Hydroxylation of Deuterated 1,4-Cineoles by *Bacillus cereus*

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The stereochemistry and mechanism of 1,4-cineole hydroxylation by the cytochrome P-450-containing strain of *Bacillus cereus* UI-1477 was examined using specifically deuterated substrates. Stereospecifically deuterated 1,4-cineoles were synthesized by Wolff–Kishner reductions of enantiomeric 2-oxo-1,4-cineoles, and by Barton–McCombie elimination of the alcohol functional group of 2-hydroxy-2-deutero-(1*R*,4*S*)-cineole. Products obtained from hydroxylations of 2-deutero-(1*R*,4*S*)-cineole and enantiomeric 2,2,3,3-tetradeutero- and 2,2,3-trideutero-1,4-cineoles were isolated and characterized by gas chromatography/mass spectrometry and by ^{1}H- and ^{2}H-NMR spectroscopy. Deuterium at the site which is oxygenated does not change hydroxylation stereospecificity, but it dramatically shifts the relative ratios of 2-*endo*- and 2-*exo*-alcohol products formed. Analysis of cineole alcohols obtained by microbial hydroxylation of multiply-deuterated-1,4-cineoles indicated that the starting materials contained deuterium at both the 2- and 6-positions, suggesting that a 2,6-proton migration occurs during the Wolff–Kishner reduction of enantiomerically pure 2-oxo-1,4-cineoles. Ratios of cineole alcohols formed from monodeuterated-1,4-cineoles reveal the likehood of a two-step mechanism involving nonspecific hydrogen atom removal followed by stereospecific hydroxylation.

Introduction

Bacillus cereus UI-1477is an interesting new biocatalyst containing a cytochrome P-450-dependent monooxygenase system that catalyzes the stereospecific hydroxylation of 1,4-cineole (1) to give enantiomerically pure 2(*R*)-*exo*- (2) and 2(*R*)-*endo*-hydroxy-1,4-cineole (3) products in high yield[1–4] (Scheme I). In this reaction, *endo*-hydroxylation is preferred over *exo*-hydroxylation by a factor of 7.5, and the high face specificity observed has been explained by invoking a single enzyme model.[3,4]

The selective hydroxylation of nonactivated hydrocarbons is a challenging and fascinating goal in synthetic chemistry. Cytochrome P-450-type enzymes were the first biocatalysts known to achieve such reactions with high selectivities and yields. For this reason, the mechanism of P-450-catalyzed hydroxylation reactions has been the subject of extensive study, and features of the catalytic cycle, the process of electron transfer, and the mechanism of oxygen activation have been well examined.[5] However, the process involving C–H bond activation, and the transfer of oxygen from the enzyme to the substrate are still not completely understood. One of the most common approaches used to elucidate the mechanism of oxygenation reactions is to use specifically deuterated substrates to measure possible deuterium isotope effects of the hydroxylation reaction.[6–10] In this paper, we describe the synthesis of stereospecifically deuterated 1,4-cineoles and the regio- and stereochemistries of their hydroxylations by the cytochrome P-450 monooxygenase enzyme system in *B. cereus*.

Scheme I

1 → 2 + 3

3 R=H
3a R = S(+)-O Acetylmandelate

Results and Discussion

^{1}H-NMR Analysis of 1,4-Cineole. Since a major objective of this work was the synthesis of specifically deuterated 1,4-cineoles, it became necessary to clearly assign proton and deuterium signals of the parent monoterpene so as to permit confirmation of the sites and stereochemistries of deuterated compounds. The chemical shifts of the proton signals for methyl and isopropyl groups in 1,4-cineole are easily assigned to δ 1.44, 2.06, and 0.96 ppm, and the ^{13}C-NMR methylene carbon resonances for positions 2,6 and 3,5 were at 37.42 and 33.24 ppm, respectively. These assignments were independently confirmed by the measured decrease in intensity of the

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Table I. Paramagnetic-Induced Shift of Proton NMR Signals of 1,4-Cineole Compared with the Distance of Protons to Oxygen

functional group	distance from oxygen, Å	Δppm/equiv of Eu(fod)
methine	2.08	5.42
methyl	2.80	3.86
2-*exo*-H	2.82	3.27
isopropyl	3.13	2.32
2-*endo*-H	3.81	2.11

signal at 37.42 ppm by the introduction of deuterium at the 2-position. Chemical shifts of protons attached to these methylene carbons were assigned by the application of carbon–proton 2D hetero-COSY NMR analysis. The *exo*- and *endo*-protons at the 3,5-positions occur as an inseparable doublet at 1.59 ppm. However, the *endo*- and/or *exo*-protons at the 2,6-positions give well-separated doublet of doublet signals at 1.66 and 1.52 ppm, respectively. The assignments for *endo*- and *exo*-protons were based on the differences in paramagnetic induced shifts of these proton signals upon addition of the metal complex, [Eu(fod)]. Paramagnetic induced shifts have been used to confirm assignments of proton NMR signals for structures similar to 1,4-cineole such as the derivatives of cylcohexane,[11] bornane,[12] and 1,8-cineole.[13]

In 1,4-cineole, the only hetero atom that can coordinate with an NMR shift reagent[14] is the bridge oxygen. The extent of the shift of proton signals upon addition of a shift reagent depends upon the distance of various protons to the bridge oxygen atom. Using the SYBYL molecular modeling program, the three-dimensional structure of 1,4-cineole was built, and the distance of every proton to the oxygen atom was measured. Since the proton-to-oxygen distance of the isopropyl group in 1,4-cineole depends upon the rotation of the σ–σ bond between carbons 4 and 8, the conformation which favored the closest distances between these atoms was used.

Proton NMR spectra of 1,4-cineole were recorded using different concentrations of deuterated Eu(fod) (Table I). The clearly assigned proton signals for the methyl and isopropyl groups served well as internal standards for the experiment. The proton signal at 1.52 ppm of unlabeled 1,4-cineole experiences a larger ΔEu (Δppm/mol equiv of Eu(fod)) than that at 1.66 ppm and was therefore assigned to the 2-*exo*-proton. The latter signal at 1.66 ppm was then assigned to the 2-*endo*-proton. Good correlation exists among ΔEu and the distances of all of the protons to the bridgehead oxygen atom, supporting the chemical shift assignments for the 2-*endo*- and -*exo*-protons.

Synthesis and Analysis of Deuterated 1,4-Cineole Substrates

Since the hydroxylation of 1,4-cineole by the P-450 system in *B. cereus* occurs predominantly at the pro-2*R* position,[3] several methods were investigated for the purpose of preparing 1,4-cineole stereospecifically deuterated at position-2. The reduction of enantiomeric 2-oxo-1,4-cineoles (4 and 7) under rigorous Wolff-Kishner reduction conditions using completely deuterated solvents and reagents gave reasonable yields (60%) of deuterated 1,4-cineoles (Figure 1). As a result of the basic conditions of the Wolff–Kishner reduction reaction, deuterium atoms were introduced at the site of the ketone and also at the adjacent methylene position.

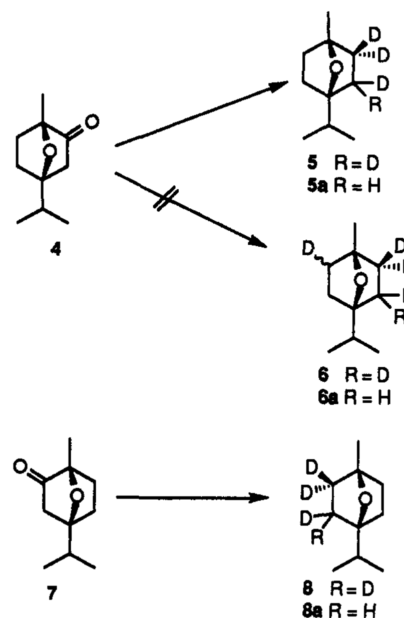

Figure 1. Specifically labeled (1*R*)- and (1*S*)-tetra- and trideutero-1,4-cineoles obtained by Wolff–Kishner Reduction of enantiomeric 2-oxo-1,4-cineoles 4 and 7.

Mass spectral analysis indicated that the products of the reduction reactions were mixtures of tri- (**5a** and **8a**) and tetradeuterated-1,4-cineoles (**5** and **8**). There were no *m/z* 160 or *m/z* 159 peaks in the mass spectra of these compounds, indicating the absence of penta- or hexadeuterated products due to homoenolate formation[15] during the Wolff–Kishner reduction reaction. The location of deuterium in the substrates can be inferred from the regiochemistry involving 2-oxo-1,4-cineole starting materials and confirmed by ^{13}C-NMR analysis of the deuterated products. In the ^{13}C-NMR spectra of the deuterated compounds, signals for carbons 2,6 and 3,5 were reduced to one-half the intensities measured for unlabeled 1,4-cineole. The reduction of intensities of the peaks for carbons bearing deuterium atoms in the proton decoupled NMR spectrum occurs because of a combination of coincidental phenomena including longer relaxation times of C–D, increased carbon multiplicities due to deuterium coupling, and the concomitant loss of nuclear Overhauser effect (NOE).[16] If the optical purities of 2-oxo-1,4-cineoles (4 and 7) are assumed to be the same as terpinen-4-ols[17] used in their syntheses, their values and those for the deuterated 1,4-cineoles **5**, **5a**, and **8**, **8a** are 96.9 and 97.3%, respectively. Thus each deuterated product contains a small percentage of up to 1.57% of the contaminating enantiomer. The measured optical purities of (*S*)-(+)-*O*-acetylmandelate esters of 2-*endo*-hydroxy-1,4-cineole products obtained from the enantiomeric ketones confirm this assumption[3] and enable an unambiguous confirmation of the enantiomeric purities of deuterated-1,4-cineole substrates.

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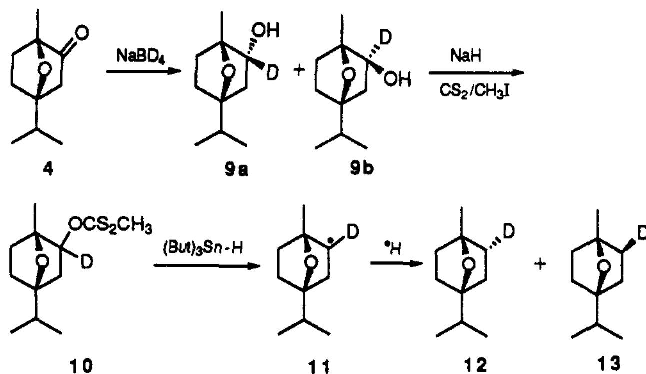

Figure 2. Synthesis of monodeuterated 1,4-cineoles **12** and **13** by the Barton–McCombie reaction.

The Wolff–Kishner method successfully incorporated deuterium at the desired sites. However, the presence of mixtures of multideuterated 1,4-cineoles could pose potential difficulties in interpreting expected deuterium isotope effects during microbial hydroxylation experiments. Therefore, different asymmetric routes were explored to introduce single deuterium atoms into 1,4-cineole.

Barton and McCombie described a free radical process for the reductive deoxygenation of secondary alcohols,[18] and applications of the reaction have been extensively reviewed.[19] The reaction is more resistant to rearrangements in many stringent ring systems such as sugars, steroids, and triterpenes[20] because it does not involve ionic intermediates. When applied to 1,4-cineole, the presumed carbon radical 11 (Figure 2) would most likely be quenched by *exo* approach of the hydrogen atom derived from tributyltin hydride.[21]

Enantiomerically pure 2-oxo-(1*R*,4*S*)-cineole (**4**) (Figure 2) was reduced with $NaBD_4$ to afford 2(*R*)-*endo*-hydroxy-*exo*-deutero-(1*R*,4*S*)-cineole (**9a**) containing 2% of 2(*S*)-*exo*-hydroxy-*endo*-deutero-(1*R*,4*S*)-cineole (**9b**). Since the deoxygenation step to follow likely involves a planar free-radical intermediate, and the conformation of the hydroxyl group at the 2-position has no effect on the stereochemistry of the product, the small amount of *exo*-alcohol was not separated from the *endo*-isomer. Derivatization to the corresponding methyl dithiocarbonate **10** and reduction with tributyltin hydride afforded an overall 95% yield of monodeuterated 1,4-cineoles (**12** and **13**). The mass spectrum of **12** and **13** gave m/z 155 for monodeutero-1,4-cineole (>98% incorporation). The proton-NMR spectrum was identical to unlabled 1,4-cineole with a slight decrease in the relative intensity of the peak at 1.65 ppm, attributed to the replacement of one of the position-2 protons by a deuterium atom. The intensity of the signal for carbons 2,6 in the ^{13}C-NMR spectrum of **12** and **13** was reduced by one-half and a low-intensity triplet at 37.28 ppm (J = 19.88 Hz) was detected and assigned to the deuterated 2-carbon. The deuterium-NMR spectrum of **12** and **13** gave peaks at 1.66 ppm (91% relative intensity) and at 1.53 ppm (9% relative intensity), which were assigned to 2-*endo*- and 2-*exo*-deuterium atoms of **12** and **13**, respectively. These assignments correlate well with 2-*endo*- and 2-*exo*-proton signals identified by Eu(fod) NMR shift reagent studies of unlabeled 1,4-cineole.

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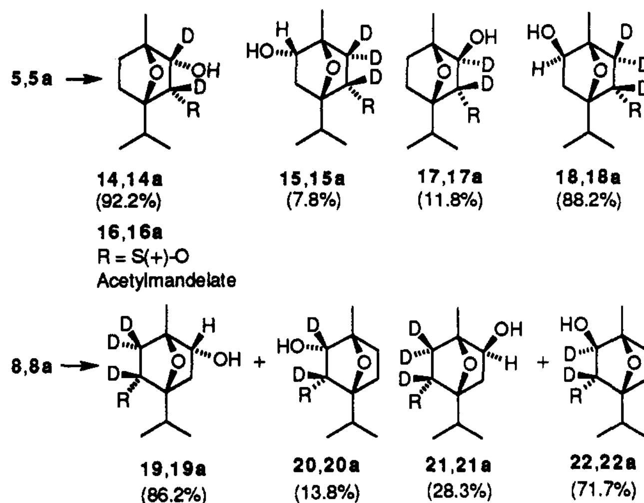

Figure 3. Products obtained in the *B. cereus* hydroxylation of tetra- and trideuterated-1,4-cineoles **5**, **5a** and **8**, **8a**.

The monodeuterated 1,4-cineole product mixture was thus identified as 2(*R*)-*endo*-deutero-(1*R*,4*S*)-cineole (**12**) containing 9% of the 2(*S*)-*exo*-deutero isomer (**13**). Deuterium was introduced into 1,4-cineole in an unambiguous way. The Barton–McCombie deoxygenation reaction involves a free-radical intermediate which could have resulted in a possible 6,2-hydrogen shift. However, unwanted deuterated isomers cannot be obtained in the present reaction because the carbon–deuterium bond introduced at the 2-position by $NaBD_4$ reduction of the ketone remains intact. The completely characterized mono- and multiply-deuterated 1,4-cineoles prepared in this work were used as substrates in probing the mechanism of hydroxylation by *B. cereus*.

Microbial Transformation of Deuterated 1,4-Cineoles

In our original work,[3] alcohols **2** and **3** obtained by microbial hydroxylation of unlabeled 1,4-cineole were the enantiomerically pure *R*-alcohols, as determined by NMR spectral analyses of the corresponding (*S*)-(+)-*O*-acetylmandelate esters. Microbial transformations of multiply deuterated cineoles (**5**, **5a**, **8**, **8a**) gave *endo*- and *exo*-deuterated metabolites which were analyzed by GC/MS. Since the enantiomeric purities of deuterated substrates were clearly established and the cineole alcohols gave no complicating $M^+ - 1$ fragment ions by mass spectrometry, we initially thought that hydroxylation stereochemistries could be established simply by measuring relative peak intensities representing tetra- and trideuterated cineole alcohols. The mass spectral results strongly suggested that mixtures of *endo* and *exo* enantiomers (Figure 3) were obtained with the deuterated substrate and that the presence of deuterium perturbed the normally high face specificity displayed by the P450 enzyme system of *B. cereus* in 1,4-cineole hydroxylations.

The deuterated 2-*endo*-hydroxy-1,4-cineole mixture obtained from **5**, **5a** was derivatized with (*S*)-(+)-*O*-acetylmandelic acid and the diastereomeric esters **16**, **16a** were subjected to proton NMR spectral analysis. The strong methyl group signal at 1.40 ppm with an almost undetectable peak (<1%) at 1.17 ppm[3] clearly indicated

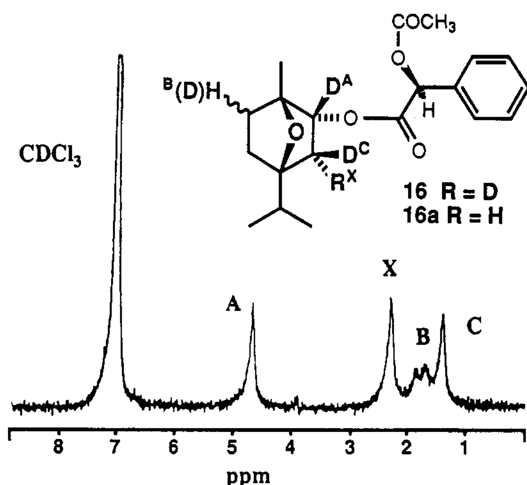

Figure 4. Deuterium-NMR spectrum of 2(*R*)-*endo*-hydroxytetradeutero-(1*R*,4*S*)-cineole (**16**) and 2(*R*)-*endo*-hydroxytrideutero-(1*R*,4*S*)-cineolyl (*S*)-(+)-*O*-acetylmandelate (**16a**).

that the *endo*-alcohols were the enantiomerically pure deutero-2(*R*)-*endo*-hydroxy-(1*R*,4*S*)-cineoles (**14, 14a**), and that less than 1% of the enantiomeric *endo*-cineoles **15, 15a** were present. The deuterium NMR spectrum (Figure 4) of **16, 16a** exhibited three major deuterium signals of similar intensity at 4.70, 2.08, and 1.10 ppm, assigned to the *exo*-deuterium atom at the 2-position and the two deuterium atoms at the 3-position. Two smaller signals at 1.58 and 1.40 ppm were assigned to *exo*- and *endo*-deuterium atoms attached to the 6-position as follows.

Deuterium and protium attached at the same carbon have essentially identical NMR chemical shifts. With standard 2(*R*)-*endo*-hydroxy-(1*R*,4*S*)-cineole (*S*)-(+)-*O*-acetylmandelic ester (**3a**),[3] proton signals for the methyl and isopropyl groups, the 2-*exo*-proton, and those from the (*S*)-(+)-*O*-acetylmandelic acid portion were easily assigned. The chemical shifts of the protons on the three remaining methylene carbons were resolved and assigned by a 2D-carbon–proton hetero-COSY experiment as follows: position-3 (2.07 and 1.09 ppm), position-6 (1.58 [H-*exo*], 1.40 [H-*endo*] ppm), and position-5 (2.13 and 1.43 ppm). The signals for position-3 were also present in the deuterium-NMR spectrum of the hydroxylated product (**14, 14a**). Those for *exo* and *endo* signals of position-6 were confirmed by a 2D proton–proton COSY experiment in which a low-intensity correlation between the two proton signals at 1.58 and 4.71 ppm was detected and assigned to W-type long-range coupling of the 6- and 2-*exo*-protons.[22] Similar results are assumed to occur with the *exo*-hydroxy metabolites and with hydroxylated products obtained from **8, 8a**. Since the amounts of these metabolites were limited a complete isolation and characterization of them was not possible.

The structure of the deuterated 1,4-cineole (*S*)-(+)-*O*-acetylmandelic ester was thus confirmed as **16,16a**, in which about 10% of the molecular population possesses an additional deuterium atom at position-6, with either *endo* or *exo* configuration. This NMR-spectral evidence also confirmed the stereochemistry of 1,4-cineole hydroxylation by *B. cereus* as pro-2*R* specific for both deuterated and unlabeled substrates. However, the presence of deuterium at the 6-position was unexpected. Mass spectrometry can only indicate the number of deuterium atoms present in such products. If for some reason the deuterium atom migrates to another position during the processes of synthesis and/or enzymatic hydroxylation, the mass spectrum would not be able to distinguish the difference.

Although the enantiomeric specificity remained the same, the regioselectivity of deuterated substrate hydroxylations changed dramatically. With **5, 5a** (Figure 3) *exo*-hydroxylation was favored over *endo*-hydroxylation by a factor of 1.06:1. However, with **8, 8a**, *endo*-hydroxylation was favored over *exo*-hydroxylation by a factor of 55.6. Those are in great contrast to the 7.5:1 ratio of *endo*/*exo* alcohols formed with unlabeled 1,4-cineole and indicate that the presence of deuterium greatly influences the face specificity of 1,4-cineole hydroxylation. In our experiments, metabolic switching[6–9] was clearly exhibited by the changes in *endo*/*exo* alcohol product ratios by factors of 7–8.

The results obtained from both mass spectral and NMR spectral analysis of the (*S*)-(+)-*O*-acetylmandelate ester (**16,16a**) strongly indicate that the products obtained by microbial hydroxylation of tetradeutero- (**5**)- and trideutero-(1*R*,4*S*)-cineole (**5a**) (Figure 3) are almost exclusively *endo* (**14, 14a**) and *exo* (**18, 18a**) metabolites and from tetradeutero (**8**)- and trideutero-(1*S*,4*R*)-cineole (**8a**), the mixture of *endo* (**19,19a**), and *exo* (**22,22a**) metabolites. However, each *endo* and *exo* product also contained another metabolite containing between 8–12% deuterium in the 6-position.

The presence of more than 10% deuterium at the 6-position can be explained only if 2,6-deuterium migration occurred during Wolff–Kishner reductions of the chiral ketones. Migration of hydrogen or deuterium has been reported for many compounds subjected to different chemical treatments.[23,24] The most related instance reports a 15% 6,2-hydride (or deuteride) shift in norbornyl cations.[25] The mechanism of Wolff–Kishner reduction[26,27] involves initial generation of a hydrazone (**23**) (Figure 5) which is reduced in the presence of strong base. Enolization of the hydrazone and addition of the first deuterium atom from solvent (forming **24**) is followed by elimination of nitrogen, leaving a 1,4-cineole anion (**25**). Most of **25** is quenched by deuterium from solvent to give **28**. However, it is possible for the p-orbital of the sp^2 anion of **25** to overlap with the σ-orbital of the 6-*endo*-proton resulting in carbanion delocalization to give **26** which can be quenched by deuterium from solvent to give **27**. Although the enantiomeric tetra/trideuterated cineoles **5, 5a** and **8, 8a** were fully characterized, they have identical chemical and spectral properties and would not be distinguishable, except by a stereospecific manipulation such as microbial hydroxylation. Since it was impossible to determine whether the apparent deuterium migration had occurred via Wolff–Kishner reduction or during enzymatic hydroxylation, it became necessary to analyze the hydroxylation products obtained from another specifically monodeuterated 1,4-cineole substrate.

Preparative scale fermentations of monodeutero-1,4-cineole isomers (**12, 13**) with *B. cereus* were used to obtain (Figure 6) pure 2(*R*)-*endo*-hydroxy-(1*R*,4*S*)-cineoles (**30,**

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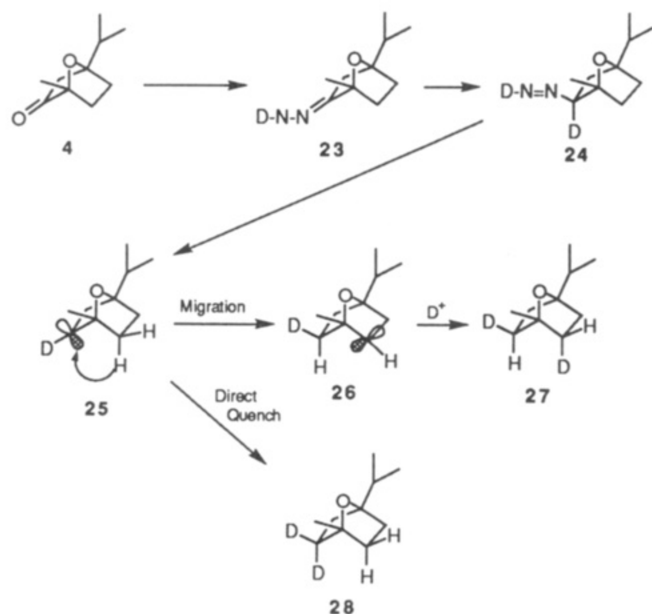

Figure 5. Proposed mechanism of Wolff–Kishner reduction of 4 accounting for the introduction of deuterium at position-6 of 27.

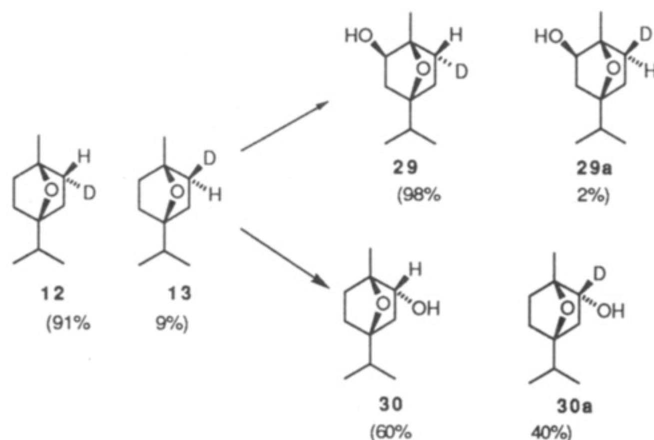

Figure 6. Microbial hydroxylation of monodeuterated 1,4-cineoles 12 and 13 by *B. cereus*.

30a) and 2(*R*)-*exo*-hydroxy-(1*S*,4*R*)-cineoles (**29, 29a**). The deuterium NMR spectrum of the (2*R*)-*exo*-alcohols (**29, 29a**) shows a single deuterium signal at 1.44 ppm, assigned to the 6-*endo*-deuterium atom as in **29**. The lack of an NMR signal at 3.78 ppm indicated that deuterium was not on the carbinol position. The near absence of **29a** by deuterium NMR indicates that **13** had been almost completely consumed during *endo*-hydroxylation. Preferential *endo*-hydroxylation of 2(*S*)-*exo*-deutero-(1*R*,4*S*)-cineole (**13**) is expected based on our previously described enzyme model[4] and accounts for the more ready microbial hydroxylation of substrate containing hydrogen at the position being hydroxylated. The deuterium NMR spectrum of the *endo*-alcohol products **30, 30a** shows a single peak at 3.87 ppm which is assigned to the retained *exo*-deuterium atom at position-2. No additional deuterium signals could be detected in these spectra. The results rule out a 2,6-migration during enzymatic hydroxylation of **12, 13** and strongly support our proposal of a 2,6-shift of deuterium during Wolff–Kishner reduction of chiral 2-oxo-1,4-cineoles.

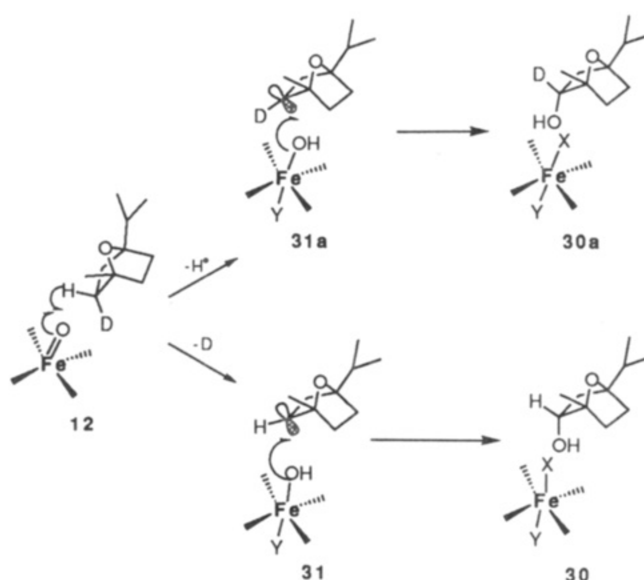

Figure 7. Proposed mechanism for nonspecific hnydrogen abstracrtion and subsequent stereospecific oxygenation of 1,4-cineole catalyzed by the cytochrome P-450 monooxygenase enzyme system of *B. cereus*.

GC/mass spectrometry clearly demonstrated that the ratio of *endo* to *exo* products obtained from monodeuterated 1,4-cineoles **12, 13** was 1.19/1, representing metabolic switching by a factor of nearly 7. Deuterium-NMR spectrometric analysis of the *exo*-alcohol products obtained with **12, 13** indicated that 2(*R*)-*exo*-hydroxy-(1*S*,4*R*)-cineole (**29**) was the major component with less than 2% (estimated detection limit) of **29a**. That was not surprising since **13** had been preferentially converted to **30a** through *endo*-hydroxylation. The *endo*-alcohols, on the other hand, gave ratios of 60 and 40% for isomers **30** and **30a**, respectively. The overall yield of **12, 13** hydroxylation was 92% (49.9% *endo*, 41.9% *exo*). Therefore, the amount of **30a** with deuterium in the 2-*exo*-methine position is greatly in excess of the theoretical amount (9%/49.9% = 18%) which could have been there. The excess of deuterium in **30a** can only arise through an enzyme hydroxylation mechanism in which initial hydrogen atom abstraction is nonstereospecific (Figure 7), followed by stereospecific hydroxylation at the enzyme active site. We believe that the substrate can orient itself at the enzyme active site to permit either *exo*-hydrogen or *endo*-deuterium abstraction to give mixtures of radical intermediates **31a** and **31** (Figure 7). It is reasonable to assume that the subsequent conformational change from an sp^3 radical to a planar radical intermediate would permit hydroxylation from the enzyme active site exclusively from *endo*-face in the second step. Similar reactions have been proposed to occur during hydroxylations of camphor catalyzed by P-450_{cam},[6] and bornylamides by the fungus *Beauveria sulfurescens*.[28]

Our results demonstrate the proficiency of the *Bacillus cereus* cytochrome P450 enzyme system to achieve hydroxylation reactions of a highly specific nature. Our findings are unlike those observed during the cytochrome P-450 catalyzed hydroxylations of norbornane,[10] camphor,[6] and norcamphor[9] in which hydroxylations proceed preferentially or specifically from the less-hindered *exo*-faces.

(28) Fourneron, J. D.; Archelas, A.; Furstoss, R. *J. Org. Chem.* **1989**, 54, 2478-2483.

Experimental Section

Instrumentation. Melting points are uncorrected. Boiling points were determined by distillation in a micro–micro short-path distillation apparatus under atomspheric pressure. Optical rotations were measured using a 0.998-cm length micro cell.

Low-resolution mass spectra or gas chromatography-mass spectra (GC-MS) were obtained at an ionization voltage of 70 eV. GC-MS was achieved on an OV-1, 3% column (6 × 1/4 in. o.d., mesh 80–100) with a helium carrier gas flow of 20 mL/min, and temperatures for injector, column, and GC transfer line which interface with the mass spectrometer were held at 200°C, 90 °C, and 220 °C, respectively.

^{1}H-NMR paramagnetic shift reagent experiments were conducted by dissolving 1,4-cineole (0.5 mmol) in 360 μL of $CDCl_3$ with 1% (w/w) TMS in a 5-mm NMR tube. Proton-NMR spectra were recorded after the addition of 0, 0.005, 0.01, 0.015, 0.02, and 0.025 mmol of Eu(fod), respectively. The chemical shifts of specific protons in each spectrum were measured and compared to the molar equivalents of NMR shift reagent added. The ΔEu (Δppm/mol equiv of Eu(fod)) for each proton signal is listed in Table I.

Chemicals. 1,4-Cineole was received from Shell Development Co., Modesto, CA. The identity of the compound was confirmed as follows: bp 165 °C (76 mmHg); ^{1}H-NMR assignments were made based on 2D H–H COSY, 2D C–H hetero-COSY, and NMR shift reagents [Eu(Fod)] as described: δ 2.06 (1H, seven peaks, J = 6.9 Hz, 8-H), 1.66 (2H, m, 2,6-*endo*-H), 1.59 (4H, m, 3,5-H), 1.52 (2H, m, 2,6-*exo*-H), 1.44 (3H, s, methyl), 0.96 (6H, d, J = 6.9 Hz, isopropyl); ^{13}C NMR δ 89.79 (1-C), 82.70 (4-C), 37.42 (2,6-C), 33.24 (3,5-C), 32.96 (8-C), 21.26 (7-C), 18.19 (9,10-C); mass spectra (70 eV) *m/z* (rel inten) 154 (M^+, 26.7), 139 (M – CH_3, 9.4), 125 (38.4), 111 (100), 96 (10.1), 93 (10.8), 86 (11.2), 83 (20.0), 81 (22.4), 71 (94), 69 (46.7).

Chromatography. Thin-layer chromatography (TLC) was performed on 0.25-mm thick layers of silica gel GF 254 (Merck) or alumina GF_{254} (Merck) prepared on 5 × 20 cm or 20 × 20 cm glass plates with a Quickfit Industries Spreader (Quickfit Industries, London, England). Chromatograms were usually developed with hexanes–ethyl acetate (4:1) and visualized by spraying with anisaldehyde. Column chromatography was performed on silica gel (Baker 3404-5, 40–140 mesh) or alumina (Fisher Scientific A-950, 80–200 mesh, activity grade II).

Gas chromatography (GC) was performed on an 3% OV-17, 100/120 mesh Supelcoport 6 ft × 0.25 in. i.d. glass column using a Hewlett-Packard 5890A gas chromatograph. Nitrogen was used as carrier gas (30 mL/min), and compounds were detected by flame ionization under the following conditions: injector temperature 120°C, ion detector temperature 180°C, initial oven temperature 80 °C for 5 min, increasing at 3 °C per min to 103 °C.

Fermentation Methods. *Bacillus cereus* UI-1477[3,4] was grown by the previously described[1] two-stage fermentation procedure using a soybean meal–glucose medium of the following composition: glucose 20 g, yeast extract 5 g, soybean meal 5 g, NaCl 5 g, K_2HPO_4 5 g, distilled water 1 L, pH adjusted to 7.0 with 6 N HCl immediately prior to sterilization. Incubations were conducted in 125-mL steel-capped DeLong culture flasks holding 25 mL of sterile medium.

Synthesis of (1*R*,4*S*)- and (1*S*,4*R*)-[2,2,3,3-D$_4$]- and [2,2,3-D$_3$]-1,4-Cineoles (5, 5a and 8, 8a). Reactions conducted to synthesize **5, 5a and 8, 8a** were conducted under identical conditions to give similar yields of products. Diethylene glycol (150 mL) was mixed with D_2O (99.8 D%, 100 g), and the mixture was distilled to remove water. The process was repeated three times to prepare deuterated diethylene glycol. KOH (40 g) was treated in the same way to prepare KOD. These deuterated compounds, tetradeuterohydrazine (MSD isotopes, 40 g), and the chiral ketone 2-oxo-(1*R*,4*S*)-cineole (4) (10 g, 0.059 mol) was heated with stirring at 115 °C for 30 min and then slowly heated to 180 °C over 4 h. The liquid which was distilled off (approx. 40 mL) was extracted with hexane (3 × 50 mL), the organic layer was washed with water (50 mL) and dried over $MgSO_4$, and the solvent was removed under reduced pressure. The product mixture was purified by flash chromatography over silica gel using hexane-ether (7:3) to afford 5.7 g (63% yield) of deuterocineoles **5, 5a**. Deuterocineoles **8, 8a** were obtained from **7** in 60% yield. The analyses of deuterocineoles were as follows.

Mixture **5, 5a**: ^{1}H-NMR ppm, 0.96 (6H, d, J = 6.71 Hz, isopropyl), 1.44 (3H, s, CH_3), 2.07 (1H, m, 8-H), 1.59 (m, 2-H, 3-H, 5-H, 6-H); ^{13}C-NMR ppm, 18.18 (C-9, 10), 21.21 (C-7), 33.15 (C-8), 33.66 (C-3, 5), 37.37 (C-2, 6), 82.91 (C-4), 89.66 (C-1); GC/MS, retention time 2.31 min, *m/z* (%rel abund) 159 (0.8), 158 (7.2), 157 (1.9), 125 (10.4), 115 (32.0), 114 (12.2), 73 (9.5), 72 (16), 71 (44.7), 43 (100); ratio of *m/z* 158/157 ion currrents over entire GC peak was measured as 3.508; composition of mixture is calculated (see supplementary material) as 77.26% tetradeutero-1,4-cineole (**5**) and 22.74% trideutero-1,4-cineole (**5a**).

Mixture **8, 8a**: ^{1}H-NMR ppm, 0.96 (6H, d, J = 6.86 Hz, isopropyl), 1.44 (3H, s, CH_3), 2.06 (1H, m, 8-H), 1.59 (m, 2-H, 3-H, 5-H, 6-H); ^{13}C-NMR ppm, 18.38 (C-9, 10), 21.41 (C-7), 33.11 (C-8), 33.24 (C-3, 5), 37.42 (C-2, 6), 83.05 (C-4), 89.79 (C-1); GC/MS, retention time 2.31 min, *m/z* (%rel abund) 159 (3.6), 158 (14), 157 (4), 143 (4), 129 (1.8), 128 (13.2), 127 (4.7), 116 (7.0), 115 (76.1), 114 (34.7), 113 (9.2), 73 (23.1), 72 (26.2), 71 (86.6), 70 (18.7); ratio of *m/z* 158/157 ion currents over entire GC peak was measured as 2.7872; composition of mixture is calculated as 72.8% tetradeutero-1,4-cineole (**8**) and 27.2% trideutero-1,4-cineole (**8a**).

Reduction of 2-oxo-(1*R*,4*S*)-cineole (4) with Sodium Borodeuteride. In a typical reaction 2-oxo-(1*R*,4*S*)-cineole (4) (4 g, 23.8 mmol) was dissolved in 50 mL of methanol-*d* (CH_3OD, 99.5+ atom % D), and a total of 5 g (120 mmol) of $NaBD_4$ (98 atom % D) was added with stirring, in five separate portions during a 1-h period. After stirring for an additional 40 min, the reaction mixture was filtered, concentrated to a small volume, diluted with 60 mL of water, adjusted to pH 6.5, and extracted with ethyl ether (4 × 40 mL). The ether extracts were combined, dried over Na_2SO_4, and concentrated to afford 4.0 g (98%) of oil which consisted (GC) of a mixture of 2(*R*)-*endo*-hydroxy-2-*exo*-deutero-(1*R*,4*S*)-cineole (**9a**) containing 2% of 2(*S*)-*exo*-hydroxy-2-*endo*-deutero-(1*R*,4*S*)-cineole (**9b**). ^{2}H-NMR (55.0 MHz, $CHCl_3$) ppm, 3.83 (s, 2-D); ^{1}H-NMR ppm, 1.40 (3H, s), 0.92 (6H, d, J = 6.8 Hz); ^{13}C-NMR ppm, 90.05, 84.55, 41.79, 33.32, 33.25, 29.26, 19.13, 17.94, 17.60.

Preparation of 2-hydroxy-2-deutero-1,4-cineole Methyl Dithiocarbonate (10). The 2-hydroxy-2-deutero-1,4-cineoles (**9a** and **9b**) (3.8 g, 22.2 mmol) were dissolved in 60 mL of THF, and 1.1 g (44.0 mmol) of NaH powder and 20 mg (29.4 mmol) of imidazole were added. The mixture was stirred at room temperature for 2 h, and 4 mL (66 mmol) of carbon disulfide was added. The reaction was continued for 1.5 h and 0.5 mL (22 mmol) of CH_3I was added. Stirring was continued for another 2 h, and the mixture was diluted with 200 mL of water and extracted with CH_2Cl_2 (3 × 100 mL). The organic extract was washed with 100 mL of saturated Na_2CO_3 and 100 mL of saturated NaCl solution, dried over anhydrous Na_2SO_4, and concentrated to afford 20 mL of oil which was further purified by chromatography over silica gel (218 g, 4 × 40 cm) with CH_2Cl_2 as eluting solvent. 2-Hydroxy-2-deutero-(1*R*,4*S*)-cineole methyl dithiocarbonate (**10**) (4.0 g, 70%) was obtained. Gas chromatography t_R 8.4 min; MS, *m/z* (%rel abund), 261 (0.15), 228 (4.5), 214 (1.3), 186 (11), 154 (100), 136 (63), 124 (40), 123 (21), 110 (62), 96 (19), 94 (35), 91 (68), 82 (31), 71 (89); ^{1}H-NMR ppm, 2.56 (3H, s, $CSCH_3$), 1.45 (3H, s, CH_3), 0.94 (6H, d, J = 4.0 Hz); ^{13}C-NMR ppm, 197.34, 90.27, 83.86, 39.60, 33.07, 32.72, 31.30, 19.51, 19.18, 17.64, 17.57.

Preparation of the Mixture of 2(*R*)-Deutero-(1*R*,4*S*)-cineole (12) and 2(*S*)-Deutero-(1*R*,4*S*)-cineole (13) by Reduction of 10 with Tributyltin Hydride. A solution of tributyltin hydride in toluene (3.0 g, 10.6 mmol, 50 mL of dry toluene) was heated to reflux under nitrogen and a solution of 2 g (7.7 mmol) 2-hydroxy-2-deutero-1,4-cineole methyl dithiocarbonate (**10**) in 50 mL of dry toluene was allowed to drop slowly into the refluxing solution over a period of 8 h. The reaction mixture was refluxed overnight, concentrated to a small volume, and passed through a silica gel column (200 g, 4 × 40 cm) slurry packed with hexanes. The column was continuously washed with hexanes to remove toluene and was then eluted with dichloromethane. Fractions containing the product were combined and carefully concentrated under reduced pressure with an acetone/dry ice condensor to afford 1.55 g (94%) of volatile oil: bp 162 °C; TLC, R_f 0.8, identical to 1,4-cineole; gas chromatog-

raphy, one major peak t_R 1.7 min. For analytical purposes, 600 mg of the oil was further purified by silica gel chromatography (silica gel 200 g, 4 × 40 cm) with CH_2Cl_2 as eluting solvent to afford 154 mg of the 2-monodeutero-1,4-cineole mixture (**12, 13**) as an oil: ^{1}H- and ^{13}C-NMR were identical to 1,4-cineole except for the intensity of the 2,6-carbon peak; ^{2}H-NMR ($CHCl_3$, 55.0 MHz) ppm, 1.66 (2-*endo*-D), 1.53 (2-*exo*-D, 9% of the intensity of the signal at 1.66 ppm); HRMS, *m/z* (% rel abund) 155.14098 (calcd for $C_{10}H_{17}O^2H$, 155.1416), 140 (M − CH_3, 6.5), 125 (17), 112 (73), 97 (8.5), 84 (19), 71 (83).

Biotransformation of Tetra- and Trideutero-1,4-cineoles by *B. cereus*. Preparative-scale incubations were conducted with four 125-mL flasks each holding 25 mL of second stage culture of *B. cereus* (UI-1477). Each flask received 42 mg of (**5, 5a**) when the second stage culture was 24 h old. After 72 h, the incubation reactions were combined and exhaustively extracted with CH_2Cl_2 (4 × 100 mL). The organic extract was dried over Na_2SO_4 and concentrated under reduced pressure to leave a residue of 65 mg (35%). The crude extract was vacuum dried for 15 min (cineole alcohols are volatile) and then dissolved in 40 mL of dry CH_2Cl_2 with 87 mg (0.45 mmol) of (*S*)-(+)-*O*-acetylmandelic acid, 91 mg (0.4 mmol) of *N,N*-dicyclohexylcarbodiimide (DCC), and 20 mg (0.16 mmol) of 4-(*N,N*-dimethylamino)pyridine. The reaction mixture was stirred at 0 °C for 72 h, before being concentrated to a small volume and filtered. The filtrate was evaporated under N_2 to afford 135 mg (100%) of a mixture of crude esters. Further purification by preparative TLC was accomplished using 100 mg of the crude esters on a 1.0 mm thick, 20 × 20 cm preparative silica gel TLC plate developed with hexanes–ethyl acetate (4:1) to afford 2(*R*)-*endo*-hydroxy-2,3,3-trideutero-(1*R*,4*S*)-cineolyl (*S*)-(+)-*O*-acetylmandelate (**16, 16a**): ^{1}H NMR ppm, 0.87 (6H, d, *J* = 6.8 Hz, isopropyl), 1.40 (3H, s, CH_3), 2.21 (3H, s, CH_3CO), 5.91 (1H, s, 2′-H), 7.43 (5H, m, ArH); ^{2}H NMR (55.0 MHz, CH_2Cl_2, $CDCl_3$ as internal standard) ppm, 4.7 (1D, 2-D), 2.15 (1D, 3-D), 1.6 (0.1D, 6-D), 1.4 (0.15D, 6-D), 1.1 (1D, 3-D) (Figure 4).

Analytical-scale experiments were carried out under the same fermentation conditions with both (1*R*,4*S*)-, and (1*S*,4*R*)-tri-, and tetradeutero-1,4-cineoles **5, 5a** and **8, 8a** as substrates. CH_2Cl_2 extracts of the hydroxy-1,4-cineoles produced after 24-h incubation were subjected to GC/MS analysis. *endo*- and *exo*-alcohols are well-separated by GC, and the relative intensities of ions at *m/z* 172, 173, and 174 for each sample were measured over entire eluted GC peaks. Isomeric compositions (see supplementary material) of deuterated hydroxy-1,4-cineoles are recorded under each of the metabolites in Figure 3.

Biotransformation of 2(*R*)-Deutero-(1*R*,4*S*)-cineoles (12, 13) by *B. cereus*. Preparative-scale incubations were conducted with 8 × 125 mL flasks holding 25 mL of second stage culture of *B. cereus* (UI-1477), each of which received 8.4 mg of monodeuterated 1,4-cineole substrate (**12, 13**) after 24 h of growth. The progress of the biotransformation reaction was monitored by taking culture aliquots at various times, extracting with CH_2Cl_2 and injecting the extracts for GC analysis. The *endo/exo* alcohol product ratio was measured as 1.19 by GC at all times ranging from 24–72 h. After 72 h (92% conversions by GC), fermentation mixtures were combined and exhaustively extracted with CH_2Cl_2 (4 × 200 mL). The organic extracts were combined, dried over Na_2SO_4 and carefully concentrated under vacuum to prevent evaporation of labeled hydroxycineole products. The concentrated extract containing volatile cineoles was purified by zinc sulfate–silica gel column chromatography[2] (adsorbant 75 g, column dimension 3 × 30 cm) eluted with hexane–ethyl acetate (4:1) to afford 24 mg (32%) of pure 2(*R*)-*endo*-hydroxy-(1*R*,4*S*)-cineoles **30** and **30a** and 6 mg (8%) of 2(*R*)-*exo*-hydroxy-(1*S*,4*R*)-cineoles **29** and **29a**. For **30, 30a**, ^{1}H- and ^{13}C-NMR are the same as that obtained for unlabeled 2(*R*)-*endo*-hydroxy-(1*R*,4*S*)-cineole (**3**):[3] ^{2}H NMR (55.0 MHz, $CHCl_3$) ppm, 3.87, 2-D; GC/MS retention time, 5.1 min; *m/z* (% rel abund) 171 (d_1, 1.47), 170 (d_0, 2.22), 154 (1.85), 153 (2.51), 125 (10.05), 119 (2.01), 112 (18.5), 97 (12.3), 83 (19.1), 71 (21.9), 43 (100). For **29**, ^{1}H-NMR ppm, 4.12 (1H, m, 2-H), 1.42 (3H, s, CH_3), 0.964 (6H, d, *J* = 6.8 Hz, isopropyl); ^{13}C-NMR ppm 89.09, 85.90, 77.09, 45.54, 32.94, 32.91, 18.57, 18.54, 16.77; ^{2}H NMR (55.0 MHz, $CHCl_3$) ppm, 1.44; GC/MS retention time 4.85 min; *m/z* (% rel abund) 171 (5.4), 170 (0.45), 154 (4.85), 153 (4.51), 138 (5.24), 126 (10.05), 120 (5.01), 112 (28.5), 97 (15.3), 83 (19.1), 71 (18.9), 59 (25.4), 43 (100).

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Supplementary Material Available: Methods for calculation of relative proportions of deuterated 1,4-cineoles (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.