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Recombinant Baker's Yeast as a Whole-Cell Catalyst for Asymmetric Baeyer−Villiger Oxidations

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Abstract: Cyclohexanone monooxygenase (E.C. 1.14.13.22) from *Acinetobacter* sp. NCIB 9871 has been expressed in baker's yeast (*Saccharomyces cerevisiae*) to create a general reagent for asymmetric Baeyer−Villiger oxidations. This "designer yeast" approach combines the advantages of using purified enzymes (single catalytic species, no overmetabolism, etc.) with the benefits of whole-cell reactions (experimentally simple, no cofactor regeneration necessary, etc.). The yeast reagent was used to systematically examine a series of 2-, 3-, and 4-substituted cyclohexanones (R = Me, Et, *n*-Pr, *i*-Pr, allyl, *n*-Bu), almost all of which were oxidized to the corresponding ϵ-caprolactones in good yields and high enantioselectivities (typically ≥ 95%). Mesomeric 4-substituted cyclohexanones were oxidized to ϵ-caprolactones in ≥ 92% ee. The engineered yeast strain also effected kinetic resolutions of 2-substituted cyclohexanones with enantioselectivity values ≥ 200 for substituents larger than methyl. The behavior of 3-substituted cyclohexanones depended upon the size of the substituent. The engineered yeast strain cleanly converted the antipodes of 3-methyl- and 3-ethylcyclohexanone to divergent regioisomers. On the other hand, for cyclohexanones with larger substituents (*n*-Pr, allyl, *n*-Bu), both antipodes were oxidized by the enzyme to a single regioisomer. In these cases, the observed enantioselectivities were due to a combination of a modest preference for one enantiomer by the enzyme and an unfavorable conformational preequilibrium required prior to binding of the less-favored antipode, a phenomenon we refer to as substrate-assisted enantioselectivity.

Introduction

Interest in integrating traditional organic chemistry and biocatalysis has increased dramatically over the past decade.[1−5] The use of isolated enzymes can be very successful when the catalyst is inexpensive, easily purified, and does not require cofactors, which is the case for many hydrolytic enzymes[6,7] and aldolases.[8−11] Because only a single catalyst is present, problems with competing reactions are avoided, and reaction conditions can be easily varied. On the other hand, whole microbial cells that produce the enzyme(s) of interest are more appropriate for enzymes that require cofactors, in particular those

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that catalyze redox reactions.[12−14] Because the growing cells provide both the enzyme and the cofactors, this approach is experimentally very simple. Unfortunately, competing enzymes and overmetabolism of the desired product can lessen the attractiveness of this strategy.[15]

In preliminary communications we have described a new and potentially general solution to these problems using common baker's yeast (*Saccharomyces cerevisiae*).[16] Standard cloning techniques are used to express a synthetically useful enzyme in yeast, which allows whole recombinant yeast cells to be used as the biocatalytic reagent. The first example of these "designer yeasts" expresses cyclohexanone monooxygenase and performs a variety of enantioselective Baeyer−Villiger oxidations.[16] In this paper, we provide full details on the use of this recombinant yeast strain for the oxidations of a complete series of 2-, 3-, and 4-substituted cyclohexanones. The corresponding lactone products are important chiral building blocks and our "designer yeast" methodology makes them accessible to all chemists, not just specialists. In addition, our results have highlighted the importance of conformational equilibria in enantioselective reactions of flexible substrates.

We chose cyclohexanone monooxygenase (E.C. 1.14.13.22) to demonstrate the potential of our "designer yeast" strategy because of its broad substrate specificity, high enantioselectivity, and underexploited potential as a synthetic reagent. Since its purification and initial characterization by Trudgill,[17] this enzyme has been shown to catalyze the asymmetric Baeyer−Villiger oxidations of a wide variety of ketones.[18] Despite these successful applications and the importance of the Baeyer−Villiger reaction,[19] cyclohexanone monooxygenase has not yet become a widely accepted part of the synthetic organic repertoire. This is partly due to the requirement for NADPH as a cofactor. While advances in NADPH regeneration technology have been reported (for example, see ref 20), virtually all of these regeneration systems require a second enzyme that increases the complexity of preparative biotransformations. Another factor is the relatively small amounts of enzyme available, either from cyclohexanone-grown *Acinetobacter* cells or from an engineered *Escherichia coli* strain.[21] Finally, cell growth and enzyme isolation require biochemical expertise and specialized equipment not found in the typical organic laboratory.

Results

Construction of the Recombinant Yeast Strain and Optimization of the Biotransformation Conditions. The *Acinetobacter sp.* NCIB 9871 cyclohexanone monooxygenase gene was amplified from genomic DNA using primers designed from the published sequence.[21] Following amplification, the structural gene was inserted into three different yeast expression vectors that differed in their plasmid copy number and type of promoter (Figure 1).[22] Each plasmid was used to transform *S. cerevisiae* strain 15C.[23] This strain was chosen because of its deficiency in a major cellular protease,[24] although later work showed that this precaution was unnecessary, and all *S. cerevisiae* strains tested to date have given satisfactory results. Preliminary experiments in which the recombinant yeast cells were used for the Baeyer−Villiger oxidation of cyclohexanone revealed that the regulated *GAL* promoter and the 2 micron circle origin of replication found in plasmid pKR001 provided the best balance between high enzyme expression level and rapid cell growth. These properties are critical because the cells must be capable of healthy growth during the biotransformation process.

We next sought to optimize the biotransformation conditions using our recombinant yeast strain and cyclohexanone as a model ketone. Because of its well-known ability to reduce carbonyl compounds,[25,26] our first goal was to favor the Baeyer−Villiger oxidation of cyclohexanone rather than its reduction to cyclohexanol by yeast enzymes, some of which have very broad substrate specificities.[27−29] Preliminary experiments demonstrated that the level of recombinant yeast cells initially present in the biotransformation mixture had a dramatic impact on the extent of cyclohexanone reduction. When the initial concentration of recombinant yeast cells was varied between 0.5 and 20 mg/mL, the final ratio of ϵ-caprolactone:cyclohexanol ranged from 88:12 to 50:50. By following the time courses for these reactions, it was clear that ketone reduction predominated at high cell densities. Thus, the key to success lay in oxidizing the ketone to completion *before* the cell density reached a high level. Using optimized conditions, cyclopentanone and cyclohexanone were oxidized by our engineered yeast to the corresponding lactones in 67 and 79% yields, respectively, and ketone reduction was limited to ca. 2.5% (Scheme 1).

During our studies with 4-alkyl-substituted cyclohexanones, we noticed a strong correlation between increasing substrate hydrophobicity and difficulties in the yeast-mediated biotransformations. Specifically, the yeast cultures failed to grow when the calculated log *P* value (partitioning of substrate between 1-octanol and water) was greater than approximately 2.0. Similar trends have been observed for biotransformations with other organisms.[30−32] To overcome these problems, stoichio-

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(21) Chen, Y.-C. J.; Peoples, O. P.; Walsh, C. T. *J. Bacteriol.* **1988**, *170*, 781−789.
(22) Plasmids used: M949 (David Stillman, University of Utah), *ARS−CEN* origin of replication, *GAL* promoter; pYES2 (Invitrogen, Inc.), 2 micron circle origin of replication, *GAL* promoter; pG-3 (Kieth Yamamoto, University of California, San Francisco), 2 micron circle origin of replication, *GPD* promoter.
(23) *S. cerevisiae* 15C: MATα, *leu*2, *ura*3-52, Δ*trp*1, *his*4-80, *pep*4-3.
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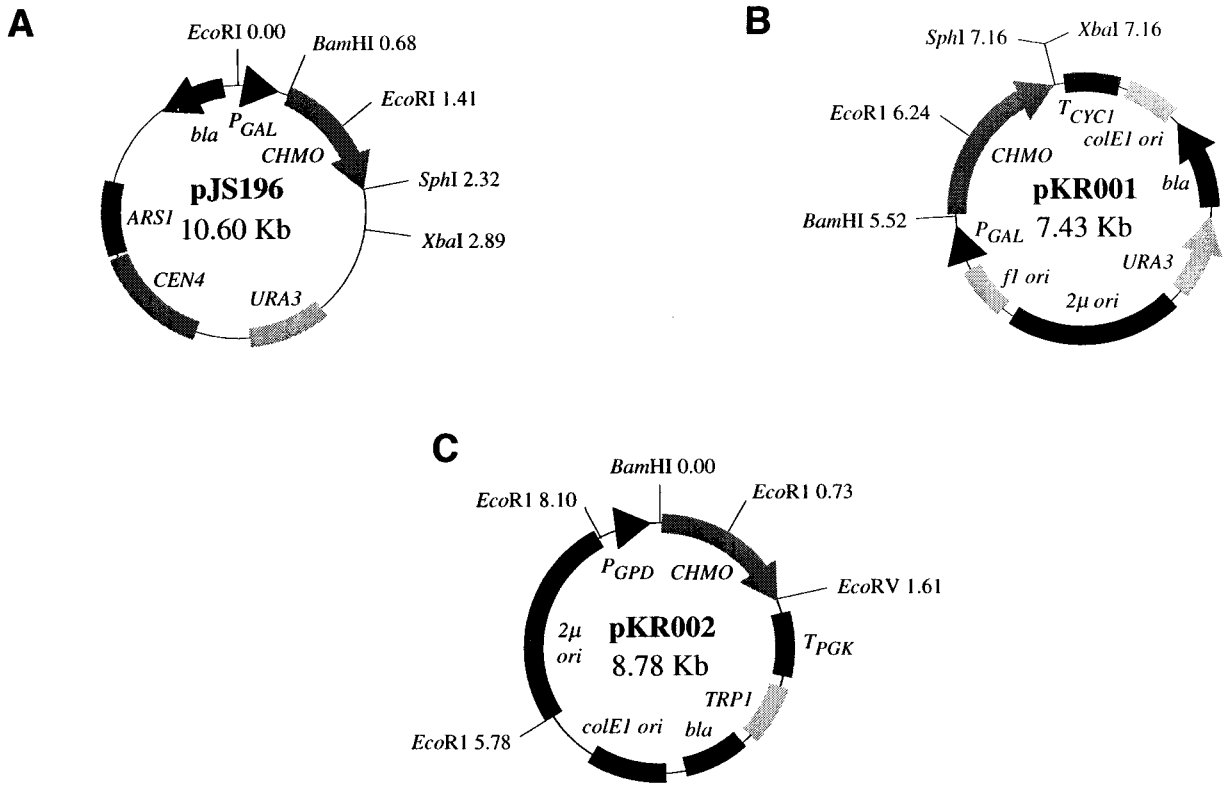
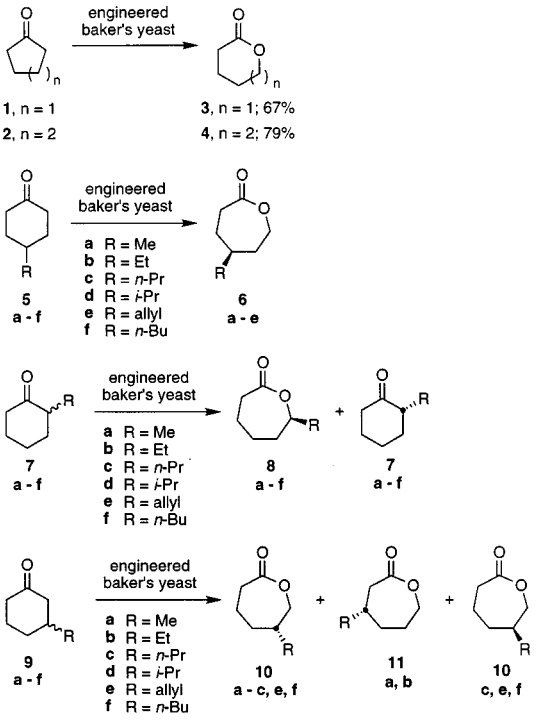


Figure 1. Structures of plasmids used in this study. A. Plasmid pJS196 contains the structural gene encoding cyclohexanone monooxygenase (*CHMO*) under control of the yeast *GAL1* promoter. The plasmid is maintained at a low copy number, and selection is accomplished by growing the yeast cells in the absence of uracil. B. Plasmid pKR001 is similar to pJS196; however, it is maintained at a higher copy number within the yeast cells by virtue of the 2 micron circle origin of replication. C. In plasmid pKR002, the *CHMO* gene encoding cyclohexanone monooxygenase is constitutively expressed from the yeast glyceraldehyde 3-phosphate dehydrogenase promoter (P_{GPD}). In addition, the plasmid is maintained at a relatively high copy number by virtue of the 2 micron circle origin of replication.

Scheme 1



metric amounts of β-cyclodextrin (relative to the ketone) were included in the biotransformation mixtures.[31,33−36] In this way, we were able to achieve substrate concentrations of 10 mM for preparative biotransformations, and this concentration was adopted as our standard throughout these studies.

Oxidations of Prochiral 4-Substituted Cyclohexanones. To demonstrate that the results from our engineered yeast strain were comparable to those obtained from purified cyclohexanone monooxygenase, we investigated a series of 4-alkyl-substituted cyclohexanones (**5a**−**f**; Scheme 1). With the exception of **5e**, which was synthesized in several steps from 1,4-cyclohexanediol,[37] the ketones were commercially available. In all cases except **5f**, the ketones were oxidized to the corresponding lactones in good yields (Table 1). Even extended reaction times failed to afford any lactone from **5f**; however, this was not unexpected since Taschner has reported that **5f** is a relatively poor substrate for the isolated enzyme.[38] The lactone structures were confirmed by comparing spectral data to racemic standards prepared by *m*CPBA oxidations.[39] In the cases of **5c**−**f**, 1 equiv of β-cyclodextrin was required in the growth medium to allow the yeast cells to grow in the presence of the hydrophobic ketones. We saw no trace of olefin oxidation in the biotransformation of **5e**, which is consistent with the oxidizing strength

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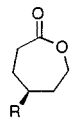
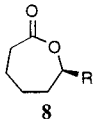
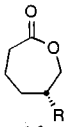
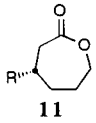
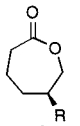
Table 1. Lactones Produced by Yeast-Mediated Ketone Oxidations

		a, R = Me	**b**, R = Et	**c**, R = *n*-Pr	**d**, R = *i*-Pr	**e**, R = allyl	**f**, R = *n*-butyl
 **6**	Yield[a]	83%	74%	63%	60%	62%	N.R.[b]
	% ee[c]	≥ 98%	≥ 98%	92%	≥ 98%	95%	---
	$[\alpha]_D$[d]	-45°, *c* 1.4	-29°, *c* 3.2	-22°, *c* 0.88	-27°, *c* 2.0	-34°, *c* 4.1	---
	Ref.	41	38	38	38	---	---
 **8**	Yield	50%	79%	54%	41%	59%	59%
	% ee	49%	95%	97%	≥ 98%	≥ 98%	≥ 98%
	$[\alpha]_D$	-6.5°, *c* 1.8	-37°, *c* 1.8	-32°, *c* 1.4	-15°, *c* 2.0	-22°, *c* 2.2	-19°, *c* 1.1
 **7**	Yield	---	69%	66%	46%	58%	64%
	% ee	---	≥ 98%	92%	96%	≥ 98%	98%
	$[\alpha]_D$	---	-24°, *c* 3.0	-15°, *c* 1.6	-49°, *c* 2.0	-10°, *c* 1.6	-29°, *c* 1.2
	E	10[e]	≥ 200	≥ 200	≥ 200	≥ 200	≥ 200
	Ref.	49	50	51	52	50	53
 **10**	Yield	71%	18%	11%	N.R.	15%	37%
	% ee	≥ 98	70%	≥ 98%	---	97%	56%
	$[\alpha]_D$	-33°, *c* 3.0	-26°, *c* 2.1	-39°, *c* 1.1	---	-25°, *c* 1.5	-23°, *c* 5.6
 **11**	Yield	60%	20%	---[f]	---	---	---
	% ee	≥ 98%	70%	---	---	---	---
	$[\alpha]_D$	-36°, *c* 2.0	-24°, *c* 2.4	---	---	---	---
 **10**	Yield	---[f]	---	8.0%	N.R.	9.3%	11%
	% ee	---	---	83%	---	≥ 98%	84%
	$[\alpha]_D$	---	---	+36°, *c* 0.8	---	+30°, *c* 0.93	+ 27°, *c* 1.1
	Overall[g] Yield			80%	N.R.	86%	77%
	10 : **11**[h]			96 : 2	N.R.	93 : 7	≥ 98 : ≤ 2
	E_{obs}[i]	2	18	61	---	77	18
	E_{actual}[j]	2	18	51	---	67	8

[a] Yields refer to chromatographically purified samples. [b] No oxidation was detectable, even after prolonged reaction times. [c] Values of enantiomeric excess were determined by chiral-phase GC analysis on a Chirasil-Dex CB column. [d] Optical rotations were measured in $CHCl_3$ solutions at ambient temperature. [e] The preference for the (*S*)-ketone isomer was confirmed by the sign of the optical rotation obtained from the lactone product isolated from a reaction that had proceeded to 60% completion. [f] This isomer was not detected in the product mixture. [g] Yields of lactones isolated from reactions that had proceeded to completion. [h] Regioisomeric composition of reactions that had proceeded to completion. [i] Enantioselectivity calculated directly from plots of ee(substrate) versus fractional conversion. [j] Enantioselectivity corrected for unfavorable conformational equilibria.

of the flavin 4*a*-hydroperoxide.[40] The yields and optical purities of lactones **6a**−**e** produced by our engineered yeast strain were similar to those reported by Taschner, who used partially purified cyclohexanone monooxygenase.[38,41] The absolute configurations of lactones **6a**−**d** were determined by comparing the signs of their optical rotations to literature values. The absolute configuration of lactone **6e** was established by hydrogenation over Pd on carbon to yield (*S*)-**6c** (as shown by chiral-phase GC analysis).

Oxidations of Racemic 2-Substituted Cyclohexanones. Chiral 6-substituted ϵ-caprolactones have been widely used as chiral building blocks in asymmetric synthesis, and several synthetic approaches have been described (see ref 16b and references therein). Because racemic 2-alkyl-substituted cyclohexanones are readily available by ketone alkylation,[42] a Baeyer−Villiger oxidation that afforded a kinetic resolution would constitute an attractive entry into this series of compounds (Scheme 1). Despite the large number of ketones that have been tested as substrates for cyclohexanone monooxygenase, the behavior of simple 2-alkyl-substituted cyclohexanones had not been investigated when we began our studies.[43] The only exception was **7a**, which Schwab and co-workers reported was

(40) Walsh, C. T.; Chen, Y.-C. J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 333−343.

(41) Taschner, M. J.; Black, D. J. *J. Am. Chem. Soc.* **1988**, *110*, 0, 6892−6893.

(42) For a summary of leading references, see: Carey, F. A.; Sundberg, R. J. *Advanced Organic Chemistry. Part B: Reactions and Synthesis*; Plenum: New York, 1983; pp 1−35.

(43) During the course of our work, Alphand et al. published their independent studies of the Baeyer−Villiger oxidations of 2-alkyl-substituted cyclohexanones by whole *Acinetobacter* cells: Alphand, V.; Furstoss, R.; Pedragosa-Moreau, S.; Roberts, S. M.; Willetts, A. J. *J. Chem. Soc., Perkin Trans 1* **1996**, 1867−1872. Our results are in good agreement.

(44) Schwab, J. M.; Li, W.-b.; Thomas, L. P. *J. Am. Chem. Soc.* **1983**, *105*, 5, 4800−4808.

oxidized by cyclohexanone monooxygenase to **8a** with modest selectivity for the (*S*)-ketone.[44]

We suspected that larger alkyl substituents might lead to greater selectivity and therefore utilized our engineered yeast strain to oxidize ketones **7a**−**f** (Scheme 1). Ketone **7a** was commercially available, **7b** was prepared by oxidation of the corresponding alcohol,[45] and the remainder were synthesized from the morpholine enamine of cyclohexanone.[46] The yeast-mediated oxidations were very specific: in all cases, the lactone regioisomer obtained was identical to that produced by oxidizing the ketones with *m*CPBA, and we were unable to detect the alternative regioisomer in the crude product mixtures from our biotransformations by ^{13}C NMR (Table 1). As in the oxidation of **5e**, the olefin of **7e** was stable to the reaction conditions, and we observed only the desired Baeyer−Villiger oxidation.

Because all the starting ketones were racemic, the yeast-mediated oxidations resulted in kinetic resolutions. To accurately determine the enantioselectivity for these resolutions, the optical purities of both the starting ketone and the lactone product were monitored during the course of each reaction by chiral-phase GC. These data was analyzed by an equation derived from the work of Sih that relates the enantiomeric excess of the residual starting material to the fractional conversion.[47] By analyzing the data in this way, there is no dependence on catalyst concentration or rates of mass transport across cell membranes. We performed nonlinear least-squares fits to the theoretical curve in which the *E* value was the only adjustable parameter. From the data in Table 1, it is clear that substituents larger than methyl gave rise to virtually complete selectivity for the (*S*)-ketone enantiomer.[48] For each entry in Table 1, the ketone and lactone were obtained from the same reaction mixture; deviations from 100% ee were mainly due to the difficulty in stopping the reactions at precisely 50% completion. The absolute configurations of the ketones were determined by comparing the signs of the optical rotation data with literature values, which in turn allowed us to deduce the absolute configurations of the lactones.[49−53] To show that it was possible to obtain both enantiomers of the 6-substituted ϵ-caprolactones from a racemic ketone in a preparatively useful fashion, **7b** was oxidized by our yeast to a mixture of (*S*)-**8b** (79% yield, 98% ee) and (*R*)-**7b** (69% yield, 94% ee). After separation, the latter was converted to (*R*)-**8b** by *m*CPBA in 80% yield with no loss of optical purity.

Oxidations of Racemic 3-Substituted Cyclohexanones. In general, the Baeyer−Villiger oxidations of racemic 3-substituted cyclohexanones are not synthetically useful reactions since they afford two enantiomeric pairs of regioisomeric lactones. Unlike the 2-alkyl-substituted cases, there is no electronic preference for migration of one of the two carbon−carbon bonds. To our knowledge, there have been only a few reports of cyclohexanone monooxygenase-catalyzed oxidations of this type of compound, and, in these cases, other substituents were also present.[41,54] However, given the success of cyclohexanone monooxygenase in discriminating between enantiomeric 2-alkyl-substituted cyclohexanones, we investigated an analogous series of 3-alkyl-substituted cyclohexanones **9a**−**f** (Scheme 1). Ketone **9a** was commercially available, while **9b**−**f** were prepared by cuprate addition to 2-cyclohexenone.[55−57]

The outcome of the yeast-mediated oxidations depended upon the size of the substituent. In the case of methyl or ethyl, the two enantiomers of the starting ketone were cleanly oxidized to alternate lactone regioisomers (Table 1). Such divergent behavior of enantiomers has also been observed in the cyclohexanone monooxygenase-mediated oxidations of racemic bicyclo[3.2.0]heptenones[58] and dihydrocarvone.[54b] The structures of the two regioisomeric lactones obtained from the yeast-mediated oxidations (**10a** and **11a**) could be confidently assigned on the basis of their 2-D NMR spectra. While the enantioselectivity of this reaction was only 1.8 when R = CH_3, optically pure (*R*)- and (*S*)-**9a** are readily available by the retro-aldol degradation of (*R*)- and (*S*)-pulegone, respectively.[59,60] Thus, one can easily prepare optically and regioisomerically pure (*R*)-**10a** and (*S*)-**11a** by our "designer yeast" approach. Similar results were obtained for the oxidation of ethyl-substituted **9b**. However, since the enantioselectivity value for this substrate was 18, enriched samples of the two lactone regioisomers (**10b** and **11b**) were obtained by isolating the products after short and long reaction times, respectively. As before, 2-D NMR spectra conclusively established the appropriate structures.

By contrast, both enantiomers of cyclohexanones bearing three- or four-carbon substituents in the 3-position were oxidized to the same lactone regioisomer. By comparing the ^{13}C NMR spectra of the crude products from biotransformations with those obtained by *m*CPBA-mediated oxidations, it was clear that the yeast reagent had produced only a single regioisomer. Interestingly, three-carbon substituents (**9c** and **9e**) afforded better enantioselectivities than 3-*n*-butylcyclohexanone **9f** (Table 1). The lactone structures were deduced from COSY NMR spectra. In all the lactones, the two diastereotopic C-6 protons were well-resolved and both showed clear coupling to only a single proton; this is only consistent with oxygen atom insertion proximal to the substituent.

In cases where the absolute configurations of the ketones were known, it was possible to show that the (*R*)-enantiomers were oxidized more quickly. In the case of methyl-substituted **9a**, where both antipodes of the starting material were available, we directly determined that the enantioselectivity was approximately 2. For **9b**, the residual ketone was isolated from a reaction that had proceeded nearly to completion, and the sign of its optical rotation was compared to literature values to show that it had been enriched in the (*S*)-ketone.[61] Finally, in the case of **9f**, the ketone was isolated after the reaction had

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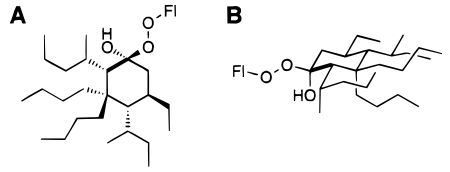

Figure 2. Summary of cyclohexanone monooxygenase substrate specificity. A. Summary of the allowed positions for alkyl substituents on cyclohexanones. The migrating carbon−carbon bond is indicated by a heavy line. B. Diamond lattice representation of the allowed positions for alkyl substituents. Note that the actual conformations of the alkyl chains within the active site of the enzyme are unknown.

proceeded to approximately 50% completion. Comparing the sign of its optical rotation to literature data showed that it had also been enriched in the (*S*)-enantiomer.[62] Unfortunately, because the absolute configurations of (+)-**9c** and (+)-**9e** have not yet been established, optical rotation data could not be used to determine the configuration of the faster-reacting enantiomer. On the other hand, because the chiral-phase GC elution patterns of the lactones were consistent with those observed for **9a**, **9b**, and **9f**, it seems likely that the (*R*)-enantiomers of **9c** and **9e** are also the preferred substrates for cyclohexanone monooxygenase.

Discussion

Since the seminal report by Dumas that baker's yeast catalyzed the reduction of elemental sulfur to hydrogen sulfide,[63] this organism has been widely used for asymmetric reductions of carbonyl compounds. Yeast is nearly ideal for organic synthesis: it is nonpathogenic and simple to grow on inexpensive media and the cells can be stored indefinitely in dried form. The major disadvantage is that its chemical repertoire is essentially limited to carbonyl reductions. However, by expressing foreign enzymes that catalyze desirable chemistry in baker's yeast, we can create novel whole-cell biocatalysts that are as simple to use as ordinary baker's yeast.

The ability to rapidly examine a systematic series of cyclohexanone homologues has allowed us to probe the active site models that have been proposed for cyclohexanone monooxygenase.[54a,64−67] The cubic space model of Ottolina et al.[66,67] requires that cyclohexanones bind in a specific chair conformation so that attack by the flavin 4*a*-hydroperoxide produces a tetrahedral Criegee-like intermediate in which the O(H) substituent is axial.[68] Based on the stereoelectronic precedents of the nonenzymatic Baeyer−Villiger reaction[19,69] and the assumption that the tightly bound flavin must occupy a fixed position within the enzyme active site, we have overlaid our substrates for cyclohexanone monooxygenase on their carbonyl carbons and defined their orientations using the carbon−carbon bonds that are known to migrate (Figure 2A). We have also summarized the allowed positions for substituents on the cyclohexanone ring in a diamond lattice representation (Figure 2B).[70] This simple model, along with calculated log *P* values, has proven useful in our laboratories for predicting the behavior of novel substrates for cyclohexanone monooxygenase.

The most striking finding in these investigations was that certain 3-substituted cyclohexanones were oxidized by cyclohexanone monooxygenase via high-energy conformations. The following discussion uses **9c** as an example, although similar logic applies to **9e** and **9f**. In all of these cases, the microscopic rate constants for the enzyme-catalyzed steps are not known; however, the following analysis is based solely on free-energy differences. In the case of (*R*)-**9c**, the enzyme can accommodate the substrate with its side-chain in an equatorial position (Figure 3).[71] The second-order rate constant, $(V/K)_R$, describes the free energy difference between free enzyme and substrate up to and including the first irreversible transition state, rearrangement of the tetrahedral intermediate (boxed region in Figure 3, top). On the other hand, (*S*)-**9c** must react via a tetrahedral intermediate whose propyl side-chain is axial. If the side-chain were equatorial, the reaction would either be forced to proceed via a chair conformation that is forbidden by the cubic space model[66,67] or by a binding mode that would afford the unobserved lactone regioisomer (Figure 3). The second-order rate constant $(V/K)_S$ therefore represents the free energy difference between free enzyme and (*S*)-$\mathbf{9c}_{ax}$ and the transition state for the irreversible rearrangement of the tetrahedral intermediate (boxed region in Figure 3, bottom). The observed *E* value (E_{obs}) for the oxidation of **9c** is thus composed of two terms: the unfavorable conformational preequilibrium ($\Delta G_{S,eq-ax}$) for the (*S*)-enantiomer as well as the actual enantioselectivity of the enzymatic conversion ($\Delta\Delta G^{\dagger}_{actual} = \Delta G^{\dagger}_{S} - \Delta G^{\dagger}_{R}$).[72] Both of these free energy terms cooperate to favor the (*R*)-enantiomer. The true *E* values were calculated by subtracting the contribution of the conformational preequilibrium (Table 1).[73] Because experimental data for the conformational equilibria of 3-alkyl-substituted cyclohexanones are sparse, the value for 3-methylcyclohexanone (1.4 ± 0.2 kcal/mol) was used as an approximation for **9c**, **9e**, and **9f**.[74] When these corrections are made, it is clear that the enzyme displays only relatively modest enantioselectivity for these substrates, particularly **9f**. However, the combination of modest enantioselectivity and an unfavorable conformational preequilibrium can still allow synthetically useful selectivity.

One assumption in the above analysis is that the energetic penalty for achieving the axial conformation is completely paid *before* formation of the tetrahedral intermediate. Since the starting ketones contain an sp^2 center that diminishes 1,3-diaxial interactions, we calculated the enthalpic differences between the diastereomeric tetrahedral intermediates. All of these values were within experimental error of $\Delta G_{S,eq-ax}$ (1.4 ± 0.2 kcal/mol).[74] The entropic contributions to the free energy differences between the pairs of diastereomeric Criegee intermediates were neglected since one would expect the side-chains of the bound intermediates to occupy single, well-defined conformations within the enzyme active site.

The notion that conformational equilibria can play an important role in enzyme-catalyzed reactions has been recognized in other studies. For example, Knowles and co-workers have proposed that chorismate mutase directly binds the less-

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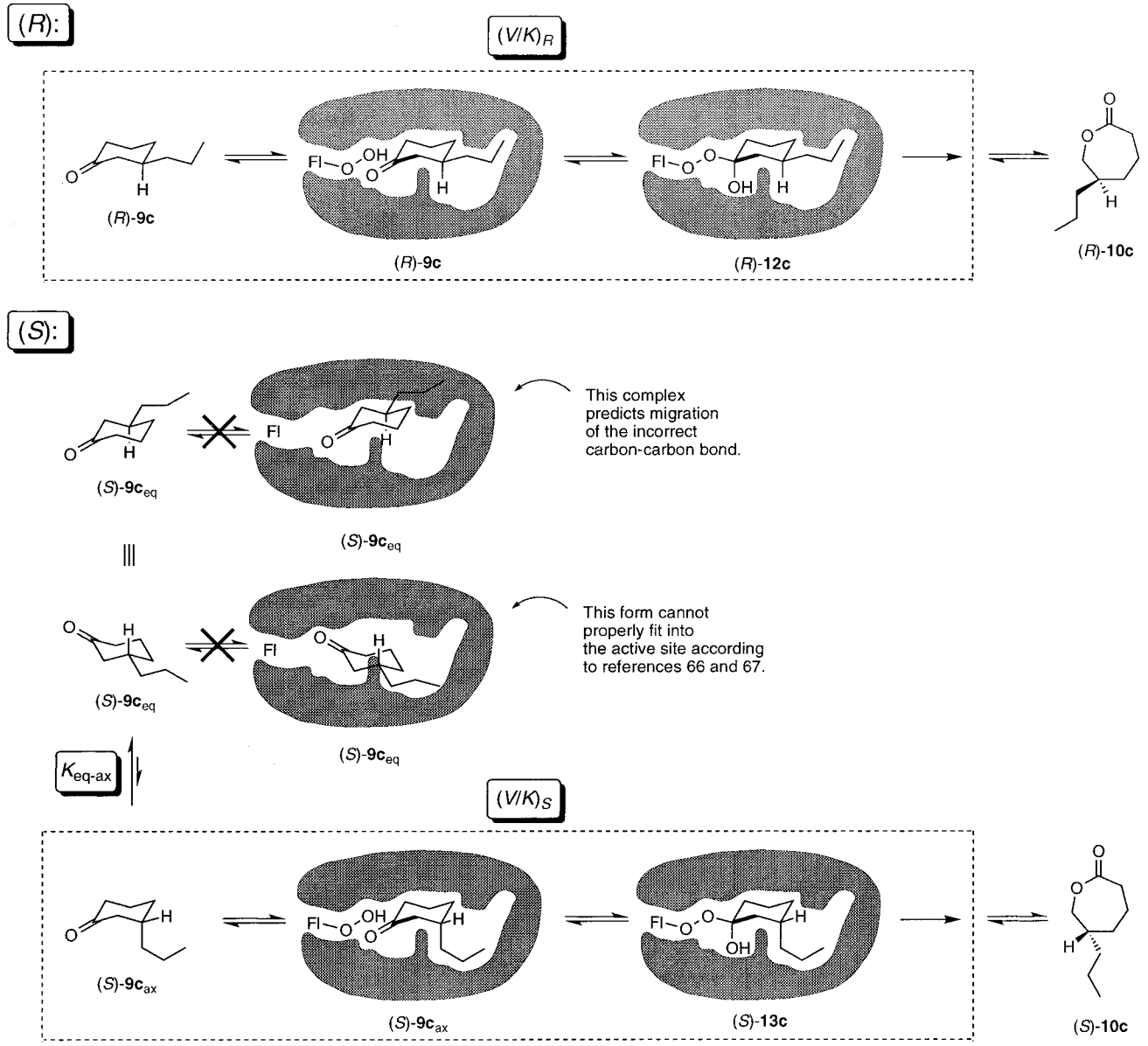

Figure 3. Oxidation of racemic 3-n-propylcylohexanone **9c**. The (*R*)-enantiomer of **9c** can productively bind to the cyclohexanone monooxygenase active site with its side-chain in an equatorial location. Thus, the $(V/K)_R$ term contains only rate constants for enzyme-catalyzed steps (boxed region, top). By contrast, (S)-**9c** must bind to the enzyme with its side-chain in an axial orientation. The $(V/K)_S$ term therefore contains rate constants for both the unfavorable conformational equilibrium as well as the enzyme-catalyzed steps.

stable diaxial substrate conformer from solution.[75] Also, in the case of horse liver alcohol dehydrogenase, Dutler proposed a similar conformational preequilibrium to explain the observed stereoselectivity in the case of 2-alkyl-substituted cyclohexanones.[76−79] We anticipate that other cases of substrate-assisted enantioselectivity will be discovered as more enzymes are applied to conformationally mobile organic substrates. This phenomenon has important implications in attempts to tailor enzyme properties such as diastereo- and enantioselectivity based on crystal structures. In such cases, directed evolution strategies may be more appropriate.[80]

Experimental Section

General Methods. Proton NMR spectra were obtained from $CDCl_3$ solutions on General Electric QE−300, Varian Gemini, or VXR-300 instruments operating at 300 MHz and referenced to residual $CHCl_3$ (7.26 ppm). ^{13}C NMR spectra were taken on a Varian Gemini 300 operating at 75 MHz in $CDCl_3$ and were referenced to solvent (77.0 ppm). IR spectra were recorded from thin films on a Perkin-Elmer 1600 FT-IR spectrophotometer. Optical rotations were measured using chloroform solutions and a Perkin-Elmer 241 polarimeter operating at ambient temperature. High-resolution mass spectra were obtained using either electron impact (70 eV) or chemical ionization with methane as the reagent gas. Packed column gas chromatography was performed on a Hewlett-Packard 5710 instrument equipped with a flame ionization detector and a 0.3 × 300 cm column of 10% OV-17 on Chromosorb WHP with helium as carrier gas. Capillary gas chromatography was performed on Hewlett-Packard 5880A or 5890A instruments equipped with flame ionization detectors and a 0.32 mm × 30 m DB-17 column for nonchiral separations and a Chrompack 0.25 mm × 25 m CP chirasil-Dex CB column for enantiomer separations. Chiral separations used the following conditions: 100 °C (2 min) to 150 °C (5 min) at

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1.0 °C/min, followed by a 10 °C/min gradient to 180 °C (5 min). The injector and detector temperatures were maintained at 250° and 220 °C, respectively. Thin-layer chromatography was performed on precoated silica gel 60 plates. Reaction products were purified by flash chromatography using 60 Å silica gel.[81] Tetrahydrofuran and diethyl ether were distilled from sodium benzophenone ketyl. All other reagents were obtained from commercial suppliers and used as received.

Recombinant DNA procedures were carried out essentially as described by Sambrook et al.[82] Restriction endonucleases were purchased from New England Biolabs or Promega. T4 DNA ligase was obtained from New England Biolabs. Plasmid DNA was purified by density gradient ultracentrifugation with CsCl in the presence of ethidium bromide.[82] Standard media and techniques for routine growth and maintenance of *E. coli*[82] and *S. cerevisiae*[83] strains were used. YPD contained 1% Bacto-Yeast Extract, 2% Bacto-Peptone, and 2% glucose. YEP contained 1% Bacto-Yeast Extract and 2% Bacto-Peptone. Yeast expression vector pYES2 was purchased from Invitrogen, pG-3 was a generous gift of Keith Yamamoto,[84] and M949 was provided by David Stillman. Oligonucleotides were obtained from Integrated DNA Technologies. *S. cerevisiae* were transformed by the lithium acetate method.[85]

Creation of Yeast Expression Plasmids for *Acinetobacter* sp. Cyclohexanone Monooxygenase. The cloning of the *Acinetobacter* sp. cyclohexanone monooxygenase gene (*CHMO*) and the construction of plasmids pJS196 and pKR001 have been described in detail elsewhere.[16a] Plasmid pKR002 was constructed in several steps. The *CHMO* gene was excised from pKR001 by sequential digestion with *Bam*HI and *Sal*I, and then it was purified by agarose gel electrophoresis. The *CHMO* gene was ligated with *Bam*HI, *Sal*I-digested pG-3. After transformation of *E. coli* XL1-Blue, the desired plasmid (designated pKR002) was identified by restriction mapping from a collection derived from 12 randomly picked transformants. The plasmid was purified by CsCl ultracentrifugation, and then it was used to transform *S. cerevisiae* strain 15C.

General Procedure for Biotransformations. *S. cerevisiae* 15C(pKR001) was maintained on SD plates containing 20 mg/L L-tryptophan, 20 mg/L L-histidine and 30 mg/L L-leucine. Fresh plates were streaked weekly from a frozen stock. A single colony was used to inoculate 25 mL of YPD in a sterile 250 mL Erlenmeyer flask, and the culture was shaken at 200 rpm at 30 °C until the OD_{600} value was between 4 and 6. Cells were then harvested by centrifuging at 3000 × g for 10 min. The cell pellet was resuspended in 10 mL of 10 mM Tris-Cl, 1 mM EDTA (pH 7.5) by vortexing. This washing procedure was repeated an additional two times. The final cell pellet was resuspended in 10 mM Tris-Cl, 1 mM EDTA, and 15% glycerol at a concentration of 0.1 g/mL (wet weight). At this stage, cells were either used directly for a reaction or frozen in aliquots at −80 °C for later use. Standard reaction mixtures for preparative biotransformations contained 90 mL YEP, 10 mL 20% galactose, and 10 mM substrate. One equiv of β-cyclodextrin (1.14 g) was added if substrate solubility or toxicity were a problem. Freshly prepared or frozen 15C(pKR001) cells were added to a final concentration of 2 mg/mL (200 μL of the above cell suspension per 10 mL of medium). Reaction flasks were shaken at 200 rpm at 30 °C, and the conversion was monitored by GC. Analytical samples were prepared by mixing 100 μL of the reaction mixture with an equal volume of ethyl acetate. After vortexing for 30 s, the mixture was centrifuged in a microcentrifuge for 2 min, the organic layer was removed, and a 1−2 μL sample was analyzed by GC. After the reaction was complete, the reaction mixture was centrifuged at 3000 × g for 10 min at 4 °C to remove yeast cells. The supernatant was extracted with ethyl acetate (4 × 100 mL). The cell pellet was vortexed with 20 mL of ethyl acetate. The organic extracts were combined, washed with brine (100 mL), dried with $MgSO_4$, and concentrated by rotary evaporation. The lactone was purified by flash chromatography on a 15 cm silica column using 1:1 ether−hexanes.

Spectral Data for New Compounds. (*S*)-5-(2-Propenyl)-2-oxepanone 6e. ^{1}H NMR δ 1.26 (br s, 1 H), 1.30−1.46 (m, 1 H), 1.46−1.58 (m 1 H), 1.62−1.77 (m, 1 H), 2.06 (br t, J = 7 Hz, 3 H), 2.55−2.74 (m, 2 H), 4.17 (dd, J = 10, 13 Hz, 1 H) 4.31 (ddd, J = 2, 6, 13 Hz, 1 H), 5.02 (d, J = 7 Hz, 1 H), 5.07 (s, 1 H), 5.67−5.82 (m, 1 H); ^{13}C NMR δ 28.6, 33.2, 35.1, 40.1, 40.8, 68.1, 117.1, 135.8, 163.1 ppm; IR (neat) 3074, 1734, 1639, 1173 cm^{-1}; HRMS calcd for $C_9H_{14}O_2$ 154.0990, M + H 155.1068, found 155.1072 (Δ = 0.4 mmu).[86]

(*R*)-6-Ethyl-2-oxepanone 10b. ^{1}H NMR δ 0.88 (t, J = 4 Hz, 3H), 1.30−1.50 (m, 4H), 1.52−1.70 (m, 3H), 2.29 (t, J = 4 Hz, 2H), 3.98 (d, J = 3 Hz, 2H); ^{13}C NMR δ 11.5, 21.4, 24.6, 34.4, 34.5, 40.3, 72.5, 173.7 ppm; IR (neat) 2963, 1735, 1460, 1169, 1101, 1012, 917, 755 cm^{-1}; HRMS calcd for $C_8H_{14}O_2$ 142.0990, M + H 143.1068, found 143.1069 (Δ = 0.1 mmu).

(*S*)-4-Ethyl-2-oxepanone 11b. ^{1}H NMR δ 0.89 (t, J = 4 Hz, 3H), 1.22−1.47 (m, 4H), 1.49−1.73 (m, 2H), 1.74−1.77 (m, 1H), 2.18−2.38 (m, 2H), 3.64 (m, 1H), 4.05 (m, 1H); ^{13}C NMR δ 11.3, 27.8, 28.9, 34.6, 35.7, 39.8, 69.3, 173.8 ppm; IR (neat) 2961, 1734, 1458, 1383, 1250, 1166, 754 cm^{-1}; HRMS calcd for $C_8H_{14}O_2$ 142.0990, M + H 143.1068, found 143.1071 (Δ = 0.3 mmu).

6-Propyl-2-oxepanone 10c. ^{1}H NMR δ 0.92 (t, J = 4 Hz, 3H), 1.20−1.48 (m, 5H), 1.61−1.70 (m, 1H), 1.79 (m, 1H), 1.88 (m, 2H), 2.63 (t, J = 3 Hz, 2H), 4.04 (dd, J = 8, 5 Hz, 1H), 4.15 (d, J = 8 Hz, 1H); ^{13}C NMR δ 14.0, 19.9, 21.3, 33.7, 34.3, 34.7, 38.3, 72.7, 176.1 ppm; IR (neat) 2930, 1736, 1459, 1282, 1169, 1046 cm^{-1}; HRMS calcd for $C_9H_{16}O_2$ 156.1146, M + H 157.1224, found 157.1254 (Δ = 3.0 mmu).

6-(2-Propenyl)-2-oxepanone 10e. ^{1}H NMR δ 1.60−1.74 (m, 2H), 1.87−1.95 (m, 3H), 2.02−2.10 (m, 2H), 2.56−2.70 (m, 2H), 4.02 (dd, J = 8, 13 Hz, 1H), 4.19 (d, J = 13 Hz, 1H), 5.05 (m, 1H), 5.10 (br s, 1H), 5.69−5.82 (m, 1H); ^{13}C NMR δ 21.4, 34.3, 34.6, 36.3, 38.4, 72.2, 117.4, 135.3, 180.5 ppm; IR (neat) 3075, 2924, 1736, 1640, 1458, 1167, 913 cm^{-1}; HRMS calcd for $C_9H_{14}O_2$ 154.0990, M + H 155.1068, found 155.1081 (Δ = 1.3 mmu).

6-Butyl-2-oxepanone 10f. ^{1}H NMR δ 0.89 (m, 3H), 1.16−1.36 (m, 4H), 1.36−1.46 (m, 1H), 1.59−1.70 (m, 1H), 1.70−1.78 (m, 1H), 1.82−1.91 (m, 2H), 2.60 (m, 2H), 4.01 (dd, J = 8, 5 Hz, 1H), 4.13 (d, J = 8 Hz, 1H); ^{13}C NMR δ 14.0, 21.4, 22.7, 29.0, 31.3, 34.4, 34.9, 38.6, 72.8, 176.1 ppm; IR (neat) 2928, 1736, 1458, 1351, 1270, 1168, 1049 cm^{-1}; HRMS calcd for $C_{10}H_{18}O_2$ 170.1302, M + H 171.1380, found 171.1406 (Δ = 2.6 mmu).

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Supporting Information Available: Table of log *P* values for substrates and products described in this study and a derivation for the quantitative analysis of substrate-assisted enantioselectivity and computational methods for calculating $\Delta\Delta H^\circ$ values for diastereomeric Criegee intermedaties (6 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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