Stereochemistry of Microbiological Hydroxylations of 1,4-Cineole

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The stereochemistries of microbial hydroxylations of 1-methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]heptane (1,4-cineole) were examined with *Bacillus cereus* and *Streptomyces griseus* as biocatalysts. Growing cultures of these bacteria introduce hydroxyl groups primarily at the 2- and 8-positions of the monoterpene substrate. Hydroxylation at position 2 destroys the C_s symmetry of 1,4-cineole and allows the possibility of creating two pairs of endo and exo enantiomers. The stereochemical purities of hydroxylated products were confirmed by proton NMR analyses of their corresponding (*S*)-(+)-*O*-acetylmandelate esters. *B. cereus* displays high face specificity in the hydroxylation of 1,4-cineole yielding only 1-methyl-4-(1-methylethyl)-[1*S*-(1β,2β,4β)]-7-oxabicyclo[2.2.1]heptan-2-ol (2(*R*)-*exo*-hydroxy-1,4-cineole) and 1-methyl-4-(1-methylethyl)-[1*R*-(1β,2α,4β)]-7-oxabicyclo[2.2.1]heptan-2-ol (2(*R*)-*endo*-hydroxy-1,4-cineole) products in an exo/endo ratio of 1/7. *S. griseus* gives 1-methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]heptan-8-ol (8-hydroxy-1,4-cineole) as the major product. This biocatalyst displays poor face specificity, affording enantiomeric mixtures of 2-*exo*- and 2-*endo*-hydroxy 1,4-alcohols. The high face specificity observed with *B. cereus* is explained by a simple enzyme model.

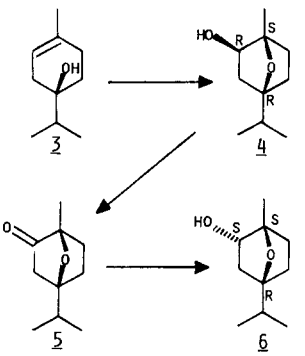
Introduction

Microorganisms and their enzymes are increasingly appreciated as a diverse group of selective reagents in synthetic organic chemistry.[1-8] Nearly every known oxidative, reductive, or hydrolytic organic reaction finds a counterpart in an enzymatic transformation. The enzymatic introduction of molecular oxygen into nonactivated carbon positions is one of the most attractive microbial reactions, and one which has been widely exploited in the synthesis of steroids[9,10] and other compounds.[4,6-8] Hydroxylation reactions are perhaps the most difficult ones to achieve by purely chemical means. The importance of such reactions has spawned efforts to identify structural and electronic features of substrate molecules that serve to direct enzymatic hydroxylations to specific remote sites. As a result, a variety of putative enzyme active site models responsible for the introduction of molecular oxygen have been postulated, particularly for the fungal hydroxylases.[11-16] Unfortunately, to date, little success has been attained in the isolation and use of fungal hydroxylases due to inherent catalyst instabilities, their particulate or membrane bound character, and the usual requirement of cofactors and several components that serve to feed electrons to the oxidizing enzymes. Much greater success has been achieved in the characterization and use of monooxygenase enzyme systems from prokaryotic (bacterial) strains.[17]

A wide range of mono-, sesqui-, and diterpenes have been subjected to microbial and enzymatic transformation experiments.[1-3,7,8,18-23] Hydroxylated terpenes possess structural features that render them attractive as synthons for other types of compounds, and they are valued for their flavoring and fragrant properties. 1,4-Cineole (1) (Figure 1) is a widely distributed, natural, oxygenated monoterpene,[24] which was examined as a substrate for microbial hydroxylation to obtain 2-*exo*-hydroxy-1,4-cineole (4 or 8, Scheme I) synthons used in the preparation of the herbicide cinmethylin (2). Screening experiments revealed that for many microorganisms, 2-endo-hydroxylation was far more common than 2-exo-hydroxylation with cineole.[25]

1,4-Cineole (1) possesses a plane of symmetry passing through carbon atoms 1, 4, 7, and 8. Hydroxylation of the 2-position of this prostereogenic[26] compound introduces local chirality to generate diastereomeric exo and endo alcohols, and it also destroys the symmetry plane of the

Scheme I. Preparation of 2(*R*)-*exo*- (4) and 2(*S*)-*endo*-Hydroxy-1,4-cineole (6) from (*R*)-Terpinen-4-ol (3)[a]



[a] The enantiomeric 2(*S*)-*exo*- (8) and 2(*R*)-*endo*-hydroxy-1,4-cineole (10) were prepared by oxidation of the enantiomeric (*S*)-terpinen-4-ol (7) and reduction of the corresponding enantiomeric ketone 9 with sodium borohydride.

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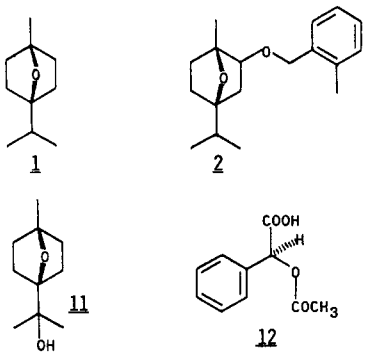

Figure 1. Structures of cineoles, cinmethylin, and (*S*)-(+)-*O*-acetylmandelic acid.

molecule to provide enantiomers of each alcohol diastereomer. GC, NMR, and mass spectral methods can easily distinguish the various cineole products formed in microbial reactions, but they cannot be used to determine the enantiospecificities of enzymatic hydroxylation.

In this paper, we establish the stereochemistry of microbial hydroxylations of 1,4-cineole.

Results and Discussion

Bacillus cereus and *Streptomyces griseus* were identified in screening experiments as reproducibly giving the best yields of hydroxylated 1,4-cineoles when 1,4-cineole was used as a starting material.[25] Both organisms afforded mixtures of 2-*exo*- (**4** and/or **8**) and 2-*endo*-hydroxy-1,4-cineoles (**6** and/or **10**) of unknown enantiomeric compositions. *S. griseus* also provided 8-hydroxy-1,4-cineole (**11**) as a major biotransformation product. To define the stereochemistry of microbial hydroxylations of 1,4-cineole, it became necessary to establish methods capable of distinguishing enantiomeric hydroxy-1,4-cineole isomers from one another.

The approach used in the preparation of standard compounds is outlined in Scheme I. Chiral 2-*exo*-hydroxy-1,4-cineoles (**4** and **8**) were readily obtained by epoxidation of (*R*)-**3** ($[M]_D$ −32.8° found, reported −33.7°,[27] optical purity 97.3%) and (*S*)-terpinen-4-ol (**7**) ($[M]_D$ +45.21° found, reported +46.67°,[27] optical purity 96.9%), followed by acid-catalyzed opening of the epoxides and intramolecular cyclization.[28] The cineole alcohols (**4** and **8**) were transformed into the enantiomeric 2-oxo-1,4-cineoles (**5** and **9**) by Swern oxidation,[29,30] and reduction of the chiral ketones with sodium borohydride afforded samples of enantiomeric 2-*endo*-hydroxy-1,4-cineoles (**6** and **10**).

Scheme II. Possible 1,4-Cineole Alcohol Enantiomers Formed by Microbial Hydroxylation and Their Corresponding *O*-Acetylmandelate Ester Derivatives

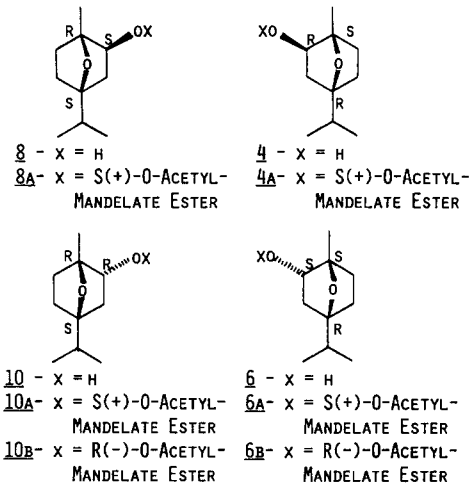

Table I. Distribution of *endo*- and *exo*-Hydroxy-1,4-cineole Metabolites by NMR Spectral Analysis of Their (*S*)-(+)-*O*-Acetylmandelate Esters

	exo, %		*endo*, %		
catalyst	**4a**	**8a**	**6a**	**10a**	ratio endo/exo
B. cereus	96.8	3.2	2.9	97.1	1/7
S. griseus	73	27	13	87	1.7/1

Most simple approaches for determining enantiomeric compositions of isomeric alcohols[31,32] proved inadequate with hydroxy-1,4-cineoles. Specific rotations of enantiomeric cineole alcohols are much less than 1°; chiral shift reagents such as EuOPT[33] failed to aid in the distinction between stereoisomers by NMR, and derivatizations with chiral reagents used by others with carbocyclic alcohols[31] led to hydroxycineole decomposition.

(*S*)-(+)-*O*-Acetylmandelic Acid Derivatization To Distinguish Hydroxy-1,4-cineole Enantiomers. The availability of enantiomerically defined, 2-*endo*- and 2-*exo*-hydroxy-1,4-cineoles as standards permitted the adaptation of a simple derivatization and NMR spectral analytical method for evaluating the stereospecificity of hydroxylation reactions catalyzed by *B. cereus* and *S. griseus*. Conditions were established for the efficient esterification[33] of hydroxycineoles with (*S*)-(+)-*O*-acetylmandelic acid (**12**) to prepare diastereomeric ester derivatives **6a** and **10a**, and **4a** and **8a**. The reactions were simple to conduct and quantitative with as little as 2 mg of cineole alcohol starting material. Furthermore, the reaction was demonstrably successful when mixtures of enantiomeric alcohols ranging in composition from 1–50% were subjected to the derivatization method. No detectable racemization occurred during derivatization or upon chromatographic purification of the ester derivatives. Proton NMR spectral properties of enantiomeric endo alcohol esters are shown in Figure 2. Diastereomeric 2-endo esters **6a** and **10a** and 2-exo esters **4a** and **8a** are

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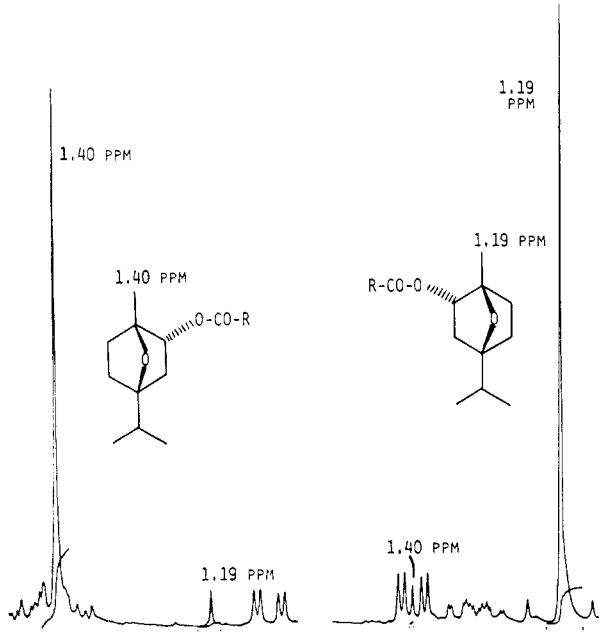

Figure 2. Portions of the 360-MHz proton NMR spectra of **6a** and **10a**, illustrating the resolution of minor contaminating amounts of enantiomeric alcohols as their (*S*)-(+)-*O*-acetylmandelate esters.

readily distinguished from one another because the methyl group chemical shift differences between these pairs of isomers was 0.23 and 0.32 ppm, respectively. Methyl-group signals were well-resolved at 360 MHz (Figure 2), and it was possible to detect as little as 1% of either ester product as a contaminant in mixtures of the enantiomeric hydroxycineoles.

Esters of **6** and **10** were prepared using both (*R*)- and (*S*)-*O*-acetylmandelic acid enantiomers. As expected,[35] methyl-group signals for **6a** and **10b**, and **6b** and **10a** occurred at 1.17 and 1.40 ppm, respectively. When the ratios of areas under these peaks are measured (Figure 2), compounds **6a** and **10a** gave ee values of 95.8% and 95.2%, respectively. These values clearly indicate that little racemization (less than 1% of sample) of *O*-acetylmandelic acid or of the esters occurred during the derivatization process. The results also define the optical purities of the starting alcohols **6** and **10** and of the ketones **5** and **9** from which they were obtained as being 95%–97%.

Hydroxylation of 1,4-Cineole by *B. cereus* and *S. griseus*. The possible 2-endo- and 2-exo-hydroxylated products of 1,4-cineole are shown in Scheme II. The pure *endo*- and *exo*-1,4-cineoles obtained from preparative-scale incubation reactions were subjected to the (*S*)-(+)-*O*-acetylmandelic acid derivatization procedure, and the 360-MHz NMR spectral properties of the derivatized metabolites were determined. With *B. cereus*, endo-hydroxylation is favored by a factor of 7:1 vs exo-hydroxylation.[25] This bacterium displays nearly complete *pro-R* face specificity for both endo- and exo-hydroxylations (Table I). The diastereomeric composition found in both endo and exo products can easily be attributed to the known enantiomeric composition of (*S*)-(+)-*O*-acetylmandelic acid used in derivatization reaction. Thus, the microbially produced exo and endo alcohols are enantiomerically pure.

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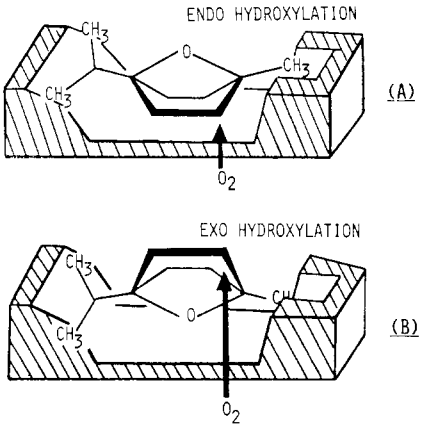

Figure 3. A model for the hydroxylating enzyme binding site of *B. cereus*.

With *B. cereus*, samples of hydroxycineoles obtained from analytical-scale cultures at 24, 48, and 72 h[24] were derivatized and analyzed by ^{1}H NMR methods to determine whether changes in hydroxylation specificity might occur with time. The relatively small scale of these reactions, and the low yields of 2-*exo*-hydroxy-1,4-cineoles precluded an analysis of their enantiomeric compositions. However, for the endo alcohol products, the ratios of methyl-group peaks at 1.17 and 1.40 ppm were essentially constant at each assay time ranging from 97% to 97.8% for **6a** throughout the sampling period. Thus, alcohol metabolites formed by *B. cereus* are not subject to further enantioselective metabolism, or to reversible oxidation/reductions to the corresponding ketones. Such additional biotransformations by viable biocatalysts can cause changes in the optical purities of alcohol products.[25]

With *S. griseus* (Table I), endo-hydroxylation is favored by a factor of 1.7:1 vs exo-hydroxylation products with 1,4-cineole.[25] The major ring hydroxylation products were the exo alcohol **4** and the endo alcohol isomer **10**. However, while *S. griseus* favors *pro-R* introduction of molecular oxygen for both the *endo*- and *exo*-hydroxy-1,4-cineoles, the enantiomeric purities of cineole alcohols formed by hydroxylase(s) of this microorganism are low.

A Model of the Hydroxylase Enzyme System of *B. cereus* and *S. griseus*. The results with both microorganisms indicate a very high preference for introduction of hydroxyl groups to *pro*-1*R* endo and *pro*-1*S* exo faces of 1,4-cineole. Endo-hydroxylation is greatly favored vs exo-hydroxylation, especially with *B. cereus*. As with most enzymes, factors that influence the outcome of catalytic reactions involve steric and electronic features of the substrate, the mechanism(s) of oxygen activation, and insertion into the substrate, and the intrinsic substrate binding properties of the enzyme active site. With 1,4-cineole, the oxygen bridge probably plays a role in substrate binding, and the methyl and isopropyl groups undoubtedly influence and limit the ways in which 1,4-cineole can become oriented at the catalytic center of the enzyme. The model shown in Figure 3 can be used to explain the results obtained, assuming that a single hydroxylating enzyme is involved in forming more than one product with each microorganism. The model is not perfect, but it invokes an oxygenation site, a pocket that accommodates the isopropyl group, and a smaller area that can accommodate a methyl group. Hydroxylation in the most favored orientation of substrate (Figure 3A) leads to preponderant *pro*-1*R* endo-oxygenation. Dissociation of the substrate, and rotation about an axis through carbons 1 and 4, and the oxygen bridge presents the substrate in an

orientation which would enable *pro*-1*S* exo-hydroxylation (Figure 3B). Such a model is particularly attractive for *B. cereus* where endo- and exo-hydroxylated cineole metabolites were essentially optically pure.

The microbial hydroxylation reactions described here were accomplished with growing cultures of *B. cereus* and *S. griseus*. As in most microbial hydroxylations, reactions do not occur in the extracellular medium surrounding microbial cells. Since catalysis required whole microbial cells, substrates must first dissolve in aqueous incubation media, penetrate microbial cell walls, diffuse to and bind with intracellular hydroxylating enzymes, undergo hydroxylation, and dissociate and diffuse from cells as polar hydroxylated products.[38] It is commonly assumed[12-15] that single enzymes may be responsible for catalyzing many different types of hydroxylation reactions even with the same substrate. However, more than one enzyme, or isoenzyme(s) can be involved in cineole hydroxylations.

The strain of *S. griseus* used in this work is a highly versatile biocatalyst with broad substrate specificities, having demonstrated abilities to catalyze aromatic and aliphatic hydroxylations, N- and O-dealkylations, epoxidations, and N-oxidation type reactions.[7] This biocatalyst introduces hydroxyl groups into positions 8, 2, and 6 of 1,4-cineole. *S. griseus* is now known to contain an inducible cytochrome P-450 enzyme system,[39] which is likely responsible for observed biocatalytic reactions. Experiments to establish the cell-free catalytic properties of the P-450 system from this microorganism are in progress. The catalytic proficiencies of *S. griseus* render it unusual for a prokaryotic (i.e. bacterial) biocatalyst. The range of reactions that it catalyzes resemble those observed for eukaryotic (i.e. fungal) monooxygenases. While *S. griseus* catalyzes many types of reactions with substrates of highly diverse structure, the present work indicates that 1,4-cineole hydroxylation reactions are poorly stereoselective.

Cytochrome P-450 systems are known in Bacilli,[40] and we have recently identified an alkane-inducible, cytochrome P-452 enzyme system in *B. cereus* UI-1477 that is responsible for 1,4-cineole hydroxylation (unpublished). With induction, yields of hydroxylated cineoles are boosted to 90% of added starting material. The identification of the enzyme system of *B. cereus*, its inducible nature, kinetic experiments, deuterium isotope effects, and aspects of the mechanism of hydroxylation of deuteriated 1,4-cineoles are the subjects of another report.

Experimental Section

Instrumental Methods. NMR spectra were recorded at 360.134 or 80.13 MHz for proton and 90.556 or 20.15 MHz for carbon-13 in deuteriochloroform solutions. Spectral data for **1**, **4–6**, and **8–10** have been reported elsewhere.[24,28]

Optical rotations were determined with acetone solutions in a 0.1-dm cell.

Gas chromatography was performed with use of an OV-17 3% Supelcoport column as described.[24] GC–MS was carried out with an OV-1 3% column ($6 \times {}^1/_4$ in. o.d., mesh 80–100) with a helium carrier gas flow of 20 mL/min. Samples were dissolved in methanol for GC–MS analysis.

Chemicals. Authentic 1,4-cineoles were prepared as described.[25,28,29] All compounds were examined for purity by optical rotation, TLC, GC, and ^1H and ^{13}C NMR spectroscopy, and mass spectrometry before use. (*S*)-(+)-*O*-Acetylmandelic acid (Sigma) exhibited the following physical properties: mp 96.5–98 °C; specific rotation +152.1° (*c* 2.4, acetone) (reported +153°[37]); ^1H NMR ($CDCl_3$) δ 2.21 (s, 3 H, CH_3), 5.95 (s, 1 H, CH), 7.39 (m, 5 H, Ar H).

Synthesis of Optically Active 2-Oxo-1,4-cineoles 5 and 9. A solution of DMSO (36 g) in dichloromethane (80 mL) was added to a stirred solution of oxalyl chloride (24.6 g) in dichloromethane (300 mL) held at −60 °C. After 10 min, a solution of chiral 2-*exo*-hydroxy-1,4-cineole (**4** or **8**, mp 84–86 °C, obtained from (−)- and (+)-terpinen-4-ol, respectively, 29.9 g) was added dropwise, and stirring was continued for an additional 20 min. Triethylamine (90 g) was added, and the mixture was allowed to come slowly to room temperature. Water (250 mL) was added, the layers were separated, and the aqueous layer was extracted with dichloromethane (2 × 100 mL). The combined organic layers were dried over anhydrous magnesium sulfate and filtered, and the solvent was removed under reduced pressure at 40 °C. The residue was distilled (bp 195–200 °C) to give the liquid chiral ketone **5** (20.9 g, 70%) or **9** (24.5 g, 82%). The liquid chiral ketones were completely characterized by GC, ^1H and ^{13}C NMR, and mass spectrometry.[29]

Reduction of 2-Oxo-1,4-cineoles to Corresponding 2-*endo*-Hydroxy-1,4-cineoles 6 and 10. In a typical reaction, 5 g (29.8 mmol) of 2-oxo-1,4-cineole (**9**) was dissolved in 40 mL of methanol at room temperature, and 5 g of sodium borohydride (132 mmol) was slowly added. After stirring for 40 min, the reaction mixture was filtered and concentrated to a dry residue, which consisted of a mixture of **10** containing about 2% **8**. The residue was purified by zinc sulfate–silica gel gravity column chromatography (400 g adsorbent, column dimensions 4 × 40 cm) with hexanes–ethyl acetate (2:1) as eluting solvent to afford 3.02 g (60%) of the liquid (bp 105–107 °C) 2-*endo*-hydroxy-1,4-cineole (**10**). Reduction of **5** afforded the enantiomeric alcohol **6**. Properties of these enantiomeric compounds have been reported before:[29] ^1H NMR ($CDCl_3$) δ 0.92 (d, J = 6.4 Hz, 6 H, isopropyl), 1.39 (s, 3 H, CH_3), 3.91 (dd, J = 10.4, 7.24 Hz, 1 H, 2-H).

Synthesis of (*S*)-(+)-*O*-Acetylmandelate Esters of Chiral *endo*- and *exo*-Cineole Alcohols.[34] In a typical reaction, 100 mg (0.59 mmol) of 2(*R*)-*endo*-hydroxy-1,4-cineole (**10**) was dissolved in 40 mL of dichloromethane containing 185 mg (0.90 mmol) of *N,N*-dicyclohexylcarbodiimide (DCC), 170 mg (0.88 mmol) of (*S*)-(+)-*O*-acetylmandelic acid, and 20 mg (0.16 mmol) of 4-(*N,N*-dimethylamino)pyridine. The reaction mixture was stirred at ice temperature overnight, and the precipitate that formed was filtered from the reaction mixture. After the filtrate was concentrated to an oil (220 mg), it was purified by silica gel column chromatography (100 g of silica gel, column 2 × 30 cm) with hexane–ethyl acetate (6:1) to afford 143 mg (70%) of 2-(*R*)-*endo*-hydroxy-1(*R*),4(*S*)-cineole (*S*)-(+)-*O*-acetylmandelate (**10a**): plates; mp 68–70 °C; HRMS (EI) for $C_{20}H_{26}O_5$ calcd 346.17802, found 346.17444; ^1H NMR δ 0.87 (d, J = 6.8 Hz, 6 H), 1.40 (s, 3 H, CH_3), 2.20 (s, 3 H, CH_3CO), 4.71 (dd, J = 10.2, 3.4 Hz, 1 H, 2-H), 5.91 (s, 1 H, 2′-H), 7.43 (m, 5 H, Ar H); $[\alpha]^{25}_D$ +36.7° (*c* 1.5, methylene chloride). 2(*S*)-*endo*-Hydroxy-1(*S*),4(*R*)-cineole (*S*)-(+)-*O*-acetylmandelate (**6a**): plates; mp 70–73 °C; HRMS (EI) for $C_{20}H_{26}O_5$ calcd 346.17802, found 346.17767; ^1H NMR δ 0.91 (d, J = 6.8 Hz, 6 H), 1.17 (s, 3 H, CH_3), 2.20 (s, 3 H, CH_3CO), 4.72 (dd, J = 10.3, 3.5 Hz, 1 H, 2-H), 5.91 (s, 1 H, 2′-H), 7.43 (m, 5 H, Ar H); $[\alpha]^{25}_D$ +67.5° (*c* 3.4, methylene chloride).

2(*R*)-*exo*-Hydroxy-1(*S*),4(*R*)-cineole (*S*)-(+)-*O*-acetylmandelate (4a) as an amorphous solid: HRMS (EI) for $C_{20}H_{26}O_5$ calcd 346.17802, found 346.17398; ^1H NMR δ 0.95 (d, J = 7.2 Hz, 6 H), 1.09 (s, 3 H, CH_3), 2.19 (s, 3 H, CH_3CO), 4.90 (m, 1 H, 2-H), 5.89 (s, 1 H, 2′-H), 7.39 (m, 5 H, Ar H).

2(*S*)-*exo*-Hydroxy-1(*R*),4(*S*)-cineole (*S*)-(+)-*O*-acetylmandelate (8a) as an amorphous solid: HRMS (EI) for $C_{20}H_{26}O_5$ calcd 346.17802, found 346.17915; ^1H NMR δ 0.87 (dd, J = 6.4, 0.85 Hz, 6 H), 1.41 (s, 3 H, CH_3), 2.19 (s, 3 H, CH_3CO), 4.90 (m, 1 H, 2-H), 5.90 (s, 1 H, 2′-H), 7.39 (m, 5 H, Ar H).

The same reaction can be conveniently conducted on samples of 2–10 mg of hydroxy-1,4-cineoles. The reaction is highly reproducible, and the procedure was satisfactorily applied to mixtures of enantiomeric alcohols ranging in composition from 98 to 2% ee to indicate that no diastereoselection occurs during

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the derivatization reaction. (*R*)-(−)-*O*-Acetylmandelate esters were prepared in the same manner as described.

Fermentation Methods. *B. cereus* UI-1477 (University of Iowa, College of Pharmacy Culture Collection), and *S. griseus* ATCC 10137 were used as biocatalysts.[25] Cultures were grown according to a standard two-stage fermentation protocol in 125-mL DeLong culture flasks holding 25 mL each of a soybean meal–glucose culture medium.[39,41] Cultures were incubated at 27 °C on New Brunswick Scientific Model G-25 Gyrotory incubator shakers operating at 250 rpm.

Hydroxylations of 1,4-Cineole with *B. cereus*. (a) Preparative-scale incubations were conducted with 16 125-mL flasks, each of which received 42 mg of 1,4-cineole substrate. After 24 h, TLC analysis indicated that the substrate had been completely converted into hydroxy-1,4-cineole products. The hydroxy-1,4-cineoles were isolated by exhaustive methylene chloride extraction (4 × 500 mL) of the combined cultures. The organic solvent was carefully removed under vacuum to leave a residue of 985 mg. The concentrated extract containing volatile cineoles was purified over a column of $ZnSO_4$–silica gel (100 g, 3 × 30 cm) eluted with hexane–ethyl acetate (4:1) to afford 158 mg (21.3%) of pure (GC) 2-*endo*-hydroxy-1,4-cineole and 28 mg (3.8%) of 2-*exo*-hydroxy-1,4-cineole. These compounds were subjected to derivatization as described, and the resulting (*S*)-(+)-*O*-acetylmandelate esters were purified by column chromatography before being examined by ^1H NMR at 360 MHz. The 2-*endo*-hydroxy-1,4-cineole ester was found to be exclusively **10a**, while the 2-*exo*-hydroxy-1,4-cineole ester was identified as exclusively **4a**.

Kinetics of 1,4-Cineole Hydroxylation. This incubation with *B. cereus* was conducted as described.[25] Duplicate flasks containing 42 mg of 1,4-cineole (**1**) were harvested at 24, 48, and 72 h following substrate additions. Cultures were extracted exhaustively with three 25-mL portions of dichloromethane; extracts were combined and dried over anhydrous sodium sulfate and analyzed by GC to show that each sample contained 14 mg (30%) of hydroxylated cineoles of which 85% was 2-*endo*-hydroxy-1,4-cineoles and 14% was 2-*exo*-hydroxy-1,4-cineoles. Each of the 24-, 48-, and 72-h samples were subjected to derivatization with (*S*)-(+)-*O*-acetylmandelic acid, and the proportions of diastereomeric esters **6a** and **10a** were determined by 360-MHz ^1H NMR analysis to be 2.2% and 97.8%, 2.3% and 97.7%, and 3.0 and 97%, respectively.

Hydroxylations of 1,4-Cineole with *S. griseus*. *S. griseus* were grown in 550 mL of medium containing 970 mg of unlabeled 1,4-cineole. TLC analysis indicated that the substrate had been consumed at 72 h at which time the cultures were combined and extracted four times with 150 mL of dichloromethane. The extracts were combined, dried over anhydrous sodium sulfate, and concentrated to a dark oil, with a composition of *endo*- (6%), *exo*- (3%), and 8-hydroxy-1,4-cineole (18%) similar to that observed before.[25] The extract was subjected to column chromatography over silica gel containing 10% zinc sulfate with hexane–ethyl acetate (4:1) as solvent to give 12 mg (1.2%) of pure 2-*endo*-hydroxy-1,4-cineoles and 5 mg (0.5%) of pure 2-*exo*-hydroxy-1,4-cineoles. These samples were esterified with (*S*)-(+)-*O*-acetylmandelic acid and the diastereomeric ester mixtures **6a** and **10a**, and **4a** and **8a** were determined by NMR analysis (Table I).

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Supplementary Material Available: ^{13}C NMR chemical shifts for (*S*)-(+)-*O*-acetylmandelate esters of enantiomeric *endo*-hydroxy-1,4-cineoles (**6a** and **11a**) (1 page). Ordering information is given on any current masthead page.

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Synthesis of 3,6-Dichloro-4-(2-chloro-1,1-dimethylethyl)pyridazine

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3,6-Dichloro-4-(2-chloro-1,1-dimethylethyl)pyridazine (**3**) is a systemic plant fungicide whose synthesis by several routes is described. Free-radical alkylations of 3,6-dichloropyridazine (**1**) gave either the 4-*tert*-butyl derivative **2** or the alcohol 3,6-dichloro-β,β-dimethyl-4-pyridazineethanol (**24**). Pyridazine **2** must be subjected to a free-radical chlorination, which resulted in other products, but alcohol **24** could be smoothly converted to **3**. Alternatively, the pyridazine ring was constructed with the side chain preattached by utilizing lactone intermediates **10** or **18**. The lactone **10** with hydrazine yielded the ether **11**, and chlorination with ether cleavage proceeded to **3**. Lactone **20** could not be transformed to **3**.

3,6-Dichloro-4-(2-chloro-1,1-dimethylethyl)pyridazine (**3**) is a systemic fungicide that controls several pathogenic *Phycomycetes* organisms in plants.[1] It was originally synthesized in our laboratories by a free-radical chlorination of **2** using sulfuryl chloride. We describe in this paper a diversity of approaches to the synthesis of **3**. One can begin with the dichloropyridazine **1** and attach the side chain, or the pyridazine ring can be formed with the side chain already attached.

Free-Radical Chlorination of 2. 3,6-Dichloro-4-alkylpyridazines such as **2** are made by a free-radical alkylation of 3,6-dichloropyridazine (**1**) with a silver-catalyzed oxidation of carboxylic acids to generate the alkyl radicals.[2] The use of pivalic acid leads to a high yield of **2**, resulting from attack of the *tert*-butyl radical on the pyridazine ring. When chloropivalic acid was used in this alkylation reaction, however, no **3** was observed. We assume the chloro-*tert*-butyl radical underwent homolytic cleavage to isobutene and chlorine radicals. This observation led to our chlorination of the *tert*-butyl group directly in order to synthesize **3**.

Random chlorination of the *tert*-butyl group of **2** does not become a problem until significant amounts of **3** are present. When the chlorination of **2** was stopped at

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