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Microbial Hydroxylation of 1,4-Cineole

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Microorganisms were examined for their potential to hydroxylate the oxygenated monoterpene 1,4-cineole. Using gas chromatography and thin-layer chromatography, screening experiments revealed that hydroxylation at position 2 was the most commonly observed microbial transformation reaction. In most microorganisms, the predominant alcohol metabolite was the 2-endo-alcohol isomer. Preparative-scale incubations were conducted in order to isolate and characterize microbial transformation products by comparison of proton nuclear magnetic resonance, mass spectrometry, and chromatography profiles with those of cineole standards. *Streptomyces griseus* yielded 8-hydroxy-1,4-cineole as the major hydroxylation product together with 2-exo- and 2-endo-hydroxy-1,4-cineoles.

The herbicide cinmethylin (compound 1, Fig. 1) is the *o*-methyl-benzyl ether of racemic 2-exo-hydroxy-1,4-cineole. As a key chemical intermediate required for the synthesis of compound 1, 2-exo-hydroxy-1,4-cineole (compound 5) may be prepared by the epoxidation of terpinen-4-ol (compound 3) and the subsequent acid-catalyzed opening of the epoxide (compound 4) to cause intramolecular etherification (G. B. Payne, U.S. patent 4,487,945, December 1984) (Fig. 1). An alternative route for the synthesis of compound 5 would be the direct enzymatic introduction of molecular oxygen by hydroxylation of position 2 of 1,4-cineole (compound 6).

1,4-Cineole is a widely distributed, natural, oxygenated monoterpene which is one of the flavor constituents of lime juice (6). This compound was deemed an attractive precursor for microbial hydroxylation on the basis of extensive precedence with biotransformations of monoterpenes (1, 5, 7, 10), including the structurally similar monoterpene, 1,8-cineole (compound 2) (8), and the carbocyclic bicyclo-[2.2.1]-heptane (11). The purpose of the present investigation was to explore microbial transformations of 1,4-cineole and to characterize the range of hydroxylated products obtainable when this simple terpene is used as a substrate.

MATERIALS AND METHODS

Chemicals. Authentic cineole samples were obtained from Shell Agricultural Chemical Company, and these samples were prepared as described previously (G. B. Payne, patent). Each compound was examined for purity by thin-layer chromatography (TLC), gas chromatography (GC), proton and ^{13}C nuclear magnetic resonance (NMR), and mass spectral evaluation before use. Compounds used in this study included the following: 1,4-cineole (compound 6), racemic 2-exo-hydroxy-1,4-cineole (compound 5), racemic 2-oxo-1,4-cineole (compound 9), racemic 2-endo-hydroxy-1,4-cineole (compound 7), and racemic 3-exo-hydroxy-1,4-cineole (compound 11). The solubilities of the following three compounds in water (in grams per liter) were determined by GC and high-performance liquid chromatography to be as follows: 1,4-cineole, 2.4; 2-exo-hydroxy-1,4-cineole, 157; and 2-endo-hydroxy-1,4-cineole, 91.5.

Spectrometric methods. NMR spectra were recorded with Bruker WH-360 (360.134 MHz for proton NMR and 90.556 MHz for ^{13}C NMR) or IBM NR-80 (80.13 MHz for proton NMR and 20.15 MHz for ^{13}C NMR) Fourier transformation-NMR spectrometers or with a Varian EM-360 (60 MHz, for proton NMR only) NMR spectrometer. Spectra were all recorded in deuterochloroform solutions by using tetramethylsilane (δ 0) as an internal standard. Proton and ^{13}C NMR spectral data obtained in $CDCl_3$ for various compounds, together with their assignments, have been reported in detail (A. Goswami, R. P. Steffek, W.-G. Liu, J. J Steffens, and J. P. N. Rosazza, Enzyme Microb. Technol., in press). Proton signals for the authentic compounds pertinent to this work were as follows: 2-exo-hydroxy-1,4-cineole (compound 5), 0.93 ppm (d, 6H, J = 6.6 Hz, isopropyl), 1.40 ppm (s, 3H, methyl), and 3.75 ppm (t, 1H, J = 9 Hz, C$\underline{H}$OH); 2-endo-hydroxy-1,4-cineole (compound 8), 0.92 ppm (d, 6H, J = 6.8 Hz, isopropyl), 1.46 ppm (s, 3H, methyl), and 3.90 ppm (dd, 1H, J = 3.7, 10.2 Hz, C$\underline{H}$OH).

GC-mass spectrometry (MS) measurements were made with a Nermag R10-10C MS linked to a 5% phenylmethyl silicone capillary column. Conditions for GC were as follows: an injector temperature of 270°C, a column temperature of 120°C, and the GC transfer line, which interfaces with the MS, held at 250°C. Nitrogen flow was at 20 to 30 ml/min, and the source temperature of the MS was 150°C. Under these conditions, the 2-exo- and 2-endo-alcohol isomers were well separated, and both compounds provided clear and reproducible mass spectral patterns. The mass spectra of the authentic 2-hydroxy-1,4-cineoles are as follows: m/z (percent relative abundance) of 2-exo-hydroxy-1,4-cineole (compound 5), 170 (37), 153 (24, M − OH), 127 (28; M − C_3H_7), 112 (75; M − C_3H_7 − CH_3), 109 (34; M − H_20 − C_3H_7), and 71 (100); of 2-endo-hydroxy-1,4-cineole (compound 7), 170 (18), 153 (16), 127 (17), 112 (44), 109 (22), and 71 (100).

Chromatography. TLC was performed on 0.25-mm-thick silica gel GF_{254} (Merck) (solvent system 1), silica gel GF_{254}

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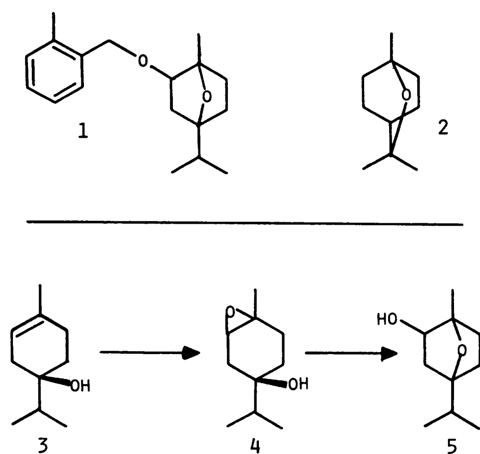

FIG. 1. Structures of cinmethylin (compound 1), 1,8-cineole (compound 2), and the chemical synthesis of 2-exo-hydroxy-1,4-cineole (compound 5) from terpinene-4-ol (compound 3).

impregnated with 10% zinc sulfate ($ZnSO_4 \cdot 7H_2O$) (solvent system 2), or aluminum oxide GF_{254} (solvent system 3) plates prepared as needed with a spreader (Quickfit Industries, London, England). Plates were air dried and oven activated at 110°C for 30 min prior to use. Solvent systems, colors obtained with visualizing reagent, and chromatographic mobilities of various compounds are shown in Table 1. Limits of detection of all terpene compounds were determined by these methods to be about 1 μg per spot. Column chromatography was performed by using silica gel (60/200 mesh; Baker 3405) heated at 110°C for 30 min prior to use. Standard silica columns (1.5 by 24 cm) containing 6 to 7 g of silica gel were eluted with hexane-ethyl acetate mixtures (17:3, vol/vol) at flow rates of 1 ml/min. Column chromatography was also performed by using alumina (neutral, 70/230 mesh, Activity II; Merck). Columns containing 10 to 20 g of alumina were eluted by using hexane-ether (4:1, vol/vol) at a flow rate of 1 ml/min.

The 2-hydroxy-1,4-cineoles were well separated by high-performance liquid chromatography. Separations were achieved by using a Waters ALC/GPC-202 unit equipped with M6000A pumps, a U6K Universal injector linked to a PRP-1 column (30.5 by 0.7 cm, 10 μm; The Hamilton Co.) with acetonitrile-water (30:70, vol/vol) as the eluent at a flow rate of 2 ml/min, and a Refractive Index detector. With this system, the retention volumes for the exo and endo compounds were 23.1 and 27.2 ml, respectively.

GC was performed on a Varian 3700 Aerograph gas chromatograph linked to a Houston Instrument Co. Omniscribe recorder. Separations were routinely achieved by using an OV-17 3% Supelcoport column (0.5-by-72-in. [1.27 by 182.88 cm] glass column, 100/120 mesh), and eluted compounds were detected by flame ionization. The flow rate of the eluent gas, nitrogen, was 50 ml/min, and typical operating conditions were as follows: injector temperature, 120°C; detector temperature, 180°C; initial column temperature, 87°C for 2 min, increased at a rate of 3°C/min to a final temperature of 103°C. Under these conditions, various cineole derivatives displayed retention times shown in Table 1. All compounds except for *9* and *10* were resolved with base-line resolution by GC. These two compounds could be readily differentiated by TLC to rule out any ambiguity as to their presence. All compounds exhibited linear peak

TABLE 1. Chromatographic properties of various 1,4-cineole derivatives

Compound	TLC R_f values[a] of: Silica gel	Silica gel-$ZnSO_4$	Alumina	Color[b]	GC retention time (min)
6	0.69	0.70	0.97	Purple	1.7
5	0.10	0.35	0.57	Pink	6.5
7	0.13	0.42	0.64	Pink	7.5
9	0.56	0.70	0.96	Yellow-green	5.8
11	0.13	0.40	0.67	Pink	5.7
8	0.45	0.50		Pink	4.0

[a] Solvent systems: 1, silica gel, hexane-ethyl acetate (17:3, vol/vol); 2, silica gel-zinc sulfate, hexane-ethyl acetate (1:1, vol/vol); and 3, aluminum oxide, hexane-ether-*n*-butanol (90:10:3, vol/vol).

[b] Developed TLC plates were sprayed with a solution of *p*-anisaldehyde-glacial acetic acid-concentrated sulfuric acid (0.5:60:0.5, vol/vol) and warmed with a heat gun to develop colors.

height/concentration relationships, and standard curves were constructed over the range of 0.15 to 0.85 μg for cineole and 0.5 to 5.0 μg for all other compounds. Limits of detection were about 0.04 μg for cineole and 0.1 μg for all other compounds, and these limits were well within experimental limits.

Cultivation methods. All cultures are maintained in the culture collection of the University of Iowa College of Pharmacy. Cultures were grown according to a standard two-stage incubation protocol (2) in sterile soybean meal-glucose medium of the following composition: glucose, 20 g; sodium chloride, 5 g; potassium phosphate dibasic, 5 g; yeast extract, 5 g; soybean meal, 5 g; and distilled water, 1,000 ml; medium was adjusted to pH 7.0 with 6 N HCl before sterilization. Incubations were conducted at 25°C on New Brunswick Scientific Co. model G25 Gyrotory shakers operating at 250 rpm.

Screening experiments were performed in 25 ml of medium held in 125-ml DeLong culture flasks. Fresh slants of microorganisms were transferred to Stage 1 culture flasks, which were incubated for 72 h before being used as inocula for Stage 2 incubations. The substrate 1,4-cineole (compound 6; 50 μl, 42 mg) was added to each 24-h-old Stage 2 culture, and controls received no terpene substrate. The extractabilities and stabilities of compounds 6, 5, 7, and 9 were established by incubating these compounds in uninoculated culture medium and in buffers held at pH 3, 7, and 8. Culture samples, 4 ml each, were withdrawn at 24, 48, 72, and 96 h after substrate addition. These samples were extracted with 1 ml of ethyl acetate, and 30-μl volumes were

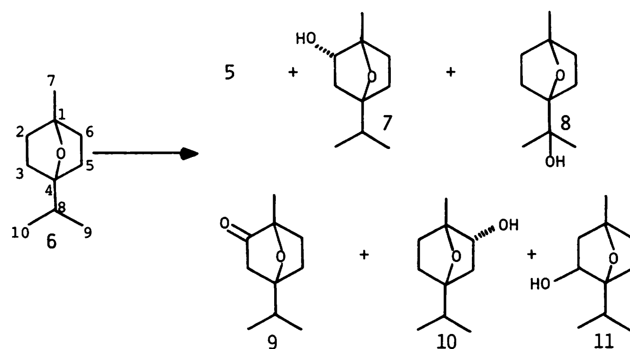

FIG. 2. Microbial transformation of 1,4-cineole (compound 6).

TABLE 2. Results of screening experiments to detect cultures capable of transforming 1,4-cineole

Culture	Compound detected in extract[a]					
	6	5	7	9	8	Unknowns
Aspergillus alliaceus NRRL 315	+ + + + +	−	+ +	−	+	−
A. niger UI 172	+ +	+ + +	−	+ + + +	−	−
Bacillus cereus UI 1477	+ +	+ +	+ + + + +	−	+ +	−
B. cereus UI 2485	+ + + + +	−	+ +	−	+ +	−
B. lichenformis UI A-5A	+ + + + +	−	+ +	−	−	−
B. megaterium UI 14581	+ + + + +	−	+ + +	−	−	+
B. subtilis ATCC 6633	+ + + + +	−	+	−	−	−
B. subtilis UI 2485	+ + + + +	−	+ +	−	+ +	−
Caldariomyces fumago ATCC 16373	−	−	−	−	−	+
Candida guillermandii NRRL 2025	+ + + + +	−	−	−	−	+ + +
C. intermedia NRRL 5159	−	−	−	−	−	−
C. lipolytica UI-Can	−	−	−	−	−	−
Cunninghamella echinulata ATCC 9244	+ + +	+ +	+ + + +	−	+ + +	+ +
Curvularia pallescens ATCC 12018	−	−	−	−	−	−
Mucor mucedo ATCC 7941	+ +	+ +	+ + + + +	−	−	−
Nematosporia sp. strain UI 314	+ + + +	+	+ +	+ + +	−	−
Penicillium stipitatum UI 336	−	+ + +	+ + +	+ +	−	−
Pseudomonas sp. strain ATCC 11299b	−	−	−	−	−	−
Rhizopus arrhizus QM-1032	+ + + +	+ +	+ + +	+ + +	−	−
Sepedonium chryosospermum ATCC 13378	+ + +	+ + +	+ +	+ + +	−	−
Streptomyces griseus NRRL B8090	+ +	+ + +	+ + + +	−	+ + + +	−
S. griseus ATCC 10137	−	+ +	+ + +	+ +	+ + + + +	−
S. griseus ATCC 13273	+ + +	+ +	+ + +	+ + +	−	−
S. griseus ATCC 13968	−	−	−	−	−	+
S. punipalus NRRL 3529	+ + + + +	+ +	+ + +	−	+	−
Streptomyces sp. strain UI 127	+ + + + +	+	+	−	−	−
Stysanus stemonites UI 2833	+ + + + +	−	+ +	−	+	+

[a] Yields are estimated by TLC as follows: −, none; +, trace; + +, 10%; + + +, 20%; + + + +, 30%; and + + + + +, 50% and higher.

spotted on TLC plates for analysis. The results of initial screening experiments are shown in Table 2.

Preparative 1,000-ml fermentations were conducted with *Aspergillus niger*, *Bacillus cereus*, *Mucor mucedo*, *Nematosporia* species, *Penicillium stipitatum*, *Rhizopus arrhizus*, *Sepedonium chrysospermum*, *Streptomyces griseus* ATCC 10137, and *S. griseus* ATCC 13273 in order to produce sufficient amounts of metabolites for isolation and verification of structure. Cultures were grown in 200 ml of medium held in 1,000-ml DeLong culture flasks, and a total of 2 g of 1,4-cineole substrate was used for each microorganism. Samples were taken and analyzed as described for screening experiments, and cultures were harvested between 48 and 96 h after substrate addition. Harvested cultures were extracted with 3 500-ml volumes of dichloromethane. The organic extracts were combined, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The reaction products were separated by repeated chromatography over silica gel and alumina. The separated products were analyzed by GC, GC-MS, and proton NMR for characterization. Details of the separation methods used for *B. cereus* and *S. griseus* ATCC 10137 fermentations are presented below.

TABLE 3. Kinetics of hydroxylation of 1,4-cineole by *B. cereus* and *S. griseus*[a]

Organism	Time (h)	% Yield of metabolite					Total recovery (%)	Exo/endo ratio
		6	8	9	5	7		
B. cereus UI 1477	24	25.3	2.0		2.1	15.1	44.6	1:7.1
	48	6.8	2.7		2.6	17.5	29.6	1:6.8
	72	2.4	2.7		2.4	16.4	24.0	1:6.7
S. griseus ATCC 10137	24	7.2	18.3	1.6	3.4	5.1	35.7	1:1.5
	48	0.3	19.7	1.8	3.3	6.2	31.3	1:1.9
	72		17.7	1.9	3.3	6.0	28.9	1:1.8

[a] Each 25-ml Stage 2 culture received 50 μl (42 mg) of 1,4-cineole as the substrate.

Separation of cineoles from the transformation by *B. cereus*. The crude extract (600 mg) obtained from the *B. cereus* culture was subjected to chromatography over a column (4.5 by 48 cm) of silica gel (200 g) and eluted with hexane-ethyl acetate (17:3, vol/vol). Elution volumes of 464 to 2,776 ml yielded 280 mg of a mixture which was further purified over a column (1.1 by 37 cm) of alumina (65 g). Elution with hexane-ether (1:4, vol/vol) yielded 114 mg of a mixture of alcohols between volumes of 504 to 632 ml. Analyses of this alcohol mixture by proton NMR, GC, and GC-MS showed that it contained about 70% 2-endo-hydroxy-1,4-cineole (compound 7), 19% 2-exo-hydroxy-1,4-cineole (compound 5), and 11% 8-hydroxy-1,4-cineole (compound 8).

Separation of cineoles from the transformation by *S. griseus*: isolation of 8-hydroxy-1,4-cineole (compound 8). The crude extract (1.5 g) obtained from the *S. griseus* culture was subjected to chromatography over a column (2 by 30 cm) of silica gel (25 g) and eluted with hexane-ethyl acetate (17:3, vol/vol). Elution volumes of 50 to 150 ml yielded a mixture (198 mg) containing the hydroxycineoles. This fraction was separated further by chromatography over a column (1 by 24 cm) of silica gel (8 g) by using the same eluent. Elution volumes of 22 to 24 ml yielded pure 8-hydroxy-1,4-cineole (4 mg; compound 8), volumes of 24 to 38 ml yielded mixtures of compounds 8, 5, and 7, and later fractions afforded mixtures of compounds 5 and 7. These fractions were all characterized by GC, GC-MS, and proton NMR by comparison with

the standards. The analytical data of the new 8-hydroxy-1,4-cineole (compound 8) were as follows: mass m/z (percent intensity), 170 (29, M^+), 155 (18, $M-CH_3$), 137 (26, $M-CH_3-H_2O$), and 111 (100, $M-C_3H_7O$); ^1H NMR, 1.23 (s, 6H, isoprophyl methyls 9, 10), 1.45 (s, 3H, methyl 7), and 1.65 (m, 8H).

Kinetics of 1,4-cineole hydroxylation. The kinetics of hydroxylation were determined with *B. cereus* (UI 1477) and *S. griseus* (ATCC 10137). The transformations were performed in 125-ml DeLong flasks holding 25 ml of the stage 2 culture containing 50 μl (42 mg) of 1,4-cineole (compound 6). The reactions were sampled by harvesting duplicate flasks from each microorganism at 24, 48, and 72 h after substrate addition. The culture samples were each extracted three times with 25 ml of dichloromethane, the organic layers were combined and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The residue was dissolved in 5 ml of methanol and diluted as necessary for GC analysis. The results of the kinetic experiments are reported in Table 3.

RESULTS AND DISCUSSION

Microorganisms were examined for their abilities to hydroxylate the pleasant-smelling liquid monoterpene 1,4-cineole (compound 6). Although terpenes such as this one are lipophilic and poorly soluble in water, they have been widely exploited as substrates for microbial transformation. Since microbial transformation reactions occur only on dissolved substrates (3), we examined the water solubilities of compound 6 and of two of the metabolites expected to be formed in biotransformation reactions. Although 1,4-cineole is only slightly soluble in water (2.4 g/liter), nearly 1/3 lb of 2-exo-hydroxy-1,4-cineole (compound 5) and 1/5 lb of 2-endo-hydroxy-1,4-cineole (compound 7) dissolved in 1 liter of water (1 lb = 453.592 g). Thus, it is possible to envision that, as hydroxylation of 1,4-cineole occurred, the reaction was driven to completion by the greatly enhanced solubilities of hydroxylated cineoles in aqueous fermentation media.

Analytical methods, including high-performance liquid chromatography, TLC, GC, GC-MS, and proton NMR spectroscopy, were established to permit the ready identification of various metabolites expected in microbial transformation experiments. TLC on silica gel plates was used in the general screening of microorganisms for their abilities to hydroxylate 1,4-cineole. This system (Table 1) enabled the separation of all expected products, except for the 2-exo- (compound 5) and 2-endo-hydroxy-1,4-cineole (compound 7) metabolites. Zinc sulfate-impregnated silica gel TLC plates were used to distinguish between these two products. The use of zinc ions as a part of the stationary phase was deemed a reasonable means of achieving the separation of the isomeric 2-hydroxy-1,4-cineoles on the basis of the well-known interaction of the zinc atom coordinated at the active site of alcohol dehydrogenases. By TLC, we expected that the exo-alcohol (compound 5) with both the oxygen bridge and the hydroxyl group on the same face of the 1,4-cineole molecule would be capable of coordinating with the zinc atom more readily than the endo alcohol (compound 7) isomer with oxygen atoms on opposite faces of the bicyclic ring system. Zinc sulfate-impregnated TLC plates enabled the ready distinction between compounds 5 and 7. GC permitted the quantitative assessment of fermentation reactions, and all compounds, except for 9 and 11 were base line resolved by this method (Fig. 2). The overlapping of GC peaks for compounds 9 and 11 was not problematic, however, because the presence of both compounds together in fermentation extracts was simple to detect by TLC. The proton NMR spectra of compounds 5 and 7 also provided clear-cut information which permitted them to be distinguished, even when both were present in reaction mixtures. The carbinol methine groups of compounds 5 and 7 at 3.75 and 3.90 ppm were well separated from each other, and the relative areas under peaks for these carbinol methine signals could be used to determine the actual ratio of the two compounds in mixtures.

Initial screening experiments were conducted by using 104 species of bacteria, fungi, and yeasts, including representatives of 39 microbial genera, among them being: *Aspergillus* (19 species), *Bacillus* (6 species), *Candida* (7 species), *Cunninghamella* (5 species), *Curvularia* (3 species), *Mucor* (3 species), *Penicillium* (7 species), *Rhodotorula* (3 species), and *Streptomyces* (16 species). Of all of the cultures examined, 27 transformed 1,4-cineole, within 96 h, to a variety of metabolites. As shown in Table 2, compound 6 was completely degraded into undetectable metabolic products by *Candida intermedia*, *Candida lipolytica*, *Curvularia pallescens*, and *Pseudomonas* species. On the basis of colors formed on visualized TLC plates, unknown 1,4-cineole metabolites were detected in extracts of *Caldariomyces fumago* and in those of one of four *S. griseus* (ATCC 13968) strains examined. The most common products formed by metabolite-accumulating strains were, in order of quantity, 2-endo-hydroxy-1,4-cineole (compound 7), 2-exo-hydroxy-1,4-cineole (compound 5), and 8-hydroxy-1,4-cineole (compound 8).

The identities of the metabolites were confirmed by conducting 1,4-cineole biotransformation reactions on a preparative scale with nine microorganisms which appeared to perform reactions in good yield. The reaction products were isolated by solvent extraction and separated by column chromatography, and the isolated products were identified by GC, GC-MS, and proton NMR comparisons with standards. In all cases, the 2-endo-hydroxy-1,4-cineole (compound 7) was present in a higher proportion than the 2-exo-hydroxy-1,4-cineole (compound 5).

1,4-Cineole appeared to be hydroxylated best by representatives of the genera *Bacillus* and *Streptomyces*. When several species of these genera were examined for their potential to catalyze the hydroxylation reaction, similar products were obtained in most cases, but the yields of metabolites varied considerably. This finding suggests that terpene hydroxylation may be a general capability within these genera.

The major biotransformation product formed by *S. griseus* (ATCC 10137) was the new compound 8-hydroxy-1,4-cineole (compound 8), which was identified by proton NMR and MS. The mass spectrum of this compound exhibited a molecular ion at m/z 170, indicating the presence of a hydroxyl group in the metabolite structure. The base peak in the mass spectrum of compound 8 occurred at m/z 111 owing to the loss of an m/z 59 mass fragment, or a protonated acetone equivalent, from its structure. The proton NMR spectrum of this compound exhibited no carbinol methine proton signal, suggesting that the hydroxyl group introduced by microbial hydroxylation might be tertiary in compound 8. The two methyl group signals of the isopropyl moiety appeared as a singlet in the spectrum of compound 8 at 1.23 ppm, shifted downfield from 0.94 ppm in the spectrum of compound 6. Since the isopropyl group normally appears as a doublet in 1,4-cineole and other metabolites, such as compounds 5 and 7, the spectrum indicates that the hydrogen atom at position 8 of 1,4-cineole was absent and most

probably replaced by a hydroxyl group. This evidence strongly supports the structure of 8-hydroxy-1,4-cineole (compound 8) for the major *S. griseus* hydroxylation product.

The kinetics of hydroxylation of 1.4-cineole were examined with *B. cereus* (UI 1477) and *S. griseus* (ATCC 10137). These two organisms were selected for further work because they gave the most consistent results in performing the hydroxylation reactions. The result of this experiment are presented in Table 3. With both microorganisms, recoveries of metabolites were best at 24 h and declined during the course of the fermentation.The combined yield of metabolites was not more than 45% with either culture. This finding indicates that 1,4-cineole was either mineralized by *B. cereus* and *S. griseus* or converted into other metabolites undetectable by TLC or GC methods developed for this work. MacRae et al. (8) described the ability of a *Pseudomonas flava* strain isolated from Eucalyptus leaves to utilize the isomeric terpene 1,8-cineole (compound 2) as a carbon source for growth. This bacterium also produced low yields of four metabolites, including the isomeric 2-endo- and 2-exo-1,8-cineole alcohols, the corresponding ketone, and a ring fission product.

With *B. cereus*, the major identified metabolite was 2-endo-hydroxy-1,4-cineole (compound 7), which was formed in an average yield of nearly 16% throughout the course of the biotransformation reaction. The 2-exo- (compound 5) and 8-hydroxy-1,4-cineole (compound 8) products were produced in average yields of 2.3 and 2.5%, respectively. It is noteworthy that the relative ratios of exo- to endo- hydroxycineole isomers remained essentially constant throughout the course of the fermentation by both microorganisms. *S. griseus* produced 8-hydroxy-1,4-cineole (compound 8) as the major cineole metabolite. Overall, yields of the 2-hydroxy-products (compounds 5 and 7) were lower than those obtained with *B. cereus*. However, relatively larger proportions of the 2-exo- isomer (compound 5) were formed by *S. griseus*. *S. griseus* also yielded small amounts of 2-oxo-1,4-cineole (compound 9), indicating the presence of oxidoreductases in this organism capable of interconverting alcohol and ketone products.

The present work demonstrates the propensities of microorganisms to hydroxylate 1,4-cineole. In most cases 2-hydroxylation was the major reaction, leading to formation of the endo compound as the major isomer. Microbial hydroxylation reactions usually proceed with retention of configuration (4), with the introduced hydroxyl group occupying the same stereochemical position as that of the hydrogen atom which is removed. Thus, with 1,4-cineole, molecular oxygen is most probably introduced from the more hindered endo face of the bicyclic terpene. This result was unexpected, and it suggests that the methyl and isopropyl groups and the oxygen bridge of 1,4-cineole play unknown steric or electronic roles in directing specific orientations of the substrate at the active site of the hydroxylating enzyme(s).

1,4-Cineole (compound 6) is a deceptively simple molecule exhibiting prochiral and prostereogenic properties (9). The compound falls within the Cs symmetry group owing to the plane of symmetry which passes through carbons 7, 1, 4, and 8 and the oxygen atom. The introduction of a hydroxyl group into position 2 of the terpene structure destroys the symmetry of the molecule and introduces a center of local chirality. Thus, it is possible to form either 2-endo-hydroxy-1,4-cineole (compound 7) or the enantiomeric product 6-endo-hydroxy-1,4-cineole (compound 10), depending upon the terpene face to which the hydroxyl group is introduced. The stereochemical features of microbiological hydroxylations of 1,4-cineole will be the subject of another communication.

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