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A New Type of Stereoselectivity in Baeyer–Villiger Reactions: Access to *E*- and *Z*-Olefins

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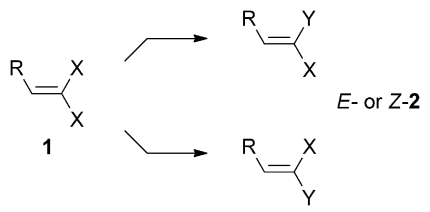
Abstract: A new concept for accessing configurationally defined trisubstituted olefins has been developed. Starting from a common ketone precursor of the type 4-ethylidenecyclohexanone, Baeyer–Villiger monooxygenases are employed as catalysts in diastereoselective Baeyer–Villiger reactions leading to the corresponding *E*- or *Z*-configurated lactones. Wild-type cyclohexanone monooxygenase (CHMO) as catalyst delivers the *E*-isomers and a directed evolution mutant the opposite *Z*-isomers. Subsequent transition metal-catalyzed chemical transformations of a key product containing a vinyl bromide moiety provide a variety of different trisubstituted *E*- or *Z*-olefins. A model based on QM/MM sheds light on the origin of this unusual type of diastereoselectivity. In contrast to this biocatalytic approach, traditional Baeyer–Villiger reagents such as *m*-CPBA fail to show any selectivity, 1:1 mixtures of *E*- and *Z*-olefins being formed.

Keywords: alkenes; Baeyer–Villiger oxidation; diastereoselectivity; directed evolution; palladium

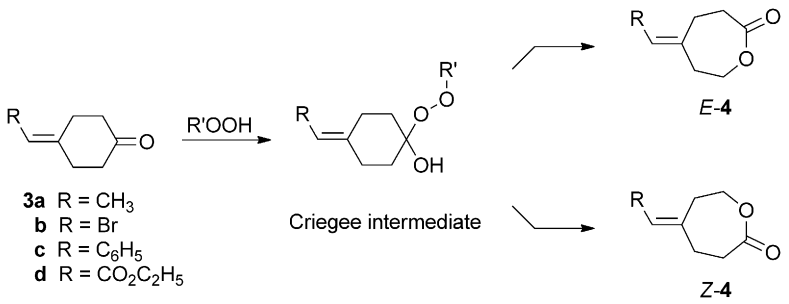
Introduction

A commonly occurring structural feature of many natural and unnatural organic compounds is the presence of configurationally well defined olefinic moieties, the respective double bonds having either the *E*- or *Z*-configuration. Controlling the *E*/*Z*-selectivity of olefin-forming processes with formation of trisubstituted products as in Wittig-type reactions, Julia–Lythgoe olefination, sigmatropic rearrangements, olefin metathesis, or C–C bond-forming reactions of alkynes has attracted a great deal of attention.[1] Variations of these themes as well as new approaches continue to be developed.[2] Making the appropriate choice of methods depends upon the particular starting material and synthetic goal under investigation.



Scheme 1.

A different approach is to start with easily accessible trisubstituted olefins **1** in which the 1,1-substituents are identical (Scheme 1). Such substituents are diastereotopic, which means that they may react with different rates leading to *E*- or *Z*-configurated olefins **2** (assignment of *E*- or *Z*-configuration depends on the priority of the particular substituents). Prominent examples include Pd-catalyzed C–C bond formation in which the *trans* C–X bond of 1,1-dihalides **1** (X = Cl or Br) reacts preferentially with formation of the respective *Z*-configurated trisubstituted olefins, as first developed by Tamao.[3] Subsequently it was generalized using a variety of coupling processes which include Kumada–Tamao–Corriu, Suzuki, Stille or Sonogashira reactions.[2f,4] It is also possible to consider a "reversed" approach in which 1,1-dimetalated reagents are utilized (e.g., X = boron in compounds **1**).[5] Another case pertains to the lipase-catalyzed hydrolysis of diesters of the type **1** ($X = CO_2Et$) which proceeds likewise in a *Z*-selective manner.[6] In all of



Scheme 2.

these systems the sterically less shielded diastereotopic group reacts preferentially, but a reversal of the stereoselectivity has yet to be achieved. Nevertheless, the possibility of subsequent chemoselective transformations makes these processes synthetically attractive.[3–6]

We envisioned a different approach to selectively activate diastereotopic groups in appropriate trisubstituted olefins bearing identical substituents at the 1,1-position. The plan foresaw Baeyer–Villiger (BV) reactions[7] of keto-olefins of the type **3**, in which the two diastereotopic σ-bonds flanking the ketone function could react with different rates, thereby diastereoselectively providing olefins *E*- or *Z*-**4** from a common precursor, ideally on an optional basis (Scheme 2). Of particular interest was the vinyl bromide **3b**, because the respective *E*- or *Z*-lactones **4b** could serve as key compounds for transition metal-catalyzed cascade reactions with formation of a variety of different configurationally defined trisubstituted olefins. This type of stereoselectivity has not been considered previously, perhaps due to the anticipated difficulty in designing selective reagents and/or catalysts for such BV reactions. The reactive diastereotopic groups in compounds of the type **3**, namely the two σ-bonds flanking the ketone function in the respective Criegee intermediate, are spatially far removed from the olefinic moiety and are thus likely to migrate with similar rates leading to an undesired mixture of thermodynamically similar *E*- and *Z*-olefins. Indeed, upon treating keto-olefin **3b** with *m*-CPBA, a 1:1 mixture of lactones *E*- and *Z*-**4b** was formed.

Results and Discussion

Utilizing Biocatalysis as an Option

We therefore turned to biocatalytic BV reactions by considering Baeyer–Villiger monooxygenases (BVMOs)[8] as potentially diastereoselective catalysts. In previous work a number of enantioselective transformations have been reported, including oxidative kinetic resolution of 2-substituted cyclohexanone derivatives and desymmerization of 4-alkylcyclohexanones using wild-type (WT) cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB 9871 (designated here as CHMO), which is the most commonly employed BVMO in enantioselective BV reactions.[8] Regiodivergent oxidation has also been observed in some cases.[8h–k] In these enzymatic transformations, dioxygen in air reacts with an enzyme-bound (reduced) flavin (FAD) to form an anionic intermediate FAD-OO$^-$ which initiates the BV reaction by nucleophilic addition to the carbonyl function, the respective Criegee intermediate then undergoing the usual fragmentation and σ-bond migration.[8] Subsequently, NADPH transforms the oxidized FAD back to the reduced form, thereby closing the catalytic cycle. When selectivity proves to be poor, the methods of directed evolution[9] can be applied with generation of enantioselective BVMO mutants.[10]

In the present study we chose WT CHMO as the catalyst in a whole cell *E. coli* system, the initial model transformation being the reaction of keto-olefin **3a**. Gratifyingly, essentially complete stereodifferentiation was observed, *E*-selectivity being favored (*E*-**4a**:*Z*-**4a** = 98:2) with excellent conversion and essentially no side-products. The other ketones were then tested under similar conditions (Table 1). Substrate **3b** likewise reacted with complete *E*-selectivity (*E*-**4b**:*Z*-**4b** ≥ 99:1). Unambiguous configurational assignment was made on the basis of the crystal structure of the key compound *E*-**4b** (Figure 1) and its transformation into *E*-**4a** by Suzuki coupling (see the Supporting Information). In sharp contrast, the other two ketones **3c** and **3d** reacted sluggishly, allowing substantial amounts of side-products to be formed which were identified as the alcohols corresponding to the reduction of the ketones. We suspect that in the whole cell process unidentified alcohol dehydrogenases are involved. From a synthetic viewpoint, poor conversion to the desired lactones **4c** and **4d** is not a serious problem, because the vinyl bromide *E*-**4b**, as already delineated, can serve as a key intermediate in the transition metal catalyzed transformation into the respective *E*-configurated products (see below).

Table 1. WT-CHMO as a biocatalyst in the Baeyer–Villiger reaction of ketones **3a–d** with formation of lactones **4a–d** in a whole-cell process.

Ketone	Product	*E*:*Z*	Conversion [%][a]	Time [h]	Other product [%][a]
3a	**4a**	98:2	>99	5	1
3b	**4b**	99:1	>99	5	4
3c	**4c**	>99:1	39	12	84
3d	**4d**	96:4	42	12	70

[a] By GC analysis of the crude products, the side-products being the alcohols corresponding to the ketones.

A possible strategy for reversing the diastereoselectivity in favor of the *Z*-isomers could be the systematic screening of different BVMOs, but a preliminary search beginning with the thermally robust phenyl acetone monooxygenase (PAMO)[11] and known mutants[10c] thereof proved to be unsuccessful. Rather than continuing along this strategy, we turned to directed evolution of WT CHMO using ketone **3b** as the model substrate, although changing such a highly *E*-selective enzyme into a completely *Z*-selective mutant appeared challenging. In earlier studies we had used error-prone PCR in order to enhance and invert the enantioselectivity of CHMO as a catalyst in the oxidative desymmetrization of 4-hydroxycyclohexanone.[10a] Later the much improved directed evolution method based on structure-guided saturation mutagenesis, the *combinatorial active-site saturation test* (CAST),[9i,12] was applied to PAMO with the creation of enantioselective mutants.[10c] Accordingly, sites around the binding pocket of an enzyme as revealed by the respective crystal structure or homology model are subjected to saturation mutagenesis, meaning the focused introduction of all of the other 19 canonical amino acids. If the initial libraries provide only moderately improved mutants, iterative saturation mutagenesis (ISM) can be applied.[9i] In the present study this structure-based approach to laboratory evolution was invoked. Since the X-ray structure of CHMO from *Acinetobacter* sp. NCIMB 9871 has not been reported to date, we used as a guide for choosing appropriate randomization sites the crystallographic data of the homologous CHMO from *Rhodococcus* recently reported by Lau, Berghuis and co-workers.[13] A number of potential randomization sites are possible, but only a limited number of experiments were performed, guided by exploratory docking simulations employing substrate **3a**. These indicated steric clashes between the methyl group and certain residues of the protein environment when considering the *Z*-selective process (see the Supplementary Information). On this basis three sites were selected, namely A (Phe432/Thr433), B (Leu143 and C (Phe505) as illustrated in Figure 2.

In the case of the single-residue sites B and C, only about 100 transformants need to be screened for 95% library coverage.[9i] In order to minimize the screening effort in the case of saturation mutagenesis at the 2-residue site A, a reduced amino acid alphabet was used comprising 12 building blocks (Phe, Tyr, Cys, Leu, His, Arg., Ile, Asn, Ser, Val, Asp and Gly) as defined by NDT codon degeneracy. This requires the assessment of only ~430 transformants for 95% library coverage (instead of about 3000 when employing the standard NNK codon degeneracy encoding all 20 canonical amino acids).[9i] Six different ISM pathways are possible in a 3-site ISM scheme. In the present case we started with site A. Upon screening 500 transformants, a double mutant Phe432Ile/Thr433Gly (variant I) was identified leading to a shift toward *Z*-selectivity, but not yet to the desired reversal of diastereose-

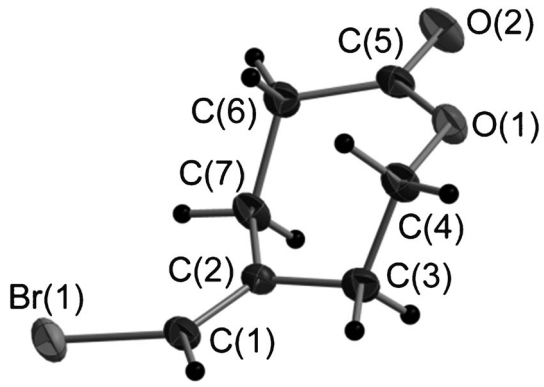


Figure 1. X-Ray structure of lactone *E*-**4b**.

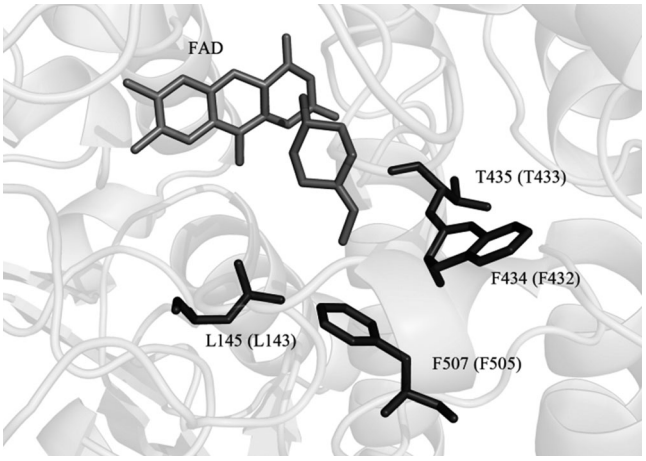


Figure 2. Possible saturation mutagenesis sites A (Phe432/Thr433), B (Leu143) and C (Phe505) in CHMO from *Acinetobacter* sp. NCIMB 9871, chosen on the basis of the crystal structure of CHMO from *Rhodococcus*,[13] PDB code 3GWD. The residue numbers not in parentheses correspond to the analogous residues in CHMO from *Rhodococcus*. The structure of cofactor FAD cofactor is marked in light grey and the substrate **3b** is marked in dark grey.

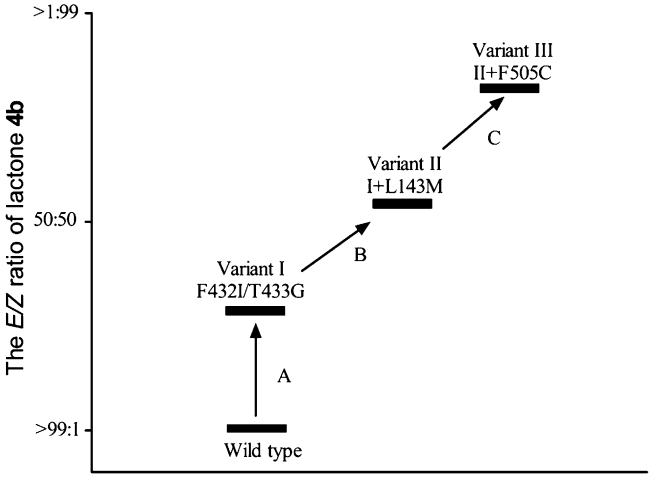


Figure 3. Chosen ISM pathway for evolving a *Z*-selective CHMO mutant as the catalyst in the BV reaction of ketone **3b**.

lectivity (*E*/*Z* = 71:29). The gene of this variant was then used as a template for saturation mutagenesis at single residue site B, this time utilizing NNK codon degeneracy. This provided a triple mutant Phe432Ile/Thr433Gly/Leu143Met (variant II) resulting in a slight reversal of selectivity (*E*-**4b**:*Z*-**4b** = 46:54). Finally, upon continuing the upward climb to site C, reversal of diastereoselectivity was further improved to 82% *Z*-selectivity (*E*-**4b**:*Z*-**4b** = 18:82) and 19% conversion after 5 h, catalyzed by quadruple mutant Phe432Ile/Thr433Gly/Leu143Met/Phe505Cys (variant III). An increased reaction time 12 h afforded conversions up to 30% with similar *Z*-selectivity (*E*-**4b**:*Z*-**4b** = 20:80) (see the Supporting Information, Table S3). Column chromatography and alternatively preparative HPLC provided essentially pure *Z*-**4b**. The arbitrarily chosen ISM pathway A→B→C is pictured in Figure 3. Exploring other pathways may well provide even better diastereoselectivities, but at this point we settled for the present results.

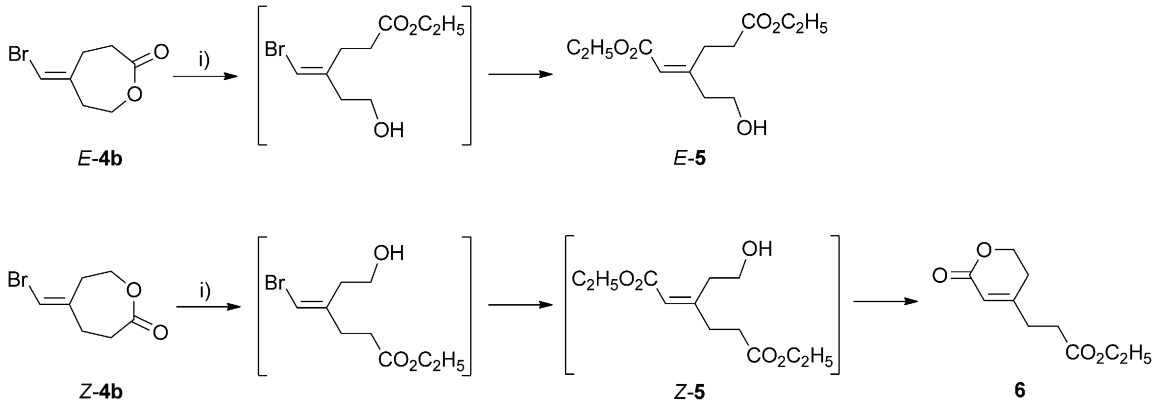
Testing the Best Mutant with Other Substrates

In order to explore the catalytic efficiency of the quadruple mutant (variant III), specifically evolved for compound **3b**, we tested it with the other ketones **3a** and **3c** and **3d**. In the case of substrate **3d**, the biocatalyst performs very well, the original *Z*-selectivity (WT CHMO) being fully reversed (*E*-**4d**:*Z*-**4d** = 4:96) at 98% conversion within 12 h and only 7% side-product (1.2 mmol scale). In the reactions of the other substrates, the results proved to be less encouraging (see the Supporting Information).

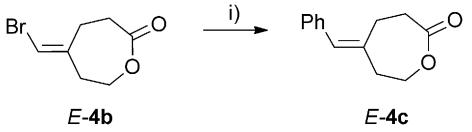
Exploiting the Vinyl Bromide 4b as a Key Compound in Cascade Reactions

As already alluded to, we planned to utilize *E*- and *Z*-**4b** as key compounds in further transformations. For example, upon subjecting them to Pd-catalyzed carbonylation[14] in the presence of ethanol, trisubstituted olefins *E*-**5** and **6** were obtained, respectively (Scheme 3). The presence of ethanol induces rapid transesterifying ring-opening under the reaction conditions, probably before the actual carbonylation occurs. We observed this kind of ring-opening for compounds of the type **5** whenever they were treated with mild nucleophiles such as water or alcohols in the absence of transition metal catalysts (see the Supporting Information). These carbonylation reactions stand in contrast to the poor results when attempting the BV reaction of **3c** (Table 1).

In other reactions, Pd-catalyzed Suzuki-type arylation[15] of *E*-**4b** provided the respective lactone *E*-**4c** again with retention of configuration (Scheme 4), which is not readily accessible starting from keto-olefin **3c** (Table 1). The products in these schemes bear functional groups which can in principle be manipulated with formation of a wide variety of different trisubstituted olefins for potential applications.



Scheme 3. Pd-catalyzed carbonylation with retention of double bond configuration. *Reagents and conditions:* i) EtOH, $Pd(PPh_3)_4$/CsF, CO, 80 °C, 48 h: *E*-**5**, 61%; **6**, 78%.



E-**4b** *E*-**4c**

Scheme 4. Stereospecific Suzuki transformation of lactone *E*-**4b** with retention of configuration. *Reagents and conditions:* i) $PhB(OH)_2$, $Pd(PPh_3)_4$/CsF, THF/70 °C/24 h; 92%.

Developing a Model to Explain the Observed Diastereoselectivity

In order to gain insight into the source of diastereoselectivity of WT CHMO on a molecular level, we first performed docking computations and molecular dynamics (MD) simulations[16] using ketone **3a** as the substrate. In this endeavor, our recent QM/MM study of CHMO as a catalyst in the BV reaction of the parent compound cyclohexanone was considered, in which it was shown that the chair form of this ketone can bind in only one way which ensures a low-energy pathway to the Criegee intermediate.[17] Analysis of this intermediate revealed a crucial characteristic, namely that only one of the two σ-bonds flanking the carbonyl group is capable of undergoing rapid migration because here the traditional stereoelectronic requirement is fulfilled (anti-periplanar arrangement of the reactive C–C–O–O segment),[7,18] in contrast to the geometric arrangement when the other σ-bond migrates. This hypothesis was then supported by QM/MM calculations of the respective transition states. In the case of enantioselective desymmetrization of 4-methylcyclohexanone, the same applies, but two different chair forms are possible, one in which the methyl group is equatorial and the other in which it is axial. The difference in energy between the two chair forms in the protein environment determines the degree of enantioselectivity (≥96% *ee* in favor of *S*-lactone).[17]

This model is not directly applicable to the CHMO-catalyzed BV reaction of ketone **3a** in the present study, because the axial/equatorial structural element is not relevant. Nevertheless, we used the geometric features of the previously calculated Criegee intermediate of cyclohexanone as a template for manually constructing the analogous intermediate involving **3a**. As shown by force field calculations and molecular dynamics (MD) simulations, there are two poses of different energy, in each case only one σ-bond reacting due to the stereoelectronic requirement. The energetically lower pose leads to the experimentally observed formation of *E*-**4a** (see the Supporting Information). In the higher energy pose, the methyl group clashes with residues in a nearby loop (Supporting Information). This conclusion was corroborated by more accurate QM(B3LYP/TZVP)/CHARMM optimizations of the respective Criegee intermediates (Figure 4, a and b). Accordingly, the difference in energy amounts to 2.3 kcal mol^{-1}, which correlates well with the experimentally observed diastereoselectivity (see the Supporting Information).

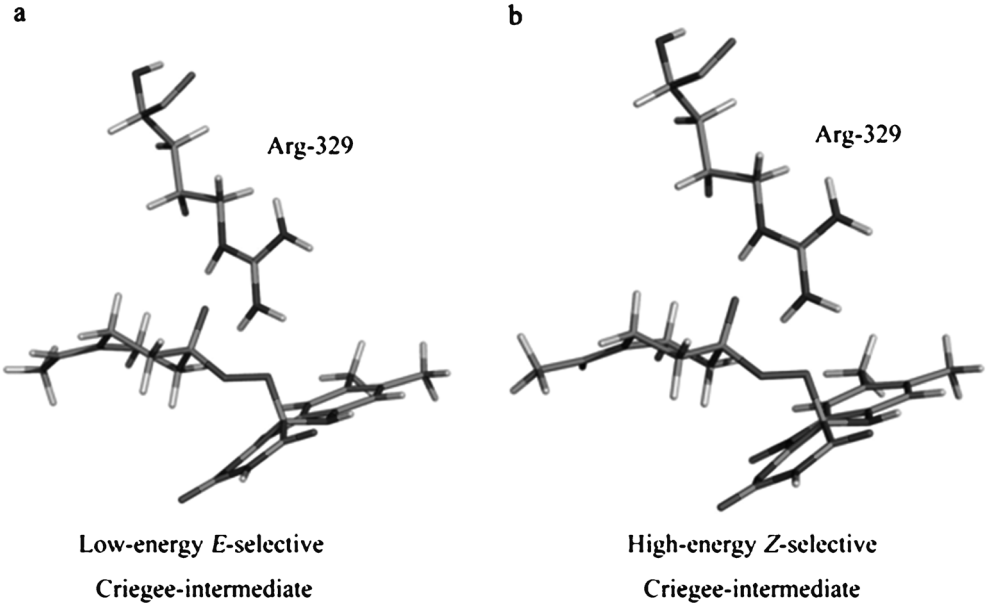


Figure 4. QM-calculated Criegee intermediates involving 4-ethylidenecyclohexanone (**3a**) in WT CHMO. **a**) Low-energy intermediate leading to the experimentally observed lactone *E*-**4a. b**) High-energy intermediate which would provide *Z*-**4a** (not observed).

Conclusions

The present study contributes to the continuing search for ways to prepare configurationally defined trisubstiuted olefins. In doing so, we have demonstrated a new type of stereoselective transformation in Baeyer–Villiger reactions, which has not been considered previously using synthetic reagents/catalysts or enzymes. Utilizing a Baeyer–Villiger monooxygenase, specifically CHMO from *Acinetobacter* sp. NCIMB 9871, a biocatalytic approach was developed which enables the control of *E*/*Z*-configuration in the formation of structurally different trisubstituted olefins starting from the respective 4-alkylidenecyclohexanone derivatives. WT CHMO is fully *E*-selective in the reaction of three different substrates. Especially the vinyl bromide **3b** proved to be a synthetically important substrate because the respective diastereoselectively formed lactone *E*-**4b** serves as a key compound in subsequent stereospecific cascade transformations such as Pd-catalyzed carbonylation or Suzuki coupling with formation of configurationally defined trisubstituted olefins. A model was developed which explains the source of diastereoselectivity on a molecular level. We also succeeded in reversing the selectivity in favor of *Z*-configurated olefins up to 82%. Future work will focus on directed evolution of the thermally stable Baeyer–Villiger monooxygenase PAMO[11] as a catalyst in this type of stereoselective transformation. It also remains to be seen whether appropriate derivatives of other even-membered cyclic ketones such as those of cyclobutanone or cyclooctanone can be used in a similar way.

Experimental Section

Biology: Creation of Mutant Libraries

The whole length of the CHMO gene from *Acinetobacter* sp. NCIMB 9871 was cloned into *E.coli* expression vector pET-22b (+) just as described previously,[19] which was also used as template for saturation mutagenesis. Mutant libraries shown in Table S1 (Supporting Information) were created by employing the QuikChange PCR method (Stratagene). Table S2 (Supporting Information) provides the oligonucleotide primers used for the creation of mutant libraries. PCR reaction mixtures (50 μL final volume) consisted of 10×KOD buffer (5 μL), $MgSO_4$ (2 μL, 25 mM), dNTP (5 μL, 2 mM each), primers (5 μL, 2.5 μM each), template plasmid (2 μL, 10 ng μL^{-1}) and KOD Hot Start DNA polymerase (1 U). The PCR reaction started at 95 °C for 3 min, followed by 18 cycles of denaturing step at 95 °C for 1 min, annealing at 52–58 °C (depending on the particular pairs of primers) for 1 min and elongation at 72 °C for 8 min. After cycling a final extension step at 72 °C for 16 min was performed. To ensure the removal of the template plasmid, PCR products were digested during 4 h at 37 °C after adding 1 unit of *Dpn*I (New England Biolabs) at 37 °C for 4 h. The digested PCR product was used to transform electrocompetent *E.coli* BL21-Gold (DE3) cells. The cells were spread on the LB agar plate with 100 μg mL^{-1} carbenicilline.

Library Screening

Single colonies were picked into 2.2-mL 96 deep-well plates containing 800 μL LB media with 100 μg mL^{-1} carbenicilline. Four wells per plate were used to culture wild-type CHMO as a control. After shaking at 37 °C, 800 rpm overnight, 100 μL of this overnight culture were used to inoculate a new 96 deep-well plate containing 700 μL LB media with relevant antibiotics. To conserve copy of libraries at −80 °C, the glycerol stock plates containing culture solution with 30% glycerol were prepared at this stage. The duplicate plates were cultivated for 1 h at 37 °C, 800 rpm. Then, 100 μL of the substrate mixture (80 μL LB with the relevant antibiotics and 20 μL of a stock solution of substrate 0.1 M in acetonitrile) supplemented with 0.2 mM IPTG were added to the plates. The incubation was continued at 30 °C shaking at 800 rpm for additional 15 h. The reaction was stopped and reaction product was extracted by adding 400 μL ethyl acetate to each well of the plates. After centrifugation, the organic phase was transferred to 96-well glass plate (Zinsser, Analytic) for GC analysis. The final positive mutants were collected and confirmed by reproducing the biotransformation in a flask scale. The plasmids of the mutants were extracted from overnight cultures and sequenced.

Biotransformations: General Upscaling Procedure for Biotransformations with the *E. coli* Wild-Type CHMO or Mutant CHMO

The wild-type CHMO or mutant CHMO were stored in the form of glycerol frozen stocks of *E.coli* BL21-Gold (DE3) (Stratagene) harboring the pET-22b (+) expression vector. Fresh LB medium (50 mL) containing carbenicillin (100 μg mL^{-1}) was inoculated with 1% of an overnight preculture of the CHMO-expressing *E.coli* strains in 300-mL Erlenmeyer flasks. The culture was incubated at 37 °C with shaking at 200 rpm until the OD_{600} reached 0.7. Then, isopropyl-thio-β-D-galactoside (IPTG) was added to a final concentration of 0.2 mM, followed by adding ketone substrate in acetonitrile with the final concentration of 20 mM. The final reaction mixture was shaken at 30 °C and 200 rpm. The whole process of the reaction was monitored by GC analysis of the aliquot samples every 2 h until the reaction completion. Then, the reaction was stopped and biotransformation mixtures were extracted with ethyl acetate. The organic phase was afterwards dried with Na_2SO_4, evaporated and the residue subjected to column chromatography for further purification and products separation.

Chemistry: General Remarks

Starting compounds and all other reagents including dry solvents were purchased from Acros, Sigma–Aldrich and Alfa and used without further purification. NMR spectra were recorded on a Bruker Avance 300 or DRX 400 (^{1}H: 300 MHz or 400 MHz, ^{13}C: 75 MHz or 101 MHz) spectrometer using TMS as internal standard ($d=0$) unless otherwise noted. High-resolution EI mass spectra were measured on a Finnigan MAT 95S spectrometer. High-resolution mass spectra

recorded in ESI and APCI mode were performed on a ThermoScientific LTQ-FT spectrometer. Conversion and diastereoisomeric composition were determined by achiral gas chromatography as described. Alternatively, product **4b** diastereomeric mixture was determined by HPLC. Diastereoisomeric assignments were determined as described in the Supporting Information. Analytical thin layer chromatography was performed on Merck silica gel 60 F254q while for column chromatography Merck silica gel 60 (230–400 mesh ASTM) was used. Reactions that required inert atmosphere (nitrogen or argon) were carried out using standard Schlenk techniques. GC analyses were performed on a HP 6890 series while HPLC data were measured on a Shimadzu LC-8 A. Crystallographic data for the structure of ***E*-4b** has been deposited with the Cambridge Crystallographic Data Centre (CCDC 880686). These data can be obtained free of charge from The Cambridge Crystallographic Data Centre *via* www.ccdc.cam.ac.uk/data_request/cifor on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. [fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

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