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Oxidative biotransformations by microorganisms: production of chiral synthons by cyclopentanone monooxygenase from *Pseudomonas* sp. NCIMB 9872

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Abstract

Cyclopentanone monooxygenase, an NADPH- plus FAD-dependent enzyme induced by the growth of *Pseudomonas* sp. NCIMB 9872 on cyclopentanol, has been utilised as a biocatalyst in Baeyer–Villiger oxidations. Washed whole-cell preparations of the microorganism oxidised 3-hexylcyclopentanone in a regio- but not enantioselective manner to give predominantly the racemic γ-hexyl valerolactone. Similar preparations biotransformed 5-hexylcyclopent-2-enone exclusively by regio- plus enantioselective oxidation to the equivalent α,β-unsaturated (*S*)-(+)-δ-hexyl valerolactone (ee = 78%), with no reductive biotransformations catalysed by either EC 1.1.*x*.*x*- or EC 1.3.*x*.*x*-type dehydrogenases.

An equivalent biotransformation of 5-hexylcyclopent-2-enone was catalysed by highly-purified NADPH- plus FAD-dependent cyclopentanone monooxygenase from the bacterium. The regio- plus enantioselective biotransformation by the pure enzyme of 2-(2′-acetoxyethyl)cyclohexanone yielded optically-enriched (*S*)-(+)-7-(2′-acetoxyethyl)-2-oxepanone (ee = 72%). The same biotransformation when scaled up again provided optically-enriched (*S*)-(+)-ε-caprolactone which was converted, using methoxide, to (*S*)-(−)-methyl 6,8-dihydroxyoctanoate (ee = 42%), thereby providing a two-step access from the substituted cyclohexanone to this important chiron for the subsequent synthesis of (*R*-(+)-lipoic acid.

Some characteristics of pure NADPH- plus FAD-dependent cyclopentanone monooxygenase were determined including the molecular weight of the monomeric subunit (50 000) of this homotetrameric enzyme, and the N-terminal amino acid sequence up to residue 29, which includes a putative flavin nucleotide-binding site.

Keywords: Synthons; Lactones; *Pseudomonas*; Lipoic acid

1. Introduction

The value of catalytic asymmetric oxidations for the synthesis of chiral building blocks is widely recognised [1]. Although the oxidative Baeyer–Villiger reaction has been recognised for almost a century, and various competent reagents and metal catalysts developed [2–4], with very few notable exceptions [5,6], such chemical reactions rarely lead to optically enriched products. Conversely, extensive use has been made of various microbial Baeyer–Villiger monooxygenase enzymes as biocatalysts able to achieve regio- and/or stereoselective ring ex-

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pansion of racemic or prochiral lactones to equivalent chiral lactones, some of which are themselves valuable biologically-active compounds [7], while others are chirons that can be used for the synthesis of target molecules [8,9].

Initially, the most extensively used microbial enzyme was the NADPH-dependent cyclohexanone monooxygenase from cyclohexanol-grown *Acinetobacter calcoaceticus* NCIMB 9871 [10]. Recently there have been some significant developments using the two NADH-dependent diketocamphane monooxygenase isozymes [9], plus the NADPH-dependent 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase(s) [11], present in *Pseudomonas putida* NCIMB 10007 after growth of the bacterium on camphor as the sole source of carbon [12].

Although it is known to be able to undertake some highly regiospecific biotransformations [13], the equivalent NADPH-dependent cyclopentanone monooxygenase (CPMO) from cyclopentanol-grown *Pseudomonas* sp. NCIMB 9872 [14] has been exploited far less extensively. In this paper we report firstly some useful characteristics of whole-cell preparations of cyclopentanol-grown *Pseudomonas* sp. NCIMB 9872 for undertaking oxidative biotransformations of unsaturated alicyclic ketones. In addition, we detail a convenient method to purify to homogeneity the relevant Baeyer–Villiger enzyme, CPMO, report various characteristics of the pure protein, and demonstrate a valuable role for a highly-specific biotransformation carried out by the pure enzyme in the chemoenzymatic synthesis of the natural biomolecule (*R*)-(+)-lipoic acid.

2. Materials and methods

2.1. Microorganisms, maintenance and growth

Pseudomonas sp. NCIMB 9872 was maintained on nutrient agar slopes at 30°C. and routinely grown as previously described [14].

2.2. Whole-cell biotransformations using Pseudomonas sp. NCIMB 9872

Cells harvested during late log phase were resuspended in 1.5 × cell volume of phosphate buffer (21 mM, pH 7.1) and the suspension used immediately for biotransformations. Unless otherwise stated, the substrate (0.5 mg ml^{-1}) which was predissolved in the minimum volume of ethanol, was added to the cell suspension, and the mixture placed in an orbital incubator (200 rpm) at 30°C. The biotransformation was monitored by routine sampling of aliquots (200 μl) which were extracted with an equal volume of ethyl acetate and analysed by GC (BP1 column, column temperature 150°C, injector temperature 250°C, detector temperature 250°C, carrier gas (He) flow rate 0.75 ml min^{-1}). When the reaction had proceeded either to the required stage (around 45% bioconversion) or had terminated, the cells were removed by centrifugation (1500*g*, 15 min) and the supernatant extracted (×3) using an equal volume of ethyl acetate. The combined extracts were dried over anhydrous magnesium sulfate, and the solvent removed under reduced pressure to give a crude residue. The residual substrate and/or products were separated and purified by flash column chromatography. The ee's were determined either by chiral GC (Lipodex E capillary column) or by derivatisation to the corresponding diastereoisomeric orthoesters [15] which were separable by GC (BP1 capillary column).

2.3. Purification of NADPH-dependent CPMO

Approximately 30 g wet weight of late log phase cells harvested from 12 l of growth medium were disrupted by a single pass through a French press. The fractured cells were suspended in 200 ml of Tris–HCl buffer (50 mM, pH 7.5) supplemented with EDTA (100 μM), β-mercaptoethanol (100 μm), and PMSF (1 μM). The cell debris was centrifuged (1500*g*, 30 min) and the clear supernatant retained. The pelleted debris was resuspended in 100 ml of

the same buffer and again centrifuged. The clear cell-free extracts resulting from both treatments were combined and then mixed with a solution of 0.1 M streptomycin sulphate in phosphate buffer (25 mM, pH 7.0) in the ratio 10 parts cell-free extract: 1 part streptomycin sulphate solution. The mixture was stirred gently for 10 min, left to stand for 1 h at 4°C, and then centrifuged (1500 g, 45 min) to remove any precipitated nucleic acids. The supernatant was taken to 35% ammonium sulphate saturation and left for 1 h at 4°C. Any precipitated protein was removed by centrifugation (1500 g, 45 min) and the supernatant then further taken to 60% ammonium sulphate saturation and the centrifugation treatment repeated. The resultant pellet of precipitated protein was dissolved in 25 ml Tris–HCl (50 mM, pH 7.5) and dialysed against three successive 2 l volumes of the same buffer. The dialysed crude enzyme preparation (70 ml) was loaded onto a Fast-Flow Q-Sepharose column (17 × 2.5 cm) which was eluted with a gradient of 0 to 0.5 M KCl in Tris–HCl buffer pH 7.5. Column fractions were assayed for NADPH-dependent CPMO activity using the method of Griffin and Trudgill [14]. Active fractions, which eluted in the 0.3–0.4 M KCl region, were pooled, the protein precipitated (70% ammonium sulphate saturation), and removed by centrifugation (30 000 g, 20 min). The pellet of protein was dissolved in 20 ml of supplemented Tris–HCl buffer, divided into 4 × 5 ml aliquots, and each aliquot in turn eluted with Tris–HCl buffer (50 ml, pH 7.5) after being loaded onto a Superose-12 column (30 × 2.5 cm). Cell fractions (2 ml) from the four separate runs that contained CPMO were pooled (38 ml) and then loaded onto a second Fast-Flow Q-Sepharose column (15 × 5 cm) which was eluted with a gradient of 0–0.5 M KCl in Tris–HCl buffer pH 7.5. Column fractions containing CPMO were pooled (25 ml) and dialysed against 1 l of phosphate buffer (50 mM, pH 6.8) containing 1.7 M ammonium sulphate. The dialysed sample was finally loaded onto a Phenyl Sepharose column (15 × 1.5 cm) previously equilibrated with phosphate buffer (50 mM. pH 6.8) containing 1.7 M ammonium sulphate. The column was then eluted with a decreasing gradient of 1.7–0 M ammonium sulphate in phosphate buffer (50 mM, pH 6.8). The fractions containing CPMO were retained (3.5 ml).

2.4. Characterisation of CPMO

The progress of the purification protocol was monitored by subjecting samples to electrophoresis on sodium dodecylsulphate polyacrylamide gels (Phastgel, Pharmacia). Gels were stained with Coomassie brilliant blue and cleared by washing with glacial acetic acid:ethanol:water (10:45:45). The N-terminal sequence of the purified protein was determined by the Protein Sequencing Facility, University of Aberdeen.

2.5. General procedures for biotransformations using purified CPMO

Unless otherwise stated the substrate (0.5 mg ml^{-1}) predissolved in the minimum volume of ethanol was stirred (orbital incubator, 200 rpm, 30°C) in Tris–HCl buffer (50 mM, pH 7.5) containing an equimolar amount of NADPH and purified CPMO (0.04–0.27 IU). The biotransformation was monitored, the reaction stopped, and the residual substrate and/or resultant product(s) isolated and characterised as described above for the equivalent whole-cell biotransformations.

2.6. Characterisation of lactone products

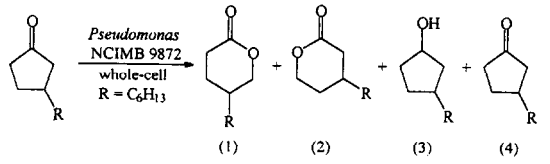
The absolute configuration of the optically active lactones was confirmed by polarimetry. The α,β-unsaturated δ-alkyl valerolactone was hydrogenated (5% Pd/C, H_2) to 6-hexyltetrahydropyran-2-one, and the sign of rotation compared to a literature value [7]. The ϵ-(2′-acetoxyethyl)-caprolactone was converted (NaOMe, MeOH) to methyl 6,8-dihydroxyoc-

tanoate, and the sign of rotation compared to a literature value [16].

3. Results and discussion

Because cyclopentanol-grown *Pseudomonas* sp. NCIMB 9872 is known to contain high induced titres of both NADH-dependent cyclopentanol dehydrogenase [17] and NADPH-dependent CPMO [14,17], the ability of this microorganism to undertake potentially useful oxidative (ketone → lactone(s)) and reductive (ketone → secondary alcohol(s)) biotransformations of carbocyclic ketones related to the growth substrate was examined. Initial studies were undertaken using washed whole-cells of the bacterium harvested in the late log phase of growth, both because of the ease of use of this form of the biocatalyst and the endogenous ability of such preparations to recycle the requisite reduced nicotinamide nucleotide coenzymes. Characterisation of the compounds isolated from the stopped reaction mixture after incubation of 3-hexylcyclopentanone with the biocatalyst for 6 h confirmed the presence of 30% unreacted ketone (isolated yield) plus 7% alcohol and 63% lactone (but not lactol) products, indicative of separate oxidative and reductive biotransformations (Table 1). The racemic nature of the residual ketone and both regioisomeric lactone products confirmed that the predominant bioconversion of this particular substituted cyclopentanone by whole-cell preparations of NCIMB 9872, (i.e, the oxygenation of the ketone unit), was not enantioselective. Potentially the most interesting aspect of this biotransformation was the production of the relatively small amount of β-hexyl valerolactone, which suggested that for this particular substrate the CPMO of NCIMB 9872 exhibited some regioselectivity (γ-hexyl valerolactone:β-hexyl valerolactone, regioisomeric excess 86%) compared to the equivalent chemical reaction (MCPBA, γ-hexyl valerolactone:β-hexyl valerolactone, regioisomeric excess 0%). Similar regioselectivity has been observed previously in the whole-cell biotransformation of norbornanone by this particular bacterium [13].

Table 1
Biotransformation of 3-hexylcyclopentanone by whole-cell preparation of *Pseudomonas* sp. NCIMB 9872



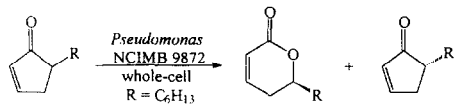
Concentration (mg/ml)	0.5
Time (h)	6
Unreacted ketone (4) (%)	30.2 [a]
Conversion to lactone (1) (%)	58.6 [a]
Conversion to lactone (2) (%)	4.4 [a]
Conversion to alcohol (3) (%)	6.7 [a]
Enantiomeric excess of recovered ketone (4) (%)	racemic
Enantiomeric excess of lactone (1) (%)	racemic
Enantiomeric excess of lactone (2) (%)	racemic
Regiomeric excess lactone (1):lactone (2) (%)	86

[a] GC on isolated crude reactants.

Attention was then focused on the whole-cell biotransformation of 5-hexylcyclopent-2-enone, a substrate with the additional potential to test the ability of cyclopentanol-grown NCIMB 9872 to undertake reductive biotransformations catalysed by EC 1.3.*x*.*x*-type enzyme(s). An equivalent biotransformation by camphor-grown cells of *Pseudomonas putida* NCIMB 10007 resulted in a complex mixture of both saturated and unsaturated lactones and ketones [18]. An added incentive for studying this particular biotransformation was to gain access to simple lactone product(s), since the equivalent chemical reaction with MCPBA proceeded with epoxidation dominating over Baeyer–Villiger oxygenation, the sole product being the racemic *anti*-epoxylactone 3,4-*exo*-oxy-6-hexyltetrahydropyran-2-one.

Incubation of the racemic ketone with the biocatalyst for 6 h yielded predominantly unreacted ketone (61% isolated yield), but interestingly no equivalent reduced ketone (Table 2). Compared to the mixture of products obtained in the equivalent biotransformation by

Table 2
Biotransformation of 5-hexylcyclopent-2-enone by whole-cell preparation of *Pseudomonas* sp. NCIMB 9872



Concentration (mg/ml)	0.5
Time (h)	6
Unreacted ketone (%)	61 [a]
Conversion to lactone (%)	34 [a]
Enantiomeric excess of lactone (%)	78
E_p of lactone [b]	~12
Enantiomeric excess of ketone (%)	~40 (calculated)

[a] GC on isolated crude reactants.

[b] Enantiomeric ratio, $E_p = \frac{\ln[1 - c(1 + ee_p)}{\ln[1 - c(1 - ee_p)}$, c = conversion.

camphor-grown NCIMB 10007 [18], the sole characterised product of biotransformation by NCIMB 9872 was the equivalent α,β-unsaturated δ-hexyl valerolactone, thereby giving the biotransformation an absolute advantage over the equivalent MCPBA-catalysed chemical reaction. The absence of any equivalent γ,δ-unsaturated α-hexyl valerolactone confirmed the previously observed [13] regioselective nature of CPMO. The failure to detect either saturated δ-hexyl valerolactone or any alicyclic alcohol(s), along with the failure to detect any saturated ketone, confirmed the inability of washed-cell preparations of NCIMB 9872 to undertake reductive biotransformations of this particular substrate catalysed by either EC 1.1.*x*.*x*- or EC 1.3.*x*.*x*-type dehydrogenases present after growth on cyclopentanol.

Of even greater interest was the enantioselective nature of the recorded oxygenative ring expansion, the predominant α,β-unsaturated δ-hexyl valerolactone product being the (*S*)-(+)-antipode (ee = 78%, E_p = ca. 12). This was the first evidence of the stereoselective nature of the NADPH-dependent CPMO from NCIMB 9872, and contrasted sharply with the racemic lactone products formed from 3-hexylcyclopentanone (vide supra).

Encouraged by the demonstrated potential of CPMO from NCIMB 9872 to undertake both regio- and enantioselective oxygenative biotransformations, a new purification protocol was developed based on the use of column chromatography materials with higher loading capacities than those originally used by Griffin and Trudgill [14]. Another advantage over the original protocol was the insignificant dissociation of the flavin prosthetic group, known to be FAD, from the enzyme during purification. Samples of the most highly purified preparations from the Phenyl Sepharose column gave an absorbance spectrum typical of a flavoprotein (λ_{max} = 437 nm). Such samples when treated with sodium dodecylsulphate, subjected to polyacrylamide gel electrophoresis, and then stained with Coomassie brilliant blue gave a single band, indicating a protein composed of only one type of monomeric subunit. When compared with a series of similarly treated monomeric marker proteins of known molecular weight, the molecular weight of the monomeric subunit from CPMO was calculated to be about 50000. As the molecular weight of the native enzyme has been shown to be approximately 200000 [14], this confirms that this particular monooxygenase is a homotetrameric flavoprotein, unlike the cyclohexanone monooxygenase from *A. calcoaceticus* NCIMB 9871 which is a structurally simple monomeric NADPH- plus FAD-binding protein [19].

N-terminal amino acid analysis of a pure sample of the protein (Fig. 1) exhibited limited

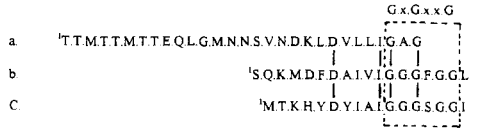


Fig. 1. N-terminal amino acid sequence of cyclopentanone monooxygenase and some related flavoproteins. Sequence alignment of nucleotide-binding regions of (a) cyclopentanone monooxygenase, (b) cyclohexanone monooxygenase from *Acinetobacter calcoaceticus*, and (c) glutathione reductase from *Escherichia coli*. Amino acid sequences are given in the standard one-letter codes. All three putative nucleotide-binding sites (G*x*G*xx*G) are located near the N-terminus of the various proteins. Homologous residues are denoted by the solid lines.

homology with the equivalent sequence of cyclohexanone monooxygenase [20]. In both proteins there is evidence for a G*x*G*xx*G nucleotide-binding domain [21]. A number of other characterised flavoproteins, including glutathione reductase and *p*-hydroxybenzoate hydroxylase are known to have an equivalent FAD-binding site located near the N-terminus of the protein [22]. One other interesting feature of CPMO, as yet unexplained, is the threonine-dominated distal sequence which shows no obvious homology with other sequenced proteins currently held in accessible databanks.

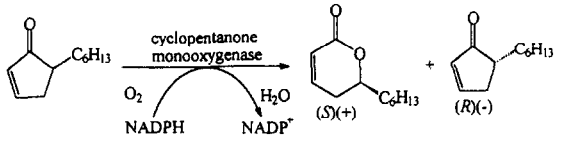
Having developed a protocol to yield significant amounts of highly purified CPMO, the homogeneous protein was then used to undertake oxygenative biotransformations of various model substrates. In each case the reaction mixtures were made up with equimolar concentrations of alicyclic ketone and NADPH, thus avoiding the complication of introducing a coenzyme recycling system.

Because of the advantageous result obtained with the whole-cell biotransformation of 5-hexylcyclopent-2-enone, this ketone was tested with the pure enzyme. The reaction was allowed to run until ca 50% of the racemic lactone had been formed, as judged by GC analysis (Table 3). Again, as with the equivalent whole-cell biotransformation (vide supra), the sole recovered product was the equivalent α,β-unsaturated (*S*)-(+)-δ-hexyl valerolactone (ee = 73%; E_p = ca. 10). Analysis of the residual ketone confirmed that the pure enzyme had accomplished a partial kinetic resolution of the racemic substrate. It seems likely that both enantiomers of the substrate are able to fit into the active site of the enzyme and undergo equivalent biooxidations to yield enantiocomplementary α,β-unsaturated δ-hexyl valerolactones, although the (*S*)-(+)-antipode is preferentially bound as evidenced by the higher recorded enantiopurity of the resultant (*S*)-(+)-lactone produced by the equivalent whole-cell biotransformation, which had consumed significantly less of the available racemic substrate (Table 2).

The enantioselectivity of the pure enzyme was then tested with racemic 2-(2′-acetoxyethyl)-cyclohexanone. A regio- plus enantioselective biotransformation of this lactone to an equivalent ϵ-caprolactone is of interest because it allows access to (*S*)-(−)-methyl 6,8-dihydroxyoctanoate, which has been shown to be a chiral synthon for a novel route (Scheme 1) to the total synthesis of (*R*)-(+)-lipoic acid [11]. Lipoic acid is a biomolecule first recognised as a growth factor for various microorganisms [23], and which is now known to be an important enzyme prosthetic group in various biological systems [24]. The previous attempt to produce the requisite (*S*)-(+)-ϵ-caprolactone by regio- plus enantioselective oxidation of the racemic ketone precursor using purified 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase, the NADPH-dependent Baeyer–Villiger monooxygenase present in the camphor-grown *P. putida* NCIMB 10007, resulted in isolation of the enantiocomplementary (*R*)-(−)-antipode in good yield (43% isolated yield, ee = 78%), which necessitated the inclusion of a Mitsunobu inversion reaction in the subsequent chemical synthesis of (*R*)-(+)-lipoic acid [11].

In a small-scale biotransformation of 2-(2′-acetoxyethyl)cyclohexanone by pure CPMO al-

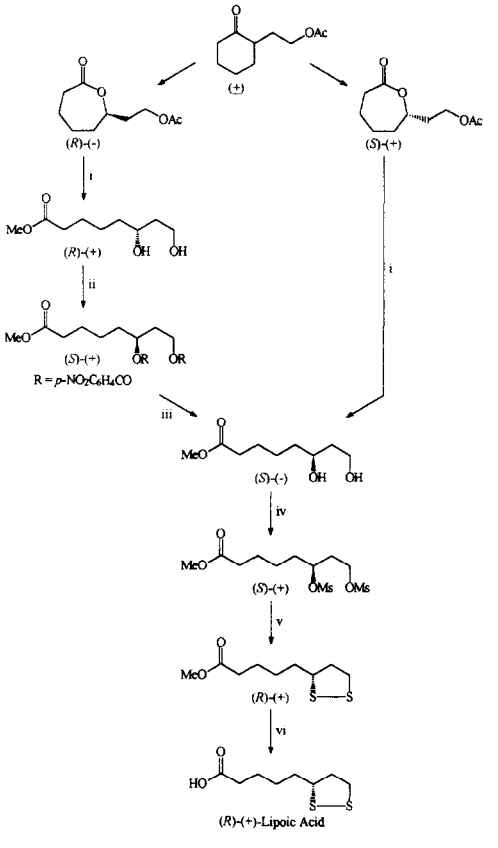
Table 3
Biotransformation of 5-hexylcyclopent-2-enone by cyclopentanone monooxygenase



Concentration (mg/ml)	0.5
Time (h)	5
Unreacted ketone (%)	43 [a]
Conversion to lactone (%)	52 [a]
Enantiomeric excess of ketone (%)	38
Enantiomeric excess of lactone (%)	75
E_p of lactone	~ 10

[a] GC on isolated crude reactants.

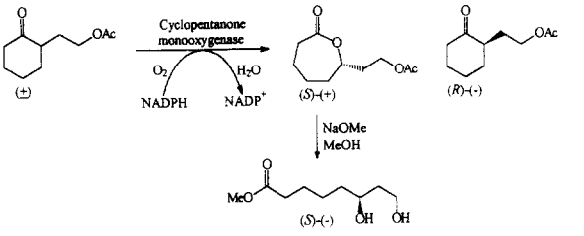
lowed to run to 30% ketone biooxidation (data not shown), the sole recovered product was indeed the equivalent optically-enriched (*S*)-(+)-ϵ-caprolactone (ee = 72%; E_p = ca. 8). The biotransformation was then scaled up (20 mg racemic ketone: Table 4), and allowed to run until the enzyme had consumed approximately 60% of the available substrate, when the sole product recovered from the stopped reaction mixture was again (*S*)-(+)-7-(2′-acetoxyethyl)-2-oxepanone (59% isolated yield: ee = 42%: E_p = ca. 5). The recovered lactone was then directly converted, using methoxide into the (*S*)-(−)-methyl 6,8-dihydroxyoctanoate (63% isolated yield: ee = 42%), thereby providing a two-step access from the substituted cyclohexanone to this important chiron for the chemoenzymatic synthesis of (*R*)-(+)-lipoic acid compared to the equivalent four-step access when using the alternative NADPH-dependent Baeyer–Villiger monooxygenase from camphor-grown NCIMB 10007.



i, NaOMe, MeOH; ii, p-$NO_2C_6H_4CO_2H$, PPh_3, DEAD, THF; iii, K_2CO_3, MeOH; iv, MsCl, Et_3N, CH_2Cl_2, 0 °C; v, $Na_2S{\cdot}9H_2O$, S, DMF, 80 °C; vi, KOH (0.1 M), MeOH.

Scheme 1. Chemoenzymatic synthesis of (*R*)-(+)-lipoic acid.

Table 4
Biotransformation of 2-(2′-acetoxyethyl)cyclohexanone by cyclopentanone monooxygenase



Concentration (mg/ml)	1
Scale (mg)	20
Time	30 min.
Crude yield (mg)	20.3
Unreacted ketone (%)	39 [a]
Isolated yield (%)	37
Enantiomeric excess (%)	68
Conversion to lactone (%)	61 [a]
Isolated yield (%)	59
Enantiomeric excess (%)	42
E_p	~ 5
Conversion lactone to diol (% isolated yield)	63
Optical rotation	$[\alpha]_D = -2°$ (c = 0.5: $CHCl_3$)
Lit. optical rotation [b]	$[\alpha]_D = -3.9°$ (c = 2.3: $CHCl_3$)
Enantiomeric excess (%)	42

[a] GC on isolated crude reactants.
[b] Ref. [16].

Clearly the NADPH-dependent CPMO from cyclopentanol-grown *Pseudomonas* sp. NCIMB 9872 warrants further investigation as a biocatalyst to gain access to chiral lactone and/or ketone synthons of value in the chemoenzymatic synthesis of useful target molecules.

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