis* DCL14

Relative activity was measured in terms of activity with (4*R*)-dihydrocarvone, where 100% = 3150 nmol·min^{-1}·[mg protein]$^{-1}$.

Substrate	Relative activity
Terpenoids	
(1*R*,4*R*)-Dihydrocarvone*	100
(1*S*,4*R*)-Iso-dihydrocarvone*†	7
(1*R*,4*S*)-1-Hydroxy-2-oxolimonene	133
(1*S*,4*R*)-1-Hydroxy-2-oxolimonene	88
(1*R*,4*S*)-Menthone	76
(1*S*,4*R*)-Menthone	82
(1*R*)-Pulegone	31
(1*S*)-Pulegone	51
(4*R*)-Carvone	2
(4*S*)-Carvone	14
(4*S*)-Piperitone	4
(4*S*)-Perillylaldehyde	< 1
8-Mercaptomenthone*	14
Norcamphor	145
(*R*,*S*)-Camphor	< 1
(1*R*)-Fenchone	4
Nootkatone	< 1
Cycloalkanones	
Cyclohexanone	151
Cycloheptanone	10
Cyclopentanone	12
2-Methylcyclohexanone‡	155
3-Methylcyclohexanone‡	151
4-Methylcyclohexanone‡	40
2-Allylcyclohexanone‡	145
2-Chlorocyclohexanone‡	112
2-Methoxycyclohexanone‡	168
2-Phenylcyclohexanone‡	148
2,2-Dimethylcyclohexanone	161
2,6-Dimethylcyclohexanone‡	108
Cycloalkanediones	
1,2-Cyclohexanedione	95
1,3-Cyclohexanedione	< 1
1,4-Cyclohexanedione	92
Others	
Acetophenone	< 1
Acetone	< 1

*, Diastereomeric mixture; †, from chiral GC analysis (see Figure 3); ‡, racemic mixture.

Table 4 Kinetic constants of MMKMO from *R. erythropolis* DCL14 with the monocyclic monoterpene ketone natural substrates

Kinetic constants were determined in incubations (30 °C and glyine/NaOH buffer, pH 9.5) with a constant enzyme concentration (40 μg protein · ml^{-1}), and were determined from Lineweaver–Burk plots ($r^2 > 0.985$).

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
(4*R*)-Dihydrocarvone*	3.9	0.13	30
(1*R*,4*S*)-1-Hydroxy-2-oxolimonene	4.9	< 0.01	> 490
(1*S*,4*R*)-1-Hydroxy-2-oxolimonene	3.9	0.13	30
(1*R*,4*S*)-Menthone	3.6	0.12	30
(1*S*,4*R*)-Menthone	6.0	< 0.01	> 600

*Diastereomeric mixture.

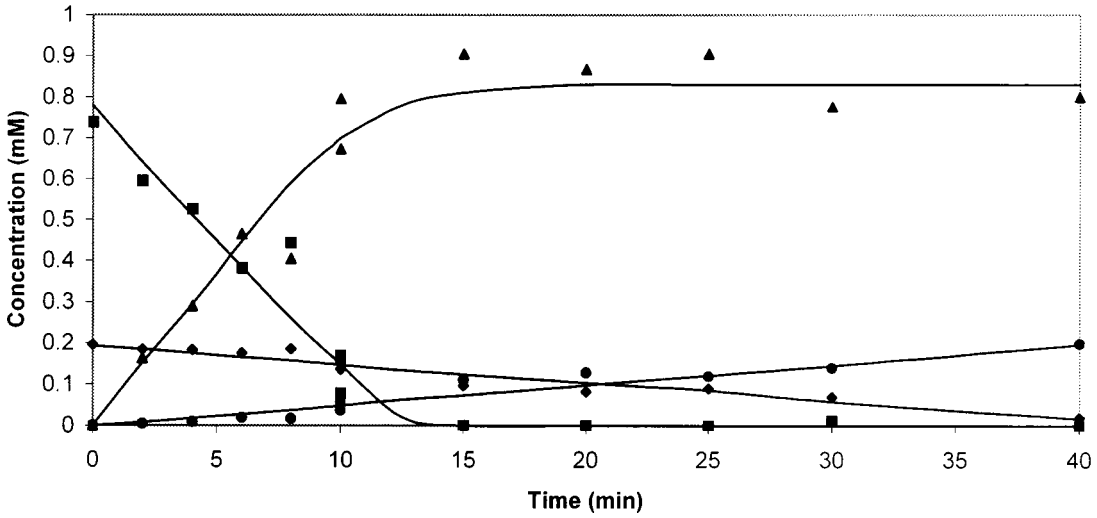

Figure 3 Stereoselectivity and regiospecificity of the conversion of (4*R*)-dihydrocarvone by MMKMO from *R. erythropolis* DCL14

Results were obtained from GC analysis. Each reaction mixture (30 °C) contained purified MMKMO (0.04 mg of protein · ml^{-1}), 50 mM Tris/HCl buffer (pH 8.5), 1.5 mM NADPH, and 1 mM (4*R*)-dihydrocarvone. ■, (1*R*,4*R*)-dihydrocarvone; ◆, (1*S*,4*R*)-iso-dihydrocarvone; ▲, (4*R*,7*R*)-4-isopropenyl-7-methyl-2-oxo-oxepanone; ●, (3*S*,6*R*)-6-isopropenyl-3-methyl-2-oxo-oxepanone.

Substrate specificity

Compounds that were tested as substrates for MMKMO are shown in Table 3. The enzyme has a broad substrate specificity and catalyses the conversion of a large number of monocyclic monoterpene ketones and substituted cyclohexanones. Cycloheptanone and cyclopentanone were much poorer substrates for the enzyme than cyclohexanone. Aldehydes, such as perillyl aldehyde, and acyclic ketones, such as acetophenone and acetone, did not act as substrates for the enzyme. Although MMKMO was essentially not active (< 0.25 %) with NADH, the K_m of MMKMO for NADPH was 0.038 mM.

MMKMO-catalyzed conversion of 1-hydroxy-2-oxolimonene, dihydrocarvone and menthone

The K_m and k_{cat} values of MMKMO for the different stereoisomers of the monocyclic monoterpene ketone natural substrates were determined (Table 4). (1*R*,4*S*)-1-Hydroxy-2-oxolimonene and (1*S*,4*R*)-menthone appeared to be the best substrates for the enzyme. However, only variations in the affinity of MMKMO for the different natural substrates were observed; the k_{cat} values were largely similar. When the kinetic constants for (1*S*,4*R*)-menthone were determined, the activity of MMKMO increased with decreasing substrate concentration, suggesting that (1*S*,4*R*)-menthone is an inhibitor of MMKMO.

(1*S*,4*R*)- and (1*R*,4*S*)-1-Hydroxy-2-oxolimonene are formed in the (4*R*)- and (4*S*)-limonene degradation pathways of *R. erythropolis* DCL14 respectively [10]. We have suggested previously that the lactone formed from 1-hydroxy-2-oxolimonene as the result of 1-hydroxy-2-oxolimonene-1,2-mono-oxygenase activity, 7-hydroxy-4-isopropenyl-7-methyl-2-oxo-oxepanone, is unstable and undergoes a spontaneous rearrangement to 3-isopropenyl-6-oxoheptanoate [10] (Scheme 1). The conversion of 1-hydroxy-2-oxolimonene by purified MMKMO confirmed this hypothesis; both enantiomers of this natural substrate were converted quantitatively into 3-isopropenyl-6-oxoheptanoate (results not shown). The intermediate lactone was not detected.

The stereoisomers of dihydrocarvone are formed in the (dihydro)carveol degradation pathways of *R. erythropolis* DCL14 [10a]. Dihydrocarvone has four stereoisomers [(1*R*,4*R*)-, (1*S*,4*R*)-iso-, (1*S*,4*S*)- and (1*R*,4*S*)-iso-dihydrocarvone], but only the diastereomeric mixture of (4*R*)-dihydrocarvone is commercially available. Both stereoisomers of (4*R*)-dihydrocarvone were converted by MMKMO; however, (1*R*,4*R*)-dihydrocarvone was converted 15 times faster than (1*S*,4*R*)-iso-dihydrocarvone (Figure 3). Two products were formed from (4*R*)-dihydrocarvone.

The ^{1}H-NMR and ^{13}C-NMR spectra recorded with this product mixture identified these products as 4-isopropenyl-7-methyl-2-oxo-oxepanone and 6-isopropenyl-3-methyl-2-oxo-oxepanone, on the basis of NMR data reported previously for these compounds [27]. From the ^{1}H-NMR peaks at $\delta = 1.23$ and 4.17 p.p.m. (specific for 6-isopropenyl-3-methyl-2-oxo-oxepanone), and at $\delta = 1.38$ and 4.47 p.p.m. (specific for 4-isopropenyl-7-methyl-2-oxo-oxepanone), the ratio of these compounds was estimated as 1:3. This ratio agrees with that calculated from the GC analysis (Figure 3). The retention times of these products, determined from the chiral GC experiments and their MS spectra, were identical with those of authentic samples of these compounds. Hence the major product of the MMKMO-catalysed conversion of (4*R*)-dihydrocarvone was identified as (4*R*,7*R*)-4-isopropenyl-7-methyl-2-oxo-oxepanone, and the minor product was shown to be (3*S*,6*R*)-6-isopropenyl-3-methyl-2-oxo-oxepanone. The substrate-conversion and product-formation profiles suggest that (1*R*,4*R*)-dihydrocarvone is exclusively transformed into (4*R*,7*R*)-4-isopropenyl-7-methyl-2-oxo-oxepanone, whereas (1*S*,4*R*)-iso-dihydrocarvone is transformed into the other regiostereoisomer, i.e. (3*S*,6*R*)-6-isopropenyl-3-methyl-2-oxo-oxepanone (Scheme 1 and Figure 3).

Although *R. erythropolis* DCL14 only grows on (1*R*,4*S*)-menthone and not on (1*S*,4*R*)-menthone [10], MMKMO converts both enantiomers of menthone. Stoichiometric accumulation of a product was observed during the conversion of both enantiomers of menthone (results not shown). The MS spectra and the retention time after chiral GC of the product formed from (1*S*,4*R*)-menthone were identical with that of authentic (4*S*,7*R*)-7-isopropyl-4-methyl-2-oxo-oxepanone, and the MS spectra and the retention time after chiral GC of the product formed from (1*R*,4*S*)-menthone were identical with those of authentic (4*R*,7*S*)-7-isopropyl-4-methyl-2-oxo-oxepanone respectively (Scheme 1).

DISCUSSION

This report describes the purification and characterization of monocyclic monoterpene ketone mono-oxygenase from *R. erythropolis* DCL14. This BVMO is induced when *R. erythropolis* DCL14 is grown on monocyclic monoterpenes (Table 1), reflecting its function in the limonene, (dihydro)carveol and (1*R*,3*R*,4*S*)-menthol degradation pathways of the bacterium [10,10a].

BVMOs are enzymes catalysing the insertion of one oxygen atom into a thermodynamically stable C–C bond between the carbonyl carbon and the adjacent carbon atom, forming lactones or esters as their products. On the basis of biochemical characteristics and the N-terminal amino acid sequences of these enzymes, two distinct types of BVMOs have been recognized [21]. Type 1 BVMOs (i) contain FAD as the prosthetic group; (ii) are NADPH-dependent; (iii) are monomeric, homodimeric or homotetrameric; (iv) have subunits of molecular mass 53–62 kDa; and (v) contain within their N-termini the characteristic GxGxxG nucleotide-binding consensus motif thought to be involved in FAD binding [23]. In contrast, Type 2 BVMOs (i) contain FMN as the prosthetic group; (ii) are NADH-dependent; and (iii) consist of two different subunits in an $\alpha_2\beta$ organization. MMKMO from *R. erythropolis* DCL14 clearly belongs to the Type 1 BVMOs, i.e. it contains FAD as the prosthetic group, uses NADPH as the cofactor, is a monomer of 60 kDa, and its N-terminus is homologous with all other known N-termini of Type 1 BVMOs.

Previously, three BVMOs involved in monoterpene degradation have been purified to homogeneity [7,8]. All three of these enzymes are from *Pseudomonas putida* PpG1 (ATCC 17453), where they are involved in the degradation of both enantiomers of the bicyclic monoterpene camphor. Two of these BVMOs are involved in the opening of the first ring of either (1*R*)-camphor (2,5-diketocamphane 1,2-mono-oxygenase [28]) or (4*S*)-camphor (3,6-diketocamphane 1,6-mono-oxygenase [7]). Both enzymes are Type 2 BVMOs, and show an absolute stereoselectivity: 2,5-diketocamphane 1,2-mono-oxygenase only converts the (1*R*)-enantiomer of camphor, whereas 3,6-diketocamphane 1,6-mono-oxygenase only converts (1*S*)-camphor [7]. The third BVMO involved in the degradation of camphor, 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA mono-oxygenase, is involved in the opening of the second ring of camphor [8]. This enzyme is a Type 1 BVMO, and has an absolute specificity for the CoA-activated natural substrate.

In marked contrast with the camphor degradation pathway of *P. putida* PpG1, the results presented here suggest that *R. erythropolis* DCL14 uses only one BVMO in three different monocyclic monoterpene degradation pathways. Not only does MMKMO act in three different pathways, it is also able to convert both enantiomers of 1-hydroxy-2-oxolimonene, menthone and dihydrocarvone [10a], in contrast with the Type 2 BVMOs from *P. putida* PpG1. However, cyclohexanone mono-oxygenase, a Type 1 BVMO from *Acinetobacter calcoaceticus* [29], is also enantioselective: it converts (1*S*,4*R*)-menthone, whereas (1*R*,4*S*)-menthone is not converted [27]. In contrast with MMKMO, cyclohexanone mono-oxygenase from *A. calcoaceticus* is also diastereoselective; it converts (1*R*,4*R*)-dihydrocarvone, but leaves (1*S*,4*R*)-iso-dihydrocarvone intact [27].

With MMKMO, the regiospecificity of the enzyme depends on the diastereomer used as the substrate (compare dihydrocarvone with iso-dihydrocarvone). Also in this respect, MMKMO differs from cyclohexanone mono-oxygenase from *A. calcoaceticus*. This BVMO converts the two enantiomers of dihydrocarvone with a different regioselectivity: whereas (1*S*,4*S*)-dihydrocarvone was converted into the chemically expected product (4*S*,7*S*)-4-isopropenyl-7-methyl-2-oxo-oxepanone, (1*R*,4*R*)-dihydrocarvone was transformed into the other regiostereoisomer, i.e. (3*R*,6*S*)-6-isopropenyl-3-methyl-2-oxo-oxepanone [27].

MMKMO has a similar broad substrate specificity, as do cyclohexanone mono-oxygenases from different microorganisms [30–32]. Since cyclohexanone is of anthropogenic origin, Donoghue et al. [29] suggested that natural cyclic molecules, such as monoterpenes, might be the natural substrate for cyclohexanone mono-oxygenase. However, although cyclohexanone mono-oxygenase from *A. calcoaceticus* converts several monoterpene ketones, including dihydrocarvone, this microorganism was not able to grow on these monoterpenes as sole source of carbon and energy [29].

In conclusion, MMKMO from *R. erythropolis* DCL14 is responsible for the initial step towards ring opening for all stereoisomers of the monocyclic monoterpene ketones in three different monocyclic monoterpene degradation pathways.

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