DOI: 10.1002/adsc.200600468

Desymmetrisations of 1-Alkylbicyclo[3.3.0]octane-2,8-diones by Enzymatic Retro-Claisen Reaction Yield Optically Enriched 2,3-Substituted Cyclopentanones

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Received: September 12, 2006

Abstract: A series of 1-alkylbicyclo[3.3.0]octane-2,8-diones was transformed by enzymatic retro-Claisen reaction using recombinant 6-oxocamphor hydrolase (OCH) overexpressed in *Escherichia coli*, to yield optically active 2,3-substituted cyclopentanones with enantiomeric excesses of up to >95%. Whilst the parent substrate, bicyclo[3.3.0]octane-2,8-dione **12**, was transformed only very slowly, derivatives **13, 14, 15, 16** and **30** with alkyl chains of varying length in the 1-position were converted rapidly to optically active products with typically 82% *de* and up to >95% enantiomeric excess. The results confirm the apparent requirement of OCH for non-enolisable diketone substrates, and offer a potential route to decorated cyclopentanone derivatives of multiple chiral centres. Computer modelling of 1-methylbicyclo-[3.3.0]octane-2,8-dione into the active site of OCH suggested that the bicyclic [3.3.0] series substrates were accommodated in the active site in similar orientation with the natural enzyme substrate, 6-oxocamphor, and would thus yield the (2*S*,3*S*)-product series.

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

Introduction

The enzymatic desymmetrisation of a prochiral substrate can offer an environmentally benign, quantitative route to an optically pure product.[1] Whilst these processes most often employ a carbon-heteroatom bond hydrolase such as lipase or esterase, to catalyse, for example the selective cleavage of one ester group of a symmetrical diacetate, the groups of Taschner[2] and Roberts[3] have reported the enzymatic desymmetrisations of symmetrical cyclic and bicyclic ketones using enzymatic Baeyer–Villiger reactions that cleave carbon-carbon bonds. In addition, we have recently reported an unusual C–C bond cleavage reaction in which an enzyme, 6-oxocamphor hydrolase (OCH) from *Rhodococcus* sp. NCIMB 9784, catalyses the desymmetrisation of bicyclic β-diketones by enzymatic retro-Claisen reaction.[4] In nature, OCH catalyses the cleavage of diketone **1** (6-oxocamphor; 2,6-bornanedione) to yield (2*R*,4*S*)-α-campholinic acid **2** and the (2*S*,4*S*)-diastereomer **3** in a 6:1 ratio, the former having an enantiomeric excess of >95% (Figure 1). Previous studies showed that OCH also accepted 2,2-alkylated cyclohexane-1,3-diones **4** and **6** as substrates, although the keto acid products **5** and **7** were racemic[4] (Figure 2). When challenged with the demethylated natural substrate analogues **8** and **10**, however, the enzyme catalysed the production of predominantly one enantiomer of keto acids **9** and **11**. In addition to early substrate specificity studies, we also purified OCH from the host strain and cloned the gene encoding the enzyme.[5] OCH was found to be

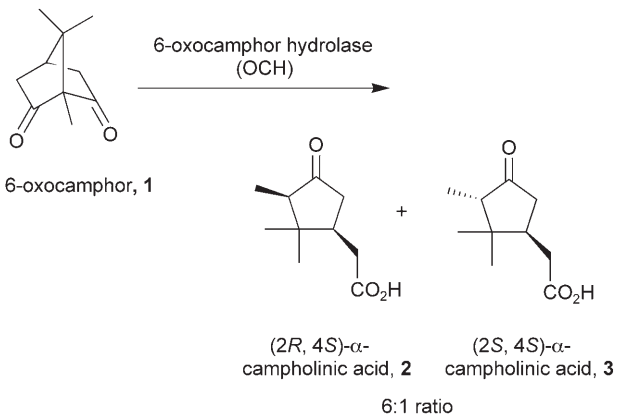

Figure 1. Natural biotransformation catalysed by 6-oxo camphor hydrolase as part of the catabolism of (1*R*)-(+)-camphor by *Rhodococcus* sp. NCIMB 9784.

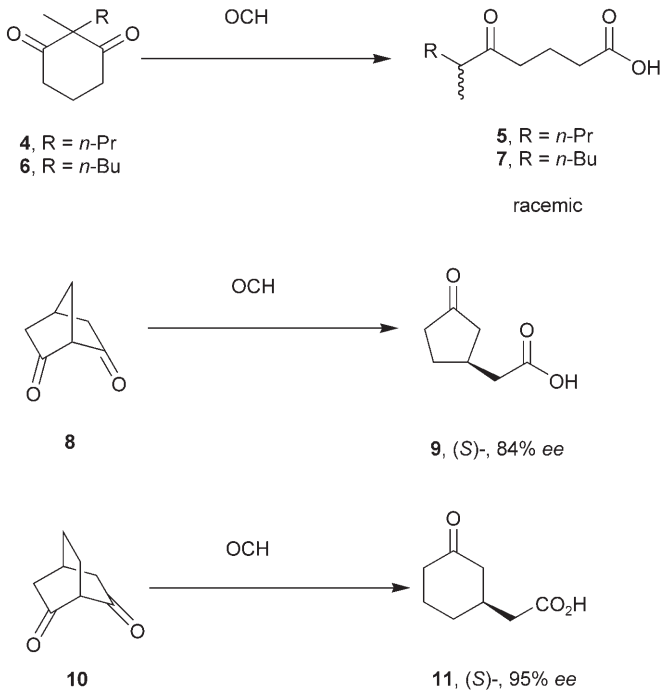

Figure 2. Application of 6-oxocamphor hydrolase to the biotransformation of xenobiotic symmetrical β-diketones (see ref.[4]).

related to the crotonase superfamily of enzymes, a group that catalyses a wide range of chemical reactions, each, until the description of OCH, accepting an acyl-CoA thioester as substrate.[6] The structure of native OCH[7] and a low k_{cat} plus low K_M mutant form, His122Ala, which was observed to bind the minor diasteromeric natural product[8] were determined and suggested that whilst the overall fold was typical of some crotonase superfamily members, the mechanism of action would perhaps be somewhat different. Enzyme kinetics experiments, in conjunction with structural studies, suggested that carbon-carbon bond cleavage was achieved by nucleophilic attack of a water molecule that had been activated by histidine 145 at the carbonyl of the *pro*-(*S*) topos of the substrate diketone. General base activation of water had already been proposed as the initiator for the mechanism of cleavage of a β-diketone in human fumarylacetoacetate hydrolase (FAAH), an enzyme involved in mammalian tyrosine metabolism,[9] but in that case, a metal ion was also required for catalysis. The mechanisms of cleavage of carbon-carbon bonds in β-diketones are emerging as intriguingly diverse,[10] but OCH is still the only one whose abilities of prochiral discrimination, allied with its cofactor independent activity appear to suggest that it may be a useful enzyme for applied biocatalysis. In this report, we have overexpressed the *camK* gene encoding OCH in *Escherichia coli* and used the enzyme to catalyse the desymmetrisation of natural substrate analogue **10**, in order to confirm the conservation of prochiral selectivity in the recombinant enzyme, and extended the substrate specificity to include 1-alkylbicyclo-[3.3.0]octane-2,8-diones, which yield chiral 2,3-substituted cyclopentanone derivatives on carbon-carbon bond cleavage.

Results and Discussion

Overexpression of 6-Oxocamphor Hydrolase

Cloning and sequencing of the gene *camK*, encoding OCH, has previously been published, and appears to form part of a larger operon for camphor metabolism in *Rhodococcus* sp. NCIMB 9784.[11] Overexpression had previously been achieved in *Escherichia coli* for the purposes of obtaining protein for crystallisation and X-ray crystallographic studies.[8] SDS-PAGE analysis of sequential ammonium sulfate fractions of the supernatant (Figure 3) obtained after cell disruption

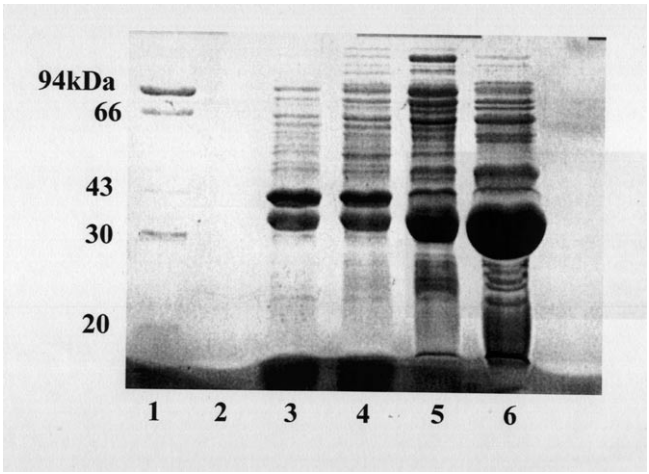

Figure 3. SDS-PAGE of sequential ammonium sulfate cuts of the cell extract of *Escherichia coli* BL21(DE3) expressing *camK*, the gene encoding OCH. Lane 1: Low molecular weight markers (BioRad); Lane 2: empty; Lane 3: 20% $(NH_4)_2SO_4$ cut; Lane 4: 40% $(NH_4)_2SO_4$ cut; Lane 5: 60% $(NH_4)_2SO_4$ cut; Lane 6: 80% $(NH_4)_2SO_4$ cut.

from expression of *camK* in pET16b(+) in *E. coli* BL21(DE3) shows that the gene is very highly expressed, and thus provides an excellent source of enzyme for biocatalysis also. The enzyme was typically used in the form of the crude extract, as further purification was time consuming and showed to be unnecessary in the context of interfering activities, of which none were detected. The crude extract yielded a specific enzymatic activity of 9.0 Units per milligram, using an assay based on the bicyclic diketone **10** as substrate,[5] where 1 Unit was equal to the trans-

formation of 1 μmol of diketone per minute per milligram of protein.

Biotransformation of Bicyclo[2.2.2]octane-2,6-dione 10 by Recombinant OCH

In an effort to establish that the prochiral selectivity of OCH had been conserved in the recombinant form, a preparative desymmetrisation of **10** was carried out. After TLC had confirmed that all the substrate had disappeared, the product was esterified using TMS-diazomethane, and a diastereomeric acetal formed using (2*R*,3*R*)-butanediol, as previously reported.[4] ^{13}C NMR of the acetal of the methyl ester, compared with the acetal of the equivalent racemic ester, confirmed that the (*S*)-acid had been obtained with an enantiomeric excess of $>95\%$, and that the prochiral selectivity of recombinant OCH was the same as the wild-type enzyme.

Biotransformation of Bicyclo[3.3.0]octane-2,8-diones 12 by Recombinant OCH

The transformation of a series of bicyclo[3.3.0] substrates **12–16** was then assessed. A scheme for the biotransformation of substrates and preparation of derivatives and racemic standards is shown in Figure 4. 10 mM substrate, dissolved in 2 mL of ethanol was added to enzyme (225 U) in a total volume of 50 mL of Tris/HCl buffer pH 7.1. Reactions were monitored by TLC until the substrate spot had disappeared, and the reaction mixture acidified to pH 3.0–4.0 before extraction into ethyl acetate. The acid was first converted to the methyl ester, then its equivalent acetal with (2*R*,3*R*)-butanediol as previously described. The diastereomeric and enantiomeric excess of derivatised products were then assessed using standard capillary GC, against standard racemic acetals, that had been derived from methyl esters themselves derived from abiotic methanolysis of the substrate diketones. As an example, a comparison of GC traces obtained for the enzyme-derived acetal of 1-ethylbicyclo[3.3.0]octane-

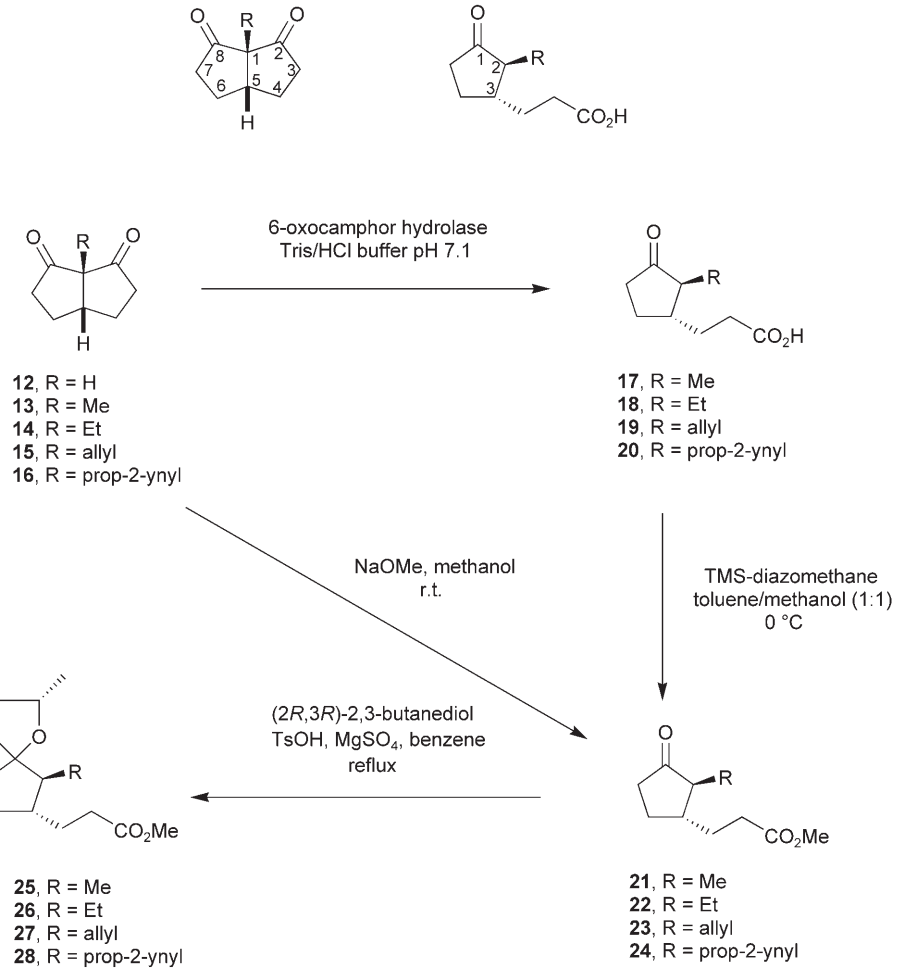

Figure 4. Enzymatic desymmetrisations of 1-alkylbicyclo[3.3.0]octane-2,8-diones: Preparation of racemic standards and derivatives.

2,8-dione **14** and its equivalent racemate is shown in Figure 5.

The results obtained for the desymmetrisation of compounds **12**–**16** are shown in Table 1. The parent

Table 1. Desymmetrisation of 1-alkylbicyclo[3.3.0]octane-2,8-diones by 6-oxo camphor hydrolase.

Substrate	Product	Diastereomeric excess (%)	Enantiomeric excess (%)
12	-	-	-
13	**17**	82	>95
14	**18**	81	>95
15	**19**	86	>95
16	**20**	78	91

compound of this series, bicyclo[3.3.0]octane-2,8-dione, is transformed only very slowly, with TLC showing only a faint accumulation of a product against buffered controls over a 48-h period. However, the methylated derivative **13** was transformed rapidly, to yield an acid, the acetal/ester derivative of which exhibited an enantiomeric excess of >95% and a diastereomeric excess of 82%. It was notable that the *de* of the enzyme-derived acetal **25** was the same as that obtained for the acetal ester acquired by abiotic methanolysis of starting material. The pattern of selectivity was repeated for the short series of compounds where the alkyl substituent was ethyl (**14**), allyl (**15**) and prop-2-ynyl (**16**), although in the case of **16**, the enantiomeric excess of products was sightly lower, perhaps owing to background hydrolysis. Düthaler and Maenfisch described in 1985 a method for the enantiotopic differentiation of equivalent prochiral substrates by abiotic desymmetrisation using stoichiometric amounts of (−)-ephedrine as nucleophile. However, the keto acid products from the transformation of methyl (**13**) and prop-2-ynyl (**16**) substituted substrates exhibited only 8% *ee* and 48% *ee*, respectively.[12]

The biotransformation of 1-(pent-2-ynyl)bicyclo-[3.3.0]octane-2,8-dione **30** was performed as precursor to obtaining the natural product (2*S*,3*R*)-oxo-2-(2*Z*-pentenyl)cyclopent-1-yl-propionic acid **29**, or its diastereomer, a jasmonate analogue previously isolated from a culture of *Botryodiploida theobromae*[13] (Figure 6). The biotransformation proceeded smoothly, although a significant if not equal level of hydrolysis was also observed in the absence of enzyme. Hydrogenation of the alkyne product **31** using Lindlar catalyst unfortunately resulted not in the *Z*-alkene, but in the saturated compound **32**, which displayed an optical rotation $[\alpha]_D$: +29.4°. Chiral GC of the enzyme-derived methyl ester **33** against the equivalent racemic compound derived by abiotic methanolysis revealed an enantiomeric excess of 75% (*de* 75%), which is lower than that obtained for the other products, having been compromised by the inherent aqueous instability of the substrate.

In each biotransformation performed in this investigation, there is strong evidence of a highly enantiotopically selective enzymatic reaction yielding, in each case, products of good to excellent optical purity. Chiral 2,3-substituted cyclopentanone derivatives form the core of a number of natural products such as the prostanoids and jasmonate series of plant growth regulators. Homologation of products such as those

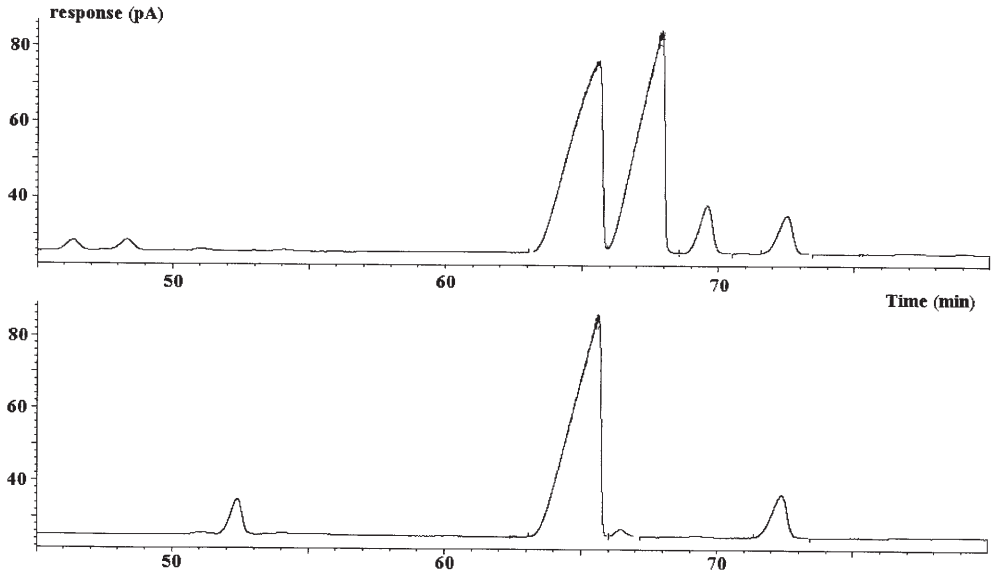

Figure 5. GC traces showing: *top*, resolution of racemic acetal derivative **26** on capillary GC; *bottom*, resolution of optically active acetal **26** derived from enzymatically acquired product carboxylic acid **18**.

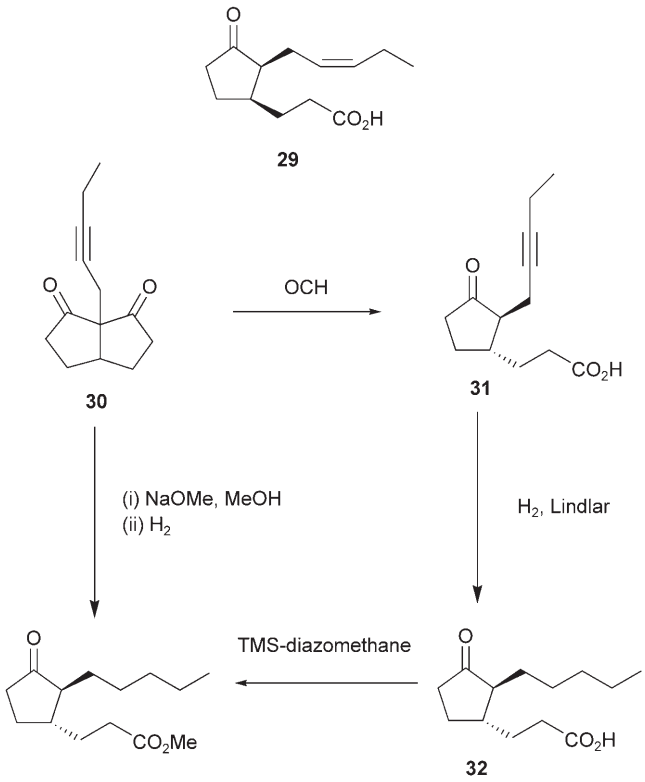

Figure 6. Biotransformation of 1-(pent-2-ynyl)bicyclo-[3.3.0]octane-2,8-dione and product analysis.

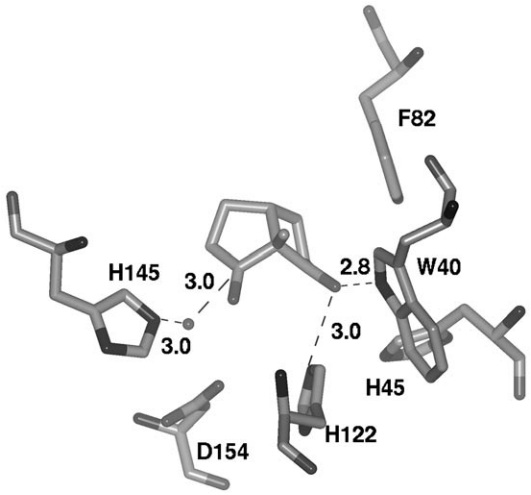

Figure 7. Model of 1-methylbicyclo[3.3.0]octane-2,8-dione in the active site of OCH, showing possible molecular determinants of prochiral selectivity. His145 is thought to activate a water molecule for attack at carbonyl group on the *pro*-(*S*)-topos (left as shown) of the substrate. A dashed line marked 3.0 (Ångstroms) indicates the distance from the water to the carbonyl carbon in the model. The cyclopentanone ring on the *pro*-(*R*)-topos (right as shown) stacks against Phe82. The carbonyl group on the *pro*-(*R*)-topos is bound in an oxyanion hole formed by the side chains of Trp40 and His122. Hydrogen bonds are shown with dashed lines, accompanied by distances (in Ångstroms) in the model.

derived from starting material **16** could serve as synthons for these or other natural products, with the crucial elements of stereoselectivity controlled by a selective enzymatic process that allows near quantitative yield of product from a symmetrical starting material.

Molecular Basis of Prochiral Selectivity – Modelling 1-Methylbicyclo[3.3.0]octane-2,8-dione into the Active Site of OCH

The structure of the low k_{cat} plus low K_M mutant of OCH, His122Ala, has revealed some of the active site mechanistic determinants of prochiral selectivity.[8] Using this and the native structure of OCH, we have used the CHARMM program to model the 1-methylbicyclo[3.3.0]octane-2,8-dione **13** into the active site of native OCH. Although the three-dimensional structure of **13** is different from that of the natural substrate, it appears that many of the interactions between substrate and active site may persist in this putative complex (Figure 7). Histidine 145, mooted to be the residue responsible for general base-catalysed activation of the putative nucleophilic water is positioned near to a candidate water molecule near the carbonyl group on the pro-*S*-topos of the diketone. The carbonyl group on the pro-*R* topos is hydrogen bonded to both histidine 122 and tryptophan 40, which together appear to form a potential oxyanion hole for the stabilisation of an intermediate enolate in the proposed mechanism for OCH. The cyclopentanone ring of the product, on the *pro*-(*R*)-topos of the substrate, is stacked against phenylalanine 82. The diastereomeric product mixtures that result from transformation of both the natural substrate and the series of 1-alkylbicyclo[3.3.0]octane-2,8-diones studied here are thought to arise from non-selective protonation of that enolate intermediate and consequent tautomerisation, leading to scrambled stereochemistry at the 2-position of the cycloalkanone ring in each case – only the stereochemistry at the 3-position appears to be determined by enzymatic enantiotopic selectivity. The diastereoisomeric mixture thus appears to be that which is the most thermodynamically stable form of the cyclic ketoacid in solution – the *trans* form that is known to be most stable for compounds of the jasmonate series.[14] Whilst the absolute configuration of each product has not been unambiguously determined, the series of 1-alkylbicyclo[3.3.0]octane-2,8-dione substrates is unlikely to invert in the active site with respect to the known orientation of the natural substrate and hence the absolute stereochemistry at the 3-position of the cyclopentanone ring would be

(*S*) as drawn, with a *trans*-relationship to the 2-position, which would thus be (*S*), in the thermodynamically favoured diastereomers. Further support is provided by well-established knowledge of the sign of optical rotation of jasmonate derivatives being determined by the configuration at the 2-position[15]; the (2*S*,3*R*)- or (2*S*,3*S*)-configuration in Figure 6 being dextrorotatory, as observed with the natural product **29**[13] and carboxylic acid **32** in this study, respectively.

Conclusions

In this report, we have demonstrated the use of recombinant 6-oxocamphor hydrolase in crude preparations as a practical biocatalyst for the desymmetrisation of bicyclic diketones. In addition, we have expanded the substrate specificity of OCH to include 1-alkylbicyclo[3.3.0]octane-2,8-diones, which, on enzymatic transformation, yield chiral 2,3-substituted cyclopentanones with high diastereomeric and enantiomeric excess. Whilst the application of this catalyst remains to be optimised in respect of enzyme stability and other process characteristics, the selectivity of the enzyme, allied to its independence from cofactors, render it an unusual and intriguing potential candidate for the preparative synthesis of optically active compounds.

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 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 carbon NMR spectra were recorded on 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. GC analysis of acetals **25**, **26**, **27** and **28** was performed on an Agilent 6890 gas chromatograph fitted with an HP5 capillary column (30 m × 0.32 mm × 0.25 μm): injector temperature 250 °C; detector temperature 320 °C; column temperature 130 °C for **25**, **26** and **28** and 180 °C for **27**. Chiral GC analysis of both racemic and enzyme-derived esters **33** 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 120 °C for 40 min, then 150 °C for 25 min, then 170 °C for 60 min.

Overexpression and Isolation of Crude Recombinant 6-Oxocamphor Hydrolase Preparations for Biotransformations

The gene encoding 6-oxocamphor hydrolase, *camK*, had been cloned previously[5] and ligated into the pET16b(+) plasmid to generate plasmid pGG3.[7] In the present study, *camK* was expressed in *Escherichia coli* strain BL21 (DE3). The plasmid pGG3 was transformed into the bacterium, which was grown on Luria Bertani (LB) agar plates containing 30 μg mL^{-1} kanamycin, which were grown overnight at 37 °C. A single colony was used to inoculate 7.5 mL of LB broth and this small culture was grown for 18 h at 37 °C with shaking. This starter culture was then used to inoculate 750 mL of LB broth in a 2-L Erlenmeyer flask. The culture was agitated at 150 rpm at 37 °C until the absorbance at 600 nm read 0.5. At this point, 1 mM isopropyl thiogalactopyranoside (IPTG) was added, and the culture grown for a further 3 h at 37 °C. After this time, the cells were harvested by centrifugation and the resultant cell paste resuspended in approximately 100 mL 50 mM Tris/HCl buffer pH 7.1 containing 20 μM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). The cell suspension was sonicated for three pulses of 45 s with 1 min intervals at 4 °C, and the cell debris removed by centrifugation. To the supernatant containing 6-oxocamphor hydrolase was added solid ammonium sulfate to 60 % saturation, and the suspension stirred for 1 h at 4 °C. The suspension was centrifuged and the resultant pellet discarded. To the supernatant was added solid ammonium sulfate to 80 % saturation and the suspension stirred for 1 h at 4 °C. The resultant suspension was centrifuged and the pellet obtained was dissolved in a minimum of cell resuspension buffer. The dissolved protein was then dialysed against two changes of the same buffer over a period of 24 h and then snap frozen in liquid nitrogen and stored at −80 °C.

Bicyclo[2.2.2]octane-2,6-dione 10

Bicyclo[2.2.2]octane-2,6-dione **10** was prepared using the procedure described by Almqvist et al.[16] Spectroscopic data were in accordance with the literature.

Bicyclo[3.3.0]octane-2,8-dione 12

Bicyclo[3.3.0]octane-2,8-dione **12** was prepared for these studies using the method of Düthaler and Maenfisch,[17] although we have recently reported a much improved method for this general class of bicyclic β-diketones.[18] ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.20$ (1 H, hept, $J = 6.0$ Hz, H-5), 3.04 (1 H, d, $J = 6.0$ Hz, H-1), 2.38–2.20 (6 H, m), 1.86–1.76 (2 H, m); ^{13}C NMR (125 MHz; $CDCl_3$): $\delta = 207.1$ (2 C=O), 63.4 (CH-1), 39.4 (CH-5), 37.9 (CH_2-3 and CH_2-7), 26.2 (CH_2-4 and CH_2-6); MS (CI; NH_3): $m/z = 138$ [100 %, $(M+NH_4)^+$], 55 (41), 110 (32), 156 (62); found for $(M+NH_4)^+$: 156.1023; $C_8H_{10}O_2$ requires: 156.1024.

1-Methylbicyclo[3.3.0]octane-2,8-dione 13

1-Methylbicyclo[3.3.0]octane-2,8-dione **13** was prepared using the method of Düthaler and Maenfisch.[17] ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.71$ (1 H, hept, $J = 6.0$ Hz, H-5), 2.38–2.20 (6 H, m), 1.86–1.76 (2 H, m), 1.19 (3 H, s, CH_3);

^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 212.8$ (2 C=O), 46.7 (CH), 36.7 (CH_2-3 and CH_2-7), 24.6 (CH_2-4 and CH_2-6), 19.3 (CH_3); MS (CI; NH_3): $m/z = 170$ [100%, $(M+NH_4)^+$], 153 (10); found for $(M + NH_4)^+$: 170.1177; $C_9H_{12}O_2$ requires: 170.1181.

1-Ethylbicyclo[3.3.0]octane-2,8-dione 14

To a suspension of NaH (61 mg, 60% suspension in nujol, 1.59 mmol) in THF (1 mL) dione **12** (220 mg, 1.59 mmol), dissolved in THF (5 mL), was added in 5 min under nitrogen. The mixture was left to stir at room temperature for a further 30 min. Iodoethane (0.25 mL, 3.18 mmol) was added and stirring at room temperature was continued for 20 h. The reaction mixture was then poured on to 10% KH_2PO_4 (50 mL) and extracted with ethyl acetate (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/ethyl acetate) gave diketone **14**; yield: 107 mg (40%); R_f (1:1 petrol/ethyl acetate): 0.84; ^{1}H NMR (400 MHz; $CDCl_3$): $\delta = 2.91$ (1 H, hept, $J = 5.0$ Hz, H-5), 2.43–2.14 (6 H, m), 1.76 (2 H, q, $J = 7.5$ Hz, CH_2CH_3), 1.92–1.70 (2 H, m), 0.84 (3 H, t, $J = 7.5$ Hz, CH_2CH_3); ^{13}C NMR (125 MHz; $CDCl_3$): $\delta = 212.4$ (2 C=O), 70.4 (C-1), 42.7 (CH-5), 37.6 (CH_2-3 and CH_2-7), 27.2 (CH_2CH_3), 25.2 (CH_2-4 and CH_2-6), 9.6 (CH_3); MS (CI; NH_3): $m/z = 167$ [8%, $(M+H)^+$], 184 (100); found for $(M+NH_4)^+$: 184.1338, $C_{10}H_{14}O_2$ requires: 184.1338].

1-Allylbicyclo[3.3.0]octane-2,8-dione 15

To a suspension of NaH (56 mg, 60% suspension in nujol, 1.45 mmol) in THF (1 mL) dione **12** (200 mg, 1.45 mmol), dissolved in THF (5 mL), was added in 5 min under nitrogen. The mixture was left to stir at room temperature for a further 30 min. Allyl bromide (0.25 mL, 2.90 mmol) was added and stirring at room temperature was continued for 18 h. The reaction mixture was then poured on to 10% KH_2PO_4 (50 mL) and extracted with ethyl acetate (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/ethyl acetate) gave diketone **15**; yield: 140 mg (54%); R_f (1:1 petrol ethyl acetate): 0.84; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.54$ (1 H, ddt, $J = 3.0$, 10.0 and 17.0 Hz, $CH{=}CH_2$), 5.08 (2 H, dd, J 10.0 and 17.0, $CH{=}CH_2$), 2.92 (1 H, hept, $J = 6.0$ Hz, H-5), 2.44–2.11 (8 H, m), 1.78–1.70 (2 H, m); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 211.7$ (2 C=O), 132.4 ($CH{=}CH_2$), 119.6 ($CH{=}CH_2$), 69.3 (C-1), 42.8 (CH-5), 38.4 (CH_2-1′), 37.5 (CH_2-3 and CH_2-7), 24.8 (CH_2CH_3), 25.2 (CH_2-4 and CH_2-6); EI-MS: $m/z = 178$ [55%, $(M)^+$], 79 (90), 122 (94), 150 (100); found for $(M)^+$: 178.0993, $C_{11}H_{14}O_2$ requires: 178.0994.

1-(Prop-2-ynyl)bicyclo[3.3.0]octane-2,8-dione 16

1-(2′-Propynyl)bicyclo[3.3.0]octane-2,8-dione **16** was prepared using the method of Düthaler and Maenfisch.[17] ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.16$ (1 H, hept, $J = 6.0$ Hz, H-5), 2.59 (2 H, d, $J = 2.5$ Hz, CH_2-1′), 2.47–2.24 (6 H, m), 1.98 (1 H, t, $J = 2.5$ Hz, CH-3′), 1.87–1.76 (2 H, m); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 210.5$ (2 C=O), 79.8 (CH-3′), 70.7 (C-1), 67.6 (C-2′), 43.7 (CH-5), 37.5 (CH_2-3 and CH_2-7), 25.2 (CH_2-4 and CH_2-6), 23.4 (CH_2-1′); MS (CI; NH_3): $m/z = 177$ [92%, $(M+NH_4)^+$], 148 (100), 194 (58); found for $(M+NH_4)^+$: 194.1181, $C_{11}H_{12}O_2$ requires: 194.1181].

1-(Pent-2-ynyl)bicyclo[3.3.0]octane-2,8-dione 30

Phosphorus tribromide (0.56 mL, 5.94 mmol) was added dropwise to a stirred, ice-cooled solution of pent-2-yn-1-ol (1 g, 11.8 mmol) and pyridine (0.1 mL) in ether (22 mL). After stirring for 22 h at room temperature, the reaction mixture was poured on to ice/water (20 mL). The organic layer was separated and the aqueous layer was extracted with ether (3×10 mL). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure to afford 1-bromopent-2-yne as a brown oil; yield: 1.52 g (88%). The product was used without further purification.

To a suspension of NaH (30 mg, 60% suspension in nujol, 0.725 mmol) in THF (1 mL) dione **12** (100 mg, 0.725 mmol), dissolved in THF (5 mL), was added in 5 min under nitrogen. The mixture was left to stir at room temperature for a further 30 min. 1-Bromopent-2-yne (321 mg, 2.17 mmol) was added and stirring at room temperature was continued for 18 h. The reaction mixture was then poured on to 10% KH_2PO_4 (50 mL) and extracted with ethyl acetate (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/ethyl acetate) gave diketone **30**; yield: 50 mg (34%); R_f (1:1 petrol ethyl acetate): 0.84; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.13$ (1 H, hept, $J = 6.0$ Hz, H-5), 2.55 (2 H, t, $J = 2$ Hz, CH_2-1′), 2.46–2.24 (6 H, m) 2.11 (2 H, qt, $J = 2$, 8 Hz, CH_2-4′), 1.83–1.74 (2 H, m), 1.08 (3 H, t, J 8, CH_3-5′); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 210.9$ (2 C=O), 84.0 (CH-3′), 74.1 (C-1), 67.9 (C-2′), 43.7 (CH-5), 37.4 (CH_2-3 and CH_2-7), 25.1 (CH_2-4 and CH_2-6), 23.8 (CH_2-1′), 14.0 (CH_3), 12.2 (CH_2-4); EI-MS: $m/z = 205$ [20%, $(M+H)^+$], 147 (100) 133 (40); found for $(M+H)^+$: 205.1228, $C_{13}H_{16}O_2$ requires: 205.1229.

Enzymatic Retro-Claisen Reaction

A solution of diketone (0.05 g) in ethanol (2 mL) was added to a stirred solution of enzyme (225 U) in buffer (50 mL). The reaction was stirred at room temperature for 24 h. When TLC indicated that all the substrate had been transformed, the reaction mixture was acidified to pH 3–4 and centrifuged at 15000 rpm for 20 min in order to remove precipitated protein. The supernatant was removed and extracted with ethyl acetate (5×50 mL), dried over anhydrous magnesium sulfate and the solvent removed under reduced pressure to give the crude product. Crude keto acids **17**, **18**, **19** and **20** derived in this way were not isolated at this stage, but were used in this form for methylation.

The acid **31** derived from biotransformation of **30** was characterised as *3-(2-pent-2-ynyl-3-oxocyclopentyl)-propionic acid* (**31**): R_f (1:1 petrol/ethyl acetate): 0.10; ^{1}H NMR: (400 MHz, $CDCl_3$): $\delta = 2.58$–2.34 (5 H, m), 2.25–2.05 (6 H, m), 1.86–1.81 (1 H, m), 1.79–1.60 (1 H, m), 1.45–1.40 (1 H, m), 1.27–1.23 (1 H, m) and 1.06 (3 H, t, $J = 7$ Hz, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 218.5$ (C=O), 179.3 (CO_2H), 83.6 (C-3′), 75.7 (C-2′), 53.7 (CH), 40.3 (CH), 37.7 (CH_2) 31.8 (CH_2), 29.2 (CH_2), 26.7 (CH_2), 17.4 (CH_2-1′), 14.0 (CH_3), 12.3 (CH_2-4); EI-MS: $m/z = 223$ [35%, $(M+$

H)$^+$], 122 (57), 107 (100); found for $(M+H)^+$: 223.1335, $C_{13}H_{18}O_3$:223.1334].

Acid **31** was then hydrogenated and the product **32** characterised, to allow comparison to natural product **29**, as *3-(2-pentyl-3-oxocyclopentyl)-propionic acid* (**32**): R_f (1:1 petrol/ethyl acetate): 0.10; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 2.54–2.32 (4H, m), 2.24–2.04 (3H, m), 1.92–1.87 (1H, m), 1.77–1.70 (1H, m), 1.59–1.53 (2H, m), 1.43–1.24 (8H, m), 0.87 (3H, t, $J=7$ Hz, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 220.7$ (C=O), 179.3 (CO_2H), 54.9 (CH), 40.8 (CH), 37.7 (CH_2), 32.1 (CH_2), 31.7 (CH_2), 29.4 (CH_2), 27.9 (CH_2), 26.7 (CH_2), 26.4 (CH_2), 22.4 (CH_2), 14.0 (CH_3); EI-MS: $m/z =$ 226 [15%, $(M)^+$], 138 (47), 83 (100); $[\alpha]_D$: +29.4°.

Preparation of Racemic Methyl Ester Product Standards

A solution of diketone (0.05 g) in methanol (2 mL) was added to a stirred solution of sodium methoxide (1.2 equiv) in methanol (2 mL). The reaction mixture was stirred at room temperature for 1 h and then poured on to water (10 mL), extracted with ethyl acetate (3×10 mL), dried over anhydrous magnesium sulfate and the solvent removed under reduced pressure to give the crude methyl ester product.

3-(2-Methyl-3-oxocyclopentyl)-propionic acid methyl ester (21): Yield: 67%; R_f (1:1 petrol/ethyl acetate): 0.55; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.66$ (3H, s, OCH_3), 2.48–231 (3H, m), 2.20–2.02 (4H, m), 1.72–1.58 (2H, m), 1.37–1.32 (1H, m), 1.06 (3H, d, $J=7.0$ Hz, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 220.5$ (C=O), 1.73.9 (CO_2Me), 51.8 (OCH_3), 50.4 (CH), 44.2 (CH), 37.3 (CH_2), 31.9 (CH_2) and 29.6 (CH_2), 26.9 (CH_2), 12.6 (CH_3); MS (CI; NH_3): $m/z =$ 202 [100%, $(M+NH_4)^+$]; found for $(M+NH_4)^+$: 202.1439, $C_{10}H_{16}O_3$ requires: 202.1443.

3-(2-Ethyl-3-oxocyclopentyl)-propionic acid methyl ester (22): Yield: 69%; R_f (1:1 petrol/ethyl acetate): 0.55; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.70$ (3H, s, OCH_3), 2.53–2.30 (3H, m), 2.20–2.00 (3H, m), 1.76–1.25 (6H, m), 0.90 (3H, t, $J=7.5$ Hz, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 217.8 (C=O), 1.73.9 (CO_2Me), 56.0 (CH), 51.8 (OCH_3), 40.2 (CH), 38.0 (CH_2), 31.9 (CH_2) and 29.9 (CH_2), 26.8 (CH_2), 20.5 (CH_2-1″), 10.9 (CH_3); MS (CI; NH_3): $m/z = 199$ [4%, $(M+H)^+$], 181 (11), 216 (100); found for $(M+NH_4)^+$: 216.1595, $C_{11}H_{18}O_3$ requires: 216.1560.

3-(2-Allyl-3-oxocyclopentyl)-propionic acid methyl ester (23): Yield: 73%; R_f (1.1 petrol/acetate): 0.55; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.69$ (1H, ddt, $J=3.0$, 10.0 and 17.0 Hz, C*H*=CH_2), 5.02 (2H, dd, $J=10.0$ and 17.0 Hz, CH=CH_2) 3.70 (3H, s, OCH_3), 2.47–2.30 (4H, m), 2.17–2.00 (4H, m), 1.91–1.79 (2H, m), 1.59–1.49 (1H, m), 1.43–1.33 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 219.6$ (C=O), 1.73.9 (CO_2Me), 135.3 (CH=), 117.4 (=CH_2), 54.6 (CH), 51.8 (OCH_3), 40.4 (CH), 37.9 (CH_2), 32.2 (CH_2-1″), 31.9 (CH_2), 29.6 (CH_2), 26.8 (CH_2); MS (CI; NH_3): $m/z = 209$ [89%, $(M+H)^+$], 94 (33), 177 (45), 226 (100); found for $(M+H)^+$, 211.1328, $C_{12}H_{18}O_3$ requires: 211.1334.

3-(3-Oxo-2-prop-2-ynylcyclopentyl)-propionic acid methyl ester (24): Yield: 49%; R_f (1.1 petrol/ethyl acetate): 0.55; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.70$ (3H, s, OCH_3), 2.62 (1H, ddd, $J=2.5$, 5.0 and 17.0 Hz, H-1), 2.53–2.36 (4H, m), 2.24–2.06 (4H, m), 1.93 (1H, t, $J=2.5$ Hz, H-3″), 1.90–1.84 (1H, m), 1.69–1.59 (1H, m), 1.47–1.37 (1H, m); MS (CI; NH_3): $m/z = 209$ [89%, $(M+H)^+$], 94 (33), 177 (45), 226 (100); found for $(M+H)^+$: 209.1176, $C_{12}H_{16}O_3$ requires: 209.1178].

3-(3-Oxo-2-pentylcyclopentyl)-propionic acid methyl ester (33): R_f (1:1 petrol/ethyl acetate): 0.75; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.67$ (3H, s, OMe), 2.50–2.30 (3H, m), 2.17–2.02 (3H, m), 1.90–1.81 (1H, m), 1.75–1.69 (1H, m), 1.58–1.52 (3H, m), 1.42–1.23 (7H, m), 0.87 (3H, t, $J=7$ Hz, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 220.5$ (C=O), 173.8 (CO_2Me), 54.8 (CH), 51.6 (OMe), 40.8 (CH), 37.8 (CH_2), 32.1 (CH_2), 31.8 (CH_2), 29.7 (CH_2), 27.8 (CH_2), 26.7 (CH_2), 26.4 (CH_2), 22.5 (CH_2), 14.0 (CH_3); EI-MS: $m/z = 240$ [30%, $(M)^+$], 84 (55), 49 (100); found for $(M)^+$: 240.1725, $C_{14}H_{24}O_3$ requires: 240.1725].

Formation of Methyl Esters of Enzyme-Derived Keto Acids using TMS-Diazomethane

To a stirred solution of the crude carboxylic acid product (1 equiv.) in a 1:1 mixture of toluene/methanol (3 mL) TMS-diazomethane (2 equivs.) was added dropwise at 0°C. The reaction mixture was then allowed to warm to room temperature and stirred for 1 h. Volatiles were removed by evaporation under reduced pressure to give the crude methyl ester products **21**, **22**, **23** and **24** in quantitative yields.

Formation of Acetals

A solution of methyl ester, (2*R*,3*R*)-2,3-butanediol (2 equivs.), tosic acid monohydrate (catalytic) and magnesium sulfate (200 mg) in benzene (7 mL) was refluxed for 5 h. The reaction was then quenched with saturated sodium bicarbonate solution, extracted with ethyl acetate (3×10 mL) dried over anhydrous magnesium sulfate and the solvent removed under reduced pressure to give the crude product acetals **25**, **26**, **27** and **28** in quantitative yields, which were purified over silica plugs prior to injection in the gas chromatograph.

Molecular Modelling

Molecular modelling was used to construct a model for the docked substrate **13** with the CHARMM program and force-field.[14] The model for the substrate **13** was built using QUANTA (Accelrys Inc., San Diego, CA., USA) and the atom types and charges were assigned using standard CHARMM parameter sets.[15] The crystal structure PDB accession code 1o8u was used to set up the initial model of the protein in its native state. All crystallographic water molecules were retained except for two in the putative active site. The substrate was initially docked using the stereochemical constraints imposed upon the reaction using the structure **13**. This led to a clash between two water molecules and the substrate and so the water molecules were removed. Of the five water molecules that were located in the active site, two with B values of 32 Å and 35 Å were removed as they clashed with the model substrate. The substrate itself was docked according to information gleaned from known OCH mechanism and the stereochemical constraints of the reaction; the structure of (2*S*,4*S*)-α-campholinic acid **3** from the crystal structure of the His122Ala mutant of the enzyme[8] was used as a guide. Hydrogen

atoms were built using the HBUILT functionality of CHARMM.[21] Examination of the energetics of hydrogen bond formation by the His side chains led to a model where two histidines (His45 and His122), both of which form part of the active site, were protonated. The geometry and energetics of the docked substrates and protein were optimised by carrying out energy minimisations. These were performed with atoms within a 10 Å radius around the substrate subject to decreasing constraints, whereas the atoms beyond this were restrained with a harmonic force of 10.0 kcal mol^{-1} Å^{-2}. Minimisations were carried out using the Steepest Descent and Adopted Basis Newton Raphson algorithms[19] and were continued until the root mean square magnitude of the forces was lower than 10^{-3} 10.0 kcal mol^{-1} Å^{-1}.

Acknowledgements

We gratefully acknowledge the award of a DTA studentship to C. L. H. from the Engineering and Physical Sciences Research Council (EPSRC). G. G. would like to thank the Innovation and Research Priming Fund, University of York, for additional funding.

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