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Enzymatic Epoxidation of *trans,trans*-1,8-Dideuterio-1,7-octadiene. Analysis Using Partially Relaxed Proton Fourier Transform NMR

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Abstract: In previous work we have established that an enzyme system from *P. oleovorans* catalyzes the conversion of terminal olefins to the corresponding 1,2-oxides. The reaction was shown to exhibit a high degree of stereoselectivity as well as specificity patterns far different from those expected on the basis of chemical reactivity in nonenzymatic epoxidation reactions. In order to probe directly the mechanism of enzymatic oxygen insertion in this system, the substrate *trans,trans*-1,8-dideuterio-1,7-octadiene was synthesized and used as a substrate in the enzymatic reaction. The 7,8-epoxy-1-octene product was isolated and its structure analyzed using partially relaxed proton FT NMR spectroscopy. While the equilibrium NMR spectrum of the epoxide product exhibited serious overlapping of signals due to the presence of fully protonated and both cis and trans deuterated species, use of the partially relaxed FT NMR technique allowed isolation of the spectra of the various species, quantitative analysis of the product mixture, and a relaxation time analysis of both the fully protonated and deuterated species which helped confirm the spectral assignments. On the basis of these analyses, it is concluded that the enzymatic product consists of 70% cis deuterated and 30% trans deuterated epoxide, while the starting olefin preparation contained only the trans deuterated species. Thus, the enzymatic reaction proceeds primarily with inversion of the original olefin geometry. In contrast, a similar analysis on the product obtained via peracid epoxidation of the trans deuterated olefin revealed the presence of only the trans deuterated epoxide. In both cases, a secondary deuterium isotope effect could not be detected via this NMR technique. Control experiments indicated that no isomerization of either the epoxide or olefin functionalities occurs under the reaction conditions. The significance of these results in terms of a simple "oxenoid" mechanism for this enzymatic epoxidation reaction, as well as the general suitability of partially relaxed FT NMR spectroscopy for analysis of complex mixtures, are discussed.

Monooxygenase reactions involve both electron transfer and oxygen insertion steps, and there has been considerable speculation about the interaction between these events on the molecular level and the nature of the "activated oxygen" species generated by these enzymes. Hamilton first suggested that an electrophilic "oxenoid" species is generated in these reactions by transfer of two electrons to oxygen prior to, or concurrent with, transfer of an oxygen atom to the substrate.[1-4] This mechanism has found particular favor in accounting for the reaction of cytochrome-P-450-containing oxygenases, and especially the "NIH shift" which occurs during aromatic hydroxylation reaction.[5,6] A number of organic model systems which undergo oxenoid-type reactions have also been designed and investigated.[1-6]

Among the most well-suited of all "oxenoid" reactions in organic chemistry is the peracid epoxidation of olefins, and this reaction clearly proceeds through electrophilic attack on the olefin[7-10] as evidenced, for example, by the well-documented effects of electron-donating or electron-withdrawing substituents. A diagnostic characteristic of peracid epoxidations is their absolute syn stereospecificity, even in cases where substituents which could stabilize a carbonium ion intermediate are present.[8-12] As Berti has pointed out,[9] among the very large number of epoxidations of known steric course, there has not been reported a single instance of a cis olefin giving threo or a trans olefin giving erythro epoxide. Similarly, among the oxo transition metal complexes, which have been considered as possible models for oxygenase reactions,[13] chromyl acetate[14] and molybdenum oxo complexes[15] are known to epoxidize olefins with retention of geometry, and the same is true of transition metal catalyzed epoxidations by alkyl hydroperoxides.[16,17]

In previous work, we have established that an enzyme system from *Pseudomonas oleovorans* catalyzes the conversion of terminal olefins to the corresponding 1,2-oxides.[18-25,48] On the basis of stereochemical studies, inhibitor binding data, and an examination of structural effects on reactivity, we have concluded that the nature of the hydrophobic interactions between the active site and a given substrate critically affect the reactivity of that substrate toward enzymatic epoxidation. Thus, it is difficult to extract mechanistic information from a direct comparison of the chemical reactivity patterns of potential

oxygenase models with that of this enzymatic system, a common problem in the analysis of enzymatic reaction pathways. We now wish to report that we have successfully utilized the substrate *trans,trans*-1,8-dideuterio-1,7-octadiene to directly probe the mechanism of enzymatic oxygen insertion in this system. The use of this deuterated substrate allows a determination of whether or not the reaction involves syn oxygen addition, despite the fact that simple internal olefins are enzymatically unreactive.[18-25] When considered together with a stereochemical analysis of the enzymatic epoxidation reaction,[21-25] our results provide information which is helpful in evaluating various mechanistic proposals for enzymatic oxygen activation.

The preparations of *trans,trans*-1,8-dideuterio-1,7-octadiene used in this study also contained significant amounts of the fully protonated 1,7-octadiene and thus gave rise to products which were mixtures of deuterated and fully protonated epoxides. The proton NMR spectra of these product mixtures exhibited serious overlapping in the spectral regions of interest, making assignments speculative, and a quantitative analysis of the mixture unreliable. In the original paper on the subject, Vold et al.[26] suggested that partially relaxed Fourier transform NMR could be used to resolve overlapping NMR signals of nuclei with different relaxation times. Since then, partially relaxed FT NMR has been used to affect spectral resolution of overlapping signals in a variety of applications, such as the resolution of overlapping signals in boron-11 spectra[27] and the removal of the residual water peak from the FT NMR spectra of D_2O solutions of biological molecules.[28]

Spectral resolution by partially relaxed FT NMR should be applicable to our enzymatic product mixture if the NMR sample is made up as a dilute solution in an aprotic solvent and is carefully degassed. The proton relaxation would then be determined almost exclusively by the intramolecular dipolar mechanism,[29] and epoxide and olefin protons in corresponding positions of deuterated and nondeuterated molecules would have substantially different relaxation rates. In fact, we demonstrate in this paper that partially relaxed proton FT NMR is ideally suited for the *quantitative* analysis of such mixtures.

Because of the nature of the partially relaxed FT NMR technique, it was a real advantage that the product mixtures contained both deuterated and fully protonated species. This allowed us to confirm spectral assignments on the basis of a comparison of the relaxation rates of the deuterated and fully protonated species. In addition, we were able to look for secondary deuterium isotope effects by comparing the relative deuterium content of the epoxide and olefin portions of the 7,8-epoxy-1-octene product.

Experimental Section

Hydrocarbon substrates and organic alcohols were purchased from various sources and were of the highest grades commercially available. 1,2-Epoxyoctane and 7,8-epoxy-1-octene were synthesized from *m*-chloroperbenzoic acid and the appropriate olefin, as described previously.[19] *P. oleovorans* cultures were grown on *n*-octane and the media and growth conditions used have been described.[30] Reaction conditions for standard epoxidation assays as well as our procedures for the extraction and quantitation of products have also been previously described.[18,19,25,30]

Syntheses. 1,3,2-Benzodioxaborole (Catecholborane).[31] Four-hundred milliliters of a 1 M solution of diborane in THF (purchased from Aldrich) was poured into a three-necked round-bottom flask. The solution was maintained under a nitrogen atmosphere at 0 °C. A solution of 44 g (0.4 mol) of pyrocatechol (recrystallized from hot benzene mp 105 °C) and 50 ml of THF (distilled over $NaAlH_4$) was added dropwise over a 30-min period with efficient stirring. The solution was stirred for an additional 30 min at 0 °C and then for 1 h at room temperature. THF was removed under vacuum (50 mm, 25 °C) and the crude product was distilled. Yield, 29.3 g of catecholborane (61%); bp 88 °C at 156 mm, 66 °C at 80 mm [lit.[32] 88 °C at 156 mm].

Bis(1,7-octadienyl)-1,3,2-benzodioxaborole. Freshly distilled 1,7-octadiyne (12.45 g; 0.117 mol) and 28.5 g (0.237 mol) of catecholborane were injected via syringe into a 100-ml three-necked flask to which a reflux condenser had been attached. The apparatus had been flame dried and cooled under N_2 before use. After several minutes of stirring, the temperature of the reaction mixture rose to 110 °C. Stirring was continued for 1 h after which a white solid was formed. The crude product was recrystallized from ethyl acetate and dried under vacuum. Yield, 22.3 g (55%); mp, 96-98 °C, NMR (δ, CD_3COCD_3), 1.0 (m, 4), 1.5 (m, 4), 5.2 (d, 2), 5.9 (m, 2), 6.4 (d of d, 8). Exact mass determination: 345.15632. The NMR splitting pattern indicated only trans olefinic protons (J = 18 Hz) corresponding to *cis* addition of the catecholborane.

***trans,trans*-1,8-Dideuterio-1,7-octadiene.** CH_3COOD (25 g; 0.409 mol) (purchased from Aldrich) was injected via syringe into a three-necked flask containing 15 g (0.043 mol) of the octadienylborole. The flask, fitted with a reflux condenser, had been flame dried and cooled under N_2 before use. During the addition and throughout the course of the reaction, a nitrogen atmosphere was maintained. After 3 h of reaction, the mixture was poured over 50 ml of ice water, extracted with 200 ml of pentane, washed successively with ice cold 1 N NaOH and brine, and then dried over $MgSO_4$. The crude product was distilled to give 5.5 ml of the deuterated product (90% yield), bp 62 °C at 140 mm.

The final product was characterized as follows: Quantitative flame ionization gas chromatographic analysis[18,19] gave a single peak with retention time identical with that obtained with an authentic sample of 1,7-octadiene. Mass spectral analysis gave a molecular ion peak at *m/e* 112. A large peak at *m/e* 96 was also observed corresponding to allylic rearrangement and cleavage of a terminal CH_2D fragment (*m/e* 16). Correspondingly, the mass spectrum of nondeuterated 1,7-octadiene showed a large peak at *m/e* 95 corresponding to the removal of a terminal CH_3 fragment. The NMR was fully consistent with the structure and the relaxation spectrum analysis showed that no cis-deuterated olefin was present (see Results section).

Epoxidation of *trans,trans*-1,8-Dideuterio-1,7-octadiene. Two 2-L Fernback flasks, each containing 1000 ml of P-1 medium,[30] 10 ml of filter sterilized octane, and a 20 ml inoculum of a resting cell suspension of *P. oleovorans* TF4-1L[30,33] (approximately 10^9 cells/ml) were incubated aseptically at 30 °C on a gyrotory shaker at 300 rpm. After 17 h the two flasks had OD_{660} of 6.4 and 6.2, respectively. The solutions were centrifuged at 4 °C and 10 000 xG for 10 min and then resuspended in 0.1 M phosphate buffer, pH 7, to a total of 300 ml. One drop of Triton X-100 and 3 ml of the deuterated substrate were added and the mixture was incubated with shaking at 30 °C. Production of epoxide was continuously monitored quantitative gas chromatography.[18,19] When epoxide production was maximal, the product was extracted with 300 ml of hexane, concentrated to 4 ml, and purified by preparative gas chromatography using a 20 ft × 0.25 in. column of 10% Carbowax 20 M on 80/100 Chromosorb W, maintained isothermally at 180 °C.

Peracid epoxidation was effected by mixing 1 g (57 mmol) of *m*-chloroperbenzoic acid and 0.583 g (52 mmol) of the deuterated diene in 30 ml of ether for 48 h at room temperature. The epoxide product (~200 mg) was collected by preparative gas chromatography as described above.

NMR Procedures. Sample Preparation. One-hundred milligrams of the enzymatic product mixture was dissolved in 0.25 ml of CCl_4 which contained 10% v/v C_6D_6 for the deuterium lock signal. The solution was made up in a standard 5 mm o.d. NMR sample tube which was fitted with a ground glass joint for attachment to a high vacuum manifold. The oxygen was removed by repeated freeze-pump-thaw cycles using a dry ice-acetone bath as the coolant.

Instrumentation. FT NMR proton spectra were obtained at 99.5 MHz using a JEOL PFT-100 FT NMR spectrometer equipped with a PG-100 computer system. The observed frequency was derived from a General Radio 1164-A frequency synthesizer which was phase locked to the 15.4 MHz D lock frequency.

Partially Relaxed Spectra. The partially relaxed spectra were obtained using a 180°-τ-90° pulse sequence.[26] The width of the 90° pulse was 55 μs, corresponding to an rf field of amplitude 4.7 kHz. An 8K transform was used with a window of 1 kHz. Each spectrum was the result of 16 accumulations with a repetition time between pulse sequences of 100 s. This repetition time is 3.4 times the longest re-

laxation time of the sample. As a check, spectra were also obtained with a repetition time of 200 s. No systematic difference in spectral intensities was observed between these spectra and those with a repetition time of 100 s.

A sequence of partially relaxed spectra was obtained in this manner with the interval time τ stepped from 1 to 25 s in 1-s steps. This enabled the null times in most cases to be determined to within ±1 second. When justified, smaller increments of τ were used to determine the null times more accurately. After the null times were determined, three spectra were obtained at each null time. In addition, three spectra were obtained with τ = 0.2 s and τ = 100 s in order to determine the fractional inversion produced by the 180° pulse. The spectra were obtained over a period of 6 days. During this time, the sample remained continuously in the NMR probe. The only spectrometer adjustments during the period were to the curvature and Y-gradient of the field homogeneity unit.

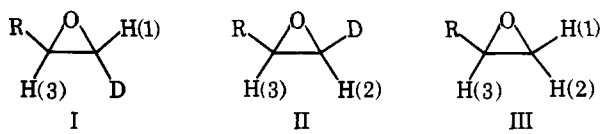
The spectra were recorded on chart paper, and the intensities of the relevant spectral features were measured with a Gelman Model 39231 compensating polar planimeter. In general, the intensities of corresponding spectral features were reproducible to better than 10% and in most cases to better than 5% (see Table I).

Results

Enzymatic Epoxidation of *trans,trans*-1,8-Dideuterio-1,7-octadiene. Previous work has established the feasibility of producing epoxides using whole cells or crude cell-free preparations of *P. oleovorans*. Thus, for example, May and Schwartz recently reported that optically active epoxides of high optical purity can be produced on a preparative scale (~2.5 g/3 L) from 1,7-octadiene via a simple procedure which uses whole cells growing on octane.[22] Resting cell preparations have been reported to produce about 0.4 mg/mL of 7,8-epoxy-1-octene after 1 h[25,33] and crude cell free preparations have been used to produce about half as much epoxide after 90 min under a given set of reaction conditions.[25] These data underscore one of the most significant advantages of this enzymatic epoxidation reaction for the mechanistic and stereochemical investigations, since relatively large amounts of products can be readily generated. In the present case, our goal was to epoxidate synthetically prepared *trans,trans*-1,8-dideuterio-1,7-octadiene, and it was thus essential to establish conditions under which sufficiently large amounts of product for physical characterization could be obtained from a limited amount of substrate. Under the conditions outlined in the experimental section, yields of 7,8-epoxy-1-octene approaching 2 mg/mL were obtained in our hands, which is several fold higher than those reported previously. Extensive optimization experiments were not carried out for the purposes of this study. After isolation and purification approximately 150 mg of final purified product was obtained from each 3-mL portion of deuterated substrate.

Partially Relaxed Proton FT NMR Spectra. Partially relaxed proton FT NMR spectra of a solution of the enzymatic product mixture in CCl_4 were obtained at 99.5 MHz using a 180°-τ-90° pulse sequence, as described in the Experimental Section. An equilibrium spectrum of the enzymatic product mixture is given in Figure 1, which also includes a structural formula of 7,8-epoxy-1-octene with the numbering of the relevant protons. Coupling constants and chemical shifts are given in the figure legend, with the lowest field line of the H(1) multiplet assigned zero frequency. Spectral features have been previously assigned.[19]

The partially relaxed spectra show that the NMR signals in the epoxide region are due to the trans deuterated epoxide I, the cis deuterated epoxide II, and the fully protonated epoxide III, and the NMR signals in the olefin region are due to the trans deuterated olefin IV and the fully protonated olefin V.[49]



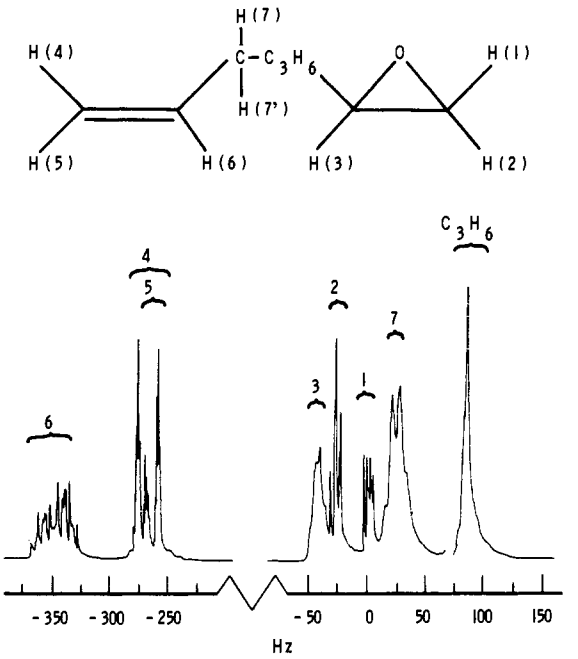
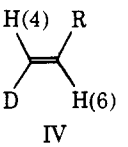
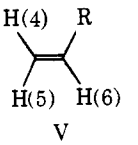


Figure 1. Top: Structural formula of 7,8-epoxy-1-octene with numbering system of protons. Bottom: Proton FT NMR spectrum of enzymatic product mixture in CCl_4 at 99.5 MHz and 28 °C, after ten accumulations with a pulse repetition time between pulse sequences of 100 s. The C_3H_6 signal has a vertical reduction of 4 with respect to the remainder of the spectrum. The NMR spectral parameters are: J_{12} = 5.4 Hz, J_{13} = 2.4 Hz, J_{23} = 3.9 Hz, J_{45} = −2.2 Hz, J_{46} = 17.1 Hz, J_{47} = 1.4 Hz, J_{56} = 10.0 Hz, J_{57} = 1.2 Hz J_{67} = 6.6 Hz, ν_{01} = 4.0 Hz, ν_{02} = −24.0 Hz, ν_{03} = −40 Hz, ν_{04} = −263.9 Hz, ν_{05} = −259.1 Hz, ν_{06} = −342.6 Hz, ν_{07} = 27 Hz, where the lowest field line of the H(1) multiplet is zero frequency.



IV



V

Partially relaxed spectra of the epoxide region are shown in Figure 2, with τ increasing from bottom to top. The bottom trace with τ = 0.05 s is an inverted spectrum. At τ = 5.7 s, a broad inverted doublet of splitting 2.4 Hz is isolated in the H(1) region. This signal is due to H(1) of I and is the X proton of an approximate AX pattern.[34,35] The broadening is due to an unresolved H-D splitting of 0.7 Hz. At τ = 6.7 s, another broad inverted doublet of 3.7 Hz splitting is isolated in the H(2) region. This signal is due to H(2) of II and is the B portion of an AB quartet. The H(2) doublet is considerably more intense than the H(1) doublet. From the τ = 5.7 and 6.7 s spectra, we see that both the trans and cis deuterated epoxides were produced but that the cis isomer was the dominant product. Therefore, we conclude that the enzymatic epoxidation reaction has occurred predominantly with inversion of the original double bond configuration. The signals due to H(1) and H(2) of the fully protonated epoxide III are isolated at τ = 13 and 19 s, respectively, and form the BX portion of an approximate ABX pattern. From these spectra we calculate J_{13} = 2.4 Hz, J_{23} = 3.9 Hz, and J_{12} = 5.4 Hz in good agreement with coupling constants reported for other epoxides.[36,37] The basis for the epoxide spectral assignment is the observation that trans coupling constants in trans disubstituted epoxides have a characteristic value of about 2 Hz.[36] It will be seen below that our relaxation time measurements and the results obtained

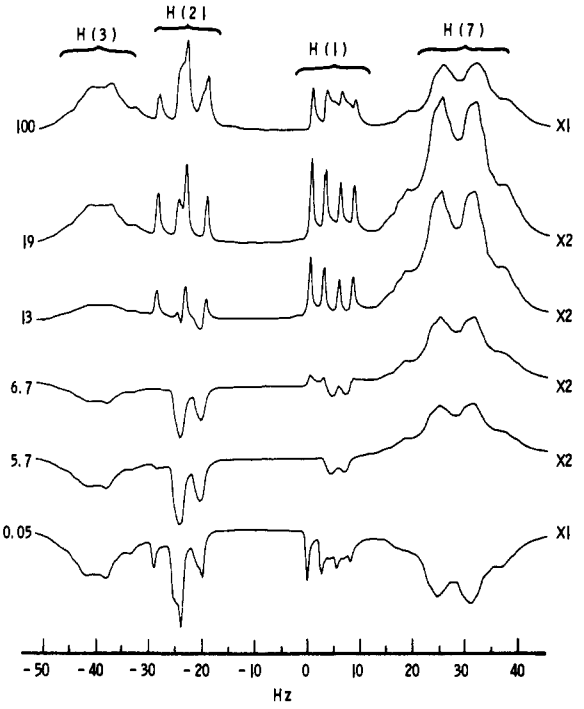


Figure 2. Partially relaxed proton FT NMR spectra of the epoxide region of the enzymatic product mixture in CCl_4 at 99.5 MHz and 28 °C. The number to the left of each spectrum is τ in seconds. Each spectrum is the result of 16 accumulations with a repetition time of 100 s. The relative vertical expansion factors are given to the right of each spectrum.

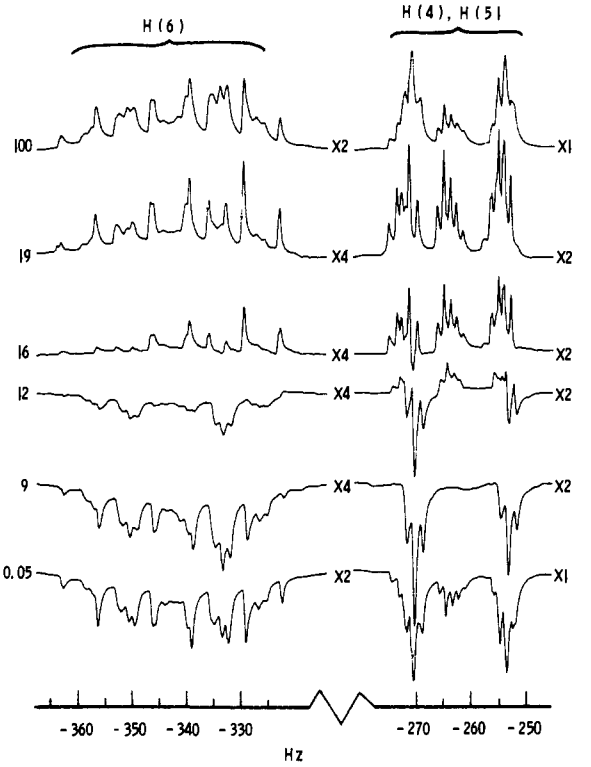


Figure 3. Partially relaxed proton FT NMR spectra of the olefin region of the enzymatic product mixture in CCl_4 at 99.5 MHz and 28 °C. The number to the left of each spectrum is τ in seconds. Each spectrum is the result of 16 accumulations with a repetition time of 100 s. The relative vertical expansion factors for the H(6) and H(4), H(5) regions of the spectra are different and are indicated to the right of the respective regions on each trace. Zero frequency is assigned to the low field line of the H(1) multiplet (see Figure 2).

with the peracid epoxidation product provide additional support for the assignment.

Partially relaxed spectra of the olefin region are shown in Figure 3. At τ = 9 s, an inverted doublet of triplets is isolated in the H(4), H(5) region with a doublet splitting of 17.1 Hz and a triplet splitting of 1.4 Hz. This signal is assigned to H(4) of IV and is the B portion of an ABX_2 pattern where A, B, X correspond to H(6), H(4), H(7), respectively. The H(4) and H(5) signals of V appear to have both relaxed to zero intensity at τ = 9 s. Actually, a vertical expansion indicated that the H(4) signal of V relaxed somewhat earlier at about τ = 8.5 s. The partially relaxed spectrum at τ = 9 s is fully consistent with our contention that no isomerization of the trans deuterated olefin functionality has occurred. If any cis deuterated olefin had been present, we would have seen substantial inverted signals at about −265 Hz. The H(4), H(5) signals of V are isolated at τ = 19 s, giving the MN portion of an approximate $AMNX_2$ pattern. At τ, 12 s, an inverted pattern is isolated in the H(6) region. This is due to H(6) of IV and is the A portion of an ABX_2Y spectrum with an AB splitting of 17.1 Hz due to H(4), AX_2 triplet splitting of 6.6 Hz due to the H(7) protons, and an AY triplet splitting of 1.5 Hz due to the deuterium. At τ = 16 s, the signal due to H(6) of structure V is isolated and is the A portion of an approximate $AMNX_2$ pattern, with an AX_2 triplet splitting of 6.6 Hz with the H(7) protons. As expected, the equilibrium spectrum can be seen to be a superposition of the various patterns isolated in the partially relaxed spectra.

Quantitative Analysis. In order to quantitatively analyze the partially relaxed spectra, we made the simplest relaxation assumption, namely that each multiplet A relaxes exponentially with an effective spin-lattice relaxation time T_{1A}. This assumption cannot be strictly correct for our sample because the proton relaxation is dominated by the intramolecular dipolar mechanism. However, effective relaxation times as defined above have proved useful in studying molecular motion[38,39] and they adequately describe the partially relaxed spectra of our sample.[50] With this assumption, the multiplet A, after inversion by the 180° pulse, relaxes to zero intensity at the null time τ_A, where

$$\tau_A = T_{1A} \ln (1 + \alpha) \quad (1)$$

In eq 1, α, the fractional inversion, is given by

$$\alpha = -I_A(0)/I_A(\infty)$$

where $I_A(0)$ is the intensity of the inverted signal (i.e., $\tau \ll T_{1A}$), and $I_A(\infty)$ is the intensity of the equilibrium signal (i.e., $\tau \gg T_{1A}$). Therefore, if A and B are overlapping multiplets, signal A is isolated at $\tau = \tau_B$, and B is isolated at $\tau = \tau_A$. From a knowledge of τ_A, τ_B, $I_A(\tau_B)$, and $I_B(\tau_A)$, we can compute the intensity of A in the inverted spectrum, $I_A(0)$, from

$$I_A(0) = \frac{\alpha I_A(\tau_B)}{(1 + \alpha) \exp(-[\tau_B/\tau_A] \ln (1 + \alpha)) - 1} \quad (2)$$

and T_{1A} from eq 1, with analogous formulas for $I_b(0)$ and T_{1B}.

The observation times, null times, and relative integrated intensities of the isolated multiplets of Figures 2 and 3 are recorded in Table I. Each intensity is an average from three spectra. The fractional inversion was determined from the olefin region to be 0.94 ± 0.02. Using this value of α, T_{1A} and $I_A(0)$ were computed for each multiplet from eq 1 and 2. The values of T_{1A} and $I_A(0)$ are given in Table I. From the $I_A(0)$ values for H(1) of I and H(2) of II, it is evident that 70% of the deuterated epoxide is the cis isomer.

We can look for deuterium isotope effects by comparing the deuterium content of the epoxide portion with that of the olefin portion of the epoxide product. From the $I_A(0)$ values for H(1) and H(2) of I, II, and III, we compute that 59% of epoxidation

Table I. Null Times, Relaxation Times, and Intensities of Epoxide and Olefin Multiplets

Multiplet	Observation time, s	Null time, s	T_1,[a] s	I^b at observe time	$I(0)^d$	Predicted T_1, s
H(1) I	5.7	13	20	−126 (2)	−265	18
H(2) II	6.7	19	29	−349 (16)	−616	
H(1) III	13	5.7	8.6	398 (27)	−649	8
H(2) III	19	6.7	10	437 (17)	−580	9
H(3) I, II, III		~10	15			11
H(4) IV	9	19	29	−457 (19)	−1039	
H(6) IV	12	16	24			
H(4) V	19	8.5	13	753 (56)[c]	−1298[c]	
H(5) V	19	9	14			
H(6) V	16	12	18			

[a] Computed from eq 1 with $\alpha = 0.94$. [b] Average of three measurements; average deviation is given in parentheses. [c] Sum of intensities for H(4) and H(5). [d] Computed from eq 2 with $\alpha = 0.94$.

product was deuterated. From the $I_A(0)$ values of H(4) of IV and H(4), H(5) of V, we calculate that 62% of the olefin substrate was deuterated. The percent deuteration in the olefin was also computed from the equilibrium spectrum by comparing the integrated intensity of the H(6) region with that of the H(4), H(5) region. These measurements gave 62% in good agreement with the partially relaxed spectra result. Therefore, within experimental error, a significant secondary deuterium isotope effect could not be detected by this technique.

Partially relaxed spectra also were obtained for a second batch of enzymatic epoxidation product. The NMR spectra showed that the substrate used for this batch contained 29 mol % of the trans deuterated and 71 mol % of the fully protonated 1,7-octadiene. This sample provided a test of the sensitivity of the partially relaxed technique because the fraction of deuterated material was low and only 50 mg of epoxidation product were available. The experimental results were virtually identical with those obtained with the first batch, with 71% of the enzymatic product being the cis deuterated epoxide.

Relaxation Analysis. The relaxation times for the gem protons can be used to compute T_{ij}, the contribution to relaxation from pairwise dipolar interactions between H(i) and H(j). T_{ij}, in turn, provides the value of the reorientational correlation time τ_R from the well-known formula[40]

$$T_{ij}^{-1} = {}^3/_2\gamma^4\hbar^2 r_{ij}^{-6}\tau_R \qquad (3)$$

where γ is the proton magnetogyrio ratio, and r_{ij} is the distance between H(i) and H(j). If it is assumed that the geometry of the olefin and epoxide portions of the product are the same as those of propylene and propylene oxide, respectively,[41] then the dimensions of the H(1), H(2), H(3) and the H(4), H(5), H(6) triangles are identical, and $r_{12} = r_{45} = 1.84$ Å. We compute $\tau_R = 3.02 \times 10^{-12}$ s for the epoxide group, using the H(1) relaxation times in Table I and eq 3. As a check, we can also compute the epoxide τ_R from the H(2) relaxation times, and again we obtain 3.02×10^{-12} s. From the H(4) relaxation times we calculate $\tau_R = 1.92 \times 10^{-12}$ s for the olefin group. Therefore, the olefin portion of the molecule reorients considerably faster than the epoxide portion.

If we assume that the difference in relaxation rates of6corresponding epoxide and olefin protons is due to the difference in correlation times, we can predict each epoxide relaxation time by multiplying the corresponding olefin relaxation time by 1.92/3.02. The epoxide relaxation times predicted in this manner are given in the last column of Table I. The agreement is satisfactory and provides strong support for the spectral assignment of the H(1) and H(2) epoxide protons.

It is interesting to note that H(1) relaxes faster than H(2) and that H(4) relaxes faster than H(5), even though H(2) and H(5) are both cis to a proton on the adjacent carbon. The cis interaction, in fact, allowed us to successfully isolate the H(6) signals of IV and V. The faster relaxation times of H(1) and H(4) can be rationalized on the basis that they are closer to the numerous protons of the methylene chains of the respective compounds than are H(2) or H(5).

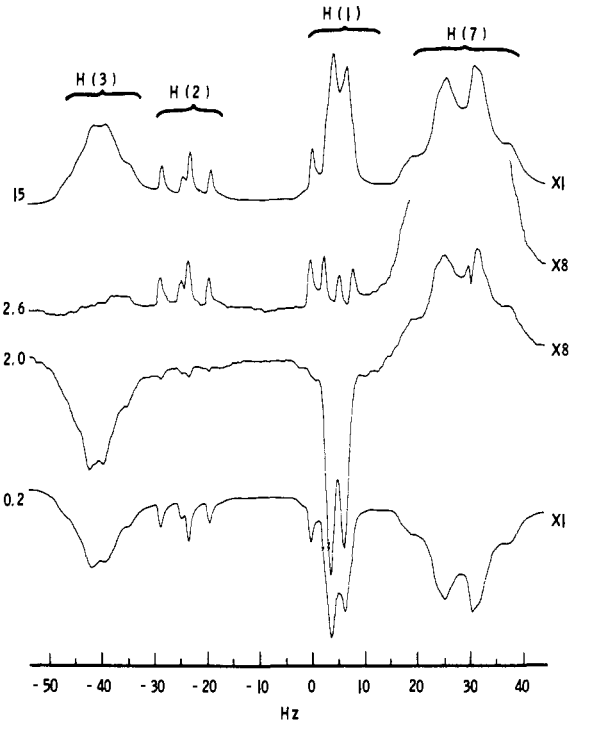


Figure 4. Partially relaxed proton FT NMR spectra of the epoxide region of the peracid product mixture in CCl_4 at 99.5 MHz and 28 °C. The sample was not degassed. The number to the left of each spectrum is τ in seconds. Each spectrum is the result of 16 accumulations with a repetition time of 15 s. The relative vertical expansion factors are given to the right of each spectrum.

Analysis of the Peracid Epoxidation Product. In order to compare directly the enzymatic and peracid epoxidation processes, the monoepoxidation product of *trans,trans*-1,8-dideuterio-1,7-octadiene was synthesized using *m*-chloroperbenzoic acid. NMR analysis showed that the starting material used in this synthesis consisted of 78% of the deuterated and 22% of the fully protonated 1,7-octadiene. Partially relaxed proton FT NMR spectra of a nondegassed sample of the peracid epoxidation product in CCl_4 were obtained. The accumulation conditions were the same as for the previous samples, except that because of the short relaxation times of the sample, a recycle time between pulse sequences of 15 s was used. The epoxide region is shown in Figure 4. The relevant multiplets

are cleanly isolated even though the dominant proton relaxation mechanism in this sample is intermolecular dipolar interaction with dissolved oxygen. The signal to noise of the isolated multiplets is lower than that of Figure 3 because the difference in null times is small.

The H(1) doublet of I is isolated at $\tau = 2.0$ s. The quartet due to H(1) of the fully protonated epoxide III is isolated at $\tau = 2.6$ s. There is no indication of an H(2) doublet arising from a cis-deuterated epoxide. Therefore, by comparison of Figures 2 and 4, we conclude, without making any assumptions whatsoever regarding spectral assignments, that the enzymatic epoxidation reaction occurs predominantly with inversion of the original double bond configuration, while peracid epoxidation occurs, as expected, with complete retention of configuration.

The deuterium content of the epoxide and olefin regions was computed in the same manner as discussed for the enzymatic product and the results showed that 80% of the epoxidation product and 78% of the olefin reactant were deuterated. Therefore, within experimental error, no significant secondary deuterium isotope effect could be detected for the peracid epoxidation reaction using this technique.

Control Experiments for Epoxide Product Isomerization. Conceptually, it might be postulated that nonenzymatic isomerization processes occur under the conditions of our experiments which could complicate or invalidate our results. It is clear from the completely trans configuration of the peracid-generated epoxide that such processes do not occur during the product isolation steps (preparative gas chromatography) or NMR analysis, and the completely trans configuration of the olefin portion in enzymatically generated 7,8-epoxy-1-octene clearly establishes that no isomerization of the olefin functionality occurs during the enzymatic reaction or subsequent steps. As far as possible isomerization of the newly enzymatically generated epoxide functionality in the resting cell suspension is concerned, it could be argued that it is extremely unlikely such a process would account for the net inversion of configuration we observe and, at most, might be responsible for our seeing 70% cis rather than, say, 90–100% cis configuration in the enzymatically produced epoxide functionality. If this were the case, our major mechanistic conclusion that a concerted mechanism of oxygen addition is untenable would be even stronger. However, despite these arguments we proceeded to run the following control experiments in order to unequivocally demonstrate that isomerization of the epoxide functionality does not occur under the conditions of our experiments.

(1) The peracid epoxidation product from *trans,trans*-1,8-dideuterio-1,7-octadiene, which we have demonstrated above (Figure 4) to contain *only* the trans deuterate epoxide functionality, was incubated with a resting cell suspension of *P. oleovorans* under conditions identical with those used for enzymatic epoxidation of deuterated octadiene. Extraction of the reaction mixture with ether followed by preparative gas chromatography allowed reisolation of the monoepoxide, which was then analyzed by NMR as described above. The reisolated compound exhibited no isomerization of either the epoxide or olefin functionalities, the NMR spectrum after incubation being identical with that of the starting material.

(2) Starting from pure samples of each of the isomeric internal olefins *cis*- and *trans*-3-hexene, the compounds *trans*-3,4-epoxyhexane and *cis*-3,4-epoxyhexane were synthesized by *m*-chloroperbenzoic acid epoxidation. A series of NMR spectra on various mixtures of the two epoxides was obtained and it was found that the presence of as little as 5% of the cis epoxide in the presence of *trans*-3,4-epoxyhexane could easily be detected. A sample of the trans epoxide was then incubated with a resting cell suspension of *P. oleovorans* under conditions identical with those previously used for epoxidation of deuterated octadiene, and the compound was then reisolated and purified by preparative gas chromatography. Once again, no isomerization of the trans epoxide was detected by careful NMR analysis.

Discussion

The results of this study establish that the configuration about the double bond in a simple olefin substrate is not maintained in the epoxide product isolated after reaction with the *P. oleovorans* enzyme system. Simple mechanisms involving the concerted addition of an electrophilic activated oxygen species do not predict such a result. Thus, our results are consistent with the conclusion that a straightforward "oxenoid" mechanism in the usual sense of the term (i.e., comparable to that operative in peracid, and possibly in transition metal oxo complex, epoxidations) is not operative in this system, although it is certainly conceivable that acceptable complex variations of such a mechanism (e.g., those involving generation of transitory intermediates with altered configurations *at the active site*) could be formulated.[46] In line with this conclusion, we have recently noted that the chemical reactivity patterns observed with the *P. oleovorans* system are far different than those observed in peracid epoxidations.[25]

It should be kept in mind that our results were obtained with resting cells, and the possible complications inherent in mechanistic analysis of such data must be considered. For example, it might be postulated that some of the isolated epoxide product is generated from octadiene by enzymes other than the "epoxidation/hydroxylation" system and these other enzymes may operate via a different mechanism. In several years of detailed reactivity, specificity and stereochemical studies using whole cells, cell free systems and purified enzymes, we have obtained no evidence for such mechanistic diversity.[47] However, even if this hypothetical situation were to exist in *P. oleovorans*, the basic conclusion that a simple concerted mechanism of oxygen addition does not predict the *major* (cis) product we obtain would still hold, since isomerization *after* product release is eliminated by our control experiments.

A second conceivable complication in this system involves the possible existence of isomerization processes which might not have been eliminated by our control experiments. For example, a specific "isomerase" could be present which functions in concert with the "epoxidase" and isomerizes only newly formed epoxides *before* their release into free solution. Although we cannot eliminate this possibility with certainty, we know of no precedent for such a situation, and it seems unlikely that such an "isomerase" would account for the net *inversion* of configuration we observe. Furthermore, we have shown elsewhere that the epoxides produced by these whole cells are opti6ally active with a very high degree of optical purity, and also that the stereochemical configuration of a preformed epoxide functionality does not undergo significant isomerization during diepoxidation by whole cells.[22-24,48] These results are not easily reconciled with the existence of such an "isomerase".

With the above caveats in mind, it is possible to make tentative evaluations of other mechanistic proposals by considering the lack of configurational retention reported here together with the highly directional attack of oxygen at the incipient asymmetric carbon (C-2) required by our stereochemical results.[22-24,48] For example, a two-step mechanism in which the activated oxygen species attacks at one of the olefin carbon atoms from a preferred direction to generate a carbonium ion or radical intermediate, which subsequently closes to the epoxide product, might be suggested. Indeed, species such as "HO·" or "HO^+" have often been considered as possible candidates for "activated oxygen" in oxygenase reactions.[42] If all of the epoxide product we isolate is generated

via one such pathway, then, of the four possible variants of such a mechanism, only prior attack at C-2 predominantly from the *si-re* face[43] to generate the intermediate, followed by closure before or after C–C bond rotation, would give both cis and trans epoxide of the correct stereochemistry. Alternatively, if multiple epoxidative pathways are indeed operative in the whole cell system, then a combination of concerted addition (giving the minor trans product) plus the two-step process initiated by *si-re* attack at C-2 and followed by closure after rotation (giving the major cis product) can account for our results, as do other similar combinations. Another interesting possibility is that a diol intermediate is formed first, and the predominance of cis deuterated product reflects the preference of the closure step for the trans orientation of attacking and leaving hydroxyl groups attained only after bond rotation. A number of variations of this mechanism involving either erythro or threo glycols and displacement of either the C-1 or C-2 hydroxyl can be envisioned and the data in hand are insufficient to distinguish between them. It is interesting to note that a cis 1,2-diol has been shown to be formed from naphthalene by an oxygenase system of *P. putida*.[44] We have obtained no evidence to date that glycol intermediates are formed during epoxidation by the *P. oleovorans* system, but it is conceivable that such a species may be formed and remain bound at the active site. Epoxides have been identified as obligatory intermediates in the conversion of simple aliphatic olefins to glycols by liver microsomes.[45,46]

Although it is evident that the detailed pathway of this enzymatic oxygenation reaction has not yet been fully defined, the results of this study have provided information which must be taken into account in formulating mechanistic proposals and in evaluating potential model systems. The question of whether the mechanism of oxygen activation operative in this system is an unusual one or is, in fact, representative of other monooxygenase systems as well must await further experimental data. However, the *P. oleovorans* system differs from the hydroxylation systems of *P. putida*, liver microsomes, and adrenocortical mitochondria in that it does not apparently involve a cytochrome P-450 containing oxygenase. Further experiments designed to evaluate potential model systems for this enzymatic epoxidation reaction are currently in progress. It is important to note that regardless of mechanistic considerations, this enzymatic epoxidation system has considerable synthetic potential, since the high stereoselectivity, the inversion of olefinic geometry, and the unusual selectivity for terminal double bonds cannot be duplicated with known chemical epoxidizing agents.

With regard to the technique of partially relaxed FT NMR used in this study, it is important to note that we were able to *quantitatively* analyze epoxidation products even though their equilibrium NMR spectra were severely overlapped. In addition, we were able to confirm the assignments of the epoxide NMR signals on the basis of relaxation analyses, to look for deuterium isotope effects, and to substantiate that the reactant did not contain cis deuterated olefin. These results demonstrate that partially relaxed proton FT NMR offers a powerful means for quantitatively studying solutions of compounds with overlapping proton NMR signals.

The epoxidation product was ideally suited to the partially relaxed FT NMR technique because the relaxation times of the H(1), H(2), and H(4) protons in the deuterated analogues were much longer than the corresponding relaxation times in the fully protonated molecules. In addition, the H(1), H(2), and H(4) peaks had an upfield deuterium isotope shift of about 1.5 Hz in the deuterated analogues. This combination of factors enabled us to cleanly isolate the epoxide and olefin signals. In less favorable cases, the technique still is applicable; however, computer simulations of the partially relaxed spectra would be required. We are currently investigating the application of partially relaxed proton NMR to cases where clean isolation of multiplets cannot be achieved. We are also studying how the analysis is affected by a detailed description of the dipole-dipole relaxation mechanism.

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tadiene is equivalent to the *si-re* face of the trans deuterio substrate, and thus we have previously noted that formation of R(+) epoxide from octadiene requires attack at the *si-si* face (ref 22).
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(50) The validity of this assumption for our samples was checked in the following ways. In the first place, the intensities of the epoxide multiplets (which had been estimated from the intensities of the low field exposed lines arising from the fully protonated species) were plotted logarithmically vs. time and in every case these plots were linear. Furthermore, the effective relaxation times obtained from these plots were in good agreement with the values computed from the experimentally measured null times. In addition the calculated $I(0)$ values listed in Table I were used to predict the total intensities of the fully inverted epoxide multiplets, and these agreed within about 5-10% with the experimentally measured intensities.

Raman Spectroscopy of Uncomplexed Valinomycin. 1. The Solid State

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Abstract: The membrane-active antibiotic valinomycin has become an important model compound for studying selective ion transport in biological and synthetic membranes. This paper reports results of the first complete Raman spectroscopic study of uncomplexed valinomycin in the solid state. Splittings in the ester and amide C=O stretch regions of valinomycin samples recrystallized from *n*-octane, CCl_4, $CHCl_3$, $CH_3(CH_2)_2Cl$, CH_3COCH_3, or CH_3CN indicate a structure resembling that obtained by x-ray crystallography. However, valinomycin recrystallized from *o*-dichlorobenzene and *p*-dioxane exhibits a considerably different structure. Comparison is made with the Raman spectra of model compounds in order to facilitate the identification of valinomycin vibrations. These results are extended to valinomycin solutions in an adjoining publication.

The antibiotic valinomycin (VM) produced by the bacteria *Streptomyces fulvissimus* is known to complex with alkali cations selectively[2] in the order $Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$ and to facilitate the transport of alkali cations across mitochondrial membranes with the same selectivity.[3a] Similar results have been obtained in model membrane systems.[3,4] Studies of valinomycin analogues[2] emphasize the importance of structural factors in complex formation, induced ionic permeability, and antimicrobial activity.

Valinomycin is a 12-membered macrocyclic depsipetide in which L-valine, L-lactic acid, D-valine, and D-hydroxyisovaleric acid (HIV) are alternately joined by amide and ester linkages.[5] The primary structure of VM (Figure 1a) suggests how it can facilitate the transport of cations across otherwise impermeable lipid barriers. There are 12 polar C=O groups, some of which can form structurally stabilizing intramolecular hydrogen bonds with the NH groups of the valine subunits, while others are free to bind a cation at the water/lipid interface via ion-dipole interactions. The nonpolar isopropyl and methyl residues can shield the hydrophilic C=O coordinated cation to facilitate its diffusion through the hydrophobic regions of membrane interiors. This structure for the K^+-VM complex has been verified by x-ray crystallography[6,7] and inferred from NMR studies of VM solutions.[2,8,9] It has recently been studied in both the solid state and in solution by laser Raman spectroscopy.[10]

The membrane activity of VM suggests that understanding its complexation mechanisms would greatly further our knowledge of the molecular basis of selective ionic permeability in biological systems. An important first step is the elucidation of the conformations assumed by uncomplexed VM, i.e., the states from which complex formation can be initiated. These states have been the subject of numerous recent investigations utilizing x-ray diffraction,[11-14] infrared spectroscopy,[2,15] nuclear magnetic resonance,[2,8,15-17] circular dichroism,[15] and optical rotary dispersion.[2]

In a preliminary paper[18] we reported the detection of two different forms of VM in the solid state, based on Raman spectroscopic observations in the 1600-1800-cm^{-1} region. We here present the first complete Raman spectra (150-3600 cm^{-1}) of uncomplexed VM recrystallized from several polar and nonpolar solvents and attempt to interpret these results in terms of VM conformations. This work also provides a basis for an accompanying Raman study of VM in solution[19] and a recent study of the VM-KSCN complex.[10]

Materials and Methods

(a) Materials. Valinomycin powder was obtained commercially from Calbiochem, San Diego, Calif. The antibiotic was prepared by the method of MacDonald and Slater,[20] the last stage of which involves slow recrystallization from warm *n*-octane. The resulting white powder is freely soluble in CCl_4, $CHCl_3$, dioxane, and acetone, somewhat soluble in hydrocarbons, and practically insoluble in water. Calbiochem also made available several larger (~1 mm^3) translucent VM crystals; these are monoclinic[12] with a space group of $P2_1$. The crystals readily cleave into thin platelets. We also recrystallized VM samples from a variety of polar and nonpolar solvents in loosely sealed Kimax capillary tubes. The model compounds D- and L-valine, L-lactic acid (lithium salt), D-α-hydroxyisovaleric acid, and poly-L-valine were also obtained from Calbiochem.

(b) Raman Spectroscopic Methods. In Raman spectroscopy, one