# Microbial Asymmetric CH Oxidations of Simple Hydrocarbons: A Novel Monooxygenase Activity of the Topsoil Microorganism *Bacillus megaterium*

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A *Bacillus megaterium* strain was isolated from topsoil by a selective screening procedure with allylbenzene as xenobiotic substrate. It is demonstrated for the first time, from analytical-scale experiments, that this microorganism hydroxylates a variety of simple *n*-alkanes (hexane through nonane) and cycloalkanes (cyclohexane and cyclooctane) to afford optically active alcohols in up to 99% enantiomeric excess (*ee*). In the case of the *n*-alkanes, the ω-1, ω-2 and ω-3 regioisomers were obtained. This enzymatic activity is unprecedented for *Bacillus megaterium* strains and is generally rarely observed in bacteria.

## Introduction

CH oxidations are some of the most versatile reactions in organic chemistry, since potentially useful oxyfunctionalized synthons may be obtained from readily accessible hydrocarbons. The currently available synthetic methods require harsh reaction conditions and yield racemic products.[1] Therefore, much effort has been expended in the development of efficient and selective catalytic oxidations of hydrocarbons.[2−5] For instance, metal-catalyzed asymmetric CH oxidations have recently been carried out with chiral auxiliaries[6] and optically active oxidants[7−8] to yield enantiomerically enriched products; however, in most cases, only low enantioselectivities have been obtained for unactivated alkanes. Moreover, biomimetic studies have been conducted to gain insight into the catalytic mechanism of the CH insertion and to improve its stereoselectivity.[9−11] Despite these efforts, the development of a broadly applicable and effective catalytic asymmetric hydroxylation of unfunctionalized hydrocarbons remains a challenge.

As an alternative, microorganisms have been successfully employed for the selective oxyfunctionalization of unactivated CH bonds in a variety of substrates.[12] So far, most of this work has focused on the hydroxylation of steroids, terpenes and other complex natural products.[13,14] In the case of simple alkanes, the substrates of interest in the present work, most of the hydroxylations have been performed by methanogenous microorganisms, like *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b, or by purified methane monooxygenases.[15,16] However, with these enzymes the highest efficiency is obtained with methane as substrate, while for substrates with increased chain length, the yields are significantly reduced.[16] Furthermore, only two species of microorganisms are known which produce P450-dependent alkane hydroxylases. These are the *Rhodococcus rhodochrous* ATCC 19067 and the *Acinetobacter calcoaceticus* EB104, both of which are ω hydroxylases and, therefore, not suitable for asymmetric oxidation.[17]

To obtain a more efficient biocatalyst for the asymmetric hydroxylation of unactivated hydrocarbons, a selective screening procedure with allylbenzene (5−25 μL) as substrate was applied to soil samples of different origin. Previously, we have reported on the oxyfunctionalization of arylalkanes with microorganisms isolated by this newly developed screening protocol.[18] In this work, we present our results on the hydroxylation of simple hydrocarbons by a *Bacillus megaterium* strain isolated from topsoil. This strain is known to hydroxylate long-chain fatty acids, fatty amides and fatty alcohols predominantly in the ω-1 position; [17,19] however, the hydroxylation of simple alkanes by *B. megaterium* appears not to have been reported to date. We demonstrate herein the unprecedented asymmetric biocatalytic oxidation of the unactivated alkanes **1a−f** to the corresponding alcohols **2** and **3**, by *B. megaterium*, in high enantiomeric excess.

## Results and Discussion

To assess the feasibility of the biocatalytic asymmetric oxidation of simple alkanes with the topsoil microorganism *B. megaterium*, the biotransformations were carried out on an analytical scale. For this purpose, cultures of the bacteria (75 mL) were grown overnight in an open system in min-

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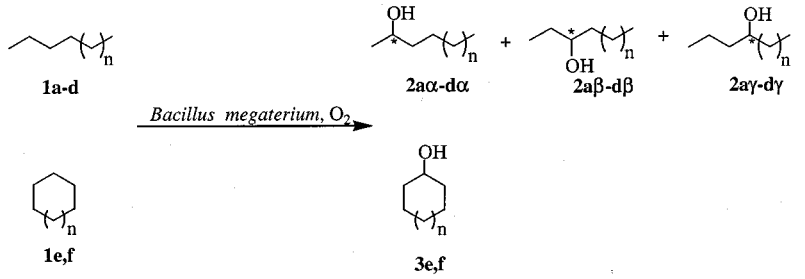
imum media and then incubated with 0.8 mmol of the respective hydrocarbon for 17 h at 30 °C. Subsequently, the bacterial broth was centrifuged and the clear medium was extracted with a pentane/dichloromethane mixture (2:1) for 24 h to recover the products and unchanged starting material from the bacterial medium. The organic solvent was carefully removed by distillation over a Vigreux column to prevent further loss of the highly volatile products, and the resulting residue was submitted to chiral GC analysis. Our results confirmed that the strain isolated from topsoil hydroxylates the *n*-alkanes **1a−d** and the cyclic derivatives **1e,f** enantioselectively to the alcohols **2** and **3** (Scheme 1). This demonstrates a novel enzymatic activity for *Bacillus megaterium* which has not been observed before.




Scheme 1. Asymmetric hydroxylation of alkanes **1** by *Bacillus megaterium*; the respective ketones of these alcohols are formed as minor products (cf. Table 1)

For this biocatalytic process, the time of substrate addition plays a crucial role with regard to the product formation, since the addition of hydrocarbon during the early log and stationary phases results in an approximately fourfold decrease of product formation over the addition during the mid-log phase (data not shown). This indicates that the substrate affects enzyme expression when added during the most pronounced growth phase, and thus induces monooxygenase activity. Consequently, the optimized biotransformation conditions were applied to the hydroxylation of the alkanes **1a−f**. The regio- and enantioselectivities of these CH oxidations by *B. megaterium* are given in Table 1.

*n*-Hexane (**1a**) yielded hexan-3-ol (**2aβ**) as major and the α-hydroxy alcohol **2aα** as minor products in an enantiomeric excess (*ee*) of 54% and 93%, respectively (Table 1, entry 1). The diminished *ee* value for hexan-3-ol (**2aβ**) is not unexpected as the molecule is more symmetric than **2aα** and, therefore, stereodifferentiation becomes worse. Low amounts of the corresponding ketones of the alcohols **2aα** and **2aβ** were also observed. However, the enantiomeric excess, in particular for **2aβ**, cannot be reconciled in terms of kinetic resolution of the respective alcohol through enantioselective oxidation to the ketone because the amount of the latter is too small to affect the enantioselectivity of the biotransformation. Therefore, the observed *ee* values are the result of direct asymmetric hydroxylation of *n*-hexane by the microorganism. In stark contrast to *n*-hexane (**1a**), the hydroxylation of the other *n*-alkanes **1b−d** predominantly affords the α-hydroxy alcohols **2bα-dα** (entries 2−4). Presumably, steric effects force the long-chain alkanes (chain length > C6) to position themselves differently in the active center of the enzyme, and thus different regioselectivities are observed.

*n*-Heptane (**1b**) was converted into the regioisomeric alcohols heptan-2-ol (**2bα**), heptan-3-ol (**2bβ**) and heptan-4-ol (**2bγ**) in a ratio of 34:21:15 with excellent enantiomeric excesses [> 99% *ee* for the *S* enantiomers of **2bα** and **2bβ** (entry 2)]. Similarly, octan-2-ol (**2cα**) and octan-3-ol (**2cβ**) are obtained as major products from *n*-octane (**1c**) with *ee* values > 99% for the *S* enantiomers, while octan-4-ol (**2cγ**) is formed in small amounts and only in 6% *ee* (entry 3). The reason for this low enantioselectivity at the γ-methylene site of *n*-octane (**1c**) is presumably the consequence of poor differentiation by the enzyme between the two sterically similar *n*-propyl and *n*-butyl substituents. For *n*-nonane (**1d**), the results are comparable to those of *n*-heptane (**1b**) and *n*-octane (**1c**), since mainly nonan-2-ol (**2dα**) and

Table 1. Regio- and enantioselectivities of the oxygen-atom insertion into the C−H bonds of *n*-alkanes **1a−d** and cycloalkanes **1e,f** by *Bacillus megaterium* cultures

| Entry[a] | Alkane | Alcohol | Selectivity [%] Regio-[b] | Enantio-[c] |
|---|---|---|---|---|
| 1 | **1a** | **2aα** | 19 (11) | 93 |
| | | **2aβ** | 57 (13) | 54 |
| 2 | **1b** | **2bα** | 34 (17) | >99 (*S*) |
| | | **2bβ** | 21 (13) | >99 (*S*) |
| | | **2bγ** | 15 | [d] |
| 3 | **1c** | **2cα** | 53 (19) | >99 (*S*) |
| | | **2cβ** | 17 (5) | >99 (*S*) |
| | | **2cγ** | 6 | 6 |
| 4 | **1d** | **2dα** | 34 (27) | >99 |
| | | **2dβ** | 23 (9) | 98 |
| | | **2dγ** | 7 | -[e] |
| 5 | **1e** | **3e** | 79 (21) | [d] |
| 6 | **1f** | **3f** | 35 (65) | [d] |

[a] Substrate (0.8 mmol) added at mid-log phase; 17 h incubation at 30 °C, 120 rpm. − [b] Ratio of regioisomeric alcohols; the product distribution is normalized to 100% and the amounts of the corresponding ketones are given in parentheses; error ±2% of stated values; conversions and yields are not given since starting materials and products are extremely volatile and evaporated in the course of the open-system experiments of these bioconversions. − [c] Enantiomeric excesses (*ee* values) were determined by multi-dimensional gas chromatography on a cyclodextrin column; absolute configurations, when given, were determined by comparison with authentic reference samples, by lipase-catalyzed kinetic resolution of the racemic alcohols, or by comparison with literature data; error ±2% of stated values. − [d] Alcohol does not possess a chiral center. − [e] The enantiomeric excess of the minor product was not determined.

nonan-3-ol (**2dβ**) are obtained, while nonan-4-ol (**2dγ**) is found only as a minor product (entry 4).

Gas-chromatographic analysis on cyclodextrin columns demonstrated that the α and β alcohols **2** of the alkanes **1b−d** are formed enantiomerically pure. For all entries, the respective ketones are observed only for the α and β regioisomers; however, in the case of *n*-nonane (**1d**), the chemoselectivity is diminished, especially for the α-hydroxy alcohol **2dα**, since higher amounts of ketones are produced.

The cyclic alkanes cyclohexane (**1e**) and cyclooctane (**1f**) are also readily hydroxylated by *B. megaterium* to yield the nonchiral alcohols **3e** and **3f** (entries 5 and 6). As with the *n*-alkanes, overoxidation to the ketones significantly increases, more so for cyclooctane (65% ketone) than for cyclohexane (21% ketone). This clearly demonstrates that the oxidative activity becomes more prevalent with increased ring size. A similar trend is apparent for the oxidation of *n*-hexane (**1a**) through *n*-nonane (**1d**) and implies that hydrocarbons with longer alkyl chains and larger rings serve as better substrates for enzymatic oxidation by bacterial cells.

## Conclusions

Our present analytical-scale experiments show that the nonpathogenic, topsoil bacterium *Bacillus megaterium* readily hydroxylates a variety of unactivated simple alkanes stereoselectively to the corresponding regiomeric alcohols in high enantiomeric excesses (up to 99% *ee*). This is the first demonstration that *Bacillus megaterium* may oxidize unfunctionalized hydrocarbons enantioselectively. The excellent stereoselectivity observed for the *n*-alkanes studied here makes further investigations of this microorganism worthwhile. Isolation and overexpression of the pertinent monooxygenase enzyme in the present *B. megaterium* strain should significantly improve the biocatalytic efficiency and facilitate preparative applications of this biotransformation for the asymmetric CH oxidation of simple hydrocarbons.

## Experimental Section

**General:** Plate-Count Agar was purchased from Creatogen (Creatogen Biosciences GmbH, Augsburg, Germany). All other compounds were acquired from Aldrich and Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). As rotary shaker, a GFL 3031 (Gesellschaft für Labortechnik mbH, Burgwedel, Germany) was used. A Wolf SanoClav-MCN autoclave was employed for sterilization (Wolf, Geisslingen, Germany) and a laminar AirFlow bench was used for sterile work (NuAire Inc., Plymouth, MN, USA). A Beckman J2-21 centrifuge was applied for cell separation (Beckman, Fullerton, CA, USA). GC analysis was performed on a HP 5890A (Hewlett−Packard, Palo Alto, CA, USA). Multidimensional gas chromatography was carried out on a Sichromat 2 with a "live-T" switching device (Siemens, München, Germany) or with a moving-column stream-switching system (MCSS) coupling device on two Fisons GC 8000 instruments (Fisons, Mainz-Kastel, Germany). Mass spectrometry was conducted on a MD 800 mass spectrometer coupled with a Fisons GC 8000 (Fisons, Mainz-Kastel, Germany).

**General Procedure for the Biotransformation of the Hydrocarbon:** Liquid minimal media (75 mL) were prepared according to Dworkin et al.,[20] 5 mL/L of a mineral salt solution[21] were added and autoclaved (121 °C, 16 min). The culture was maintained under sterile conditions during the addition of glucose (5 g/L). For inoculation of the liquid media, cultures were taken from freshly grown plates. In an open system and under sterile conditions, ca. 0.8 mmol of the hydrocarbon substrate was added and the liquid culture was allowed to grow for ca. 18 h at 30 °C. Control experiments without bacteria were carried out to verify the stability and authenticity of the starting material; no oxidation products were observed under these conditions. After ca. 17 h, the culture was worked up by sonication of the bacterial broth for 15 mins. and centrifugation at 15000g for 20 min. The supernatant solution was extracted with 120 mL pentane/dichloromethane (2:1) by liquid-liquid extraction for 24 h. The extract was dried over $Na_2SO_4$ and distilled over a Vigreux column (40 °C, 990 mbar) to prevent loss of volatile compounds by evaporation. The products were submitted to gas chromatographic analysis, coupled with mass spectrometry, and their identity was verified by comparison with authentic reference samples. Conversions were not determined due to the volatility of the substrates, which evaporated during the course of the bioconversion. The enantiomeric excess of the alcohols was determined by multidimensional gas chromatography (MDGC) on the following columns:

a) 2,3-*O*-Diacetyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin column for **2aα**, **2aβ**, **2bα**, **2bβ**, **2cβ**, **2cγ**, **2dα**

b) 2,3-*O*-Diethyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin column for **2cα**, **2dβ**

Assignment of the absolute configuration was done by lipase-catalyzed kinetic resolution of the alcohols **2** and, wherever possible, confirmed by comparison with authentic enantiomerically pure reference samples.

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