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E. coli cells expressing the Baeyer-Villiger Monooxygenase 'MO14' (*ro*03437) from *Rhodococcus jostii* RHA1 catalyse the gram-scale resolution of a bicyclic ketone in a fermentor

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Benjamin D. Summers,[a] Muhiadin Omar,[a] Thomas O. Ronson,[a] Jared Cartwright,[b] Michael Lloyd[c] and Gideon Grogan[a]

The Baeyer-Villiger monooxygenase (BVMO) 'MO14' from *Rhodococcus jostii* RHA1, is an enantioselective BVMO that catalyses the resolution of the model ketone substrate bicyclo[3.2.0]hept-2-en-6-one to the (1*S*, 5*R*)-2-oxa lactone and the residual (1*S,* 5*R*)- substrate enantiomer. This regio- plus enantioselective behaviour is highly unusual for BVMOs, which often perform enantiodivergent biotransformations of this substrate. The scaleability of the transformation was investigated using fermentor-based experiments, in which variables including gene codon optimisation, temperature and substrate concentration were investigated. *E. coli* cells expressing MO14 catalysed the resolution of bicyclo[3.2.0]hept-2-en-6-one to yield (1*S*, 5*R*)-2-oxa lactone of >99% e.e and (1*S,* 5*R*)-ketone of 96% e.e. after 14 h at a temperature of 16°C and a substrate concentration of 0.5 g L^{-1} (4.5 mM). MO14 is thus a promising biocatalyst for the production of enantio-enriched ketones and lactones derived from the [3.2.0] platform.

Introduction

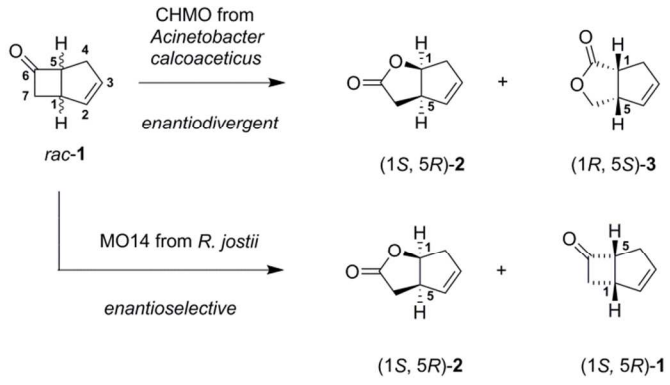
Baeyer-Villiger monooxygenases (BVMOs) are flavin-containing enzymes that catalyse the Baeyer-Villiger reaction, in which an oxygen atom is inserted adjacent to a carbonyl group in a ketone to yield the corresponding ester or lactone.[1] BVMOs represent an attractive biological alternative to abiotic Baeyer-Villiger reactions, which typically use peracids such as *meta*-chloroperbenzoic acid, as the enzymes have the ability to catalyse the oxygen insertion reaction both regio- and enantio-selectively, using molecular oxygen as an oxidant and producing only water as a waste product. These characteristics make the investigation of BVMOs highly desirable from both an academic and an industrial perspective.[2-4]

The activity of the best-studied BVMOs, which are part of the 'Class B' subgroup of the larger class of flavoprotein monooxygenases[5] relies on two co-factors: a nicotinamide coenzyme (usually NADPH) and a flavin (usually FAD).[6] Catalysis involves NADPH-dependent reduction of the flavin group to produce $FADH_2$, which then reacts with O_2 to form a (hydro)peroxy intermediate, which then then mimics the peracid species in the abiotic Baeyer-Villiger reaction, attacking the carbonyl group and forming a tetrahedral 'Criegee' intermediate. This is followed by a rearrangement, involving the migration of one of the ancillary groups attached to the acetal carbon.[7] The interaction of enzyme, nicotinamide cofactor, flavin and substrate throughout the course of the reaction is complex, and is currently being elucidated with the help of structural studies that present 'snapshots' of the reaction coordinate.[6-10]

Amino acid sequences of NADPH-plus-FAD dependent BVMOs are usually approximately 500-550 amino acids long, and are characterised by the presence of a number of sequence motifs.[11] These include two Rossman fold motifs [GXGXX(G/A)] involved in recognition of the ADP moiety of either cofactor, and a 'BVMO motif' as described by Janssen and co-workers [FXGXXXHXXXW(P/D)],[11] which resembles a motif [(FXGXXXHXXX(Y/F)] found in other flavin-dependent monooxygenases. Mutational analysis of one representative BVMO, 4-hydroxyacetophenone monooxygenase (HAPMO) suggested that the central histidine in this motif is important for the enzymes' function,[11] although structural studies of BVMOs[6-10] have revealed that this histidine residue is somewhat distant from the flavin binding site.

The BVMO sequence motif has been employed very productively in the identification of novel BVMOs from genome sequence data, revealing members of a diverse family of enzymes that display useful and distinct catalytic properties. BVMOs have been identified for example, with a substrate preference for aliphatic,[12] aromatic[13] or bulky substrates,[14] and with different catalytic characteristics, such as thermostability,[15] and the ability to use NADH as cofactor.[16,17] The characterisation of this range of BVMOs has shown that each may be useful as complementary starting points for *in vitro* evolution experiments that are designed to improve or alter enzyme activity.

One such genome mining experiment involving BVMOs was performed on the genome of the bacterium *Rhodococcus jostii* sp. RHA1, which is known for its huge catabolic capacity, especially in respect of the degradation of hydrocarbons.[18] The genome of RHA1 contains many genes encoding oxygenases, and in excess of twenty gene sequences that contain either the BVMO motif, or one that is closely related.[18,19] In previous work by our group, 23 native genes encoding BVMOs from *R. jostii* were cloned and expressed in *E. coli*, yielding thirteen soluble BVMOs, whose activity was assayed against familiar BVMO substrates.[19] Subsequently, Fraaije and co-workers have expanded the library of soluble BVMO gene products from *R. jostii* RHA1 using constructs based on a modified pBAD vector.[20] Initial screens of the activity of *R. jostii* BVMOs revealed complementary substrate specificity and regio/stereoselectivity, notably with the model BVMO substrate bicyclo[3.2.0]hept-2-en-6-one **1** (**Figure 1**).[19]



Scheme 1. Biotransformations of racemic bicyclo[3.2.0]hept-2-en-6-one **1** by cyclohexanone monooxygenase from *A. calcoaceticus* (*Ac*CHMO)[21] and MO14 from *R. jostii* RHA1.[19]

In one of the most well-known Baeyer-Villiger biotransformations, cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* was shown to catalyse an enantiodivergent biotransformation of the racemic substrate **1**, in which each ketone enantiomer was transformed to a complementary lactone regioisomer **2** and **3**, each with high e.e.[21] (Figure 1) The stereochemical complexity of this reaction has meant that it has become a useful model for BVMO activity ever since**.**

Amongst BVMOs from *R. jostii* were identified enzymes that catalysed the regioselective biotransformation of **1**, to lactone **2** of low e.e., and also activity which duplicated that of *Ac*CHMO.[19] The activity of the enzyme encoded by *ro*03437, and named 'MO14' (Uniprot code Q0SB46) was the most interesting, however, in that it appeared to catalyse the regio-plus enantioselective *resolution* of **1** to yield (1*S*, 5*R*)-**1** and a single lactone enantiomer (1*S*, 5*R*)-**2**, thus exhibiting catalytic behaviour previously not described for a BVMO on this substrate. MO14 possesses classical BVMO-type motifs, but, notably, has a leucine in the position of the otherwise well-conserved histidine in the BVMO sequence motif. The activity of MO14 presented a potentially new enzymatic route to single enantiomers of **1** and **2**, each of which is of potential interest as a chiral building block. In this report, the practical utility of a recombinant strain expressing the gene for the resolution of **1** was investigated using fermentor-based biotransformations on a gram scale. In addition, the molecular basis of regioselectivity in MO14 was investigated using targeted mutation of a close sequence homolog, MO15, from the same organism.

Results and discussion

Expression of the gene *ro*03437 encoding MO14

In our previous work, native genes encoding BVMOs from *R.jostii* RHA1 were each cloned into the pET-YSBLIC-3C vector, a derivative of pET28a (Novagen) that features modification suitable for ligation independent cloning, each producing a protein equipped with an N-terminal hexahistidine tag.[19] Each was then expressed in the *E. coli* Rosetta™2 (DE3) pLysS strain and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an optical density (A_{600}) of 0.8. In a first attempt at optimising expression of the MO14 gene, and with a view to future fermentation experiments, experiments were conducted using Rosetta™2 (DE3) pLysS at different temperatures. Protein production in the soluble fraction of cells following sonication and centrifugation was optimal at the lower temperature of 16°C (**Figure 1**).

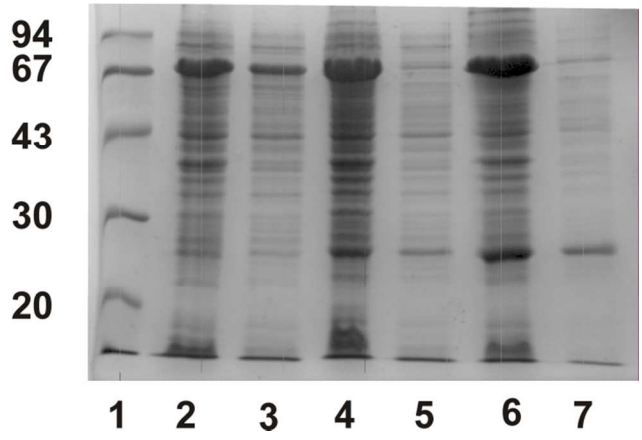


Figure 1. SDS-PAGE showing expression of native *ro*03437 gene in *E. coli* Rosetta™2 (DE3) pLysS strain at different temperatures. Lane 1: Low molecular weight Marker (Biorad) with molecular weights given on the left-hand side in kiloDaltons (kDa); Lane 2: Total cell fraction 16°C; Lane 3 soluble cell fraction 16°C; Lane 4: Total cell fraction 30°C; Lane 5 soluble cell fraction 30°C; Lane 6: Total cell fraction 37°C; Lane 7 soluble cell fraction 37°C. MO14 can, in the case of Lanes 2, 3, 4 and 6 be observed as the intense band near the 67 kDa marker (The molecular weight of MO14 plus the His-tag is approximately 62 kDa).

In a further study, a version of the gene *ro*03437 was synthesised with the sequence optimised for expression in *E. coli*. The codon-optimised gene was also ligated into the pET-YSBLIC-3C vector and expressed in Rosetta™2 (DE3) pLysS. SDS-PAGE analysis of expression using the codon-optimised versus native genes showed that, despite the GC-rich nature of the latter, the strain expressing this gene gave superior levels of both expression overall, and soluble protein production (**Figure 2**).

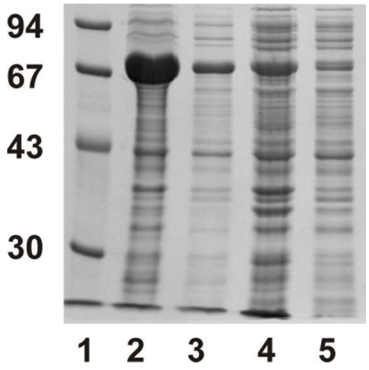


Figure 2. SDS-PAGE showing expression of native and codon-optimised *ro*03437 gene in *E. coli* Rosetta™2 (DE3) pLysS strain. Lane 1: Low molecular weight Marker (Biorad) with molecular weights given on the left-hand side in kiloDaltons (kDa); Lane 2: Native gene, total cell fraction 16°C; Lane 3: Native gene: soluble cell fraction 16°C; Lane 4: Codon-optimised gene, total cell fraction 16°C; Lane 5: codon-optimised gene, soluble cell fraction 16°C.

It was concluded that the native N-terminally His-tagged construct, expressed in Rosetta™2 (DE3) pLysS provided the best opportunity for optimal soluble expression of MO14 for scale-up studies.

Biotransformation of racemic-1 in a fermentor

In order to assess the practical utility of MO14 for scaleable resolution of **1**, biotransformation experiments were conducted on a 2 L scale in a fermentor. It was intended to examine factors such as substrate concentration, temperature of incubation, time of substrate addition, and also the comparative performance of gene products encoded by native and codon-optimised genes, with substrate addition at the point of induction. Fermentations at this stage were conducted with growing cells, following the work of Mihovilovic and co-workers, who have routinely demonstrated that growing cells of *E. coli* expressing BVMOs are effective catalysts for the biotransformations of ketone substrates.[22, 23]

Temperature of incubation

The first variable to be examined was temperature. Fermentations of *E. coli* Rosetta™2 (DE3) pLysS expressing MO14 were conducted at 16°C and 30°C post induction. After growth at 37°C to an optical density of 0.8, 1 mM IPTG was added as inducer and directly afterwards, the substrate (0.5 g L^{-1}; 4.5 mM) was added. At 16°C, after a lag time of some 4 h, oxygenation of the substrate was observed to commence, and proceeded over the next ten hours to approximately 49% conversion (solid triangles in **Figure 3**). A distinct second phase of biotransformation was then observed, as would be expected for an enantioselective process, with the oxygenation slowing as the less favoured ketone enantiomer was consumed. The reaction then progressed to approximately 60% conversion after 20 h. A detailed analysis of the enantioselectivity of the biotransformation is presented below (*vide infra*). At the higher temperature of 30°C the conversion was very low. This was almost certainly due to the poorer levels of soluble expression observed in this strain at 30°C. Conversion in this instance did not exceed single integer figures over the 20 h period. Interestingly, Woodley and co-workers report the use of 37°C[24] or 30°C[25, 26] for their fermentor-based investigations of the biotransformation of *rac*-**1** using *Ac*CHMO expressed in resting cells of *E. coli.*

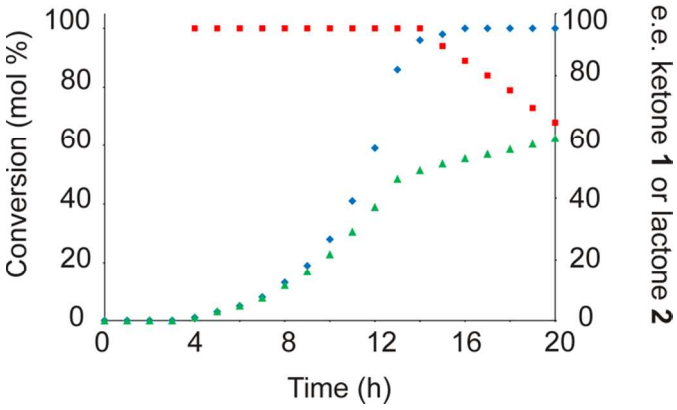


Figure 3. Enantiomeric excess of substrate and product in biotransformation of racemic **1** in a fermentor using cells of *E. coli* Rosetta™2 (DE3) pLysS expressing *ro*03437 using strain expressing native *ro*03437 gene at 16°C after induction of gene expression with substrate at 4.5 mM; ▲ = conversion (mol %); ♦ = e.e. ketone (1*S*, 5*R*)-**1**; ■ = e.e. lactone (1*S*, 5*R*)-**2.**

Substrate concentration

Having established a suitable temperature for further fermentations, the next parameter to be studied was the concentration of substrate. Fermentors were inoculated and grown to an O.D of 0.8 as previously, and substrate was again added at the point of IPTG induction of gene expression. At 1.0 g L^{-1} (9.0 mM), the extent of biotransformation were markedly lower, with only approximately 9% conversion achieved after 24 h, and no prospect of a substrate resolution at this concentration The enantiomeric excess of the lactone (1*S*, 5*R*)-**2** was still high (>99%), however, and equalled the final concentration of lactone achieved at a concentration of 4.5 mM, if only by virtue of higher substrate concentration. At 2 g L^{-1} (18 mM), conversions were very low and substantial toxicity effects were observed. For *E. coli* Top10 cells expressing the *Ac*CHMO gene it was also reported that substrate concentrations over 0.2-0.4 g L^{-1}, and also lactone product concentrations of above 3.5 g L^{-1} were toxic to the organism,[24,25] necessitating substrate feeding strategies[24-26] and the application of *in situ* product removal techniques.[26]

Native gene versus codon-optimised gene

SDS–PAGE analysis of the comparative expression levels of native and codon-optimised genes (**Figure 2**) suggested that the latter was

expressed more poorly in the *E. coli* strain of choice. The lower levels of expression of the native gene were reflected in the poorer performance of the strain expressing it as a biocatalyst. Although conversion was linear, it achieved a level of only 12% after 14 h, whereas the strain expressing the wild type gene had reached 49%.

Substrate Addition 1 h after induction

Mihovilovic and co-workers have shown that biotransformations using BVMO genes expressed in *E. coli* can be achieved with addition of substrate either 30 min after induction of gene expression[22] or at the point of induction itself.[23] The delay in the initial conversion of the substrate after induction in our previous experiments was noted and thought to be related to the simultaneous addition of IPTG and substrate. In order to explore the effect of waiting for BVMO gene expression to become estabished, experiments were conducted in which induction with IPTG was followed by a 1 h delay prior to the addition of substrate. However, in this case, while conversion of **1** was again observed to proceed in a linear fashion, only 14% conversion was achieved after 20 h, suggesting that the joint addition of inducer and substrate is actually beneficial to the rate of biotransformation of **1.**

Enantioselectivity during the course of biotransformation

The enantioselectivity of the biotransformation of **1** by MO14 was studied in detail through extraction of samples during the reaction and by analysing the enantiomeric excess of residual (1*S, 5R*)-**1** (solid diamonds) and product (1*S*, 5*R*)-**2** (solid squares) as a function of time (**Figure 3**) using chiral gas chromatography. Preliminary observations of both the regio- and enantioselectivity of MO14[19] were confirmed by the results, which showed that the lactone e.e. is >99% up to approximately 49% conversion at 14 h, with the corresponding ketone e.e. rising steadily to approximately 96% at this time, representing an *E* value for the resolution of >700. After this point, the much slower biotransformation of (1*S*, 5*R*)-**1** is observed to make a contribution to the e.e. of the lactone product, resulting in a steady decrease beyond 49% conversion as a result of the production of (1*R*, 5*S*)-**2**.

Investigating the molecular basis for regioselectivity in MO14 versus the non-regioselective homolog MO15

The peculiar regio- and enantioselective properties of BVMOs towards substrate **1** has stimulated investigations into mutation of the active sites of BVMOs that would shed light on the determinants of both regio– and enantioselectivity in these enzymes.[27,28] In our previous studies on the BVMOs from *R. jostii* RHA1, we showed that, in the closely related 'Group II' BVMOs, comprising MO9, MO14 and MO15 each catalysed the resolution of **1** through preferential oxygenation of the (1*R, 5S*)-enantiomer, but whilst MO14 was also regioselective, giving only the 2-oxa lactone, MO15 (*ro*02492, Uniprot Q0SDT9) catalysed the conversion of **1** to (1*S*, 5*R*)-**2** and the 3-oxa lactone (1*S*, 5*R*)-**3** in approximately equimolar amounts.[19] A sequence alignment of MO14 and MO15 revealed that they are 50% sequence identical with a further 17% strong similarity.

The similarity of MO14 and MO15 allows the rare opportunity to directly probe the active-site determinants of BVMO selectivity using highly sequence-related natural variants with very contrasting properties; in this case the different regioselective behaviours of MO14 and MO15. In order to identify residues that might be significant in determining regioselectivity, the structure of the closely-related phenylacetone monooxygenase (PAMO)[6] was used to identify residues within 9 Å of the C4a atom of the FAD that were different between MO14 and MO15 (**Figure 4**).

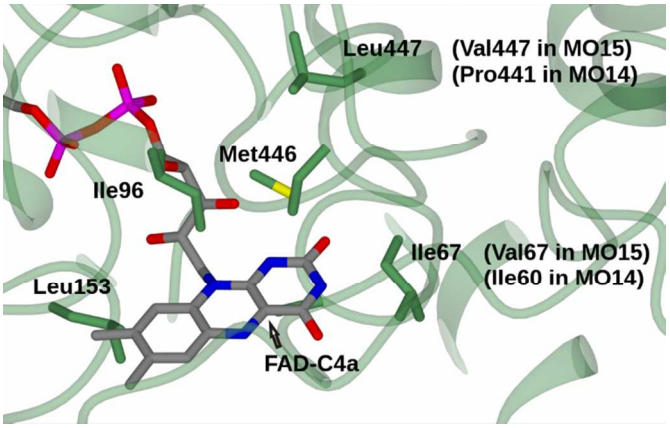


Figure 4. Representation of structure of phenylacetone monooxygase (PAMO; PDB code 1W4X)[6] illustrating some of the conserved amino acids at sites near the FAD between PAMO, MO14 and MO15 and location and variation in amino acids in positions PAMO 67 and 447 between the three enzymes. The protein backbone and relevant side-chains are shown in green in ribbon and cylinder format respectively. FAD is shown in cylinder format with carbon atoms in grey.

The analysis suggested that, of the relevant residues, MO14 Cys58 (MO15 Cys65) Ile89 (Ile96), Met440 (Met446), Leu146 (Leu153) and Val436 (Val442) and others were all conserved between MO14 and MO15, but that Ile60 and Pro441 in MO14 were replaced in MO15 by valine residues Val67 and Val447 respectively (**Table 1**). Consequently, we first prepared the MO14 mutants Ile60Val and Pro441Val and the double mutant Ile60Val/Pro441Val to see if regioselectivity would be destroyed by these mutations. Transformation of **1** by cell extracts of *E. coli* strains expressing the mutant genes showed that there was very little change in the product profile of these biotransformations however, with the lactone (1*S*, 5*R*)-**2** still formed with high e.e. We then prepared the reciprocal mutants Val67Ile and Val447Pro of MO15 and also the double mutant Val67Ile/Val447Pro, and determined the regioselectivity of transformation of **1** in a similar way. In this case, the regioselective excess for the wild-type MO15, in favour of the 2-oxa lactone was 23%, but this rose to 34% and 42% for the Val67Ile and Val447Pro mutants respectively, and to 49% for the Val67Ile/Val447Pro double mutant, suggestive of an additive effect (**Figure 5**), and a progression towards the more regioselective behaviour of MO14.

E	26	55	65	66	67	72	96	153	200	337	441	442	445	446	447
PAMO	F	W	C	D	**I**	Y	I	L	Q	R	S	A	N	M	**L**
MO14	F	W	C	D	**I**	Y	I	L	Q	R	S	V	N	M	**P**
MO15	**F**	**W**	**C**	**D**	**V**	**Y**	**I**	**L**	**Q**	**R**	**S**	**V**	**N**	**M**	**V**

Table 1. Conservation of residues in MO14 and MO15 suggested to be within 9 Å of the FAD as determined using the structure of PAMO[6] as a model. **E** = enzyme. Numbers refer to amino acid positions within the PAMO sequence.

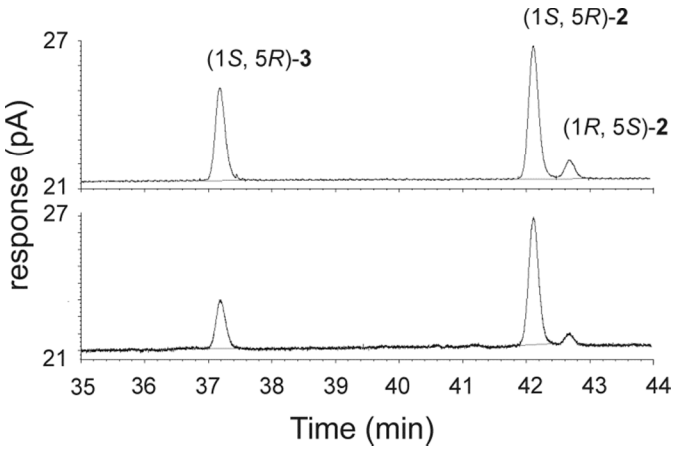


Figure 5. Chiral GC analysis of biotransformation of **1** by wild-type MO15 (top) and the Val67Ile/Val447Pro double mutant (bottom). The mutation to a more 'MO14'-like active site improves the regioselectivity of the biotransformation from a regioselective excess of 23% to 49%, as revealed by the reduction in amount of the 3-oxa lactone (1*S*, 5*R*)-**3** relative to 2-oxa lactone (1*S*, 5*R*)-**2**. MO14 (not shown) produces exclusively (1*S*, 5*R*)-**2**.[19]

Despite the apparent conservation between MO14 and MO15 of active site residues at the substrate oxygenation site beneath the FAD, it appears that a measure of enzyme selectivity in MO15 at least is governed by the region beside and above the pyrimidinedione ring of FAD, as shown in **Figure 4**. This region is adjacent to the P[437]GSP loop in PAMO and related enzymes that had already been suggested to have a role in the selectivity of biotransformation of **1** on the basis of sequence-activity relationships[19] and targeted saturation mutagenesis.[27] Moreover, both positions 67 and 447 in PAMO had previously been targeted for mutagenesis by Fraaije and co-workers, who demonstrated, for example, that mutation of Ile67 to the smaller threonine resulted in a switch in regioselectivity in that enzyme from 3:1 (2-oxa:3-oxa lactone) to approximately 1:1.[28] However, the same effect was achieved through point mutation of Ser441 to Ala, and yet Ser441 is conserved between regioselective MO14 and non-regioselective MO15. These observations, coupled with the failure to mutate MO14 to have MO15 selectivity, and the limited success in mutating MO15 to have MO14 selectivity completely, are illustrative of the fact that selectivity in BVMOs is achieved through the combination of many amino acid residues and cooperative effects, and that these may combine in different ways in different enzymes to achieve individual enzyme selectivity overall. However, as the number of sequences of BVMOs and their characterisation increases, progressive data on the targeted mutation of individual residues may be useful in informing rational redesign of BVMOs in the future.

Conclusion

The application of Baeyer-Villiger monooxygenases in industrial synthetic chemistry requires that new and useful catalysts are identified, and that their catalytic characteristics are studied under performance conditions. The successful expression and application of the BVMO MO14 under fermentation conditions suggests that this enzyme may have valuable potential for the resolution of important bicyclic ketone structures which are difficult to access using other, more established enzymes. The application of MO14 in this vein would benefit from the application of detailed process modelling studies[29] and techniques to ameliorate substrate and product toxicity[24-26] that have been applied to the fermentor-based biotransformation of **1** by the CHMO from *Acinetobacter*.

Experimental

Genes and Plasmids used in this study

A description of the cloning of genes *ro*03437, encoding MO14 and *ro*02492 into the pET-YSBLIC-3C vector, with which the gene product is equipped with an N-terminal hexahistidine tag, is given in reference.[22] The version of gene *ro*03437 codon-optimised for expression in *E. coli* was synthesised by GeneArt, and subcloned into pET-YSBLI3C in the same way. Mutants of MO14 and MO15 were generated using a Clontech InFusion™ kit, according to the manufacturer's instructions, using the primers listed in **Table 2**:

Gene Expression Studies

1 µL of the relevant plasmid was incubated with 25 µL of *E. coli* Rosetta™ 2 (DE3) pLysS cells in 500 µL Eppendorf tubes and kept on ice for 30 min. The samples were then heat shocked at 42°C for 45 s and returned to ice for 2 min. Samples were subsequently added to 1 mL of LB medium and incubated at 37°C for 1 h, after which they were plated onto LB agar containing 30 µg

mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol. Plates were incubated at 37°C for 18 h.

Primer	Sequence
MO14: Ile60Val For	GCCCGCTGCGACGTGGAGAGCGTCCACTATTCGTACTC
MO14: Ile60Val Rev	CACGTCGCAGCGGGCACCCGGATAGCGATTCCAGAAC
MO14: Pro441Val For	CTCTACAACATGGTGCTCGCCATCGAGGACCACGTC
MO14: Pro441Val Rev	CACCATGTTGTAGAGCACCGACGGCGACTGCGGTC
MO15: Val67Ile For	GCCCGGTGCGACATCGAAAGCGTCGACTATTCGTACTC
MO15: Val67Ile Rev	GATGTCGCACCGGGCGCCGGGATAGCGGTTCCAG
MO15: Val447Pro For	CTGGCGAACATGCCACTCGGCGCCGAACAGCAC
MO15: Val447Pro Rev	CTGGCGAACATGCCACTCGGCGCCGAACAGCAC

Table 2. PCR primers used for the generation of MO14 and MO15 mutants in this study.

Colonies were picked and added to 5 mL LB media containing 30 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol and the cultures incubated at 37°C for 18 h. These starter cultures were then added to 500 mL LB media containing 30 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol and incubated at 37°C until the absorbance of the culture, measured at 600 nm, was aproximately 0.6, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The temperature of the incubator was then reduced to 16°C and the cultures were then grown at this temperature for 18 h. The cells were harvested by centrifugation for 15 min at 4225 g in a Sorvall GS3 rotor in a Sorvall RC5B Plus centrifuge. In order to make cell extracts for gel analysis, cell pellets were resuspended in one-tenth the growth volume of of 50 mM Tris-HCl buffer pH 7.5. and these suspensions sonicated for 3 x 60 s bursts at 4°C with 1 min intervals. The soluble and insoluble fractions were separated by centrifugation for 30 min at 26,892 g in a Sorvall SS34 rotor

Fermentation

Fermentations were carried out using a 7 L capacity Fisher BC10 fermenter with conditions controlled by an Applikon Bio Controller ADI 1010. 2 L of Terrific Broth (TB) containing 30 mg/L kanamycin and 2 mL Antifoam C. 50 mL of a starter culture of cells expressing the relevant gene encoding MO14 were added to the fermentor and the mixture stirred at 37°C to maintain oxygen levels at 30% of optimum. Once the solution reached an OD_{600} of ~0.6, gene expression was induced by addition of IPTG to a concentration of 1 mM and the temperature controlled according to the experiment (see Results). In most experiments, the required amount of the substrate **1** was then added to the desired concentration. 500 μL samples were removed at hour intervals and each extracted into 500 μL ethyl acetate, to yield samples for analysis by gas chromatography.

Biotransformations Using Cell Extracts

For biotransformations by cell extracts of *E. coli* expressing MO14, MO15 and the MO15 mutants, prior to sonication the cell density was adjusted to an OD_{600} of 50 with buffer. For each biotransformation, to 1 mL of cell lysate was added bicyclo[3.2.0]hept-2-en-6-one (**1**, 1 mg, 9 x 10^{-3} mmol) and 10 μL of 50 mM Tris-HCl buffer pH 7.5 containing NADPH (1 mg, 1.2 x 10^{-3} mmol), glucose-6-phosphate (0.6 mg, 2 x 10^{-3} mmol) and glucose-6-phosphate dehydrogenase (0.1 mg). Samples were incubated at 25°C for 18h with shaking. 500 μL of reaction mixtures were then extracted and each was mixed with 500 μL of ethyl acetate, vortexed, and centrifuged for 2 min at 13300 g for a Genfuge 24D tabletop centrifuge. The organic layer was then removed to a GC vial for analysis.

Non-chiral capillary GC analysis

For GC analysis of biotransformation mixtures, an Agilent HP-6890 gas chromatograph was employed, which was fitted with an HP-5 column (30m x 0.32 mm x 0.25μm). Helium was used as the carrier gas at a pressure of 12 Psi. The injector temperature was 250°C and the detector temperature was 320°C. The gradient programme used for analysis of biotransformation reactions with **1** was: 50°C to 250°C at 10°C min^{-1}. The retention times for **1** and **2** were 4.04 min and 7.70 min respectively.

Chiral capillary GC

For chiral GC analysis a BGB-173 column (30 m x 0.25 mm x 0.25 μm) and a BGB-175 column (30 m x 0.25 mm x 0.25 μm), each obtained from BGB Analytik, were employed. Helium was used as carrier gas at a pressure of 12 Psi. Tthe injector temperature was 250°C and the detector temperature was 320°C. Enantiomers of ketone substrate **1** were resolved using the BGB-175 column and a gradient of 100°C to 127°C with 2°C min^{-1}. Retention times for enantiomers were: (1*R*, 5*S*)-(+)-**1,** 11.6 min.; (1*S*, 5*R*)-(-)-**1**, 12.4 min. Enantiomers of lactones **2** and **3** were resolved using the BGB-173 column with a gradient of 90°C to 134 °C at 1°C min^{-1}. Retention times of enantiomers were: (1*R*, 5*S*)-(-)-**3**, 36.7 min.; (1*S*, 5*R*)-(+)-**3**), 37.0 min.; (1*S*, 5*R*)-(-)-**2**, 41.8 min.; (1*R*, 5*S*)-(+)-**2**, 42.4 min.

(1*S*,5*R*)-2-Oxabicyclo[3.3.0]oct-6-en-3-one (2)

Data were in agreement with the literature.[30] Waxy colourless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (dq, *J* = 4.5, 2.0 Hz, 1H), 5.58 (dq, *J* = 6.0, 2.0 Hz, 1H), 5.16–5.11 (m, 1H), 3.51 (ddq, *J* = 9.5, 6.0, 2.0 Hz, 1H), 2.77 (dd, *J* = 18.0, 9.5 Hz, 1H), 2.72 (sext, *J* = 2.0 Hz, 2H), 2.45 (dd, *J* = 18.0, 2.0 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.91, 131.40, 129.93, 83.19, 45.72, 39.69, 33.44; IR (thin film)/cm^{-1} 2928w, 1770s, 1418w, 1346w, 1295w, 1256m, 1229w, 1171s, 1046m, 1015m, 949w, 920m, 897w, 864w, 805w, 723m; MS (ESI$^+$) *m/z* (rel. %) 271 ([2M+Na]$^+$, 30), 147 ([M+Na]$^+$, 100), 125 ([M+H]$^+$, 5); HRMS (ESI$^+$) 147.0419 [M+Na]$^+$, $C_7H_8NaO_2$ requires 147.0417.

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Notes and references

[a] B.D. Summers, M. Omar, T. Ronson, Dr. G. Grogan, York Structural Biology Laboratory, Department of Chemistry University of York, York YO10 5DD U.K. Tel: (+44) 1904 328256; Fax: (+44)1904-328266; e-mail gideon.grogan@york.ac.uk

[b] Technology Facility, Department of Biology University of York, York YO10 5DD U.K.

[c] Chirotech Technology Ltd., Dr. Reddy's Laboratories (EU) Ltd, Unit 410, Cambridge Science Park, Milton Road, Cambridge, CB4 0PE

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