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New Bioorganic Reagents: Evolved Cyclohexanone Monooxygenase—Why Is It More Selective?

Margaret M. Kayser* and Christopher M. Clouthier

Department of Physical Sciences, University of New Brunswick, Saint John, New Brunswick, E2L 4L5 Canada

kayser@unbsj.ca

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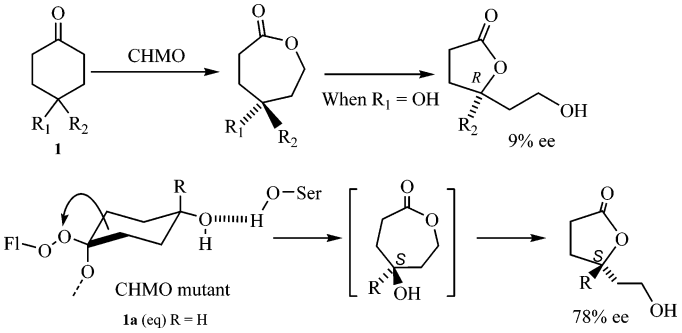

Four mutants of the cyclohexanone monooxygenase (CHMO) evolved as catalysts for Baeyer−Villiger oxidation of 4-hydroxycyclohexanone were investigated as catalysts for a variety of 4-substituted and 4,4-disubstituted cyclohexanones. Several excellent catalytic matches (mutant/substrate) were identified. The most important, however, is the finding that, in a number of cases, a mutant with a single exchange, Phe432Ser, was shown to be as robust and more selective as a catalyst than the wild-type CHMO. All biotransformations were performed on a laboratory scale, allowing full characterization of the products. The absolute configurations of two products were established. A model suggesting a possible role of the 432 serine residue in enantioselectivity control is proposed.

Introduction

Baeyer−Villiger oxidation of cyclic or linear ketones to the corresponding lactones or esters is an important reaction that has been used by chemists for over a century.[1] Enantiopure lactones are useful building blocks for a variety of valuable compounds, and many strategies, chemical and biochemical, have been devised to access these compounds.[2] In recent years, Baeyer−Villiger monooxygenases (BVMOs) from several microorganisms have been shown to be potential bioreagents for Baeyer−Villiger oxidations and sulfoxidations of an array of substrates.[3] Among BVMOs, cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 has been the most thoroughly investigated because of its ability to accept a remarkably broad range of substrates and its generally high enantioselectivity.[4] CHMO was also the first Baeyer−Villiger monooxygenase to be overexpressed in bakers' yeast[5,6] and in *E. coli*,[7,8] allowing general and convenient access to this useful bioreagent.[9]

CHMO's broad substrate base and high enantioselectivity invites a question: how can an enzyme achieve such versatility coupled with catalytic prowess? As excellent as it is, CHMO has limitations. For example, it readily oxidizes prochiral

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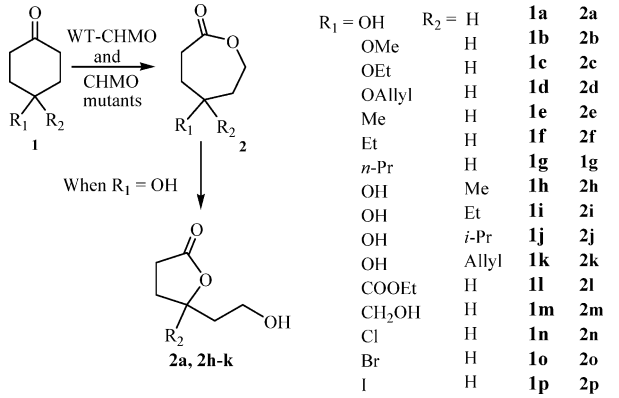
TABLE 1. Bayer−Villiger Oxidation of Prochiral Ketones 1a−1p to the Corresponding Lactones 2a−2p Catalyzed by WT-CHMO and the First Generation Mutants Evolved for Improved Enantioselectivity of the Baeyer−Villiger Oxidation of 1a

		% conv[a] (% ee)[b]				
ketone	R_1/R_2	WT-CHMO	Phe432Ser	Phe432Ile	Leu143Phe	Asp41Asn/Phe505Tyr
1a	OH/H	61[c] (9*R*)	100 (79*S*)	100 (49*R*)	100 (40*R*)	100 (46*S*)
1b	OMe/H	84[c] (78*S*)	100 (99*S*)	100 (99*S*)	100 (47*S*)	100 (80*S*)
1c	OEt/H	NR[e]	NR	NR	NR	NR
1d	OAllyl/H	NR	NR	NR	NR	NR
1e	Me/H	100 (95*S*)	100 (99*S*)	100 (99*S*)	100 (99*S*)	97 (99*S*)
1f	Et/H	100 (95*S*)	100 (99*S*)	100 (99*S*)	96 (99*S*)	97 (99*S*)
1g	*n*-Pr/H	89 (92*S*)	100 (99*S*)	NR	NR	NR
1h	OH/Me	100 (96*R*)	100 (87*R*)	NR	20 (46*R*)	17 (63*R*)
1i	OH/Et	100 (94*R*)[d]	100 (97*R*)	NR	NR	NR
1j	OH/*i*-Pr	100 (97*R*)[d]	100 (99*R*)	NR	NR	NR
1k	OH/Allyl	100 (27*R*)[d]	100 (97*R*)	NR	NR	NR
1l	COOEt/H	15 (93*S*)	100 (99*S*)	NR	NR	27 (99*S*)
1m	CH_2OH/H	100 (98*S*)	ND[f]	ND	ND	ND
1n	Cl/H	100 (95*S*)	100 (97*S*)	100 (97*S*)	100 (97*S*)	100 (97*S*)
1o	Br/H	100 (97*S*)	100 (97*S*)	100 (97*S*)	100 (97*S*)	100 (97*S*)
1p	I/H	100 (97*S*)	100 (97*S*)	100 (97*S*)	100 (97*S*)	100 (97*S*)

[a] Conversion based on GC analysis. [b] Values for enantiomeric excess from chiral-phase GC analysis. [c] Isolated yield. [d] Absolute configuration assigned by analogy with lactone **2h**. [e] NR = no reaction under growing conditions. [f] ND = not determined.

4-hydroxycyclohexanone to the corresponding lactone but with only marginal enantiopreference (9% (*R*)). This result stands in contrast to the reaction of other 4-substituted cyclohexanone derivatives, such as 4-methyl- or 4-chlorocyclohexanone, which are known to provide the corresponding (*S*) lactones with almost perfect enantioselectivity (>96% ee). To understand why such a broadly selective enzyme fails to be selective toward a certain closely related compound may be as revealing as to understand why it is selective in a multitude of other cases. Since the characteristics of the active site and the 3-D structure of CHMO were unknown, the directed evolution approach was the most promising route to a better insight into CHMO's catalytic behavior and to a discovery of more selective bioreagents for the Baeyer−Villiger oxidation of 4-hydroxycyclohexanone. Therefore, in an earlier directed evolution study, we subjected CHMO to random mutagenesis using error-prone polymerase chain reaction (PCR) at various mutation rates.[10] Subsequently, the resulting library of mutant genes generated in the process was inserted into the *E. coli* host and screened for enhanced enantioselectivity. The initial 10000-membered library contained several mutants showing enhanced (*R*) selectivity as well as a few mutants displaying reversed enantioselectivity, that is, (*S*) selectivity. The best mutant gene of CHMO, encoding an improved (*R*)-selective mutant, was subjected to a second cycle of mutagenesis, expression, and screening, leading to the identification of the most highly (*R*)-selective variant obtained so far (90% ee). The most (*S*)-selective mutant (79% ee), carrying a single amino acid change (Phe432Ser), was found to be particularly robust and was chosen for further study. It was gratifying to discover that it oxidizes 4-methoxycyclohexanone with selectivity superior to that of the wild-type CHMO (78% (*S*) to 98.6% (*S*))[10] as well as a number of bicyclic ketones, cyclobutanones, and trisubstituted cyclohexanones.[11] In another study, the same mutant performed a highly enantioselective oxidation of methyl-*p*-methylbenzyl thioether to (*R*) sulfoxide (98.7%).[12]

SCHEME 1



These results demonstrate that enhanced selectivity for one substrate was not at the expense of the enzyme's robustness and broad substrate tolerance and suggest that the single amino acid exchange was likely to be in, or in the vicinity of, the active site. This was further supported by the fact that several mutants with enhanced selectivities identified in the screening carried the mutation at the 432 position, for example, Phe to Ile. In the present paper, we describe an evaluation of the best mutants as catalysts in Baeyer−Villiger oxidations for a series of 4-substituted cyclohexanones and strategies directed at gaining an understanding of the enantioselectivity of the oxidation process itself.

Results

The first generation CHMO mutant enzymes[10] that showed the highest enantioselectivities, combined with good activity, in the oxidations of 4-hydroxycyclohexanone (Table 1) were chosen for further evaluation. The test substrates were selected in view of the usefulness of their lactonic products and their potential as probes in the study of the CHMO's active site (Scheme 1). Thus, the substituents R_1 and R_2 in substrate **1** include a cross section of polar, apolar, and mixed substituents, specifically chosen to explore possible H-bonding interactions that might be involved in the positioning of a substrate in the active site and to monitor changes in the size and nature of the active site's "pockets" upon productive mutations.

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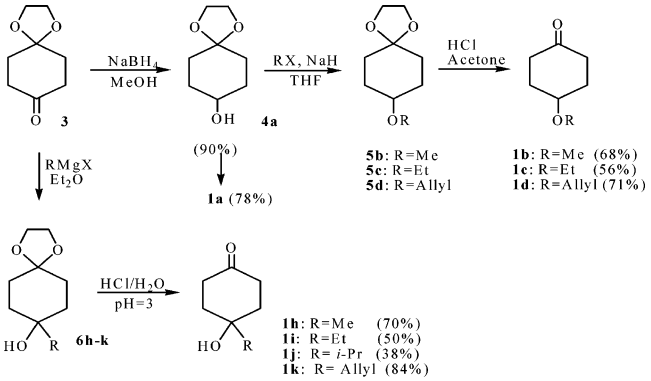
SCHEME 2



Preparation of Substrates 1a−1k. Ketones **1e**−**1g** were purchased from Sigma-Aldrich; **1l** was obtained from TCI America Co. The synthesis and characterization of compounds **1n**−**1p** were reported earlier.[13] Commercially available 1,4-cyclohexanedione monoethylene ketal was the starting material for the preparation of **1a**−**1d**, as shown in Scheme 2. The synthesis of 4,4-disubstituted cyclohexanones **1h**−**1k** was accomplished via the reaction of appropriate alkylmagnesium halides with ketal **3**, followed by acid hydrolysis (Scheme 2).

The construction of the *E. coli* strain overexpressing wild-type cyclohexanone monooxygenase (WT-CHMO) and the CHMO mutants has been described previously.[7,10] All WT-CHMO-catalyzed reactions reported here were carried out with the *E. coli* BL21(DE3)(pMM4) expression system,[7] and those for CHMO mutants were carried out with the JM109(pET-22b)-(CHMO mutants) expression system.[10] The general procedure for all biotransformations is described in the Experimental Section.

Screening of CHMO Mutants. The results of the Baeyer−Villiger oxidations of the 4-substituted ketones by WT-CHMO and CHMO mutants are reported in Table 1. Control fermentations with *E-coli* host strains BL21 and JM109 showed no competing reactions within 24 h. All transformations carried out with growing cells were monitored by GC and were analyzed by chiral phase GC; product lactones were not isolated in the screening experiments unless indicated otherwise. Chemical oxidations with *m*-chloroperbenzoic acid were performed on all substrates prior to biotransformations to establish appropriate conditions for the clean GC resolution of all lactones.

The screening of 4-alkoxycyclohexanones **1b**−**1d** demonstrated that the CHMO mutants, like WT-CHMO, accepted only substrate **1b** with a short, methoxy chain in position 4. Two of the mutants showed increased enantioselectivity compared to WT-CHMO: from 78% ee (*S*) to 99% ee (*S*) for both Phe432Ser and Phe432Ile. In both mutants, there was only a single amino acid exchange in position 432 where phenylalanine was replaced with serine and isoleucine, respectively. A decrease in selectivity was observed for the reaction catalyzed by mutant Leu143Phe, while the double mutant Asp41Asn/Phe505Tyr provided a product with the same selectivity as that of WT-CHMO. The amino acid modifications did not improve the mutants' capacity to accept 4-alkoxycyclohexanones with longer chains, such as **1c** and **1d**; these compounds (Table 1) were clearly not suitable substrates for either WT-CHMO or the mutants. We were gratified to see that carboethoxy-substituted **1l**, which was a poor substrate for WT-CHMO, was rapidly and enantioselectively oxidized by Phe432Ser. High selectivity was also achieved with mutant Asp41Asn/Phe505Tyr, but the conversion was low.

Ketones with relatively small, nonpolar groups in position 4 (**1e**, **1f**, **1n**−**1p)** were oxidized rapidly and with high (*S*) selectivity by WT-CHMO and the mutants alike. The 4-*n*-propyl-cyclohexanone **1g**, which is effectively oxidized by WT-CHMO (92% ee), was converted only by Phe432Ser, with a slight enhancement in enantioselectivity (99% ee). These results suggest the existence of a midsize hydrophobic "pocket", which has not been significantly affected by the mutations. The limited size of the "pocket" is confirmed by the fact that a bulky 4-*tert*-butylcyclohexanone is a poor substrate[14] for either WT-CHMO or any of the mutants (not shown in Table 1).

The 4-disubstituted substrates (**1h**−**1j**) with hydroxy and nonpolar chains were expected to provide further information on the characteristics of the active site. In this series, again, the Phe432Ser mutant turned out to be a winner. It accepted and transformed the four substrates with excellent enantioselectivity. Neither WT-CHMO nor Phe432Ser accepted 4-hydroxy-4-phenylcyclohexanone (not shown). It was particularly gratifying to obtain an essentially enantiopure lactone **1k**, which was oxidized by WT-CHMO efficiently but with low selectivity (Table 1).

Absolute Configuration of Lactones 2m and 2h. The absolute configurations of the monosubstituted lactones shown in Table 1 have been established earlier,[7] with the exception of carboethoxy-substituted compound **2l**. The latter lactone was assigned an (*S*) configuration when, upon reduction with $LiAlH_4$, it gave triol (*S*)-**7**, identical to that obtained for the reduction of a previously reported (*S*)-**2m**[15] (Scheme 3).

The specific rotations for **2h**−**2k** produced in oxidations catalyzed with WT-CHMO and Phe432Ser indicated that the same configuration was obtained in both series of biotransformations (Table 2); since there were no data in the literature on the absolute configuration of any of the disubstituted lactones or their derivatives, the structures had to be determined.

To establish the absolute configuration of **2h**, we carried out an asymmetric synthesis of the acylated lactone (*R*)-**2h** as shown in Scheme 4. Ichihara and co-workers reported the preparation of enantiopure (*R*)-**2h** as an intermediate in the total synthesis of (−)-betaenone C.[16] Unfortunately, no rotation or experimental details were provided. In our synthetic sequence, the commercially available natural product nerol **8** was converted to (*S*,*R*) epoxide **9** (79% ee) via Sharpless expoxidation.[17] The reduction with $LiAlH_4$ in THF at −40 °C generated (*R*)-3,7-dimethyl-oct-6-ene-1,3-diol. Acylation of the primary hydroxyl group, followed by oxidative cleavage of the double bond,[18] gave acid (*R*)-**11**, which spontaneously closed to the five-member γ-lactone (*R*)-**12**. In the course of a biotransformation of **1h**, the resulting seven-member lactone also spontaneously rearranged to the five-member lactone **2h**, which was acetylated to give compound **12**. Direct comparison of the chiral phase

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SCHEME 3

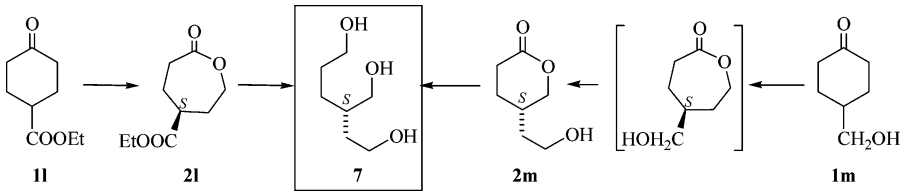

TABLE 2. Enantiomeric Excess Values (% ee) and the Corresponding Specific Rotations of Lactones Obtained in Baeyer−Villger Oxidations Catalyzed by WT-CHMO and Mutant Phe432Ser

		WT-CHMO		Phe432Ser	
lactone	R_1/R_2	% ee	$[\alpha]_D$	% ee	$[\alpha]_D$
2h	OH/Me	96 (*R*)[a]	−5.67 (*c* = 1.53)	87	−4.64 (*c* = 0.73)
2i	OH/Et	94 (*R*)[b]	−3.51 (*c* = 0.73)	97	−3.68 (*c* = 1.26)
2j	OH/*n*-Pr	97 (*R*)[b]	−5.21 (*c* = 1.30)	99	−5.56 (*c* = 1.10)
2k	OH/Allyl	27 (*R*)[b]	−3.07 (*c* = 1.53)	98	−10.0 (*c* = 3.00)

[a] Absolute configuration established experimentally. [b] Absolute configuration assigned by analogy with compound **2h**.

GC traces for compound **12** from the chemical reactions with those from the biotransformation established, unambiguously, the absolute configuration of the biotransformation product as being (*R*).

Discussion

The ability of CHMO to transform a remarkable variety of substrates, frequently with a very high enantioselectivity, has fascinated chemists for years, and several substrate-based active site models have been proposed.[19−23] To rationalize the results of CHMO-catalyzed oxidations of a series of 2-, 3-, and 4-substituted cyclohexanones, Stewart et al.[3,24] proposed a diamond lattice model of the allowed positions for *alkyl* substituents (Figure 1a). Based on the stereoelectronic requirements of the nonenzymatic Baeyer−Villiger reactions[25,26] and the fact that tightly-bound flavin occupies a fixed position in the active site, the overlaying of numerous substrates allows for the definition of the migrating carbon−carbon bond as shown in Figure 1a.

This simple model succinctly predicts the (*S*) configuration obtained in the CHMO-catalyzed oxidations of 4-alkyl-, 4-halogen-, and 4-methoxy-substituted cyclohexanones (Figure 1b). It also works for the 4-alkyl- and 4-hydroxy-substituted ketones **1h**−**1k**. In the latter case, the alkyl substituent can be expected to occupy the equatorial position, leaving the hydroxyl group in an axial position (Figure 1c). The model then correctly predicts the preferential formation of the (*R*) lactone using WT-CHMO. Note that the (*S*) to (*R*) "change" is due to a switch in priority, according to the Cahn−Ingold−Prelog convention.

Interestingly, the model can also be extended to explain the low (*R*) selectivity observed for 4-hydroxycyclohexanone. One assumption in the model is that the reacting conformation of the substrate is assumed *before* the formation of the tetrahedral intermediate. This notion is in agreement with recent mechanistic studies of cyclohexanone monooxygenase, indicating that the substrate's oxidation rapidly follows the formation of flavin peroxide. If the substrate is not available, the flavin peroxide anion slowly equilibrates with its protonated form (hydroperoxide), which itself is unreactive *vis à vis* ketonic substrates.[27]

The NMR study on the effects of the transannular substituents in cyclohexanols showed that electron-attracting groups increase the proportion of molecules with OH in an axial conformation. In the case of 4-hydroxycyclohexanone, the distribution is 56:44 in favor of the axial conformation.[28] Thus, a slightly higher proportion of reacting molecules with OH in the axial position will lead to a slightly higher proportion of the (*R*) lactone; this is in agreement with the observed 9% enantiomeric excess produced in the WT-CHMO-catalyzed oxidation of ketone **1a** to lactone (*R*)-**2a**.

A similar argument may be made for the enantioselectivity observed in the WT-CHMO-catalyzed oxidation of 4-hydroxy-4-methyl ketone **1h**; the conformation with the axial hydroxy and equatorial methyl groups is energetically favored, and according to the model, the (*R*)-**2h** lactone should be a major product; this prediction is confirmed experimentally. It is reasonable to assume that the absolute configurations of the related lactones **2i**−**2k** are (*R*) for the same reason.

In an attempt to quantify differences in the stability of conformations, we calculated the structures and relative stabilities of the axial and equatorial conformations of **1a** and **1h**. Initial geometry optimization and frequency analysis were performed at the Hartree−Fock (HF) 3-21G* level[29] for both conformations to ensure that the starting input geometries gave true ground-state structures. Starting with the "true minima", higher-level calculations using the 6-311+G** basis set[29] were carried out (see Supporting Information). The energy difference (ΔE) between the geometry-optimized structures of **1a**(ax) and **1a**(eq) is 0.50 kcal/mol in favor of the axial conformation. This difference conforms to the NMR results[28] and the observed enantioselectivity of CHMO. The same calculations for **1h**(ax) and **1h**(eq) show much greater stability of the axial (OH) conformation; ΔE = 2.34 kcal/mol. In this case, only the formation of a very strong H-bond could make the conformation with OH in an equatorial position more favorable.

In most cases, with the exception of 4-hydroxycyclohexanone (**1a**), WT-CHMO and mutant Phe432Ser lead to the same absolute configuration, usually with the degree of enantiose-

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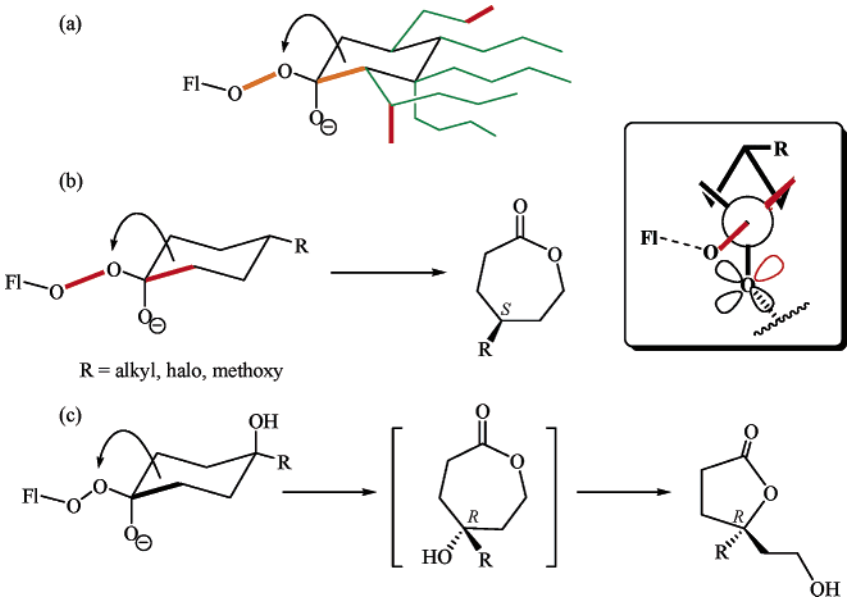
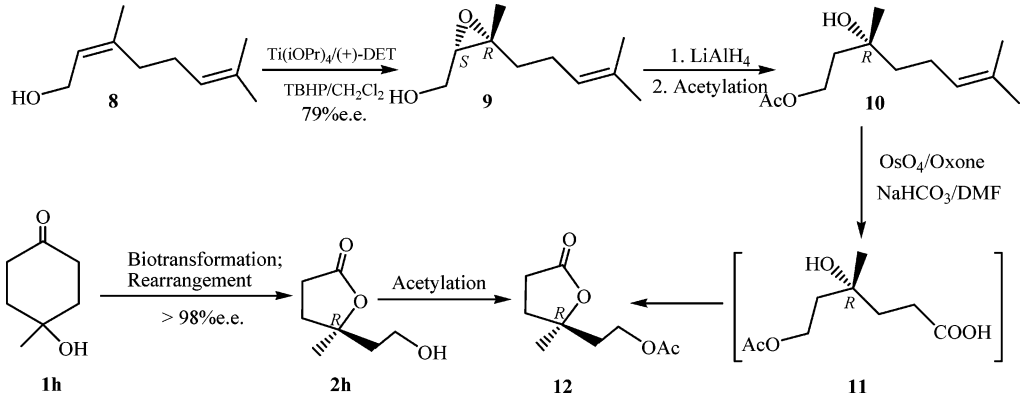


FIGURE 1. (a) A diamond lattice model of the allowed (green) and forbidden (red) positions for alkyl substituents.[3,24] (b) The model-allowed lower energy conformation for 4-alkyl-substituted cyclohexanone places the substituent in an equatorial position. Under stereoelectronic requirements of a Criegee intermediate, only the bond (shown in red) antiperiplanar to the O−O bond migrates (see insert). This imposes the (*S*) configuration on the lactonic product. In the case of 4-hydroxycyclohexanone **1a**, a higher proportion of molecules have an axial configuration. Such a population, according to the model, will lead to the (*R*) lactone **2a**. (c) The model-allowed lower energy conformation of the 4-alkyl-4-hydroxycyclohexanones (**2h**−**k**) places the alkyl substituents in an equatorial position, resulting in the (*R*) lactone.

SCHEME 4



lectivity being similar (Table 1). Notable improvements were observed in the reactions of **1b** (increase from 78% to 99% ee) and **1k** (increase from 27% to 97% ee). A remarkable change in enantioselectivity toward **1a**, shown by mutant Phe432Ser, with a single amino acid mutation suggested that this residue is located within the active site. A recently reported X-ray crystal structure of a closely related phenylacetone monooxygenase (PAMO)[30] and homology modeling[31] confirm that the 432 residue is in the proximity of the CHMO's active site. If one assumes that a H-bond between Ser432 of the mutant and the 4-hydroxy group of **1a** occurs only when the latter is in the equatorial position (Figure 2), a shift of the axial/equatorial equilibrium in favor of the equatorial conformer will occur. Thus, the stabilizing effect of a H-bond may explain the dramatic enantioselectivity switch from (*R*) to (*S*) configuration in the lactone product.

The disubstituted substrate **1h** was designed to probe the putative H-bond between the 432 serine and the OH group of the substrate. With the strong H-bond, we expected to see a significant, if not complete, formation of (*S*)-**2h**, because stabilization of the OH group in the equatorial position would compensate for the methyl group being forced into an axial conformation. The transformation of **1h** with I-K2-F5 gave (*R*)-**2h** in 87% enantiomeric excess, indicating a relatively small contribution from the H-bond-stabilized equatorial conformation and, consequently, only a weak H-bond (Figure 2). The fact that an increase in the size of the 4-alkyl group from **1h** to **1j** is accompanied by enhanced (*R*) selectivity bolsters the above argument (Table 1).

It is important to point out that in the diamond model the "reacting" conformation of a 4-substituted cyclohexanone determines the absolute configuration of the product lactone; the proposed H-bond stabilization is only *one* of several effects that can influence the conformation and position[32] of the substrate in the active site of the enzyme. In the case of the alkyl-substituted ketones, the "reacting" conformation (equato-

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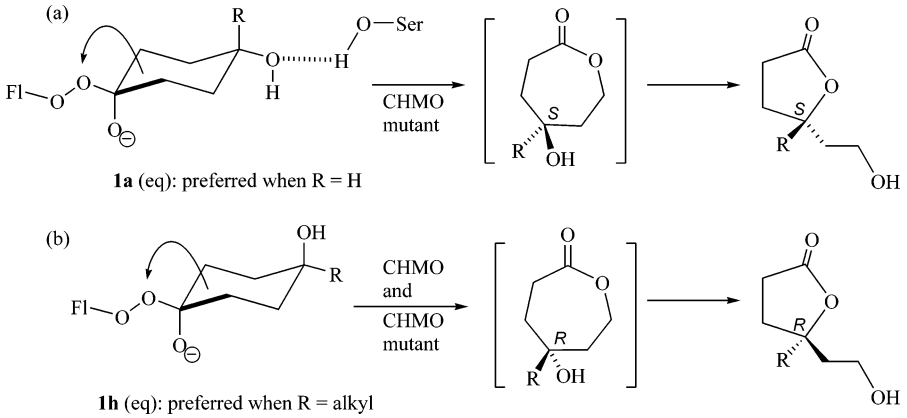

FIGURE 2. (a) A proposed H-bond between Ser432 of the mutant and the 4-hydroxy group of **1a** can occur only when the latter is in the equatorial position. (b) In the more stable conformation, alkyl R occupies the equatorial position. The formation of a strong H-bond might compensate for alkyl R being in the axial conformation. As this is not observed, we may conclude that the postulated H-bond should be relatively weak.

rial) corresponds to their conformational preference. This is not the case for the 4-methoxy- and 4-halo-substituted cyclohexanones. NMR studies indicate that in the latter compounds, as in 4-hydroxycyclohexanone, the axial conformation is slightly preferred.[33] The fact that the "reacting" conformation is equatorial in **1b** and **1n**−**1p** suggests that the equatorial conformer fits better into the active site, probably because of superior stabilizing interactions of the substituents with the residue in position 432 and/or with its neighbors. It is also possible that the axial position cannot accommodate groups larger than the ethyl substituent.[7,34] Thus, the 4-methoxy substituent is preferentially axial[33] when in solution, but within the active site of the CHMO, the equatorial conformer fits better and reacts faster;[35] in the Phe432Ser mutant, the additional stabilization through the H-bond with serine may be, therefore, responsible for the observed enhanced enantioselectivity (Table 1). In the case of the 4-halocyclohexanones, which also have a slight preference for the axial conformation,[33] the interaction with serine in the mutant may be just as stabilizing as the interaction of these substituents with the 432 phenylalanine in the WT-CHMO. Furthermore, the fact that the 432 position is not the only "hot spot" in CHMO, and that mutations at other residues do influence the enantiomeric ratio of lactonic products, suggests that the shift of the substrate position within the active site may also have an important effect on selectivity.[32]

In summary, the diamond model was successfully used to explain the effects of the Phe432Ser mutation on the enantioselectivity of 4-hydroxy- and 4-hydroxy-4-methylcyclohexanones. It is more difficult to interpret the results of the transformations with the remaining substrates and mutants without further information on the structure and dynamics within the active site. A more general model will emerge as the structure and interactions within the active site of CHMO and the related Baeyer−Villiger monooxygenases are unraveled.

Conclusion

The evolution of CHMO, carried out specifically to improve the enantioselectivity of 4-hydroxycyclohexanone oxidation, produced robust mutants with diverging selectivities for the target substrate and enhanced selectivity for several other substrates. Of the mutants selected for further investigation, Phe432Ser, with a single amino acid exchange, turned out to be the most versatile and most selective. The directed evolution of the enzyme with an unknown active site was successful in identifying one residue crucial for enantioselectivity control, placing it within the active site region. The diamond model may be used to predict selectivity changes in many of the substrates studied here, but more work is necessary to understand enantioselection at a molecular level. From a practical point of view, two biotransformations merit particular attention: 4-carboethoxycyclohexanone **1l** was completely converted to lactone **2l** with >99% ee, and 4-allyl-4-hydroxycyclohexanone **1k** gave the corresponding lactone **2k** with complete conversion and 97% ee.

Experimental Section

General Procedure for Grignard Reactions. Magnesium metal (2.6 g, 110 mmol) and 50 mL of anhydrous ether were placed in a 250 mL flask equipped with a dropping funnel and a reflux condenser. Alkyl halide (80 mmol) dissolved in 40 mL of anhydrous ether was added dropwise at a rate sufficient to maintain a gentle reflux; this was continued for an additional 1.5 h at room temperature. Ketal **3** (15 mmol) dissolved in 40 mL of anhydrous ether was added dropwise, and then stirring was continued for an additional 3.5 h at room temperature. The reaction was quenched with 70 mL of water. The organic layer was separated, and the aqueous layer was extracted with ether (3 × 150 mL). The combined organic layers were dried with anhydrous $MgSO_4$, and the solvent was removed by rotatory evaporation. The crude product was dissolved in 100 mL of aqueous HCl (pH ≈ 3) and stirred at room temperature for 24 h. After extraction with EtOAc (3 × 150 mL), the combined organic layers were dried with anhydrous $MgSO_4$, and the solvent was removed by rotatory evaporation. The crude product was purified by chromatography on silica gel using petroleum ether/ethyl acetate (4:1).

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(34) For example, 1,4-dioxaspiro[4.5]decan-8-one is not a substrate for CHMO (Iwaki, H.; Hasegawa, Y.; Wang, S.; Kayser, M. M.; Lau, P. C. K. *Appl. Environ. Microbiol.* **2002**, *68*, 5671−5684), and neither is spiro[5.5]-undecan-3-one (ref 7). Halogen-substituted substrates might not fit into the active sites in their axial conformation because of the long carbon−halogen bonds (C−Cl (1.77 Å), C−Br (1.94 Å) vs C−C (1.54 Å)).

(35) NMR results (ref 32) estimate that for 4-methoxycyclohexanone the axial conformation is favored by approximately 0.5 kcal/mol. In the calorimetric studies, the hydrogen-bonded interaction OH···O is given as 4.8 kcal/mol (Letcher, T. L.; Bricknell, B. C. *J. Chem. Eng. Data* **1996**, *41*, 166−169). In view of that, we may expect that even a relatively weak (long) H-bond will determine the conformation of the reacting 4-methoxycyclohexanone.

4-Hydroxy-4-methylcyclohexanone (1h). Magnesium metal (2.6 g, 110 mmol), methyl iodide (5.7 g, 2.5 mL, 40 mmol), and ketal **3** (2.4 g, 15 mmol) were reacted according to the general procedure to give a colorless oil (1.42 g, 75% yield). IR (in $CHCl_3$) ν_{max}: 3405 (m), 2962 (m), 2927 (m), 2856 (m), 1703 (vs), 1416 (m), 1375 (m), 1251 (m), 1133 (s), 909 (m) cm^{-1}. ^1H NMR δ: 2.76 (2H, ddd, $J_1 = 14.1$, $J_2 = 7.6$, $J_3 = 2.1$ Hz), 2.26 (2H, m), 1.97 (2H, m), 1.87 (2H, m), 1.38 (3H, s). ^{13}C NMR δ: 212.3, 68.4, 38.7, 37.1, 29.7.

Biotransformations with *E. coli*/CHMO and *E. coli*/CHMO Mutants. The *E. coli* strain BL21(DE3)(pMM4) (or JM109(DE3)(pET-22b)) was streaked from frozen stock on LB-Ampicillin plates and incubated at 37 °C until the colonies were 1−2 mm in size. One colony was used to inoculate 10 mL of LB-Ampicillin medium in a 50 mL Erlenmeyer flask, and then the flask was shaken (250 rpm) at 37 °C overnight. This culture was used at a 1:100 (v/v) ratio to inoculate LB-Ampicillin medium supplemented with 2% glucose in a baffled Erlenmeyer flask. The culture was incubated at 37 °C and shaken at 250 rpm until OD_{600} was approximately 0.3−0.4. A stock solution of isopropyl-β-D-thiogalactopyranoside (IPTG) (200 mg/mL) was added (0.1 μL per mL of medium), and then the flask was shaken for another 30 min at 24 °C. The substrate was then added; if cyclodextrin was necessary to alleviate solubility or toxicity problems, it was added at this stage. The culture was shaken (250 rpm) at 24 °C and monitored by GC analysis until the reaction was complete. The culture was then saturated with NaCl and extracted with ethyl acetate or dichloromethane. The combined extracts were washed once with brine and dried with anhydrous $MgSO_4$. The solvent was removed on a rotary evaporator, and the residue was purified by flash chromatography on silica gel.

5-Methoxypan-2-one (2b). *E. coli* JM109(DE3)(pET-22b)-mediated oxidation of ketone **1b** (100 mg, 0.78 mmol) was performed in the presence of β-cyclodextrin (0.5 g) according to the general procedure. Chromotography on silica gel using petroleum ether/acetone (4:1) as eluent gave **2b** as a colorless oil (59 mg, 53% yield); 99% ee by chiral phase GC, $[\alpha]_D = +17.140$ (*c* 0.9, in CH_2Cl_2). IR (neat) ν_{max}: 2938 (s), 2854 (m), 1743 (vs), 1435 (s), 1318 (s), 1135 (m), 1078 (m) cm^{-1}. ^1H NMR δ: 4.42 (1H, ddd, $J_1 = 13.0$, $J_2 = 10.4$, $J_3 = 2.5$ Hz), 3.98 (1H, ddd, $J_1 = 13.4$, $J_2 = 6.7$, $J_3 = 2.5$ Hz), 3.50 (1H, h, $J = 3$ Hz), 3.28 (3H, s), 2.89 (1H, m), 2.35 (2H, m), 1.98 (3H, m), 1.79 (1H, m). ^{13}C NMR δ: 175.9, 75.3, 63.1, 55.7, 33.2, 27.5, 26.6.

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Supporting Information Available: Theoretical calculation of energy preferences in 4-mono- and 4,4-disubstituted cyclohexanone species. Full experimental procedures and characterization of the products shown in Schemes 2−4, and GC traces for compounds **2h**−**2m**. Selected NMR plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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