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STEREOSPECIFIC HYDROXYLATION OF 1,8-CINEOLE USING A MICROBIAL BIOCATALYST

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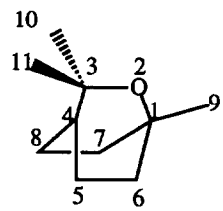
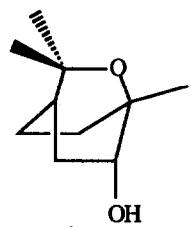
Abstract: The new bacterial catalyst, Bacillus cereus hydroxylates 1,8-cineole (1) to give 6R-exo-hydroxy-1,8-cineole, [(1R,4S,6R)-1,3,3-trimethyl-2-oxabicyclo(2,2,2)-octane-6-ol], (3). The regio- and stereochemical outcome of this reaction was predicted using a model for the B. cereus hydroxylase.

The stereospecific introduction of molecular oxygen to nonactivated carbon atoms remains a challenging goal in synthetic organic chemistry[1]. Microorganisms with intact and highly organized monooxygenase enzyme systems activate molecular oxygen, and achieve such reactions under very mild reaction conditions. Microbial hydroxylation reactions are usually regio- and stereospecific, and they have been widely exploited in the industrial synthesis of steroid hormones[2]. Controlled hydroxylations also hold great potential for use with other groups of compounds[3].



(1)



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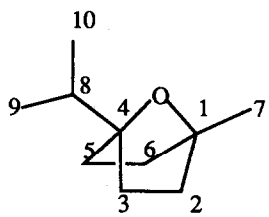

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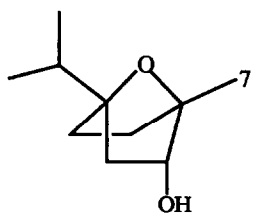
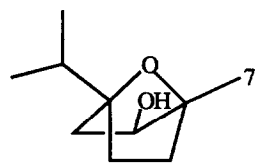


(2a)



(2b)

1,8-Cineole [1,3,3-trimethyl-2-oxabicyclo(2,2,2)octane] (1) is the major component of the oil from leaves of Eucalyptus radiata var.

Australiana[4]. It is also an important representative of the class of fragrant monoterpenes which are available for derivatization and use as chiral synthons for chemical syntheses[5]. Microbial oxidations have been reported for 1,8-cineole by species of Rhodococcus[6] and Pseudomonas flava[7] which grow by using 1 as a carbon source in place of sugar, and by Aspergillus niger[8]. All three of these biocatalysts formed multiple degradation products of 1,8-cineole in low yield. Rhodococcus sp. gave 6-endo-hydroxy-1,8-cineole and 6-oxo-1,8-cineole, both of unspecified stereochemistries as products, while P. flava gave 6S-exo-, 2R-endo-hydroxy-1,8-cineoles, 1S,4R-6-oxocineole and a lactone metabolite. A. niger gave racemic 5-exo-, 5-endo-, and 6-exo-hydroxy-1,8-cineole, as well as the 6- and 5-ketone derivatives.

Bacillus cereus (UI-1477) was identified as a useful new biocatalyst in the hydroxylation of 1,4-cineole (2)[1d,9]. Good yields of essentially pure 2R-endo- (2a), and 2R-exo-hydroxy-1,4-cineole (2b) were obtained, and the major alcohol product 2a was consistently obtained in yields eight times larger than 2b. The high stereochemical purities of both compounds, and the relative yields of hydroxylated 1,4-cineoles 2a and 2b permitted the description of a model for the B. cereus hydroxylase enzyme system. We assumed that a single enzyme was responsible for catalyzing both hydroxylation reactions, and that the isopropyl, methyl and oxygen bridge moieties of 2 all favored terpene binding to the enzyme surface for preponderant pro-1R-endo-oxygenation. With the B. cereus enzyme model, one would predict that 1 would bind to the catalyst to favor pro-1R-exo-hydroxylation, and that the bulky gem dimethyl-oxygen bridge of 1 would preclude substrate binding to enable pro-1S-endo-1,8-cineole hydroxylation.

Using our standard biocatalysis protocol[9], B. cereus was grown in 500 ml of medium containing 500 mg of 1,8-cineole for 72 hours before the reaction was stopped. The methylene chloride extract (366 mg) was purified by silica gel column chromatography (hexanes:ethyl acetate, 4:1, v/v). Similar fractions were combined to afford 154 mg of white crystals (from hexanes) of 3 which gave the following values: mp, 94° (lit.[7a], 98°); $[\alpha]_D^{25°}$ = -31.28° (c = 1.6 in ethanol), (lit.[7a], 31°); high resolution mass spectrum, m/z 170.1313 for $C_{10}H_{18}O_2$ (calc. 170.1307) with major fragment ions at m/z 152 ($M^+ - H_2O$), 137 ($M^+ - H_2O$, $- CH_3$); IR, λ_{max} (cm^{-1}) 3385, 2900 - 2982, 1458, 1368, 1133, 1063, 977; ^{13}C-NMR[8] (55.7 MHz, $CDCl_3$) ppm, 73.4 (C-8), 72.5 (C-1), 71.1 (C-2), 34.6 (C-3), 34.2 (C-4), 29.0 (C-9), 28.6 (C-10), 24.9 (C-6), 24.1 (C-7), 22.2 (C-5); ^{1}H-NMR (360 MHz, $CDCl_3$) ppm, 1.10, 1.19 and 1.28 (three methyl groups), and 3.73 (1H, carbinol

methine, ddd, J = 10, 7.5, 2 Hz). As the chemical shift of the methine proton for 5-hydroxy-1,8-cineole is always larger than 4.0 ppm[8,10], the physical data for 3 suggested a 6-hydroxylated 1,8-cineole product. It is possible to discriminate between 6-endo- and 6-exo-hydroxy-1,8-cineoles by high field proton NMR[11]. The NMR signal of the 6-endo-hydroxy-1,8-cineole exo-carbinol methine proton usually resonates as a triplet, or a doublet of doublets, while the endo-carbinol methine proton of the corresponding 6-exo-hydroxy-1,8-cineole is split by W-type long range coupling with one of the 7-protons to appear as a ddd signal[12]. Based upon these considerations, and the observed physical properties of the isolated B. cereus product, the structure of the only product formed by hydroxylation of 1 is optically pure 6R-exo-hydroxy-1,8-cineole [(1R,4S,6R)-1,3,3-trimethyl-2-oxabicyclo(2,2,2)octane-6-ol] (3)[13].

The rate and yield of 1,8-cineole hydroxylation by B. cereus were determined by GC analyses[9] of methylene chloride extracts of reaction samples taken at different times. A 60% yield was obtained within 15 hours, and the maximum yield of the hydroxylation reaction was 74% by 24 hours. Levels of the 6R-exo-hydroxy-1,8-cineole product remained constant for 48 additional hours indicating that the bacterial catalyst does not utilize 3 for growth and energy.

It is possible to utilize cell-free, crude enzyme preparations from B. cereus to catalyze the hydroxylation reaction. Cultures are grown as usual[9], cells are harvested by centrifugation, and disrupted by French pressure homogenization. The resulting homogenate is centrifuged at 50,000 x g for 40 minutes and examined for the presence of cytochromes using difference spectroscopy[14]. Cell free extracts contain a cytochrome P_{452} enzyme by this method, and they catalyze the hydroxylation reaction when NADH, H^+ is added as a reducing cofactor together with 1,8-cineole.

This work describes the first high-yielding and stereospecific hydroxylation of 1,8-cineole by a microbial catalyst, and it illustrates the potential for predicting the site and stereochemistry of hydroxylation. Much more experience is required to validate the previously reported enzyme model, and we have screened additional mono-, sesqui- and diterpene substrates, most of which yield new and presumably hydroxylated products with B. cereus. The identification of these products is in progress.

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13. Reference samples of various 1,8-cineole metabolites including 3 were requested from the only laboratory in the world possessing these compounds (see refs 7a and 7b). Professor Carman informed us that his entire supply of reference terpene samples were destroyed by an arson in a laboratory fire.
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