New Oxyfunctionalization Capabilities for ω-Hydroxylases: Asymmetric Aliphatic Sulfoxidation and Branched Ether Demethylation

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Abstract: Due to inherent difficulties in the chemical generation of aliphatic synthons, the stereo- and regioselective oxyfunctionalization of simple aliphatic substrates represents an area where chemical applications of biocatalysis would be particularly useful. The hydrocarbon monooxygenase from *Pseudomonas oleovorans* is a prototypical "ω-hydroxylase" known to carry out hydroxylation at the terminal methyl of alkanes as well as epoxidation of terminal olefins. It is now demonstrated that this enzyme system catalyzes stereoselective sulfoxidation of methyl thioether substrates, representing the first clear example of oxygenase-produced chiral aliphatic sulfoxides yet reported. In addition, it is shown that this enzyme system catalyzes oxygenative O-demethylation of branched alkyl methyl and branched vinyl methyl ethers to secondary alcohols and ketones, respectively. These findings establish new oxyfunctionalization capabilities, and thus a significantly expanded biotechnological potential, for the hydrocarbon monooxygenases.

Due to inherent difficulties in the chemical generation of aliphatic chirons,[1] the stereo- and regioselective oxyfunctionalization of simple aliphatic substrates represents an area where chemical applications of biocatalysis would be particularly useful. Since such applications typically require processing of multifunctional substrates, the hydrocarbon "ω-hydroxylases",[2] whose regioselectivity effectively protects susceptible functionalities not near the substrate terminus, are quite attractive, provided reasonable chemical versatility can be demonstrated for these enzymes. We have demonstrated facile, highly stereoselective epoxidation by the *Pseudomonas oleovorans* alkane monooxygenase (POM),[3] a competence subsequently demonstrated for other analogous ω-hydroxylases.[4] We now report stereoselective sulfoxidation of methyl thioether substrates by POM, representing the first clear example of oxygenase-produced chiral aliphatic sulfoxides yet reported. In addition, we report POM-catalyzed oxygenative O-demethylation of branched alkyl methyl and branched vinyl methyl ethers to secondary alcohols and ketones, respectively. These findings establish new oxyfunctionalization capabilities, and thus a significantly expanded biotechnological potential, for the hydrocarbon monooxygenases.

Experimental Section

Enzyme Preparations and Assays. *P. Oleovorans* cultures were maintained and grown as described previously.[5] Rubredoxin and *P. oleovorans* monooxygenase (POM) were purified from *P. oleovorans* paste as reported earlier,[3g,6] with the exception that for POM purification the 30–35% ammonium sulfate pellet was dissolved in 300 mM sucrose buffer instead of glycerol/detergent buffer. Assays were performed by incubating the reconstituted system of POM for 10 min at 25 °C. The assay mixture contained 2 mg of partially purified POM, 80 μg of *P. oleovorans* rubredoxin, 14 μg of spinach ferredoxin reductase, 1 mg of NADPH, and 20 μL of substrate solution (100 μL in 1 mL of acetone) in a total of 1 mL of 20 mM Tris-Cl, pH 7.5. Products were extracted with 200 μL of $CHCl_3$ and quantitated by GC, using 2-octanol as an internal standard.

For preparative-scale sulfoxide production, the assay quantities were multiplied 100-fold, an NADPH recycling system was added (100 units of glucose 6-phosphate dehydrogenase and 200 mg of glucose 6-phosphate), and the mixture was incubated for 2 h at 25 °C. It was then centrifuged, saturated with NaCl, and extracted twice with 100 mL of $CHCl_3$. The organic layer was dried and concentrated, and the components were separated by flash chromatography on a silica column, using acetone as eluant. Fractions were pooled on the basis of TLC analysis, concentrated, and analyzed by GC, using 2-octanol as an internal standard. The yields of sulfoxide products varied, depending on the sulfide substrate. In a typical large-scale incubation, using methyl octyl sulfide as substrate, about 40 mg of sulfoxide was collected, an isolated yield of 25%. In general, the yields corresponded to the activities shown in Table I for various substrates. Optical rotations of the enzymatic sulfoxides were obtained at the sodium line with a 10-cm cell and a digital polarimeter.

For large-scale 2-octanol production, the assay mixture was multiplied 10-fold and NADPH recycling was added. The mixture was incubated for 40 min at 25 °C, then centrifuged, saturated with NaCl, and extracted twice with 10 mL of pentane. The organic phase was then dried and concentrated to dryness under a stream of nitrogen. The enzymatic product was treated with freshly prepared (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride[7] in 1 mL of CH_2Cl_2 and 100 μL of pyridine in a hermetically sealed vial for 5 h at room temperature. The reaction mixture was washed with dilute HCl and dilute NaOH and dried. Analyses of the MTPA derivatives were performed by GC, using a 30-m DB5 open tubular column (J&W Scientific) and a temperature program of 3 °C/min from 110 to 185 °C. MTPA derivatives of authentic (*R,S*)-, (*R*)-, and (*S*)-2-octanol were prepared in a similar manner and were used for GC peak assignment; GC–MS analysis (molecular ion peak at *m/z* 346) also confirmed the peak assignments.

Substrate Syntheses. The saturated alkyl sulfides were prepared by one of two methods. The first method consisted of the reaction of the appropriate thiol (0.1 mol) with 2 equiv of NaOH in methanol and then addition of excess MeI. After evaporation of methanol, the residue was dissolved in methylene chloride, the mixture washed with water, and the

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(2) (a) For a general review, see: May, S. W. *Enzyme Microb. Technol.* **1979**, *1*, 15. (b) For examples in yeast, see: Delaisse, J. M.; Martin, P.; Verheyn-Bouvy, M.-F.; Nyns, E.-J. *Biochim. Biophys. Acta* **1981**, *676*, 77 and references therein. (c) ω-Hydroxylases are centrally involved in important mammalian processes. For examples, see: Shak, S.; Goldstein, I. M. *J. Clin. Invest.* **1985**, *76*, 1218. Holm, K. A.; Kupfer, D. *J. Biol. Chem.* **1985**, *260*, 2027.

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(4) (a) Ohta, H.; Tetsukawa, H. *J. Chem. Soc., Chem. Commun.* **1978**, *19*, 849.

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(6) May, S. W.; Lee, L. G.; Katopodis, A. G.; Kuo, J. Y.; Wimalasena, K.; Thowsen, J. R. *Biochemistry* **1984**, *23*, 2187.

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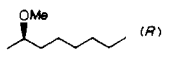
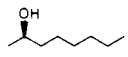
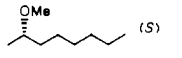
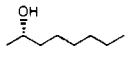
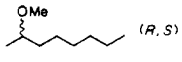
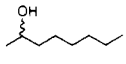
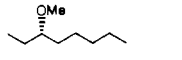
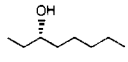
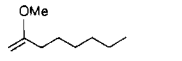
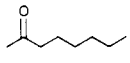
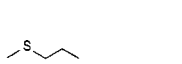
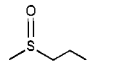
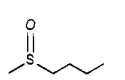
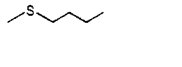
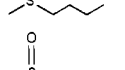
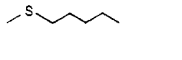
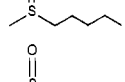
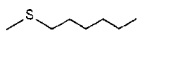
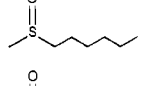
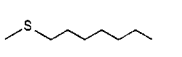
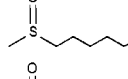
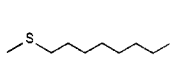
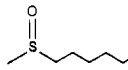
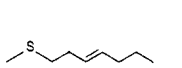
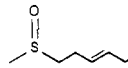
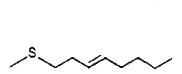
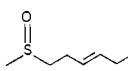
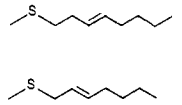
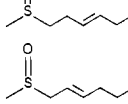
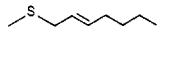
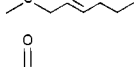
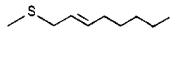
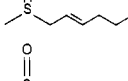
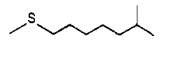
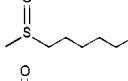
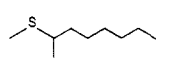
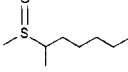
Table I

substrate	product	amt,[a] μmol/10 min	ee,[b] %
		0.35	100 (*R*)
		0.35	100 (*S*)
		0.35	9 (*R*)
		0.04	?
		0.18	
		0.11	88
		0.25	80
		0.61	60
		0.77	30
		0.36	70
		0.99	48
		0.71	86
		0.40	88
		0.66	6
		0.48	4
		0.93	2
		0.10	52
		0.34	30
		0.11	52

[a] Assays were performed with 2 mg of partially purified POM (see the Experimental Section). The assay mixture was saturated with NaCl and extracted with chloroform with 2-octanol as an internal standard. [b] Enantiomeric excess of the alcohols was determined by comparison of the integrated GC peaks. For the sulfoxides, the integrated methyl signals after chiral shift reagent addition were compared. Absolute configurations of the alcohols were assigned by comparison with authentic standards. All sulfoxides were assigned the *R* configuration by comparison with (*S*)-methyl *n*-butyl sulfoxide.[13] We note that allylic sulfoxides have been reported to undergo racemization via reversible 2,3-sigmatropic rearrangement to achiral sulfenates.[14,15]

organic layer dried and distilled. In the second method, 0.1 mol of the appropriate alkyl halide was treated with an equivalent amount of sodium methanethiolate, generated in situ by bubbling excess methanethiol into a solution of sodium methoxide in methanol. The product was then isolated as in the first method. Olefinic substrates were synthesized by treating 0.1 mol of the appropriate alkynyl alcohols with sodium in liquid ammonia to obtain the trans olefinic alcohols. Treatment with PBr_3 in benzene gave the corresponding bromides, which were then treated with sodium methanethiolate as indicated above. Distilled product yields were high, typically better than 80%. Products were characterized by GC, GC–MS, and NMR. Authentic samples of sulfoxides were produced by treating 100 μL of the appropriate sulfide in 5 mL of ethanol with 250 mg of sodium periodate in 3 mL of water. After 1 h at room temperature the sulfoxide was extracted into chloroform and analysed by GC, or separated on a silica column as indicated for the enzymatic products, and characterized by NMR.

2-Methoxy- and 3-methoxyoctane were prepared by treating the corresponding alcohols (0.1 mol) with excess NaH and MeI in dry THF. After being cooled and quenched with water, the product mixture was extracted with ether, washed with brine, dried, and distilled. Residual alcohol, if any, was removed by adding a small amount of NaH in the still pot. Both products were pure by GC and GC–MS (molecular ion peak at *m/z* 144), contained no trace of the starting alcohols, and had NMR spectra consistent with the expected structures. The same procedure was used to synthesize (*R*)- and (*S*)-2-methoxyoctane, starting with the respective chiral alcohols.

2-Methoxy-1-octene was synthesized by a method reported previously.[8] The literature procedure was successful only when modified to include addition of excess base before addition of $NaBH_4$. 1-Octyne (60 nmol) was added dropwise to an equivalent amount of $Hg(OAc)_2$ in dry methanol at 0 °C. Cold pentane and cold 3 M NaOH in excess were added, followed by addition of 2.5 g of $NaBH_4$ in 3 M NaOH at 10–20 °C. After filtration, the separated aqueous layer was extracted twice with pentane. The combined organic phases were dried over $MgSO_4$, concentrated, and distilled. The colorless product (27% yield) had the following properties: bp 32–33 °C (3 mm); ^1H NMR (60 MHz, CCl_4) δ 3.78 (s, 2 H), 3.45 (s, 3 H), 2.07 (t, 2 H), 1.6–1.0 (m, 8 H), 0.92 (t, 3 H); MS, molecular ion peak at *m/z* 142. Analysis by GC gave a single peak.

NMR Methods. (*R*)-(−)-*N*-(3,5-Dinitrobenzoyl)-α-phenethylamine was synthesized from 3,5-dinitrobenzoyl chloride and (*R*)-α-phenethylamine as reported by Deshmukh et al.[9] NMR samples of the sulfoxide products were prepared by dissolving 5–10 μL of the purified sulfoxide in 600 μL of $CDCl_3$. NMR spectra were obtained at 400 MHz before and after the addition of 1.2 equiv of the shift reagent. Enantiomeric excess was determined by comparing the integrated areas of the shifted methyl signals.

Results and Discussion

The ω-hydroxylation system of *P. oleovorans* consists of three proteins: *P. oleovorans* monooxygenase (POM), rubredoxin, and a flavoprotein reductase.[10] POM, a prototypical non-heme iron monooxygenase, has long been known to carry out hydroxylation at the terminal methyl group of straight-chain alkanes, and we have previously demonstrated its facility in effecting stereospecific epoxidation of terminal olefins[3] and oxygenation of terminal methyl ethers.[3g]

When either 2- or 3-methoxyoctane was incubated with POM under standard assay conditions, GC–MS analysis confirmed production of the corresponding 2- or 3-octanol product (table). As expected for a POM-catalyzed monooxygenase process, product formation was abolished by omission of any of the three protein components of the POM system, NADH, or oxygen. Quantitative colorimetry[11] confirmed stoichiometric coproduction of formaldehyde, as expected from an oxygenative O-demethylation pathway. A simple displacement pathway would have produced a C-1 product at the oxidation state of methanol and *is* thus incompatible with stoichiometric appearance of formaldehyde, concomitant with the appearance of the 2- or 3-ol. Finally, O-demethylation and epoxidation were mutually competitive even under conditions of saturating electron flux through rubredoxin, as expected for reactions occurring at the same active site of the monooxygenase.

To investigate the possibility of chiral 2-octanol production, enzymatically generated and authentic (*R*)- and (*S*)-2-octanol

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were converted to MTPA esters that were resolved by GC, as indicated in the Experimental Section. Enzymatically produced 2-octanol gave rise to both diastereomeric peaks (table); authentic (*R*)- and (*S*)-2-octanol each gave rise to only the single expected MTPA ester peak. Both (*R*)- and (*S*)-2-methoxyoctane were found to be equally good substrates, each exhibiting the same reactivity as the racemic mixture. Thus, a lack of enzymatic discrimination, and not racemization during turnover, fully accounts for the stereochemical result.

Incubation of the vinyl methyl ether 2-methoxyoct-1-ene with POM resulted in production of 2-octanone (table), with the reaction exhibiting the expected component requirements. Once again, concomitant formaldehyde production confirmed an oxygenative pathway of enzymatic processing, while a relatively minor background hydrolysis of this substrate produced 2-octanone and methanol.

While the above results establish the competence of POM for oxygenation of branched chain substrates and demonstrate that chiral products were not obtained, methyl alkyl sulfides, which we have shown to be POM substrates,[12] allow a determination of whether the reactivity of POM can be directed toward a penultimate nucleophilic moiety in a chiral manner. When methyl *n*-octyl sulfide was incubated with POM, GC analysis indicated that methyl *n*-octyl sulfoxide was produced in an enzyme-dependent manner (table). As was the case for the ether substrates, sulfoxidation required all protein components, NADH, and oxygen and was fully competitive with epoxidation of 1,7-octadiene. To further examine the POM sulfoxidation reaction, a series of substrates with varying chain length and substituents was synthesized. As shown in the table, POM sulfoxidized a wide range of aliphatic methyl sulfides with varying efficiency, with methyl *n*-octyl sulfide being the most active substrate under our standard assay conditions.

Preparative quantities of methyl sulfoxides were readily produced from large-scale POM incubations and fully characterized. Optical purities were determined by integration of the *S*-methyl NMR signal of the enantiomers by use of chiral shift reagent as indicated in the Experimental Section; isomeric ratios are shown in the table. Chemical shifts of the *S*-methyl group for all sulfoxides examined were similar; for example, butyl methyl sulfoxide produced an *S*-methyl signal at 2.573 ppm and, after the addition of shift reagent, a signal at 2.550 and 2.538 ppm. In all cases the downfield signal was the most abundant. All sulfoxides with significant enantiomeric excesses exhibited strong negative rotations and analogous CD spectra over the entire 400–220-nm region, whereas the sulfoxides prepared via periodate oxidation exhibited no rotation or CD bands. Enzymatically produced butyl methyl sulfoxide, which by NMR contained 80% ee of one isomer (table), exhibited $[\alpha]^{25}_{D}$ of −55° in acetonitrile. Controls with enzymatically produced methyl *n*-octyl sulfoxide confirmed that neither racemization nor enantioselective degradation of the sulfoxide occurred under the reaction conditions. Since (*S*)-(+)-methyl *n*-butyl sulfoxide exhibits $[\alpha]^{25}_{D}$ of +42° in isooctane,[13] we presume that our enzymatic products are of the *R* configuration.

The utilization of enzymatic systems to carry out stereoselective sulfoxidations is a subject of much current interest. There are a number of reports dealing with aryl, benzyl, or cyclic sulfides. This laboratory has reported highly stereoselective sulfoxidation of phenyl 2-aminoethyl sulfide by dopamine-β-monooxygenase, the first example of sulfoxidation by a "specific hydroxylase",[16] and moderate to high degrees of stereoselectivity have been obtained in oxidation of these types of sulfides by other microbial or enzymatic systems.[17] However, very poor, if any, stereoselectivity has heretofore been achieved for simple aliphatic sulfide substrates.[18-21] The maximal stereoselectivity reported here for aliphatic sulfoxidation by POM is far higher than in any previous reports where microbial systems were evaluated for this purpose. Moreover, in contrast to whole-cell systems where complications due to enantioselective metabolism are present,[17-19] we have clearly demonstrated that the sulfoxides arise from POM catalysis. While detailed mapping of the POM binding site would obviously be required for optimized biotechnological exploitation, it is apparent that high stereoselectivity accompanied by regioselectivity has been successfully demonstrated with active substrates. Taken together, the results reported here establish important, new chemical versatility for the hydrocarbon monooxygenases in the difficult area of generation of simple aliphatic chirons.

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