rotation in a micellar urethane system in aqueous solution that are similar to those found for the protein systems here,[57] and we tend to believe that an appreciable positive $\Delta S^{\ddagger}$ parameter is simply characteristic of the rotation of this functional group within organized structures in water. With these considerations in mind, it is striking what little influence the native protein structure has on the rotation of the diphenylcarbamoyl group of the enzyme. Although the evidence discussed above suggests a rather highly ordered protein structure about the aromatic rings of this group, the rotation kinetics indicate that the group can easily become unencumbered of the enzyme and carry out rotational motion about the carbamyl carbon–nitrogen bond in essentially the same way as the denatured enzyme or the urethane model II. At least two easily accessible conformational states for the acylated enzyme are thereby suggested (eq 5). In one state (E_F), the diphenyl-

$$E_F \overset{\text{unfolding}}{\rightleftharpoons} E_U \overset{\text{rotation}}{\rightleftharpoons} E_U \qquad (5)$$

carbamoyl group takes up the position in the structure suggested by the chemical shift data and is essentially immobilized. In a second state (E_U), the enzyme is locally unfolded so that rotation can take place easily; the chemical shifts and relaxation processes characteristic of this second state are likely different from those of the first. If the free energies of the two states, E_F and E_U, are different by ~4 kJ/mol and equilibrium between them is rapid, the (averaged) NMR parameters observed would largely be those of the first state. This free energy separation of the two states would add to the inherent barrier for rotation, but the increment is of the order of our experimental errors. In the denatured enzyme highly ordered states such as E_F would disappear, with the result that the large protein-induced chemical shift differences for the fluorophenyl rings would be greatly reduced. The diphenylcarbamoyl rotation process represented by the last part of eq 5 would take place at a rate largely defined by the local electronic structure of this group and should be similar for both native and denatured systems if structural constraints on this motion are removed.

The proposed conformation excursion E_F to E_U, or more elaborate schemes involving more conformational substates are compatible with notions of local mobility in protein structures that derive from a variety of experiments.[1,2] Our results allow us to put some rather broad limits on the time scale for these local motions in that the local unfolding must be more rapid than the time required for rotation but must be appreciably slower than the time required for molecular tumbling. Thus, the rate constant for overall unfolding must be between 2×10^2 s^{-1} and 2×10^7 s^{-1}.

Acknowledgment. This work was supported by Grant GM-25975 from the National Institutes of Health. The Southern California Regional NMR Facility was supported by the National Science Foundation (Grant CHE 79-16324A1), and the purchase of the 300-MHz instrument was aided by Grant CHE 80-018438 from the NSF. We tender our appreciation to these sponsors.

Registry No. II, 85710-94-7; *N,N*-bis(4-fluorophenyl)amine, 330-91-6; *p*-fluoroacetanilide, 351-83-7; *p*-bromofluorobenzene, 460-00-4; *N,N*-bis(4-fluorophenyl)carbamoyl chloride, 85710-93-6.

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Cyclohexanone Oxygenase: Stereochemistry, Enantioselectivity, and Regioselectivity of an Enzyme-Catalyzed Baeyer-Villiger Reaction[†1,2]

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Abstract: Cyclohexanone oxygenase, from *Acinetobacter* NCIB 9871, has been incubated with (2*S*,6*S*)-[2,6-$^2H_2$]- and (2*R*)-[2-$^2H_1$]cyclohexanone. The resulting labeled ϵ-caprolactone (2-oxepanone) samples were degraded to 1-pentanol, which was esterified by using (−)-camphanyl chloride. Analysis of the camphanates by deuterium NMR spectroscopy, using Eu(dpm)$_3$, showed that the conversion of ketone to lactone had in each case proceeded with complete retention of configuration at the migrating carbon center. A similar degradation of (2*R*)-[2-$^2H_1$]cyclohexanone itself showed that reduction of [2-$^2H_1$]-cyclohex-2-enone by *Beauveria bassiana* ATCC 7159 is also completely stereoselective. A method has been developed for assessing the enantioselectivity of enzymes toward racemic substrate mixtures. (2*R*)-2-[*methyl*-$^2H_3$]- and (2*S*)-2-[*methyl*-^{13}C]methylcyclohexanone were synthesized and mixed in equal amounts, and the resulting mixture (a virtual racemate) was incubated with cyclohexanone oxygenase. The course of the reaction was followed by both ^{13}C and ^{2}H NMR spectroscopy, showing that the initial rate of oxidation of the 2*S* enantiomer was nearly twice that of the 2*R* enantiomer. (2*R*)- and (2*S*)-2-methylcyclohexanone were both converted by cyclohexanone oxygenase to 6-methyl-ϵ-caprolactone (7-methyl-2-oxepanone). Advantages of the virtual racemate/multinuclear NMR technique over existing methodology are described.

While the Baeyer-Villiger reaction[3] has been a standard tool of organic chemistry for the better part of a century, only in recent years has it become clear that nature makes widespread use of this reaction in biodegradative pathways.[4] Studies in several laboratories[4c-g,5] have revealed that Baeyer-Villiger enzymes are flavin-dependent monooxygenases, not requiring metal ions for

† Dedicated to Dr. Ulrich Weiss on the occasion of his 75th birthday.
‡ The Catholic University of America.
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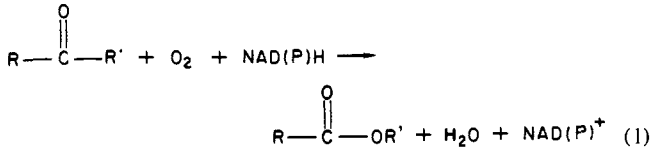
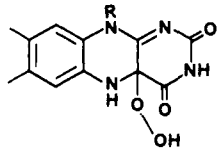
catalytic activity. The generalized stoichiometry is shown in eq 1.



(1)

Flavins are remarkably versatile cofactors,[6] taking part in a broad spectrum of biological redox processes. Despite extensive study by a number of research groups,[6] our understanding of the mechanisms of flavoprotein catalysis remains incomplete. While there is substantial evidence supporting the intermediacy of a flavin C-4a hydroperoxide (FlHOOH) in flavin monooxygenase reac-



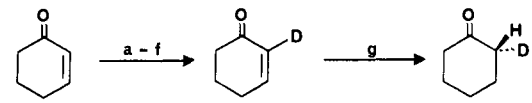
tions,[6b,7] the specific role of this intermediate is not clear at present. In fact, plausible biochemical Baeyer-Villiger mechanisms can be written which portray FlHOOH functioning either as a nucleophilic or as an electrophilic oxygen transfer reagent (vide infra).

A salient feature of the nonbiochemical, peracid-mediated Baeyer-Villiger reaction is that it is accompanied by complete retention of configuration at the site of oxygen insertion.[8] While several biochemical Baeyer-Villiger reactions have long been known[5] which, similarly, exhibit retention of configuration, the substrates for these reactions possess multiple chiral centers, leading to innate stereochemical biases. That is, the possible products are diastereomers; hence, the transition states leading to them are diastereomeric.

In the mid 1970s, a report from Trudgill's group described[4e] the isolation of cyclohexanone oxygenase, an enzyme from *Acinetobacter* which catalyzes the conversion of a wide variety of cyclic ketones to the corresponding lactones. As cyclohexanone oxygenase was readily available and had been purified to homogeneity and rather well characterized by Trudgill and his co-workers, it provided an opportunity for in-depth study as a representative Baeyer-Villiger enzyme. Our interest was especially strong because the use of cyclohexanone, a substrate with no chiral centers, would make it possible to investigate reaction stereochemistry in a system isolated from potential steric perturbations of the type described above.

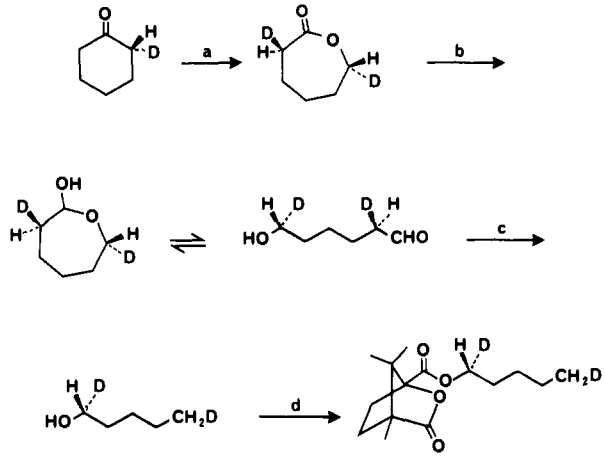
Noting the broad substrate specificity of cyclohexanone oxygenase[4c] as well as our own observation[2a] that the reaction which it catalyzes is the equivalent of a stereospecific remote functionalization reaction, we have also set out to investigate its potential as a general tool for the production of chiral, bifunctional synthons of high enantiomeric purity. (Many enzymes are known which discriminate quite efficiently between enantiomeric substrates.[9]) We now report the results of these experiments and describe a new application of isotopic substitution and multinuclear NMR spectroscopy to the study of enzyme enantioselectivity. As noted below, this methodology offers some substantial benefits over more traditional techniques.

Scheme I[a]



[a] (a) Br_2, CCl_4. (b) Et_3N, CCl_4. (c) $HOCH_2CH_2OH$, *p*-TsOH, benzene, reflux. (d) *n*-BuLi, THF, −76 °C. (e) ^2H_2O. (f) $(COOH)_2$, CH_2Cl_2, H_2O. (g) *B. bassiana* ATCC 7159.

Scheme II[a]



[a] (a) MCPBA, $CHCl_3$. (b) DIBAL, THF, −76 °C. (c) $(Ph_3P)_3RhCl$, benzene, reflux. (d) (−)-Camphanyl chloride, pyridine.

Results

Substrate/Product Stereoselectivity. Solution of the overall reaction stereochemistry required incubation with cyclohexanone oxygenase of samples of cyclohexanone, chirally labeled at C-2, followed by analysis of the chirality at C-6 of the resulting labeled ϵ-caprolactone (2-oxepanone).

(1) Labeled Substrate Synthesis. For a number of reasons,[10] we chose to use deuterium for isotopic labeling, and the synthetic route leading to (2*R*)-[2-$^2H_1$]cyclohexanone was inspired by the report by a group of French workers[11] on the stereoselective reduction of a variety of cyclic and acyclic enones by cultures of *Beauveria bassiana* (formerly[12] *Beauveria sulfurescens*) ATCC 7159. Accordingly, [2-$^2H_1$]cyclohex-2-enone was synthesized by the procedure of Guaciaro et al.[13] (Scheme I) and was incubated overnight with a *Beauveria* culture, affording a sample of deuterium-substituted cyclohexanone.

(2) Substrate Configurations and Enantiomeric Purities. Based on the previous report,[11] it seemed likely that our [2-$^2H_1$]cyclo-

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hexanone was of the *R* configuration. Nevertheless, as the absolute configurations and enantiomeric purities of the ketones produced by the microbial enone reduction had originally[11] been assigned by examination of optical rotation values, we elected to perform our own analysis (Scheme II).

A sample of our [2-$^2H_1$]cyclohexanone was subjected to standard Baeyer-Villiger conditions, giving ϵ-caprolactone which, owing to the symmetry of the parent ketone, bore equal amounts of deuterium at C-2 and C-6. Reduction with diisobutylaluminum hydride gave the lactol, which, as the hydroxyaldehyde tautomer, was decarbonylated[14] to pentanol by use of Wilkinson's catalyst. The pentanol, labeled at C-1 and at C-5, was not isolated; rather, the reaction mixture was filtered to remove solids [including $(Ph_3P)_2CORhCl$], and then pyridine and (−)-camphanic acid chloride were added.[15] Workup and chromatography gave pentyl camphanate, a chloroform solution of which was examined by proton NMR spectroscopy. The diastereotopic pentyl C-1 protons appeared as a well-defined triplet at 4.22 ppm. By addition of $Eu(dpm)_3$ this signal was separated into two distinct but broad multiplets (owing to geminal H–H and H–D coupling) at 5.94 and 6.26 ppm. (The shift reagent addition was monitored not by weight but by the extent of shifting of the signals of interest.) As previously noted, the ester was labeled at both C-1 and C-5 of the pentyl moiety, with ca. half of the total at each position (exchange at the hydroxyaldehyde stage would affect the distribution). Thus, we did expect to see two shifted proton resonances. In fact, our [2-$^2H_1$]cyclohex-2-enone was only 70% d_1 (as shown by integration of the proton NMR spectrum), owing largely to the poor quality of the deuterium oxide used in the quench of the vinyl anion. Accordingly, proton NMR spectroscopy did not clearly indicate whether the deuterium at pentyl C-1 of the pentyl camphanate was in the *pro-R* (upfield) or *pro-S* (downfield) position[15] or if both positions were deuterated. It was clear to us that we could derive far more information from experiments designed to detect the *presence* of deuterium rather than the *absence* of protium. We therefore changed spectrometer probes and acquisition parameters and measured the deuterium NMR spectrum of the identical $Eu(dpm)_3$-shifted sample. Only three signals were observed: at 0.95 ppm ($-CH_2D$), 5.83 ppm (–OCHD–), and 7.26 ppm ($CDCl_3$ at natural abundance in the solvent $CHCl_3$; this signal was used as the internal standard). Thus, the chemical shift of the methylene deuteron corresponded rather well to that of the *pro-R* proton. Nevertheless, we were not convinced at this stage that we could even observe separate *pro-R* and *pro-S* deuterons, owing to the broad lines and limited signal dispersion inherent in deuterium NMR spectra.[16]

As a control, pentyl camphanate stereorandomly deuterated at pentyl C-1 was synthesized from (2*RS*)-[2-$^2H_1$]cyclohexanone (made by reaction of cyclohexene oxide with $LiAl^2H_4$,[17] followed by Jones oxidation), and the $^1H/^2H$ NMR experiment was performed in the identical manner as previously. The shifted methylene proton resonances were at 5.87 and 6.17 ppm, and the corresponding deuterium signals were observed at 5.80 and 6.10 ppm. There was nearly base-line resolution, even with 1.0 Hz of line broadening. We could thus say with confidence that the previously described deuterium NMR spectrum of our chirally labeled pentyl camphanate had shown no evidence for deuterium in the *pro-S* position at C-1. Assuming retention[8] of configuration in the degradative, MCPBA-mediated Baeyer-Villiger reaction (Scheme II), the precursor labeled cyclohexanone was of the 2*R* configuration and was enantiomerically pure.

At the time we were completing these experiments, Prof. Thomas Hellman Morton presented us with a substantial quantity of (2*S*,6*S*)-[2,6-$^2H_2$]cyclohexanone, prepared from [2,2,6,6-$^2H_4$]cyclohexanone by acetoacetate decarboxylase-mediated exchange.[18] An identical degradation and spectral analysis were performed on a small sample of this material, confirming the configuration which Prof. Morton and his co-workers had assigned based on an independent degradation.[18,19]

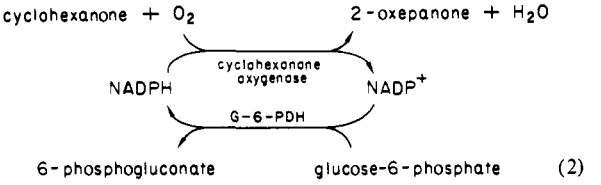
Table I. Data from $Eu(dpm)_3$-Shifted ^2H NMR Spectra of Chirally Labeled Pentyl Camphanate Samples (See the Text and Scheme II)

substrate	chemical shifts, ppm $-CH_2D$	*pro-R* –OCHD–	*pro-S* –OCHD–
(2*R*)-[2-$^2H_1$]cyclohexanone	0.94	5.95	
(2*R*)-[2-$^2H_1$]cyclohexanone[a]	0.94	6.06	6.41
(2*S*,6*S*)-[2,6-$^2H_2$]cyclohexanone	0.93		5.72
(2*S*,6*S*)-[2,6-$^2H_2$]cyclohexanone[a]	0.93	5.48	5.70

[a] After addition of pentyl camphanate stereorandomly substituted with deuterium at pentyl C-1.

(3) Preparative Incubations and Product Analyses. *Acinetobacter* NCIB 9871 was grown[7] on a medium containing cyclohexanol as the sole carbon source, and cyclohexanone oxygenase was isolated and purified through the DEAE-cellulose chromatography stage.[4e] The enzyme thus obtained was not homogeneous (as shown by $NaDodSO_4$–polyacrylamide gel electrophoresis) but proved to be sufficiently pure for our purposes.

Owing to the cost of NADPH, we required preparative incubation conditions which would allow recycling of the product $NADP^+$. After much experimentation to find optimal conditions, it was found that efficient conversion of ketone to lactone could be brought about in the presence of a catalytic amount of NADPH, with cofactor recycling by glucose-6-phosphate dehydrogenase (eq 2).



(2)

As mentioned, our cyclohexanone oxygenase preparations were not homogeneous but contained a lactone hydrolase (vide infra) in addition to other unidentified proteins. Although recoveries from preparative incubations were modest, enough labeled lactone was obtained from each that we were able to carry out degradations as per Scheme II.

Spectral analysis of our labeled camphanates this time was solely by deuterium NMR spectroscopy. The variations between proton and deuteron chemical shifts observed in our earlier work (vide supra) along with our confidence in the clean separability of deuterium NMR signals differing in chemical shift by 0.3 ppm led to this approach. Thus, to each camphanate sample (dissolved in $CHCl_3$) was added sufficient $Eu(dpm)_3$ to shift the methylene signal to ca. 6 ppm. A "good" spectrum was obtained under these conditions, and then a small portion of our stereorandomly C-1 deuterated pentyl camphanate was added, to serve as an internal reference. As can be seen in Table I, incubation of (2*R*)-[2-$^2H_1$]cyclohexanone with cyclohexanone oxygenase gave pentyl camphanate with the *R* configuration at pentyl C-1; hence, the precursor caprolactone was of the *R* configuration at C-6. Pentyl camphanate from (2*S*,6*S*)-[2,6-$^2H_2$]cyclohexanone conversely had deuterium in the *pro-S* position at pentyl C-1 (Table I). We can therefore conclude unambiguously that *the cyclohexanone oxygenase-catalyzed Baeyer-Villiger reaction had proceeded with retention of configuration.* In fact, the spectra provide no evidence of label epimerization; thus, *the reaction is stereospecific.*

Enantioselectivity and Regioselectivity. Cyclohexanone oxygenase has very broad substrate specificity,[4e] catalyzing the

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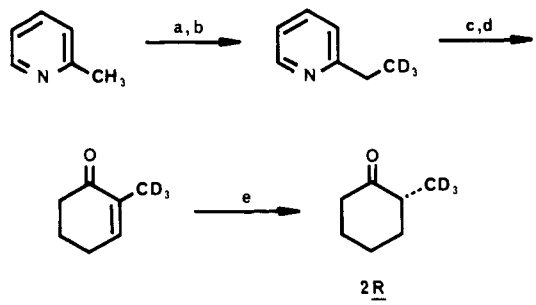
Scheme III[a]



[a] (a) LDA, THF. (b) C^2H_3I. (c) Li, NH_3(liquid), EtOH. (d) OH^-. (e) *B. bassiana* ATCC 7159.

oxidation of such dissimilar compounds as cyclooctanone, camphor, 1,2-cyclohexanedione, and dihydrocarvone, as well as cyclohexanone. In light of the stereospecificity of the lactonization reaction as well as the essentially perfect discrimination shown by many enzymes toward substrate and/or product enantiomers,[9] we saw potential utility in cyclohexanone oxygenase as a general vehicle for the production of chiral, bifunctional molecules of high enantiomeric purity. Such an agent could be of substantial value in synthetic organic chemistry.[20]

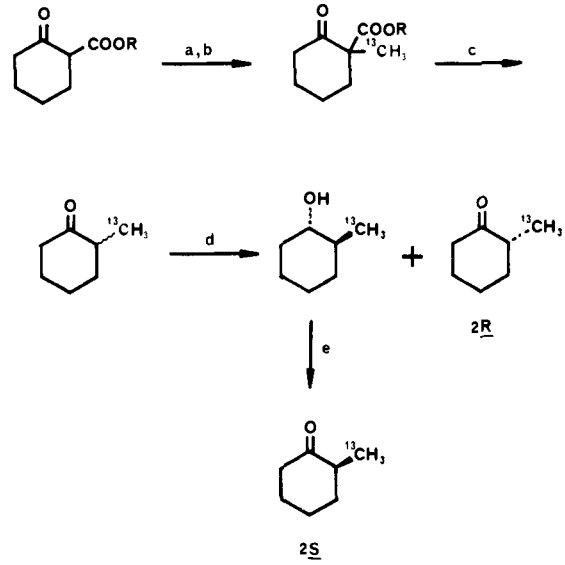
In order to rigorously test enzyme regio- and enantioselectivity, we chose the minimally asymmetric substrate 2-methylcyclohexanone, and we asked the following set of questions: (1) On which side of the carbonyl does the oxygen atom become inserted? (2) Does one substrate enantiomer react faster, and if so, which one? (3) Is the enzyme sufficiently enantioselective that, given a *racemic* substrate, enantiomerically pure lactone or residual ketone is produced?

In an approach to the regiochemistry problem, 2-methyl-ϵ-caprolactone and 6-methyl-ϵ-caprolactone were synthesized and found to exhibit essentially identical gas chromatographic retention times. GC-based methods for evaluation of regioselectivity were therefore inappropriate.

The standard method for the study of enantioselectivity involves synthesis of substrate enantiomers and measurement of the K_m and V_{max} values for the two compounds in the usual manner. Accordingly, (2*R*)- and (2*S*)-2-methylcyclohexanone were synthesized (by methods described below) and incubated separately with cyclohexanone oxygenase under standard spectrophotometric assay conditions.[4e] The rates of reaction were found to be rather similar. Assessment of enantioselectivity was complicated by substantial variations in the observed rates from run to run, a phenomenon attributed to inadequate control of the extent of aeration during mixing of the assay components. (Vigorous shaking of a cuvette containing all assay components leads to a far more rapid initial rate of reaction than if the cuvette is only gently inverted.[21]) Significant enantioselectivity was suggested by these experiments, but we could not quantify the extent using spectrophotometric assays. It was clear that we needed a method for testing enantioselectivity which would necessarily give identical conditions for incubation of both the *R* and the *S* enantiomers. Furthermore, in order to be useful for preparative-scale resolutions, cyclohexanone oxygenase would need to exhibit substantial enantioselectivity when administered a *racemic* substrate. Our approach therefore became to synthesize (*R*)- and (*S*)-2-methylcyclohexanone samples, each bearing a unique isotopic modification, mix these samples in equal quantities, and incubate the resulting "virtual racemate" with cyclohexanone oxygenase, monitoring the reaction by NMR spectroscopy at two observation frequencies. Using this technique we felt it would be possible to answer in a *single* experiment all three questions posed above.

(1) Synthesis of Substrates. The synthesis of (2*R*)-2-[*methyl*-$^2H_3$]methylcyclohexanone once again utilized *B. bassiana* for the final step. The precursor enone was made by the method of Danishefsky and Cain[22] (Scheme III). α-Picoline was deprotonated with lithium diisopropylamide and the resulting anion quenched with C^2H_3I, giving labeled 2-ethylpyridine. The latter was reduced to the dihydropyridine (a masked ketoaldehyde), which was converted with base to 2-[*methyl*-$^2H_3$]methylcyclohex-2-enone. Incubation with *B. bassiana* gave (2*R*)-2-[*methyl*-$^2H_3$]methylcyclohexanone.

Scheme IV[a]



[a] (a) NaH, THF. (b) $^{13}CH_3I$. (c) $Ba(OH)_2$, EtOH, H_2O, reflux. (d) Horse liver alcohol dehydrogenase, EtOH. (e) CrO_3, H_2SO_4, H_2O, acetone.

(2*S*)-2-[*methyl*-^{13}C]Methylcyclohexanone (Scheme IV) came from the enantioselective reduction of the corresponding racemic, labeled ketone using horse liver alcohol dehydrogenase (HLADH),[23] separation of (1*S*,2*S*)-2-[*methyl*-^{13}C]methylcyclohexanol, and oxidation. Racemic ketone was, in turn, made by alkylation of commercially available β-keto ester, followed by saponification and decarboxylation.[24]

(2) NMR Experiments. Our NMR investigations of cyclohexanone oxygenase enantioselectivity were carried out on the Bruker WH-400 located at the South Carolina Magnetic Resonance Laboratory, utilizing the 10-mm ^{13}C probe. In addition to being able to observe ^{13}C nuclei in the usual fashion, by means of a simple electronics changeover (switching of cables and preamplifiers) and adjustment of spectral parameters, it was also possible to observe deuterium nuclei, via the lock coils. The spectrometer was operated unlocked, and all spectra were measured with complete proton decoupling.

We first carried out a control experiment designed to test the comparability of kinetic data obtained from a series of ^{13}C NMR spectra to those obtained from a series of ^{2}H NMR spectra. A 10-mm NMR tube was charged with oxygen-saturated 80 mM glycine–NaOH (pH 8.0), NADPH, a trace of flavin adenine dinucleotide, and 2 μL each of ^{2}H- and ^{13}C-labeled (2*R*)-2-methylcyclohexanone. Reaction was initiated by addition of enzyme. As we had hoped would be the case, the data show (Figure 1) that the initial rates of disappearance of the substrate ketones were essentially identical, thus confirming the validity of our approach. Factors such as NOE or relaxation time differences or isotope effects would not complicate or prevent direct comparison of ^{13}C and ^{2}H data in the actual enantioselectivity experiment.

The same NMR experiment was then run, this time with 1.5 μL each of (2*R*)-2-[*methyl*-$^2H_3$]methylcyclohexanone and

(20) Reference 4a, pp 69–107.
(21) Schwab, J. M.; Li, W.-b., unpublished observations.
(22) Danishefsky, S.; Cain, P. *J. Org. Chem.* **1975**, *40*, 3606–3608.
(23) Helmchen-Zeier, R. E. Ph.D. Thesis, ETH, Zurich, 1973.
(24) Brady, S. F.; Ilton, M. A.; Johnson, W. S. *J. Am. Chem. Soc.* **1968**, *90*, 2882–2889.

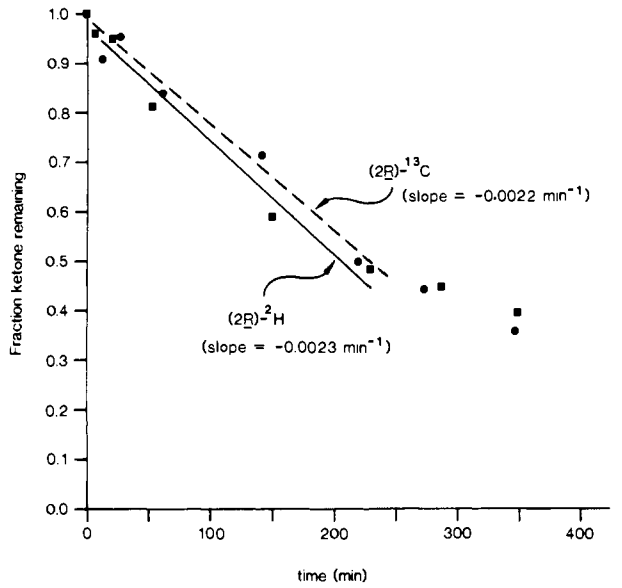


Figure 1. Time course for incubation of (2*R*)-2-[*methyl*-$^2H_3$]- (■) and (2*R*)-2-[*methyl*-^{13}C]methylcyclohexanone (●) with cyclohexanone oxygenase. (See the text for reaction conditions.) Ordinate values were determined by dividing the area under the ketone peak by the total area (residual ketone plus products). The "cut and weigh" method was used to determine relative peak areas.

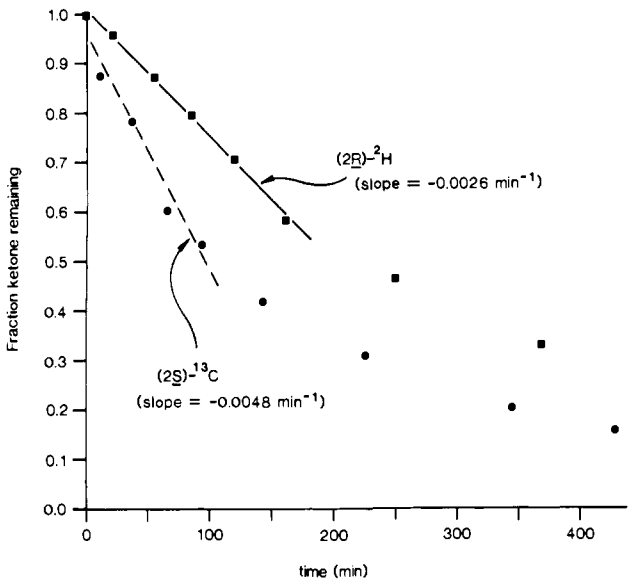


Figure 2. Time course for incubation of (2*R*)-2-[*methyl*-$^2H_3$]- (■) and (2*S*)-2-[*methyl*-^{13}C]methylcyclohexanone (●) with cyclohexanone oxygenase. (See the text for reaction conditions.) Ordinate values were determined by dividing the area under the ketone peak by the total area (residual ketone plus products). The cut and weigh method was used to determine relative peak areas.

(2*S*)-2-[*methyl*-^{13}C]methylcyclohexanone. This time the data (Figure 2) show a substantial difference, nearly a factor of 2, between the initial rates of lactonization of the ketone enantiomers.

(3) Identification of Products. Incubation of 2-methylcyclohexanone with partially purified cyclohexanone oxygenase gives rise to two main products, as illustrated in the spectra (Figure 3) and in Table II. For product identification, the two possible isomeric lactones were synthesized and their proton and ^{13}C NMR spectra measured. In addition, 6-methyl-ϵ-caprolactone was hydrolyzed to the hydroxy acid and its NMR spectra taken. From the data in Table II it is clear that 6-methyl-ϵ-caprolactone is a product and that it is undergoing enzymatic hydrolysis. (The rate of appearance of hydroxy acid, produced at the expense of lactone, is far too great for this to be a nonenzymatic process. Indeed, Prof. Trudgill has described[25] the presence of a lactone hydrolase as an impurity in cyclohexanone oxygenase preparations.) At extended reaction times, small ^{13}C and ^2H NMR peaks are observed in roughly the vicinity of the methyl ^{13}C and ^2H resonances of 2-methyl-ϵ-caprolactone. Nevertheless, the deviations between the chemical shifts of authentic material and the enzymatically produced minor component are substantial, and this minor component remains unidentified.

Table II. Incubation of 2-Methylcyclohexanone with Cyclohexanone Oxygenase: ^1H, ^2H, and ^{13}C Chemical Shifts of Substrate and Product Methyl Groups

	synthetic standards		enzyme products	
compound	$^1H^a$	$^{13}C^a$	$^2H^b$	$^{13}C^b$
2-methylcyclohexanone	0[e]	14.819[e]	0[f]	14.819[f]
3-methyl-2-oxepanone	0.148[e]	18.255[e]	(0.82)	(18.799)
7-methyl-2-oxepanone	0.354[e]	22.413[c]	0.365	22.341
6-hydroxyheptanoic acid sodium salt	0.192[e]	22.790[d]	0.191	22.738

[a] No enzyme present; ^2H_2O. [b] Enantioselectivity experiment; enzyme present; H_2O. [c] Dioxane used as internal standard (set to 67.400). [d] 7-Methyl-2-oxepanone used as internal standard. [e] 2-Methylcyclohexanone used as internal standard. [f] Used as the standard.

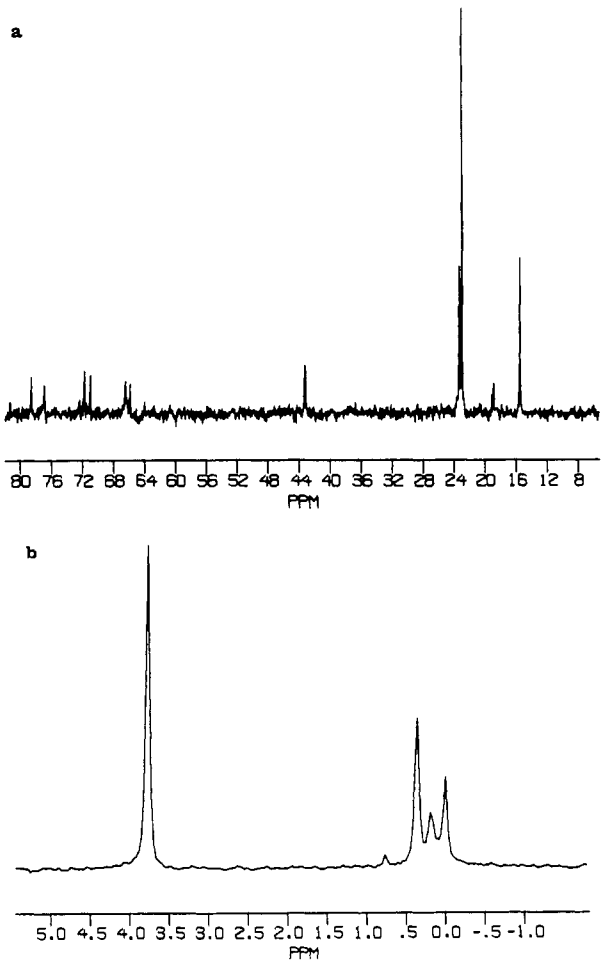
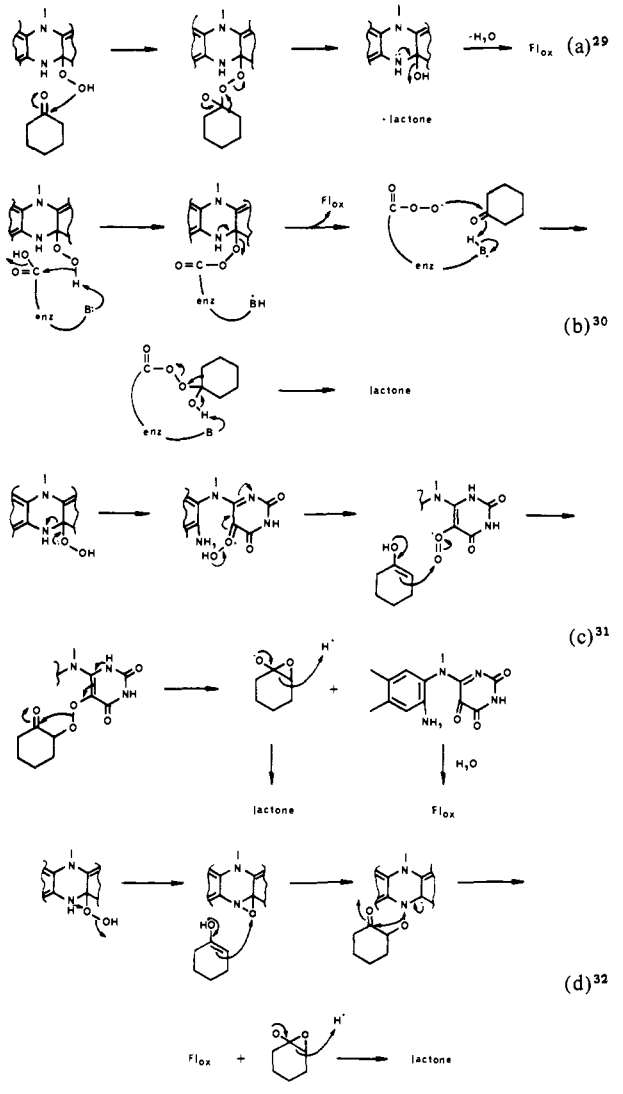


Figure 3. (a) A typical 100.614-MHz ^{13}C NMR spectrum taken from a virtual racemate enantioselectivity experiment. See Table II for spectral assignments. Spectral width, 20 000 Hz; pulse width, 8 μs; 1000 scans; 4-Hz line broadening. (b) A typical 61.423-MHz ^2H NMR spectrum taken from a virtual racemate enantioselectivity experiment. See Table II for spectral assignments. Spectral width, 1000 Hz; pulse width, 45 μs; 1000 scans; 2-Hz line broadening. 2-[*methyl*-$^2H_3$]-Methylcyclohexanone signal used as the reference (arbitrarily set to 0.0 ppm). Peak at 3.75 ppm is due to HDO at natural abundance in the buffer.

(25) Trudgill, P. W., personal communication to John M. Schwab.

Scheme V. Hypothetical Mechanisms of Action for Cyclohexanone Oxygenase



Discussion

Of the various classes of bacterial flavin-dependent monooxygenases, by far the greatest amounts of experimentation and mechanistic speculation have been directed toward the aromatic hydroxylases,[26] luciferases,[27] and liver microsomal oxidases;[28] by contrast, the Baeyer-Villiger monooxygenases have received relatively little attention. Nevertheless, adaption of hypothetical monooxygenase mechanisms to the biological Baeyer-Villiger reaction is straightforward. Ryerson et al. have recently shown[7] that C-4a FlHOOH is formed as an intermediate during the cyclohexanone oxygenase catalytic cycle; plausible mechanisms for cyclohexanone oxygenase action incorporating this feature are exemplified in Scheme V. While other reasonable mechanisms can be written, this scheme does serve to illustrate the fact that can one portray the substrate ketone functioning as an electrophile (e.g., mechanisms a and b) or as a nucleophile, via the enolate (e.g., mechanisms c and d).

An evaluation of these hypothetical mechanisms should really begin by comparison of the characteristics of the Baeyer-Villiger monooxygenase reaction to those of the nonenzymatic, peracid-mediated Baeyer-Villiger reaction. As mentioned in the introduction, the nonenzymatic Baeyer-Villiger reaction proceeds with retention of configuration at the site of oxygen insertion. Another well-known characteristic of this reaction concerns migratory tendencies. If the substrate is an unsymmetrical ketone (RCOR′), then the tendencies of the substituents R and R′ to migrate to oxygen follows the order 3° alkyl > 2° alkyl, aryl > 1° alkyl > methyl.[33] A third salient feature is that the oxygen of the substrate ketone functionality remains in the carbonyl position in the product ester. The "ether bridge" oxygen atom comes from the peracid reagent.[34] We have now proven that cyclohexanone is converted to ϵ-caprolactone by cyclohexanone oxygenase with complete retention of configuration, so far as we can detect. We have also shown that enzyme-catalyzed lactonization of 2-methylcyclohexanone conforms to the migratory trend noted above for the nonenzymatic reaction; i.e., oxygen insertion occurs between the carbonyl carbon and the more highly substituted α-carbon center. The source of the ether bridge oxygen atom in lactone produced by cyclohexanone oxygenase has not been determined to date. Nevertheless, in at least two other Baeyer-Villiger enzyme systems,[4c,5b] the ether linkage has been found to derive from molecular oxygen, and no scrambling has been observed. We expect that the same will be found for cyclohexanone oxygenase. All of the foregoing facts are suggestive of substantial mechanistic parallels between the peracid- and the enzyme-mediated Baeyer-Villiger reactions. In addition, both Ryerson et al.[7] and we[2a] have observed that [2,2,6,6-$^2H_4$]cyclohexanone shows negligible loss of deuterium in the cyclohexanone oxygenase catalyzed conversion to lactone. Although the failure to observe exchange at C-2 of the substrate ketone would seem to favor mechanisms which do not involve enolization (i.e., a and b rather than c and d), there are well-documented examples[35] of enzyme-catalyzed reactions which involve fast, shielded proton transfers. Thus, while there is a strong temptation to conclude that the mechanisms of the chemical and biochemical reactions parallel one another,[7] there is not enough evidence available at this time to provide clear proof. If the mechanisms are found to be parallel, then the Baeyer-Villiger enzymes would be the only class of bacterial flavoprotein monooxygenases examined to date (with the possible exception of the luciferases[6b,27]) in which the distal oxygen of FlHOOH functions nucleophilically.

The enantioselectivity experiments are noteworthy for several reasons. Admittedly, the degree of enantioselection (2-fold) shown by cyclohexanone oxygenase toward 2-methylcyclohexanone enantiomers is rather modest when considered alone. In retrospect, though, it seems rather surprising that an enzyme which will accept substrates as sterically diverse as cyclooctanone, dihydrocarvone, and camphor, in addition to cyclohexanone, shows any substantial enantioselectivity toward a substrate as minimally chiral as 2-methylcyclohexanone. Based on the present positive result, it seems entirely possible that cyclohexanone oxygenase would show far greater discrimination toward enantiomers of a substrate which bears a bulkier C-2 substituent.

Although cyclohexanone oxygenase shows clear enantioselectivity toward 2-methylcyclohexanone early in the incubation, the data (Figures 1 and 2) show that after ca. 50% reaction there is

(26) (a) Entsch, B.; Massey, V.; Ballou, D. P. *Biochem. Biophys. Res. Commun.* **1974**, *57*, 1018–1025. (b) Entsch, B.; Ballou, D. P.; Massey, V. *J. Biol. Chem.* **1976**, *251*, 2550–2563. (c) Entsch, B.; Husain, M.; Ballou, D. P.; Massey, V.; Walsh, C. *Ibid.* **1980**, *255*, 1420–1429. (d) Husain, M.; Massey, V. *Ibid.* **1979**, *254*, 6657–6666.

(27) (a) Hastings, J.; Eberhard, A.; Baldwin, T.; Nicoli, M.; Cline, T.; Nealson, K. In "Chemiluminescence and Bioluminescence"; Cormier, M.; Hercules, D.; Lees, J., Eds.; Plenum Press: New York, 1973; p 369. (b) Hastings, J.; Balny, C.; LePeuch, C.; Douzou, P. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3468–3472. (c) Hastings, J. W.; Balny, C. *J. Biol. Chem.* **1975**, *250*, 7288–7293.

(28) See: Ziegler, D. In "Enzymatic Basis of Detoxification"; Jakoby, W., Ed.; Academic Press: New York, 1980; Vol. 1, pp 201–227.

(29) cf. ref. 7.

(30) Bruice, T. C. *Prog. Bioorg. Chem.* **1976**, *4*, 1–87.

(31) Hamilton, G. A. *Prog. Bioorg. Chem.* **1971**, *1*, 83–157.

(32) Orf, H. W.; Dolphin, D. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 2646–2650.

(33) March, J. "Advanced Organic Chemistry"; McGraw-Hill: New York, 1977; p 1011.

(34) Doering, W. von E.; Dorfman, E. *J. Am. Chem. Soc.* **1953**, *75*, 5595–5598.

(35) Glyoxalase I has, for example, been shown to mediate a shielded proton transfer process. See: Hall, S. S.; Doweyko, A. M.; Jordan, F. *J. Am. Chem. Soc.* **1976**, *98*, 7460–7461. Other leading references can be found in footnote 3 of ref 7, cited above.

a progressive decrease in both the degree of enantioselectivity as well as the absolute rates of lactonization of the two substrate enantiomers. In light of the failure of enantiomerically pure [2-$^2H_1$]cyclohexanone samples to epimerize under similar reaction conditions (vide supra), we seriously doubt that the present observation of similar rates at extended reaction times results from racemization of the labeled 2-methylcyclohexanone enantiomers. The reasons for the diminishing rates of reaction are not clear either. Donoghue et al. have found the K_m for racemic 2-methylcyclohexanone to be 12 μM (determined at pH 9.0, though).[4e] In our virtual racemate, each substrate enantiomer was initially present at a concentration of ca. 4.9 mM; hence, the change in rate may be attributable to depletion of one of the *other* substrates (oxygen or NADPH), change of the pH of the medium, a time-dependent change in the structure of the enzyme (via proteolysis or denaturation), or inhibition by $NADP^+$.[7] ($NADP^+$ is a competitive inhibitor; however, lactone has been found[7] not to effect inhibition.) We have, in fact, observed striking rate differences between oxygenations carried out in oxygen-saturated buffer vs. buffer at atmospheric oxygen concentration, although no attempt has been made to quantify this effect.

Another important aspect of these enantioselectivity experiments lies in the methodology which was employed. Our experiment was more difficult than originally anticipated owing to the similarities of the rates of reaction of the enantiomeric substrates and to difficulties in exactly duplicating reaction conditions from run to run. Furthermore, determination of K_m and V_{max} values for individual enantiomers was not our goal. The use of the virtual racemate/multinuclear NMR technique provides a number of advantages over traditional approaches for the study of enzyme enantioselectivity. Most importantly it is assured that both "halves" of the enantioselectivity experiment (i.e., incubation with enzyme of both substrate enantiomers) are conducted under identical conditions. In addition, the method is nondestructive, allowing monitoring without the need for isolation and purification of products and/or residual starting materials. This method is also sensitive, requiring only small amounts of both enzyme and substrates, either of which might be difficult to procure in quantities sufficient for preparative-scale incubations. Finally, NMR spectroscopy provides information on product identity along with extent of reaction and allows observation of changes in enantioselectivity with time.

The use of virtual racemates (often referred to as "pseudoracemates"[36]) in studying the enantioselectivity of biological processes is not in itself a new concept. Leistner et al. have described[37] a particularly elegant virtual racemate based approach for the studying of enantioselectivity in secondary metabolic pathways of higher plants. The method as described, however, utilizes radioisotopes rather than stable isotopes. Another group has described[38] the use of virtual racemates for the study of cyclophosphamide metabolism, with analysis by mass spectrometry. Because of the nature of the biological systems under study (intact plants and mammals), neither of these investigations would have been readily amenable to analysis by NMR spectroscopy. For the study of enzyme stereochemistry in a cell-free system or enantioselective nonbiochemical processes, however, the application of NMR spectroscopy has clear advantages, as we have shown.

Experimental Section

NMR spectra were recorded on five machines, a JEOL FX-90Q(II) (^1H, ^{13}C), a Varian EM-360A (^1H), a Bruker WP-200 (^1H, ^2H, ^{13}C), a Bruker WM-300 (^2H), and a Bruker WH-400 (^2H, ^{13}C), with solvents and internal standards as noted. Gas chromatographic analyses were conducted on Varian 2100 and Packard 428 instruments with 6 ft × 2 mm (i.d.) columns packed with 7.5% Carbowax 20M on Chromosorb W AW-DMCS and with flame ionization detection. All chemicals used were of reagent quality, and tetrahydrofuran was distilled from lithium aluminum hydride prior to use (unless otherwise noted). All petroleum ether used was of the low-boiling (30–60 °C) variety. Isotopically labeled compounds were purchased from Merck & Co., Inc., or Aldrich Chemical Co.

(2*RS*)-[2-$^2H_1$]Cyclohexanol. Cyclohexene oxide (3.9 g; 40 mmol) in 5 mL of THF was added over a period of 15 min to a stirred suspension of 462 mg (11 mmol) of $LiAl^2H_4$ (Aldrich) in 20 mL of THF, under N_2. After initial reaction, the mixture was heated at reflux overnight and then cooled to 0 °C. Cautious addition of water (1 mL) in THF (4 mL) gave a gelatinous mass which was diluted with Et_2O to facilitate stirring. The mixture was poured into 10% aqueous H_2SO_4 and extracted several times with Et_2O. The combined Et_2O extracts were washed with brine, then dried ($MgSO_4$), and filtered. Concentration of the filtrate in vacuo afforded ca. 5 g of a yellow liquid, which was distilled (bp 56–7 °C; 8 torr) to give 3.31 g (82%) of [2-$^2H_1$]cyclohexanol, as a colorless liquid. The GC retention time of this material was identical with that of authentic cyclohexanol. ^2H NMR (^1H decoupled) showed singlets at 1.21 (5% of the total, C-2 ^2H cis to -OH), 1.79 (85%, C-2 ^2H trans to -OH), and 3.50 ppm (10%, C-1 ^2H).[39]

(2*RS*)-[2-$^2H_1$]Cyclohexanone. [2-$^2H_1$]Cyclohexanol (258 mg) in 5 mL of acetone was treated with Jones reagent[40] (0.75 mL) at 0 °C for 5 min. After workup, 185 mg of labeled cyclohexanone was isolated. This material was used without further purification.

2-(3*RS*,7*RS*)-[3,7-^2H]Oxepanone. (2*RS*)-[2-$^2H_1$]Cyclohexanone (185 mg) was dissolved in a few milliliters of $CHCl_3$. The solution was filtered (Whatman No. 1) to remove water and then added dropwise to a stirred solution of 375 mg of *m*-chloroperbenzoic acid in 3 mL of $CHCl_3$. After overnight reaction at room temperature, a heavy white precipitate had separated. Gas chromatography of the supernatant showed the reaction to be complete. The mixture was added to 10 mL of half-saturated Na_2CO_3 and the aqueous phase extracted with two aliquots of CH_2Cl_2. The organics were washed twice with water and filtered to remove residual water, and the solvent was removed in vacuo. The residue was purified by flash chromatography (6 in. × 20 mm; 7:3 Et_2O–petroleum ether), yielding 114 mg of labeled lactone as a colorless liquid.

(2*RS*,6*RS*)-6-[2,6-^2H]Hydroxyhexanal. The labeled lactone (114 mg; 1.00 mmol) was dissolved in 9 mL of THF in a flame-dried 25-mL round-bottomed flask equipped with a magnetic stir bar, capped with a rubber septum, and flushed with N_2. The solution was cooled to −75 °C, and 0.91 mL (1.37 mmol) of a 1.5 M solution of diisobutylaluminum hydride (in toluene) was added dropwise from a syringe, over a period of 2.5 min. The mixture was stirred for 30 min and then added to ca. 5 mL of saturated aqueous NH_4Cl. The resulting suspension was extracted 3 times with Et_2O. The combined extracts were dried over anhydrous $MgSO_4$, affording a semigelatinous mass, which was filtered through a Celite pad. Unfortunately, at this point the ether solution was spilled, and only 25% of the original amount was recovered. The solvent was removed in vacuo, the residue was dissolved in a small amount of benzene, and the solution was filtered through cotton. Removal of solvent gave 26 mg of a colorless gum which had a GC retention time similar to but slightly longer than that of authentic lactone. No lactone appeared to be present.

(1*RS*)-1-[1,5-^2H]Pentanol. The foregoing labeled hydroxyaldehyde (26 mg; 0.22 mmol) was dissolved in 2 mL of dry, N_2-saturated benzene. To this solution, in a 4-mL round-bottomed flask outfitted with a reflux condenser and a magnetic stirrer, was added (after the system had been purged with N_2) 224 mg (0.24 mmol) of $(Ph_3P)_3RhCl$. The reaction mixture was heated at reflux for 3.75 h, after which time GC analysis showed the starting material to have been consumed. Five microliters of pentanal was then added in order to consume excess Wilkinson's catalyst, and the solution was heated at reflux for an additional hour. The mixture was allowed to stand at room temperature overnight following which time another 5 μL of pentanal was added, and heating was resumed for 1 h. By this time the color of the solution had changed from deep red to orange. After the solution was cooled to room temperature, yellow crystals of $(Ph_3P)_2CORhCl$ separated, and these were removed by filtration through cotton. The labeled pentanol in the filtrate was esterified without further manipulation.

(1*RS*)-[1,5-^2H]Pentyl Camphanate. To the benzene solution of labeled pentanol (assumed to be 0.22 mmol) from the decarbonylation reaction was added 3 mL of dry pyridine (distilled from CaO and stored over molecular sieves). The resulting solution (ca. 5 mL total) was stirred,

(36) See, however: Eliel, E. L. "Stereochemistry of Carbon Compounds"; McGraw-Hill: New York, 1962; p 106.

(37) Leistner, E.; Gupta, R. N.; Spenser, I. D. *J. Am. Chem. Soc.* **1973**, *95*, 4040–4047.

(38) (a) Cox, P. J.; Farmer, P. B.; Foster, A. B.; Griggs, L. J.; Jarman, M.; Kinas, R.; Pankiewicz, K.; Stec, W. J. *Biomed. Mass Spectrom.* **1977**, *4*, 371–375. (b) Jarman, M.; Cox, P. J.; Farmer, P. B.; Foster, A. B.; Milsted, R. A. V.; Kinas, R. W.; Stec, W. J. In "Stable Isotopes: Proceedings of the Third International Conference"; Klein, E. R.; Klein, P. D., Eds.; Academic Press: New York, 1979; pp 363–370.

(39) For interpretation of these NMR data, see: Schwab, J. M *J. Org. Chem.* **1983**, *48*, 2105–2106.

(40) Bowden, K.; Heilbron, I. M.; Jones, E. R. H.; Weedon, B. C. L. *J. Chem. Soc.* **1946**, 39–45.

under N_2, and 72 mg (0.33 mmol) of (−)-camphanic acid chloride (Fluka) was added. After 90 min, the reaction mixture was poured into 1 N HCl, and Et_2O was added. The organic layer was washed several times with dilute HCl until the washes were strongly acidic to pH paper and then once with half-saturated $NaHCO_3$. The Et_2O solution was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography (6 in. × 12 mm; 9:1 petroleum ether–EtOAc), affording 15 mg of a colorless oil, identified by chromatographic and spectral characteristics as pentyl camphanate: NMR (200 MHz) δ 0.91 (br t), 0.97 (s), 1.06 (s), 1.11 (s), 4.22 (t).

[2-$^2H_1$]Cyclohex-2-enone. Several samples of this compound were made by the method described by Guaciaro et al.[13] The sample which was used to make the (2*R*)-[2-$^2H_1$]cyclohexanone which, in turn, was used for proof of the reduction stereochemistry (oxidation to lactone with MCPBA) was shown by proton NMR spectroscopy (60 MHz) to be only 70% enriched in deuterium at C-2. [The integral of the signal centered at 6.01 ppm (doublet of triplets, J = 10 Hz, J = 2 Hz, C-2 H) was only 30% of that of the complex multiplet at 7.0 ppm (C-3 H).] Low deuterium enrichment was a result of the poor quality of the ^2H_2O used in the quench of the vinyl anion. The sample of [2-$^2H_1$]cyclohex-2-enone used to make the chirally labeled ketone which was ultimately incubated with cyclohexanone oxygenase had only a small amount (ca. 5%) of protium at C-2.

(2*R*)-[2-$^2H_1$]Cyclohexanone. A typical microbial enone reduction is described. *B. bassiana* ATCC 7159 was maintained on Sabouraud dextrose agar slants and was grown preparatively on a minimal medium comprising $(NH_4)_2SO_4$ (2 g), K_2HPO_4 (1 g), dextrose (30 g), $MgSO_4$ (0.5 g), $ZnSO_4$ (0.3 g), KCl (0.5 g), $Fe(NH_4)_2(SO_4)_2$ (0.01 g), and tap water (1000 mL). The ferrous ammonium sulfate was sterilized separately, by ultrafiltration of a concentrated stock solution. Fifty milliliters of culture medium was inoculated from a slant and the culture incubated at 27 °C, with shaking at 100 rpm. After 4 days the culture was thick and was used to inoculate 500 mL of medium. The preparative culture was incubated at 20 °C, aerated at a rate of 5 mL/min, and shaken at 150 rpm. After 1 day, the temperature was readjusted to 27 °C. (The change to 20 °C had originally been a mistake; nevertheless, we hesitated to deviate from procedures which gave excellent results.) After a total of 3 days, a solution of 250 mg of labeled cyclohexenone in 1.0 mL of Me_2SO was added and the incubation temperature reduced to 20 °C. After 1 day, workup of an aliquot showed the reduction to be complete. The culture was filtered through Celite and the filtrate saturated with $(NH_4)_2SO_4$. After extraction with several aliquots of Et_2O, the extracts were dried over anhydrous $MgSO_4$, and the sample was concentrated. Rotary evaporation was used in our early work. In later runs, we removed the bulk of the solvent by flash distillation and last traces by cooling the stirred sample in an ice bath and evacuating the flask via a water aspirator. Reduction of enones by *B. bassiana* often gives substantial amounts of the doubly reduced alcohol.[11] The overall yield can be increased by brief treatment of the crude product with Jones reagent.[40] Purification of cyclohexanone is accomplished by flash chromatography. In one run, ca. 200 mg of [2-$^2H_1$]cyclohex-2-enone gave, following Jones oxidation, ca. 120 mg of (2*R*)-[2-$^2H_1$]cyclohexanone, 91% pure (GC).

Degradation of (2*R*)-[2-$^2H_1$]Cyclohexanone and (2*S*,6*S*)-[2,6-$^2H_1$]-Cyclohexanone: Determination of Enantiomeric Purity. Chirally labeled samples of cyclohexanone were degraded in the same manner as was (2*RS*)-[2-$^2H_1$]cyclohexanone (vide supra). Eu(dpm)$_3$ was added incrementally to a $CHCl_3$ solution of labeled pentyl camphanate while monitoring changes in the proton NMR spectrum. When the methylene protons had shifted from 4.2 to about 6 ppm, the probe was changed, and a deuterium NMR spectrum of the same sample was taken. All spectra were run at 50 °C, in order to enhance solubility of the shift reagent and to minimize the effect of solution viscosity on line widths.

Preparation of Cyclohexanone Oxygenase. Cultures of *Acinetobacter* NCIB 9871 were grown as described by Ryerson et al.,[7] and the purification of cyclohexanone oxygenase was carried out according to Donoghue et al.,[4c] through the DE-52 (DEAE-cellulose) step. $NaDodSO_4$ gel electrophoresis showed one major protein band along with various protein impurities. We express our sincere gratitude to Drs. Carol Ryerson and Christopher Walsh for sending us their detailed procedures for cell culture prior to publication and for a replacement sample of *Acinetobacter* NCIB 9871. We thank Dr. Peter Trudgill for samples of several ketone monooxygenase producing strains.

Cyclohexanone Oxygenase Incubations: Conversion of Labeled Cyclohexanone to Lactone. A typical preparative incubation mixture contained 80 mL of 100 mM glycine–NaOH at pH 8.0, 19 mL of deionized water, 251 IU of glucose-6-phosphate dehydrogenase (Sigma type XV, 176 IU/mg) in 177 μL of buffer, 7.3 mg of NADPH (Sigma) in 179 μL of buffer, 250 mg of $MgCl_2 \cdot 6H_2O$, 49 μL of labeled cyclohexanone, and 150 μL of cyclohexanone oxygenase solution (3.81 IU/mg; 50 mg/mL). Glucose-6-phosphate (Sigma; 198 mg in water; total volume 2.81 mL) was added via syringe pump at a rate of 13.5 μL/min. The reaction was allowed to proceed in the dark, with gentle stirring, at 30 °C, for 18 h. At this time solid NH_4Cl was added until the solution was saturated, and the solution was extracted with three aliquots of Et_2O. The combined Et_2O extracts were dried over $MgSO_4$ and filtered. Ether was removed from the ice-chilled filtrate by means of a gentle stream of N_2, giving ca. 15 mg of a colorless oil, comprising 95% ε-caprolactone (GC). (The more volatile, unreacted cyclohexanone had been blown off by the nitrogen.) Under less fortuitous circumstances, lactone was purified by flash chromatography.

2-[*methyl*-$^2H_3$]Ethylpyridine.[41] To 20.7 mmol of LDA in THF at −78 °C was added dropwise 1.68 g (18 mmol) of α-picoline. The resulting solution was allowed to warm to 0 °C (assuming a bronze color as it was stirred for 1 h) and then to room temperature. The solution (now a deep burgundy color) was stirred at room temperature and then cooled to −78 °C. Iodo[$^2H_3$]methane (3.0 g; 20.7 mmol; Merck & Co., Inc.) was added dropwise and the reaction mixture stirred for an additional 45 min before being poured into water. The resulting solution was extracted 3 times with Et_2O, and the combined extracts were washed with saturated $NaHCO_3$ and then dried over anhydrous K_2CO_3. After filtration, the solution was concentrated by flash distillation, and last traces of solvent were removed in vacuo at reduced temperature. The crude product (1.785 g of an orange liquid) was purified by flash chromatography (1:1 Et_2O–petroleum ether), leading to the isolation of 607 mg of colorless liquid, 91.3% pure (GC) and containing 8.2% solvent. The retention time of the main fraction was identical with that of authentic 2-ethylpyridine.

2-[*methyl*-$^2H_3$]Methylcyclohex-2-enone. 2-[*methyl*-$^2H_3$]Ethylpyridine (554 mg; 5.0 mmol) was converted to labeled enone by the method of Danishefsky and Cain,[22] yielding 460 mg of a slightly yellowish oil, comprising 73.0% of the desired enone (as identified by its GC behavior, relative to that of authentic cyclohex-2-enone) and 26.1% ethanol. This mixture was administered to *B. bassiana* without further purification.

(2*R*)-[*methyl*-$^2H_3$]Methylcyclohexanone. *B. bassiana* ATCC 7159 was grown as described above, and 250 mg of 2-[*methyl*-$^2H_3$]methylcyclohex-2-enone (weight corrected for the presence of solvent) in 1.0 mL of Me_2SO was added to the mature culture. After 20 h the culture was worked up, yielding 74 mg of product, 97% 2-methylcyclohexanone, 2% 2-methylcyclohex-2-enone, and 1% solvent. NMR data are cited in the text.

(2*RS*)-2-[*methyl*-^{13}C]Methylcyclohexanone. 2-Carboalkoxycyclohexanone (Aldrich, mixture of methyl and ethyl esters; 1.155 g; ca. 7 mmol) in 1.4 mL of THF (dried by filtration through Woelm alumina, activity super I) was added slowly to a magnetically stirred, ice-cooled suspension of 169 mg (7.04 mmol) of oil-free NaH in 7 mL of THF. (The reaction was run in a flame-dried 25-mL round-bottomed flask under N_2 atmosphere.) After the addition had been completed, the suspension was stirred for an additional 15 min. A solution of 1.00 g of iodo[^{13}C]methane (90% ^{13}C; 7.0 mmol) in 1.4 mL of THF was added dropwise, affording a deep yellow solution containing a powdery white precipitate. After being stirred at room temperature overnight, the reaction mixture was heated at reflux for 1 h, then cooled to room temperature, diluted with water, and extracted 3 times with a total of 30 mL of Et_2O. The combined organics were washed with brine and dried over anhydrous $MgSO_4$, which was removed by filtration. The solvent was removed in the usual manner. Gas chromatographic analysis showed the residue (1.7 g) to be 61% esters and 37% THF. The crude β-keto ester, in a 50-mL round-bottomed flask, was dissolved in 5.25 mL of EtOH, and 14.7 mL of water plus 4.42 g of $Ba(OH)_2 \cdot 8H_2O$ was added. The resulting mixture was stirred magnetically, under N_2, and heated at reflux overnight, during which time a thick white precipitate appeared. The cooled reaction mixture was partitioned between dilute HCl and Et_2O. When CO_2 evolution had ceased, the aqueous phase was extracted 3 times with Et_2O. The combined extracts were washed with saturated $NaHCO_3$ solution followed by brine and then dried over anhydrous $MgSO_4$. After filtration, volatiles were removed as before, affording crude ketone as a yellow liquid. This material was purified by flash chromatography (20% Et_2O in pentane), following which 495 mg of the labeled ketone was obtained (97.5% pure by GC). The overall yield, corrected for purity of the product, was 60.9%.

(2*S*)-2-[*methyl*-^{13}C]Methylcyclohexanone. A 250-mL round-bottomed flask was charged with $Na_2HAsO_4 \cdot 7H_2O$ (549 mg), glycine (603 mg), K_2HPO_4 (234 mg), water (27 mL), cysteine hydrochloride hydrate (455 mg), fructose-1,6-bisphosphate trisodium salt (1.23 g), NAD^+ (10.7 mg), fructose-bisphosphate aldolase (24.6 IU), and glyceraldehyde-3-phosphate dehydrogenase (214 IU). The total volume was adjusted to 94 mL, and the pH brought to 8.0 by addition of 2 N KOH. (2*RS*)-2-[*methyl*-^{13}C]Methylcyclohexanone (450 mg) was added, followed by horse liver

(41) We thank Prof. Paul Hudrlik and Tilahun Yimenu (Howard University) for sharing this procedure with us.

alcohol dehydrogenase (6.6 IU). The reaction mixture was stirred, at room temperature, in the dark. The following day, horse liver alcohol dehydrogenase (5.5 IU), NAD^+ (10 mg), glyceraldehyde-3-phosphate dehydogenase (214 IU), and fructose-1,6-bisphosphate aldolase (24.6 IU) were added, and stirring was continued. After a total of 4 days, horse liver alcohol dehydrogenase (6 IU) and NAD^+ (10 mg) were added. After 6 days (total), the reaction mixture was saturated with solid NH_4Cl and extracted with three portions of Et_2O. The combined Et_2O extracts were dried over anhydrous $MgSO_4$, filtered, and concentrated in the usual manner, giving 284 mg of a colorless liquid (41.9% 2-methylcyclohexanone and 55.6% 2-methylcyclohexanol, as shown by GC). Ketone and alcohol were separated by flash chromatography (1:1 Et_2O–petroleum ether). Removal of solvent afforded 145 mg of (1*S*,2*S*)-2-[*methyl*-^{13}C]methylcyclohexanol as a colorless oil. Gas chromatography showed the alcohol to be 94.0% pure (5.9% residual solvent). The alcohol (136 mg, corrected for purity) was oxidized by the Jones procedure[40] (vide supra), giving 120 mg of the ketone as a colorless oil, 96.3% pure (1.3% residual alcohol, 2.1% solvent). The ^{13}C NMR spectrum ($CDCl_3$, 22.49 MHz) showed a single peak (14.79 ppm, relative to Me_4Si).

Virtual Racemate Enantioselectivity Experiment. A 10-mm NMR tube was charged with 34.4 mg of NADPH, 1 mg of FAD, 0.5 mL of water (O_2 saturated), 2.0 mL of 100 mM glycine–NaOH (pH 8.0, O_2 saturated), and 1.5 μL of each labeled ketone enantiomer. Solution of the ketones in the buffer was effected by brief immersion of the NMR tube in an ultrasonic cleaning bath. Enzyme (80 μL; 8.8 mg/mL; 12.5 IU/mg) was added after the initial ^{13}C and ^{2}H NMR spectra had been run. The Bruker WH-400 spectrometer, probe equilibrated at ca. 37 °C (ascertained by immersion of a thermocouple in a sample which contained enzyme, buffer, and NADPH but no ketone), was operated unlocked. The 10-mm ^{13}C probe had been modified to allow for irradiation and observation of ^{13}C nuclei in the usual manner, as well as observation, through the lock coils, of ^{2}H nuclei. A minute or two was needed for changeover of the connections. For each ^{2}H spectrum 1000 scans were taken, over a period of 17 min. For each ^{13}C spectrum 3000 scans were taken, over a period of 10 min. ^{2}H spectra were run at 61.423 MHz by utilizing 2K data points over a spectra width of 1000 Hz. ^{13}C spectra were run at 100.614 MHz by utilizing 8K data points over a spectral width of 20 000 Hz. Both ^{2}H and ^{13}C spectra were run with broad-banded proton decoupling. The incubation mixture was kept in the spectrometer at all times between spectra.

2-Methyl-ε-caprolactone (3-Methyl-2-oxepanone). 2-Methyl-ε-caprolactone was made by the method of Herrman and Schlessinger,[42] using CH_3I as the alkylating agent: ^{13}C NMR (^2H_2O, relative to dioxane internal standard; multiplicities from off-resonance decoupled spectrum in parentheses) δ 18.284 (q), 28.332 (t), 28.823 (t), 32.259 (t), 37.919 (d), 70.692 (t), 184.315 (s); ^{1}H NMR (^2H_2O, relative to dioxane internal standard) δ 0.89 (d, J = 6.6 Hz, 3 H, $-CH_3$), 1.05–1.8 (m, 6 H, methylene protons), 2.65–2.8 (m, 1 H, $-CO-CH(CH_3)-$), 4.05–4.25 (m, 2 H, $-O-CH_2-$).

6-Methyl-ε-caprolactone (7-Methyl-2-oxepanone). MCPBA (2.46 g; ca. 11.4 mmol; Aldrich, 80–90%) was suspended in 25 mL of $CHCl_3$. 2-Methylcyclohexanone (1.11 g; 9.9 mmol) was dissolved in a few milliliters of $CHCl_3$ and the resulting solution added dropwise to the stirred MCPBA suspension. A white precipitate was observed within 30–45 min. After 3.5 h, the reaction mixture was poured into 150 mL of half-saturated Na_2CO_3. The mixture was extracted with three aliquots of CH_2Cl_2. The combined extracts were filtered (to remove water) and concentrated under reduced pressure. The remainder (2–3 mL) was purified by careful bulb-to-bulb distillation from a grain of K_2CO_3. The distillate (1.24 g) was shown by GC to be pure (no solvent present) and was stored over K_2CO_3: ^{13}C NMR (^2H_2O, relative to dioxane internal standard) δ 22.413 (q), 23.135 (t), 28.217 (t), 35.204 (t), 36.186 (t), 79.759 (d), 181.600 (s); ^{1}H NMR (^2H_2O, relative to dioxane internal standard) δ 1.09 (d, J = 6.4 Hz, 3 H, $-CH_3$), 1.15–1.85 (m, 6 H, methylene protons), 2.28–2.45 and 2.5–2.75 [m, each 1 H, $-CO-CH_2-$ (they are diastereotopic)], 4.4–4.6 [m, 1 H, $-O-CH(CH_3)-$].

6-Hydroxyheptanoic Acid Sodium Salt. 6-Methyl-ε-caprolactone (0.5 g; 3.9 mmol) was saponified by heating at reflux with 10.8 mL of 0.37 N NaOH. The pH of the resulting solution was adjusted to 8.0 with dilute HCl. An aliquot of the neutralized solution was combined with a similar aliquot of 2-methylcyclohexanone and the ^{1}H NMR spectrum run at 90 MHz, with homonuclear gated decoupling to eliminate the water peak. The difference in the chemical shifts of the two methyl groups was 0.192 ppm (the ketone methyl group was at higher field). In the virtual racemate enantioselectivity experiment, the ^{2}H resonance assigned to 6-hydroxyheptanoate is found at 0.191 ppm relative to the $-C^2H_3$ signal of labeled 2-methylcyclohexanone. Addition of the hydroxy acid solution (0.7 mmol of hydroxy acid) to a virtual racemate reaction mixture which had been allowed to incubate with enzyme overnight resulted in striking enhancement of an existing ^{13}C resonance 7.92 ppm downfield from the $-^{13}CH_3$ of labeled 2-methylcyclohexanone. The identity of this enzymatic reaction product was thus established.

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Registry No. Cyclohexanone oxygenase, 52037-90-8; cyclohexanone, 108-94-1; [2-$^2H_1$]cyclohexanol, 85954-61-6; cyclohexene oxide, 286-20-4; (2*RS*)-[2-$^2H_1$]cyclohexanone, 85954-60-5; 2-(3*RS*,7*RS*)-[3,7-^{2}H]oxepanone, 85893-47-6; (2*RS*,6*RS*)-6-[2,6-^{2}H]hydroxyhexanal, 85893-48-7; (1*RS*)-1-[1,5-^{2}H]pentanol, 85893-49-8; [1,5-^{2}H]pentyl camphanate, 85893-50-1; (−)-camphanic acid chloride, 39637-74-6; cyclohex-2-enone, 930-68-7; [2-$^2H_1$]cyclohex-2-enone, 77102-46-6; (2*R*)-[2-$^2H_1$]cyclohexanone, 75348-02-6; (2*S*,6*S*)-[2,6-$^2H_1$]cyclohexanone, 78680-00-9; 2-[*methyl*-$^2H_3$]ethylpyridine, 85893-51-2; α-picoline, 109-06-8; iodomethane, 74-88-4; 2-[*methyl*-$^2H_3$]methylcyclohex-2-enone, 85893-52-3; 2-methylcyclohexanone, 583-60-8; (2*R*)-[*methyl*-$^2H_3$]methylcyclohexanone, 85541-91-9; (2*RS*)-2-[*methyl*-^{13}C]methylcyclohexanone, 85954-62-7; (2*S*)-2-[*methyl*-^{13}C]methylcyclohexanone, 85541-90-8; (1*S*,2*S*)-2-[*methyl*-^{13}C]methylcyclohexanol, 85893-53-4; 2-methyl-ε-caprolactone, 2549-61-3; 6-methyl-ε-caprolactone, 2549-59-9; 6-hydroxyheptanoic acid·Na, 85893-54-5.

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