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On the Resolution of Chiral Substrates by a *retro*-Claisenase Enzyme: Biotransformations of Heteroannular Bicyclic β-Diketones by 6-Oxocamphor Hydrolase

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Abstract: The enzyme 6-oxocamphor hydrolase (OCH) from *Rhodococcus* sp. NCIMB 9784 catalyses the cleavage of a carbon-carbon bond between two carbonyl groups in both mono- and bicyclic non-enolisable β-diketone substrates. In this mode OCH has been shown to effect the desymmetrisation of both bridged symmetrical bicyclic [2.2.1] and [2.2.2] systems and a series of 1-alkylbicyclo[3.3.0]octane-2,8-diones, yielding chiral substituted cyclopentanone and cyclohexanone products in high optical purity. In the present study, OCH has been challenged with a series of heteroannular substrates including 1-methylbicyclo[4.3.0]nonane-2,9-dione (7a-methylhexahydroindene-1,7-dione) in an effort to assess the competence of the enzyme for kinetic resolutions of asymmetric, racemic substrates. OCH was shown to catalyse the resolution of 1-methylbicyclo[4.3.0]nonane-2,9-dione with an *E* value of 2.9. The effect of increasing the length of the alkyl chain in the 1-position, or enlarging one of the rings, was to increase the enantioselectivity of the enzyme to 5.7 and 3.1 for the substrates 1-allylbicyclo[4.3.0]nonane-2,9-dione (7a-allylhexahydroindene-1,7-dione) and 1-methylbicyclo[5.3.0]decane-2,10-dione (8a-methyloctahydroazulene-1,8-dione), respectively. 1-Methylbicyclo[5.4.0]undecane-2,10-dione (9a-methyloctahydrobenzocycloheptene-1,9-dione) was not a substrate for OCH. These experiments constitute the first description of the resolution behaviour of such a *retro*-Claisenase enzyme, and suggest a maximum steric limit for substrate recognition by OCH.

Keywords: biotransformations; chemoenzymatic synthesis; β-diketones; enzyme catalysis; enzymes; lyases

Introduction

We have recently been evaluating the potential of the C–C bond cleaving 6-oxocamphor hydrolase, involved in the metabolism of (1*R*)-(+)-camphor by *Rhodococcus* sp. NCIMB 9874, for the preparative desymmetrisation of bicyclic β-diketones.[1] The transformation catalysed by OCH in nature is the cleavage, by a *retro*-Claisen reaction, of the bicyclic diketone 6-oxocamphor (**1**), to α-campholinic acid (**2**), a reaction that proceeds with high diastereoselectivity and enantioselectivity in favour of the (2*R*,4*S*)-enantiomer of the product (Figure 1). This represented an unusual enzymatic desymmetrisation reaction, and we have since applied the enzyme to the desymmetrisation of a series of symmetrical bicyclic β-diketones of the [2.2.1],[1] [2.2.2][1] (**3**) and [3.3.0][2] (**5**) series, and demonstrated that, in each case, it is possible to obtain quantitative yields of optically enriched keto acid products (**4** and **6**) from these biotransformations. We have also obtained an X-ray crystal structure of both native OCH[3] and a low activity mutant, in which one of the natural products of reaction was observed.[4] The latter structure has helped to shed light on the molecular determinants of both mechanism and enantiotopic selectivity in reactions catalysed by OCH. At this stage, we have proposed a mechanism for the activity of OCH that is initiated by general base activation of a water molecule by histidine 145. The hydroxide nucleophile was postulated to attack the carbonyl group on the *pro*-(*S*) topos of the substrate only, as the carbonyl group on the other topos is held by an oxyanion hole formed by the side chains of histidine 122 and tryptophan 40. A tetrahedral oxyanion is formed, that rearranges to form an enolate intermediate that is protonated to give the final product. An

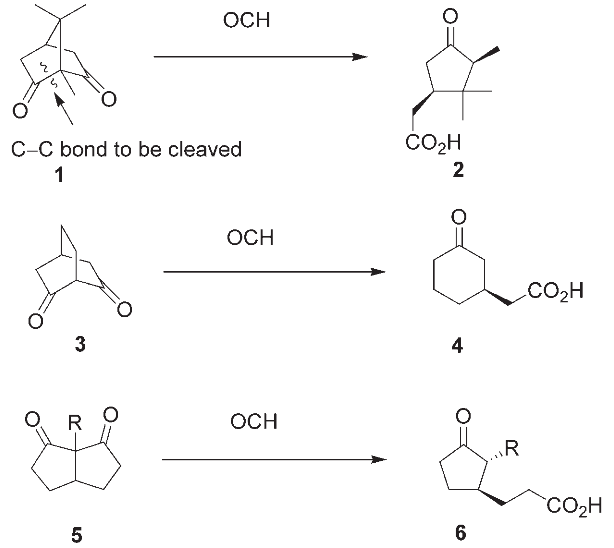

Figure 1. Desymmetrisations of prochiral bicyclic β-diketones by OCH.

additional measure of prochiral control is afforded by the nascent cyclopentane ring of the product forming a stacking interaction with phenylalanine 82.[4]

With a view to examining the wider substrate specificity of OCH, we decided to synthesise a series of asymmetric, heteroannular bicyclic β-diketones that would test the ability of the enzyme to catalyse the *resolution* of racemic substrates. As the substrate specificity of OCH had proven to be quite narrow, accepting only mono- or bicyclic diketones that were non-enolisable, either through restrictions imposed by Bredt's rule (in the case of bicyclo[2.2.2]octane-2,6-dione) or by alkylation at the carbon atom between the carbonyl groups (as in the series of 1-alkylbicyclo-[3.3.0]octane-2,8-diones,[2] it was decided to synthesise compounds based around bicyclo [4.3.0], [5.3.0] and [5.4.0] systems (Figure 2), as these would present the best opportunities for successful substrate recognition. A general route to this class of bicyclic β-diketones was recently published.[5] In this report we detail studies into the biotransformation of compounds **9**, **10** and **12** by OCH, and describe some characteristics of the kinetic resolution exhibited by the enzyme.

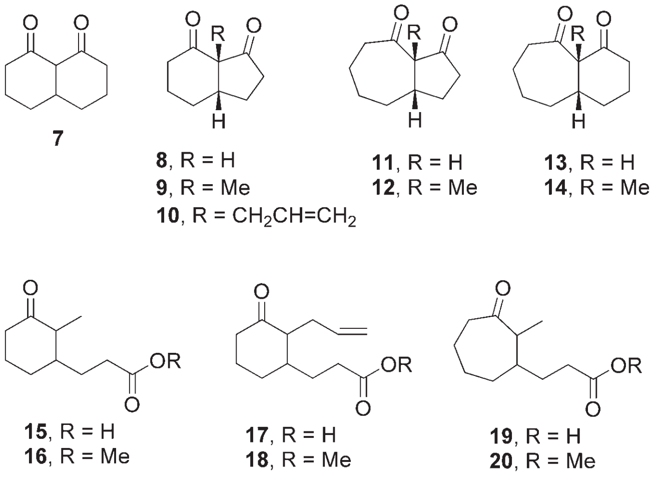

Figure 2. Compounds described in the present study.

Results and Discussion

As part of a continuing study into the wider substrate specificity of OCH, the decalin analogue substrate **7** was synthesised according to the published method.[5] Disappointingly, it did not prove possible to perform alkylations of substrate **7** using the standard techniques, and predictably, **7** itself was not a substrate for OCH, nor were the unsubstituted [4.3.0] (**8**), [5.3.0] (**11**) and [5.4.0] (**13**) bicyclic diketones, the syntheses of which were reported in our previous paper,[5] that form the basis of compounds **9**, **10**, **12** and **14**. Substrates **9**, **10**, **12** and **14** were synthesised by alkylation of the parent bicyclic diketones using the procedures described in the Experimental Section. Milligram quantities of compounds **9**, **10**, **12** and **14** were tested for their suitability as substrates, and gratifyingly, reactions analysed by TLC against the appropriate controls revealed disappearance of substrates **9**, **10** and **12** over a 16–72 h period. Substrate **14** proved not to be a substrate for OCH.

The substrates under consideration had been synthesised in an attempt to explore the substrate specificity of OCH with respect to the kinetic resolution of racemates. It is useful at this point to consider the possible stereochemical course of the biotransformation of these asymmetric substrates by the enzyme. In the case of heteroannular substrates such as **9**, **10**, **12** and **14**, it is conceivable that a) the bond between carbonyls that may be cleaved may be in either the larger or smaller ring, or both, and that b) two pairs of enantiomeric product forms may result from cleavage of either bond. Hence, the maximum possible number of products from the reaction is eight (Figure 3). The ideal outcome of any such biotransformation of a heteroannular ketone would be one product of eight therefore, in which case the enzyme would display first *enantio*-selectivity, selecting one enantiomer of the diketone for transformation; thence *regio*-selectivity for the bond to be cleaved. The diastereomeric mix of the final product has previously been found not to be determined by the enzyme, as, whilst the attack of base-activated water at one topos is enzyme-controlled, the protonation of the enolate is not. This is evidenced by the same diastereomeric mixture of enantiomerically enriched products being recovered from biotransformations of both the natural substrate **1**[1] and the 1-alkylbicyclo-[3.3.0]octane-2,8-dione series **5**.[2]

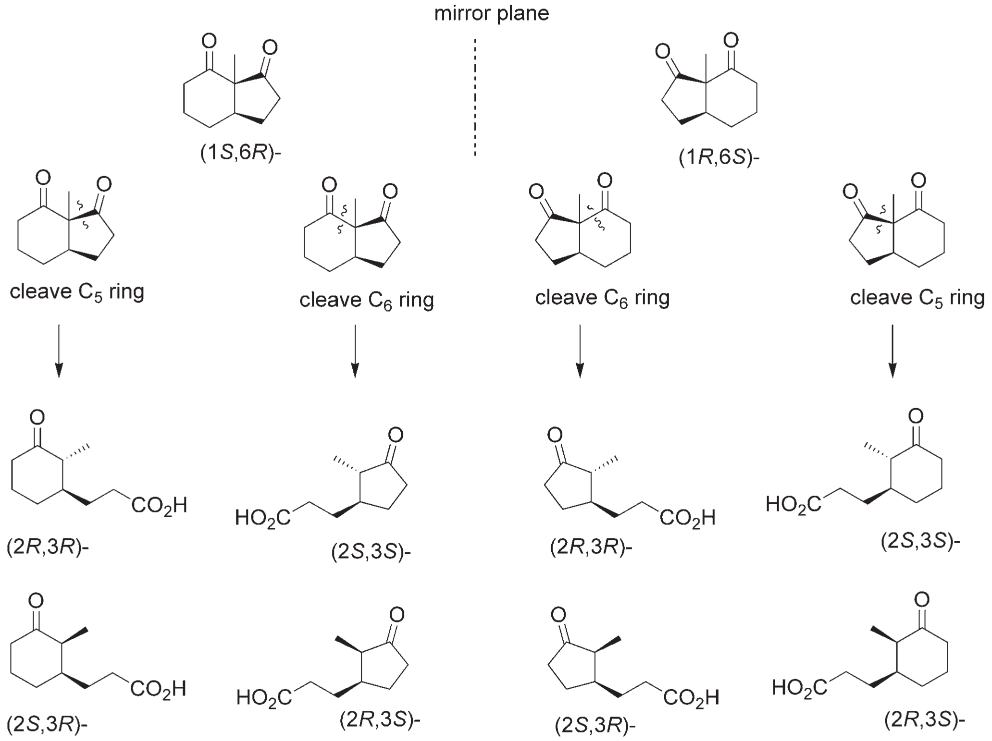

Figure 3. Possible stereochemical outcomes of *retro*-Claisen cleavage of heteroannular bicyclic diketone **9** by OCH.

In the case of substrates **9**, **10** and **12** therefore, the racemic keto ester products obtained from abiotic methanolysis of these compounds were to prove valuable in assessing the enantioselectivity of the enzyme. First, the product mixtures from the methanolysis of **9**, **10** and **12** revealed that in each case, as may be expected, it was the smaller C_5 ring of each substrate that had been cleaved. This was revealed by a fragmentation pattern for ester **16** that clearly showed a major peak at $m/z = 111$ corresponding to a six-membered ring with a methyl substituent. A peak at $m/z = 125$ was observed for ester **20** was due to the presence of a seven-membered ring with a methyl substituent. ^{1}H NMR of the product mixtures resulting from these reactions revealed possible diastereomeric mixtures, and these were resolved by chiral GC into four enantiomeric products. The diastereomeric ratios for the methyl esters **16**, **18** and **20** were 2.7:1, 1.7:1 and 1.1:1, respectively. A chiral GC trace illustrating the resolution of the four enantiomeric forms of **16** is shown in Figure 4.

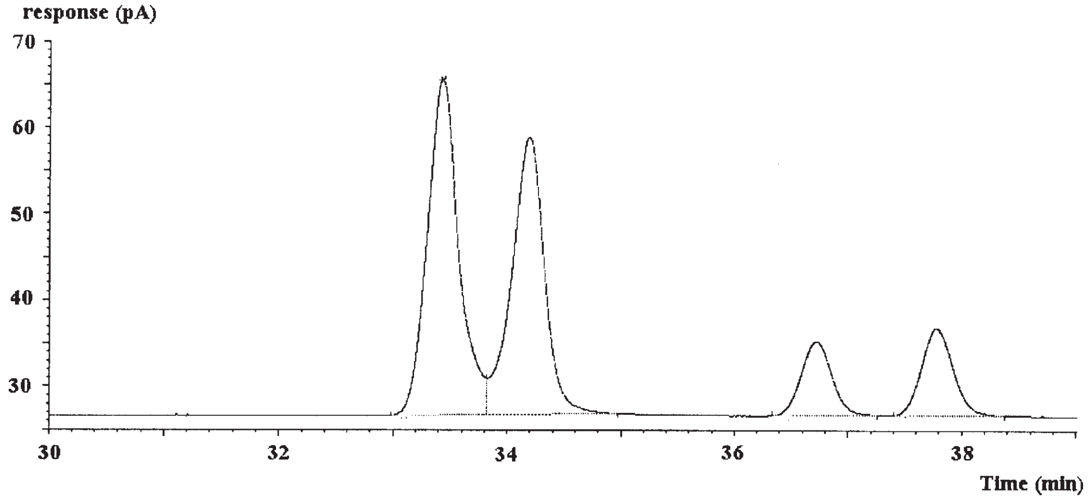

Figure 4. Separation of diastereoisomers and enantiomers of ester **16** on Cyclosil-B chiral GC column run isothermally at 150 °C.

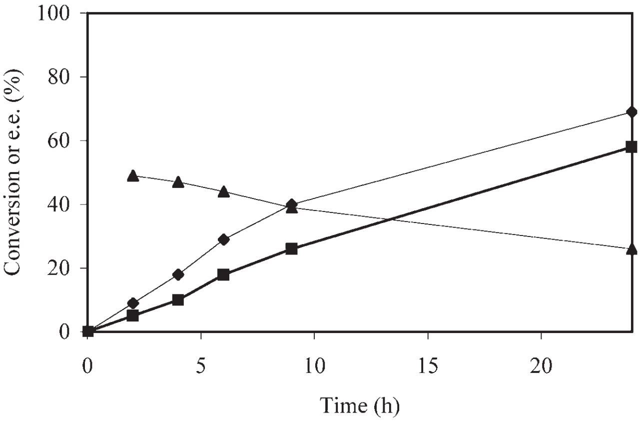

Figure 5. Resolution of 1-methylbicyclo[4.3.0]nonane-2,9-dione **9** by 6-oxocamphor hydrolase. ◆ = % conversion; ■ = % *ee* diketone substrate* **9**; ▲ = % *ee* ester product **16**. * This value determined by abiotic methanolysis of recovered residual ketone (see Experimental Section).

The resolution behaviour of OCH with substrates **9**, **10** and **12** was then tested through the periodic sampling of enzymatic reactions, using the protocol described in the Experimental Section. In each case, GC analysis of the methyl ester products **16**, **18** and **20** revealed that the diastereomeric mixtures recovered from both abiotic methanolysis and enzyme catalysed *retro*-Claisen reaction plus methyl esterification, exhibited the same diastereomeric excess. This is consonant with previously observed results that suggest that such molecules are present in the most thermodynamically stable form as a consequence of protonation/deprotonation of their enol tautomers. A graph describing the resolution of substrate **9** is shown in Figure 5. The enantiomeric excess of the residual substrate increases with time, consistent with OCH displaying a measure of enantioselectivity with respect to **9**, yet both enantiomers had been consumed after 48 h. The enantioselectivity as determined by the *E* value, using the method of Rakels and co-workers[6] was only 2.9 (*E* values were calculated using data obtained at the time point closest to 50% conversion). The biotransformation of **10**, in which the 1-methyl substituent was replaced with an allyl group, proceeded at a slower rate than that of **9**, yet the enantioselectivity of the reaction increased to 5.7 indicative of a greater level of discrimination of the enzyme for each substrate enantiomer. Substrate **12** was processed much more slowly than **9**, and not to completion under the conditions described, an *E* value of 3.1 was calculated, and an enantiomeric excess of 84% of the residual diketone at a conversion of approximately 75% observed.

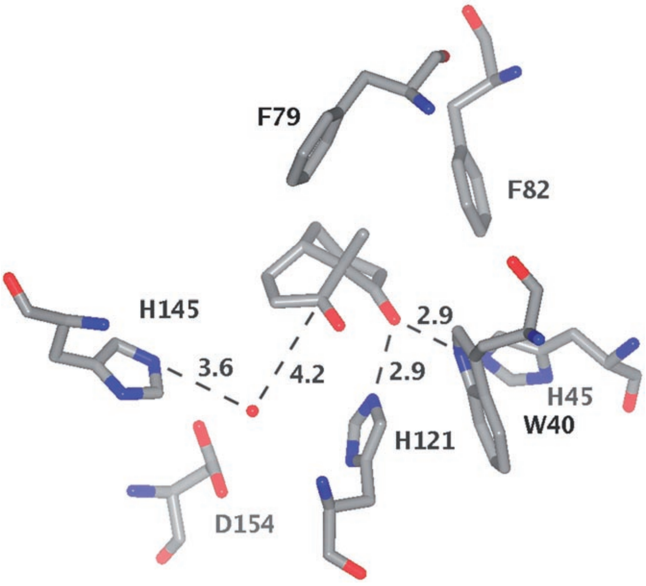

Figure 6. Model of the (1*R*,6*S*)-enantiomer of substrate **9** in the active site of OCH. Distances of hydrogen bonding interactions (dashed lines) are shown in Ångstroms, including the accommodation of the carbonyl group of the six-membered ring in the oxyanion hole formed by H121 and W40. A candidate water molecule for attack at the carbonyl group of the five-membered ring is shown at a distance of 4.2 Ångstroms (dashed line) from the relevant carbon atom.

In order to gain further insight into the enantioselectivity of OCH with respect to the chiral substrates, we have modelled the structures of both enantiomeric forms of substrate **9** and used them as virtual ligands in energy-minimised CHARMM models of the OCH active site in the complex (see Experimental Section). In the model with the (1*R*,6*S*)-enantiomer bound (Figure 6), the carbonyl group of the six-membered ring was constrained in the oxyanion hole formed by histidine 122 and tryptophan 40 as suggested by the previously obtained ligand complex.[4] The 6-membered ring stacks against phenylalanine 82, in a similar fashion to the nascent cyclopentane ring of the cycloalkanoic acid products derived from the [3.3.0] series of substrates.[2] In this arrangement, the methyl group of the substrate points toward the entrance to the active site marked by the gate residue phenylalanine 79, analogous to the proposed binding of the symmetrical 1-methylbicyclo[3.3.0]octane-2,8-dione substrate presented previously.[2] This would allow for the attack of a water molecule activated by histidine 145 at the carbonyl carbon of the five-membered ring as previously suggested. Transformation of this favoured enantiomer would result in a mixture of the (2*S*,3*S*)-*trans*- and (2*R*,3*S*)-*cis*-keto acid product enantiomers of **15**. This outcome would be consistent with the stereochemical outcome of the biotransformations of the bicyclo[3.3.0] series of ketones as suggested by both modelling studies and optical rotation measurements.[2]

The poor *E* values recorded for the resolutions indicate that the disfavoured enantiomer is also recog-

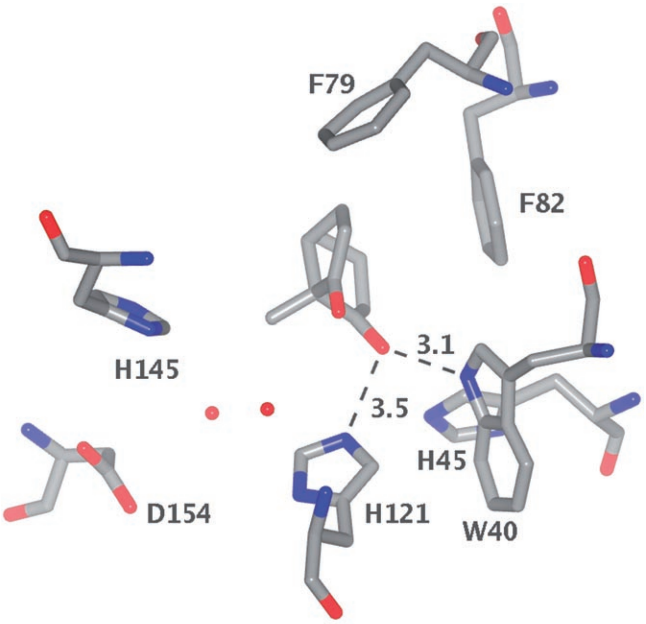

Figure 7. Model of the (1*S*,6*R*)-enantiomer of substrate **9** in the active site of OCH. The carbonyl group of the six-membered ring has once again been modelled to sit in the oxyanion hole formed by H121 and W40. Hydrogen bonding interactions are indicated by dashed lines and labelled in Ångstroms. However, in this instance, both candidate water molecules are somewhat distant from the carbonyl group of the five-membered ring, suggesting that this enantiomer would be the disfavoured substrate in the transformation of the racemate.

nised by OCH. In order to explain the recognition of the (1*S*,6*R*)-enantiomer in the active site, it would need to be accommodated in a significantly different conformation to allow attack at the carbonyl carbon of the five-membered ring. In a model of the (1*S*,6*R*)-enantiomer in the active site (Figure 7), the methyl group points towards and pushes away the aspartate 154 that is proposed to form part of a putative dyad that activates histidine 145 for general base catalysis. When the binding of the carbonyl group of the six-membered ring in the oxyanion hole was retained throughout the simulation, the geometries on the 5-membered ring are such that the attack by a water activated by histidine 145 would not be as readily undergone as for the (1*R*,6*S*)-enantiomer, as the now distorted geometry of any putative Asp154-His145 dyad will make this event less likely. Hence, although the absolute configuration of the favoured enantiomer in these resolutions is not known, a knowledge of the structure of the active site in complex with the natural product[4] in conjunction with the modelling studies are suggestive of the (1*R*,6*S*)-enantiomer as being that transformed most rapidly.

Conclusions

In this report, we have further examined the biocatalytic properties of the *retro*-Claisenase 6-oxocamphor hydrolase and have established both some steric limits of substrate acceptance and described for the first time the catalytic behaviour of this unusual enzyme with respect to the resolution of chiral substrates. When challenged with a small series of heteroannular substrates, OCH was shown to display a measure of enantioselectivity, that we have attributed, through the use of modelling, to comparatively poor molecular recognition of the disfavoured enantiomer in the active site of the enzyme. The results suggest that, whilst OCH in its native form would not be a useful catalyst for the resolution of bicyclic diketone substrates, enough enantioselectivity exists to suggest that mutation either by rational or random means may yield an enzyme capable of improved performance.

Experimental Section

General Remarks

All solvents were distilled before use. All non-aqueous reactions were carried out under oxygen-free nitrogen. Petrol refers to the fraction of petroleum ether boiling in the range of 40–60 °C. Flash chromatography was carried out using Davisil Flash Silica 60, 35–60 micron. Thin layer chromatography was carried out on commercially available Merck F_{254} aluminium-backed silica plates. Proton and other NMR spectra were recorded on a Jeol EX 400 (400 MHz) instrument. Chemical shifts are quoted in parts per million. Carbon NMR spectra were assigned using DEPT experiments. Chemical ionisation mass spectra were recorded on a Fisons Analytical (v6) Autospec spectrometer. Chiral GC analysis of both racemic and enzyme-derived esters **16**, **18** and **20** was performed on the same instrument, fitted with an Agilent Cyclosil-B capillary column (30 m × 0.25 mm × 0.25 μm); injector temperature 250 °C; detector temperature 320 °C; column temperature 150 °C isothermal for **16**; 120 °C isothermal for **18** and **20**.

Preparation of Biocatalyst

A crude cell extract from a recombinant strain of *E. coli* BL21 (DE3) expressing the *camK* gene that encodes OCH was used as the biocatalyst in all experiments. The preparation of this crude biocatalyst[2] and the assay used to determine specific activity of the enzyme have been described previously.[7] The specific activity of the enzyme preparation with respect to the assay substrate, bicyclo[2.2.2]octane-2,6-dione, was 9 U mL^{-1}, the cell extract routinely having a protein concentration of approximately 5 mg mL^{-1}.

1-Methylbicyclo[4.3.0]nonane-2,9-dione (7a-Methylhexahydroindene-1,7-dione) (9)

A solution of diketone **8** (280 mg, 1.84 mmol), potassium carbonate (1.53 g, 11.0 mmol) and iodomethane (1.5 mL, 24.8 mmol) in THF (15 mL) was heated to 50 °C for 5 h under nitrogen. The solution was then cooled and water (15 mL) added. The aqueous phase was extracted with Et_2O (3×10 mL). Combined organic extracts were washed with brine (10 mL), dried ($MgSO_4$), filtered and solvent evaporated under reduced pressure to give the crude product. Purification by flash column chromatography on silica (9:1) petrol/EtOAc), gave diketone **9**; yield: 200 mg (61%); R_F = 0.71 (1:1 petrol/EtOAc); ^1H NMR (400 MHz; $CDCl_3$): δ = 2.52–2.26 (4H, m), 2.23–2.16 (1H, m), 2.12–1.99 (3H,m), 1.87–1.78 (2H, m), 1.67–1.56 (2H, m) and (3H, s, CH_3); ^{13}C NMR ($CDCl_3$): δ = 215.3 (C=O), 208.5 (C=O), 64.2 ($\mathit{C}CH_3$), 47.1 (CH), 39.4 (CH_2), 34.2 (CH_2), 26.8 (CH_2), 23.9 (CH_2), 18.1 (CH_3), 14.2 (CH_2); EI-MS: *m/z* = 166 [55%, (M)], 138 (25), 123 (54), 110 (100), 55 (63); HR-MS: *m/z* = 166.0994 [$C_{10}H_{14}O_2$ $(M)^+$ requires*:* 166.0994].

1-Allylbicyclo[4.3.0]nonane-2,9-dione (7a-Allylhexahydo-7a*H*-indene-1,7-dione) (10)

To a suspension of NaH (74 mg, 60% suspension in nujol, 1.97 mmol) in THF (1 mL) dione **8** (300 mg, 1.97 mmol), dissolved in THF (5 mL), was added over a period of 5 min under nitrogen. The mixture was left to stir at room temperature for a further 30 min. Allyl bromide (0.5 mL, 5.91 mmol) was added and stirring at room temperature was continued for 18 h. The reaction mixture was then poured onto 10% (w/v) aqueous KH_2PO_4 (50 mL), extracted with EtOAc (3×15 mL), dried ($MgSO_4$) and the solvent removed under reduced pressure to give the crude product. Purification by flash column chromatography on silica (1:1 petrol/EtOAc) gave diketone derivative **10**; yield: 208 mg (54%), R_F = 0.84 (1:1 petrol/EtOAc); ^1H NMR (400 MHz; $CDCl_3$): δ = 5.54 (1H, ddt, *J* = 3.0, 10.0, 17.0 Hz, $C\mathit{H}{=}CH_2$), 5.08 (2H, dd, *J* = 10.0, 17.0 Hz, $CH{=}C\mathit{H}_2$), 2.92 (1H, hept, *J* = 6.0 Hz, H-5), 2.44–2.11 (10H, m), 1.78–1.70 (2H, m); ^{13}C NMR ($CDCl_3$): δ = 214.0 (C=O), 207.2 (C=O), 133.2 ($\mathit{C}H{=}CH_2$), 118.8 ($CH{=}\mathit{C}H_2$), 68.0 (C-1), 42.3 (CH_2), 39.8 (CH_2), 35.7 (CH_2), 34.7 (CH_2), 26.5 (CH_2), 23.9 (CH_2), 23.2 (CH_2); EI-MS: *m/z* = 192 [75%, $(M)^+$], 136 (85), 79 (100); HR-MS: m/z = 192.1148 [$C_{12}H_{16}O_2$ $(M)^+$ requires*:* 192.1150].

1-Methylbicyclo[5.3.0]decane-2,10-dione (8a-Methyloctahydroazulene-1,8-dione) (12)

A solution of diketone **11** (280 mg, 2.67 mmol), potassium carbonate (1.53 g, 11.0 mmol) and iodomethane (1.6 mL, 36.0 mmol) in THF (15 mL) was heated to 50 °C for 5 h under nitrogen. The solution was then cooled and water (15 mL) added. The aqueous phase was extracted with Et_2O (3×10 mL). Combined organic extracts were washed with brine (10 mL), dried ($MgSO_4$), filtered and solvent evaporated under reduced pressure to give the crude product. Purification by flash column chromatography on silica (9:1 petrol/EtOAc) gave diketone **12**; yield: 300 mg (62%); R_F = 0.7 (1:1 petrol/EtOAc); ^1H NMR (400 MHz; $CDCl_3$); δ = 2.66–2.60 (1H, m) 2.55–2.46 (1H, m), 2.40–2.28 (2H, m), 2.24–2.17 (1H, m), 2.10–2.02 (1H, m), 1.95–1.57 (7H, m), 1.34 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$): δ = 217.0 (C=O), 209.4 (C=O), 65.7 ($\mathit{C}CH_3$), 45.5 (CH), 41.0 (CH_2), 37.4 (CH_2), 31.3 (CH_2), 25.7 (CH_2), 25.2 (CH_2), 24.7 (CH_2), 21.0 (CH_3); EI-MS: *m/z* = 180 [27%, (M)], 152 (77), 123 (100), 110 (100), 55 (88); HR-MS: m/z = 180.1155 [$C_{11}H_{16}O_2$ (M^+) requires: 180.1150].

1-Methylbicyclo[5.4.0]undecane-2,11-dione (Octahydro-9a-methyl-9a*H*-benzo[7]annulene-1,9-dione) (14)

To a suspension of NaH (53 mg, 60% suspension in nujol, 1.39 mmol) in THF (1 mL) dione **13** (250 mg, 1.39 mmol), dissolved in THF (5 mL), was added over a period of 5 min under nitrogen. The mixture was left to stir at room temperature for a further 30 min. Iodomethane (0.3 mL, 4.17 mmol) was then added and stirring at room temperature was continued for 2 h. The reaction mixture was then poured onto 10% (w/v) aqueous KH_2PO_4 (25 mL), extracted with EtOAc (3×10 mL), dried ($MgSO_4$) and the solvent removed under reduced pressure to give the crude product. Purification by flash column chromatography on silica (1:1 petrol/EtOAc) gave diketone derivative **14**; yield: 110 mg (41%); R_F = 0.81 (1:1 petrol/EtOAc); ^1H NMR (400 MHz; $CDCl_3$): δ = 2.77–2.70 (1H, m), 2.58–2.38 (3H, m), 2.06–1.98 (1H, m), 1.92–1.67 (10H, m), 1.36 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$): δ = 213.7 (C=O), 209.4 (C=O), 67.6 ($\mathit{C}CH_3$), 45.4 (CH), 44.3 (CH_2), 39.5 (CH_2), 28.4 (CH_2), 25.1 (CH_2), 25.0 (CH_2) 23.7 (CH_2), 19.6 (CH_3).

3-(2-Methyl-3-oxocyclohexyl)propionic Acid Methyl Ester (16)

Enzyme-catalysed reaction: A solution of diketone **9** (30 mg, 0.18 mmol) in ethanol (2 mL) was added to a stirred solution of enzyme (225 U) in 50 mM Tris/HCl buffer (50 mL, pH 7.1). The reaction was left stirring at room temperature for 24 h. When TLC analysis confirmed the disappearance of substrate, the mixture was acidified to pH 3–4 and centrifuged at 15000 rpm for 20 min to remove precipitated protein. The supernatant was poured off and extracted with EtOAc (5×50 mL), dried ($MgSO_4$) and the solvent removed under reduced pressure to give the crude product, acid **15**; yield: 30 mg (90%); R_F = 0.15 (1:1 petrol/EtOAc).

To a stirred solution of **15** (30 mg, 0.16 mmol) in a 1:1 mixture of toluene/methanol (3 mL) TMS-diazomethane (0.2 mL, 0.54 mmol) was added dropwise at 0 °C. The reaction mixture was then allowed to warm to room temperature and left to stir for 1 h. Volatiles were removed by evaporation under reduced pressure to give the crude product. Purification by flash column chromatography on silica (9:1 petrol/EtOAc) afforded ester **16**; yield: 28 mg (87%); R_F = 0.63 (1:1 petrol/EtOAc); ^1H NMR (400 MHz; $CDCl_3$): δ = 3.68(3H, s, OCH_3), 2.45–2.24 (4H, m), 2.15–1.90 (3H, m), 1.72–1.35 (5H, m), and (3H, d, *J* 6, CH_3); ^{13}C NMR ($CDCl_3$): δ = 212.9 (C=O), 173.9 ($\mathit{C}O_2Me$), 51.7 (OCH_3), 49.8 (CH), 44.2 (CH), 41.4 (CH_2), 31.1 (CH_2), 29.9 (CH_2), 28.9 (CH_2), 25.6 (CH_2), 11.9 (CH_3); EI-MS: *m/z* = 198 [32%, (M)], 167 (44), 111 (100), 55 (42); HR-MS: *m/z* = 198.1248 [$C_{11}H_{18}O_3$ (M^+) requires*:* 198.1256].

Methanolysis: A solution of diketone **9** (50 mg, 0.30 mmol) in methanol (2 mL) was added to a stirred solution of sodium methoxide (18 mg, 0.33 mmol) in methanol (3 mL). The reaction was left to stir at room temperature for 1 h and then poured onto water (10 mL), extracted with EtOAc (3×10 mL), dried ($MgSO_4$) and the solvent removed under reduced pressure to give the crude product. Purification by flash column chromatography on silica (9:1 petrol/EtOAc) gave ester **16**; yield: 40 mg (72%).

3-(2-Allyl-3-oxocyclohexyl)propionic Acid Methyl Ester (18)

Enzyme-catalysed reaction: A solution of diketone **10** (30 mg, 0.16) in ethanol (2 mL) was added to a stirred solution of enzyme (225U) in 50 mM Tris/HCl buffer (50 mL, pH 7.1). Reaction, product isolation and esterification were performed as for **16**. Purification by flash column chromatography on silica (9:1 petrol/EtOAc) gave ester **18**; yield: 28 mg (85%); $R_F=0.68$ (1:1 petrol/EtOAc); ^1H NMR (400 MHz; $CDCl_3$): $\delta=5.75$ (1H, ddt, $J=3.0$, 10.0, 17.0 Hz, $CH{=}CH_2$), 5.03 (2H, dd, $J=10.0$, 17.0 Hz, $CH{=}CH_2$), 3.68 (3H, s, OCH_3), 2.61–1.55 (14H, m); ^{13}C NMR ($CDCl_3$): $\delta=$ 222.0 (C=O), 173.8 (CO_2Me), 136.0 (CH), 116.4 (CH_2), 54.7 (CH) 51.7 (OCH_3), 41.2 (CH), 41.0 (CH_2), 31.5 (CH_2), 31.2 (CH_2), 28.4 (CH_2), 24.6 (CH_2), 15.3 (CH_2).

Methanolysis: A solution of diketone **10** (50 mg, 0.26) in methanol (2 mL) was added to a stirred solution of sodium methoxide (15 mg, 0.27 mmol) in methanol (3 mL) and the reaction carried out as for **16** above. Purification by flash column chromatography on silica (9:1 petrol/EtOAc) gave racemic ester **18**; yield: 39 mg (67%).

3-(2-Methyl-3-oxocycloheptyl)propionic Acid Methyl Ester (20)

Enzyme-catalysed reaction: A solution of diketone **12** (30 mg, 0.17 mmol) in ethanol (2 mL) was added to a stirred solution of enzyme (20 mL) in 50 mM Tris/HCl buffer (50 mL, pH 7.1). Reaction, product isolation and esterification were performed as for **16**. Purification by flash column chromatography on silica, (9:1 petrol/EtOAc) gave ester **20**; yield:29 mg (91%); $R_F=0.68$ (1:1 petrol/EtOAc); ^1H NMR (400 MHz; $CDCl_3$): $\delta=3.69$(3H, s, OCH_3), 2.85–2.79 (1H, m), 2.62–2.55 (1H, m), 2.38–2.18 (4H, m), 1.75–1.32 (8H, m), 1.07 (3H, d, $J=7$ Hz, CH_3); ^{13}C NMR ($CDCl_3$): $\delta=$ 215.3 (C=O), 173.8 (CO_2Me), 51.6 (OCH_3), 49.4 (CH), 43.2 (CH_2), 39.8 (CH), 32.4 (CH_2), 32.0 (CH_2), 25.7 (CH_2), 24.8 (CH_2), 24.1 (CH_2), 13.1 (CH_3); EI-MS: $m/z=212$ [64%, (M)], 181 (63), 125 (100), 55 (64); HR-MS: $m/z=212.1413$ [$C_{12}H_{20}O_3$ (M^+) requires: 212.1412].

Methanolysis: A solution of diketone **12** (50 mg, 0.28 mmol) in methanol (2 mL) was added to a stirred solution of sodium methoxide (18 mg, 0.31 mmol) in methanol (3 mL) and the reaction carried out as for **16** above. Purification by flash column chromatography on silica (9:1 petrol/EtOAc) gave racemic ester **20**; yield: 38 mg (64%).

Procedure for Detailed Monitoring of Resolutions of 9, 10 and 12

In each case, a solution of diketone (100 mg) in ethanol (2 mL) was added to a stirred solution of enzyme (450 U) in 50 mM Tris/HCl buffer (100 mL, pH 7.1). At the intervals shown in Figure 5 for the biotransformation of **9**, a 20 mL aliquot of reaction mixture was removed. This was first extracted with ethyl acetate (3×10 mL) in order to remove the residual ketone. The residual ketone was subjected to methanolysis as above, in order to obtain a methyl ester product that would be easily analysed by chiral GC. The enantiomeric excess of the residual ketones were thus determined from analysis of their methyl ester derivatives, as it did not prove possible to resolve the ketone substrates on the chiral GC columns available. After the first extraction, the aqueous phase of the reaction was acidified as above and again extracted with ethyl acetate in order to obtain the keto acid product. The crude product was esterified using TMS-diazomethane as above, in order to obtain methyl ester products that could be analysed by chiral GC. Enantiomeric excess measurements for methyl esters derived from residual ketone substrate and keto acid product were then plotted in Figure 5 to give an overall picture of the resolution behaviour of the enzyme.

Computer Modelling

Extensive modelling of the separate enantiomeric forms of substrate **9**, including detailed molecular dynamics simulations, were carried out and will be presented elsewhere. The models illustrated in Figure 6 and Figure 7 were built by using 6-oxocamphor hydrolase pdb coordinate files 1o8u (native structure) and 1szo (ligand complex). The structure of the ligand was altered using Quanta (Accelrys Inc, San Diego), removing any overlapping structural water molecules in the process. The two enantiomeric states of the ligand were thus generated and subjected to further optimisations using CHARMM.[8] This was carried out by adding hydrogen atoms to the protein, followed by a restrained energy minimisation to remove any remaining steric clashes. The dynamics that characterise the two enantiomeric states were probed by carrying out molecular dynamics simulations lasting 4 ns using NAMD software[9] with the CHARMM27 force field. For this, the model structures were solvated to a distance of 6 Å from all protein atoms in a rectangular box of water, and sodium cations were added to each box to neutralise the overall negative charge of the structures. Molecular dynamics simulations were performed with a 2 fs time step at a constant temperature of 300 K and a constant pressure of 1 atmosphere under periodic boundary conditions. The Particle Mesh Ewald (PME) method was used for electrostatics, and a 12 Å cut-off was used for van der Waals interactions. The TIP3P water model[10] was used to model the solvent. The ensemble was minimised for 1500 steps using the conjugate gradient method. This was followed by a heating protocol where the ensemble temperature is increased from 0 K to 300 K over 5 ps. Afterwards, the backbone atoms of the structure were fixed, while the side chains and solvent were allowed to move unrestrained for a further 30 ps, followed by totally unrestrained equilibration for 10 ps. The production run was then performed for 4 ns, with the SHAKE algorithm[11] implemented to restrain all hydrogen motions. Coordinates were saved every 1 ps.

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References

[1] G. Grogan, J. Graf, A. Jones, S. Parsons, N. J. Turner, S. L. Flitsch, *Angew. Chem. Int. Ed. Engl.* **2001**, *40*, 1111–1114.

[2] C. L. Hill, C. S. Verma, G. Grogan, *Adv. Synth. Catal.* **2007**, *349*, 916–924.

[3] J. L. Whittingham, J. P. Turkenburg, C. S. Verma, M. A. Walsh, G. Grogan, *J. Biol. Chem.* **2003**, *278*, 1744–1750.

[4] P. M. Leonard, G. Grogan, *J. Biol. Chem.* **2004**, *279*, 31312–31317.

[5] C. L. Hill, M. McGrath, T. Hunt, G. Grogan, *Synlett* **2006**, 309–311.

[6] J. L. Rakels, A. J. Straathof, J. J. Heijnen, *Enzym. Microb. Technol.* **1993**, *15*, 1051–1056.

[7] J. P. Bennett, J. L. Whittingham, A. M. Brzozowski, P. M. Leonard, G. Grogan. *Biochemistry* **2007**, *46*, 137–144.

[8] B. R. Brooks, R. E. Bruccoleri, B. D. Olafson, D. J. States, S. Swaminathan, M. Karplus, *J. Comp. Chem.* **1983**, *4*, 1878–2217.

[9] L. Kalé, R. Skeel, M. Bhandarkar, R. Brunner, A. Gursoy, N. Krawetz, J. Phillips, A. Shinozaki, K. Varadarajan, K. Schulten, *J. Comp. Phys.* **1999**, *151*, 283–312.

[10] W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey, M. L. Klein, *J. Chem. Phys.* **1983**, *79*, 926–935.

[11] W. F. van Gunsteren, H. J. C. Berendsen, *Mol. Phys.* **1977**, *34*, 1311–1332.